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Innervation périphérique et réparation cutanée : rôle de l'innervation dans la cicatrisation après brûlure et sur l'activité cellulaire des fibroblastes dermiques

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Abréviations

ASIC	Acid-Sensing Ion Channel
ATP	Adenosine Triphosphate
α-ML	Actine alpha-Musculaire Lisse
CGRP	Calcitonin Gene-Related Peptide
CRLR	Calcitonin-Receptor Like Receptor
CSMs	Cellules Souches Mésenchymateuses
DAPI	4',6-Diamidino-2-Phenylindole
DKK-1	Dickkopf 1
DMEM	Dulbecco's Modified Eagle Medium
EGF	Epithelial Growth Factor
FNIEs	Fibres Nerveuses Intraépidermiques
GAP-43	Growth Associated Protein 43
GDNF	Glial cell-Derived Neurotrofic Factor
GDRs	Ganglions Dorso-Rachidiens
IB4	Isolectine B4
IL-1α	Interleukine 1 alpha
IL-8	Interleukine 8
INF-γ	Interféron gamma
MEC	Matrice Extracellulaire
MMP	Matrix MetalloProteinase
NF	Neurofilaments
NGF	Nerve Growth Factor
P/S	Pénicilline-Streptomycine
PBS	Phosphate Buffer Saline
PFA	Paraformaldéhyde
PGP 9.5	Proteine Gene Product 9.5

RAMP1	Receptor Activity-Modifying Properties 1
RTX	Résinifératoxine
SKP	Skin Derived-Precursor
SNC	Système Nerveux Central
SNP	Système Nerveux Périphérique
SP	Substance P
STT	Somatostatine
SVF	Sérum de Veau Fœtal
TGF-β1	Transforming Growth Factor-β1
TIMP	Tissue Inhibitor of Metalloproteinase
TRPV1	Transiant Receptor Potential Vanilloid 1
VEGF	Vascular Endothelial Growth Factor
VIP	Vasoactive Intestinal Peptide

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Chapitre 1 : Etude bibliographique

I - La peau

La peau est un organe indispensable à la survie du corps humain. Chez l'adulte, la surface totale de la peau est d'environ 2 m^2 et son épaisseur varie de 0,5 à 5 mm en fonction de sa localisation, du sexe et de l'âge de la personne. La peau est fine au niveau des paupières ou du mamelon mais elle est beaucoup plus épaisse au niveau de la paume des mains ou de la plante des pieds.

La peau possède 4 fonctions principales.

Sa fonction première est son rôle de protection : elle sert de barrière vis-à-vis de l'environnement mais permet également de limiter les pertes hydriques.

Elle est également impliquée dans la perception de stimuli sensoriels (Schmelz, 2011) détectés grâce aux terminaisons des fibres nerveuses sensorielles présentent dans l'ensemble des compartiments de la peau.

La peau participe à la thermorégulation par plusieurs mécanismes. Le principal moyen pour réguler la température interne du corps est une balance aux niveau des capillaires cutanés entre vasoconstriction (pour conserver la chaleur) et vasodilatation (pour éliminer l'excès de chaleur) (Charkoudian, 2003). Le tissu adipeux et les poils permettent une isolation thermique, la transpiration participe quant à elle à la thermolyse.

Enfin, la peau a une fonction métabolique. En effet, grâce au rayonnement UV B du soleil, la peau est impliquée dans la synthèse de la vitamine D indispensable pour la fixation du calcium sur les os (Bikle, 2012).

Pour réaliser toutes ces fonctions, la peau est divisée en plusieurs compartiments : l'épiderme (au contact de l'environnement), le derme, l'hypoderme mais aussi les annexes cutanées (Figure 1).

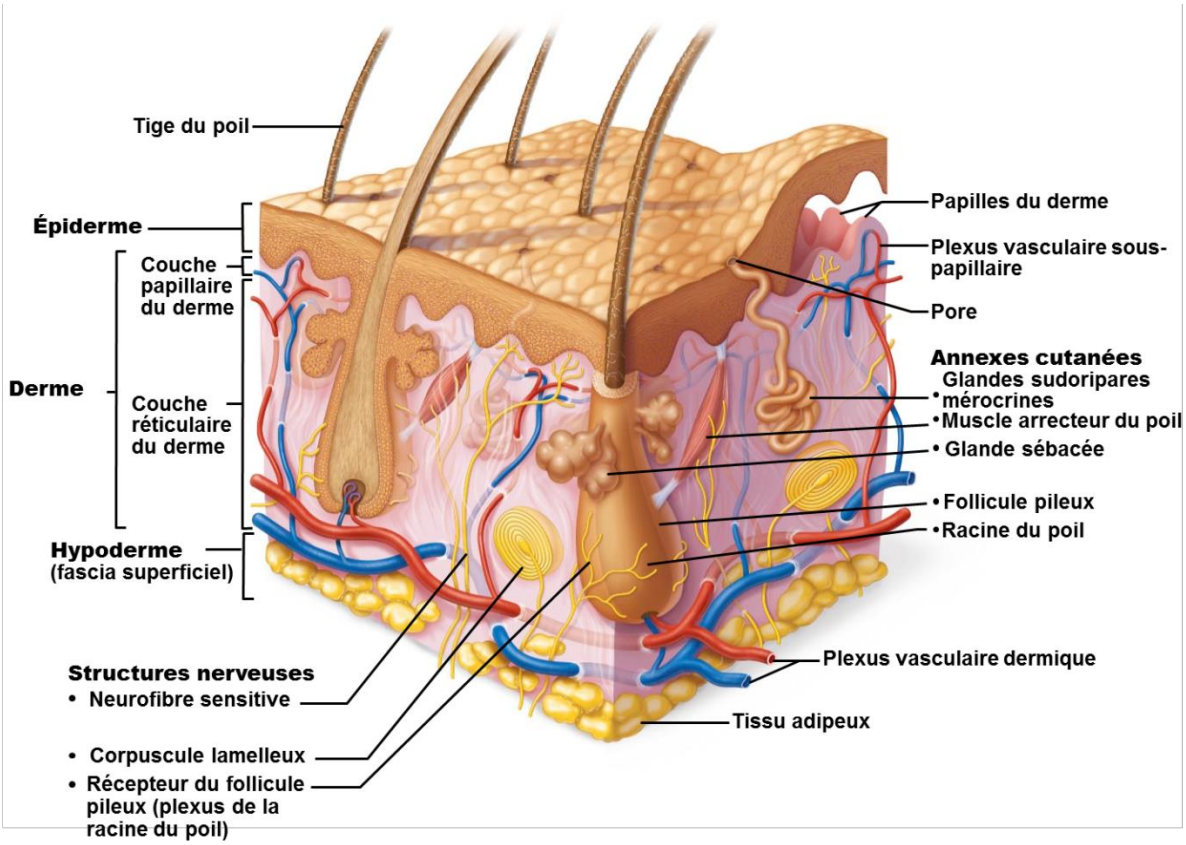


Figure 1 : **Représentation schématique de la structure de la peau** (d'après ERPI, 2013)**.**

I – 1 L'épiderme

L'épiderme est un épithélium pluristratifié kératinisé. Les différentes couches de l'épiderme sont impliquées dans la fonction barrière de la peau. En plus des kératinocytes qui représentent 90 à 95 % des cellules de l'épiderme, on note la présence de mélanocytes, de cellules de Merkel et de cellules de Langerhans.

Au sein de cet épiderme, on distingue plusieurs couches (Figure 2). La couche la plus profonde ou couche basale est composée de kératinocytes avec de larges noyaux cubiques qui se divisent. Parmi ces kératinocytes se trouvent des cellules souches épidermiques qui permettent le renouvellement de l'épiderme. Les kératinocytes de cette couche expriment les kératines 5 et 14 (Nelson and Sun, 1983).

En remontant vers la surface, on retrouve la couche épineuse composée de 5 à 15 assises de kératinocytes polygonaux et volumineux exprimant les kératines 1 et 10 (Fuchs and Green, 1980).

La couche granuleuse est composée de 1 à 3 assises de kératinocytes aplatis et fusiformes disposés parallèlement à la surface de la peau. Les kératinocytes de la couche granuleuse

expriment l'involucrine et la profillagrine, marqueurs terminaux de différenciation kératinocytaire.

Au contact de l'environnement, on retrouve la couche cornée constituée de 5 à 10 assises de cellules non viables éliminées par desquamation. Cette desquamation est compensée par la migration de kératinocytes provenant des couches inférieures. Le cycle de différenciation kératinocytaire depuis la couche basale jusqu'à la couche cornée s'effectue en une vingtaine de jours.

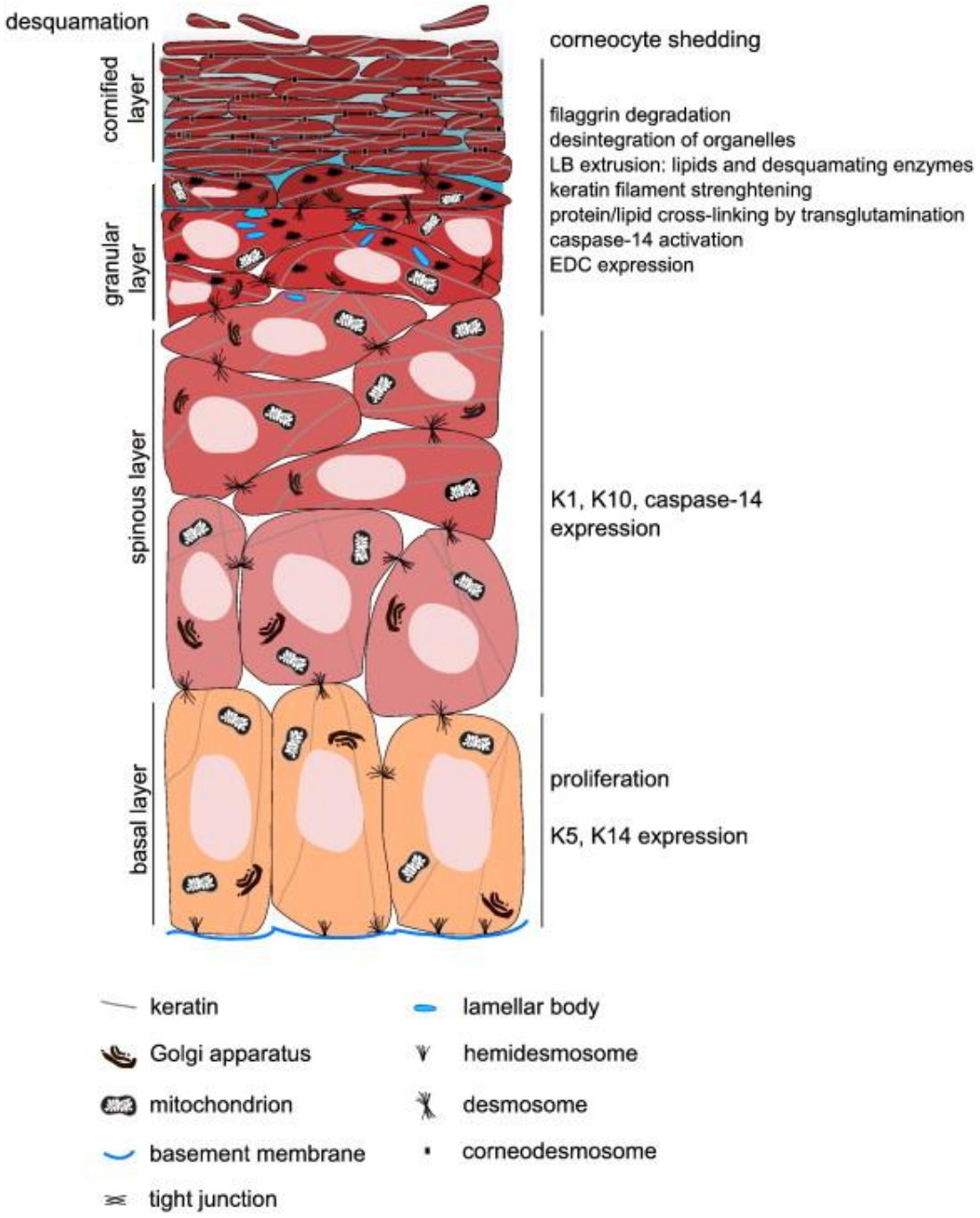


Figure 2 : Structure de l'épiderme et processus de différenciation des kératinocytes (d'après Eckhart et al., 2013).

L'épiderme ne contient ni vaisseaux sanguins ni vaisseaux lymphatiques. Les cellules de l'épiderme ont donc besoin des vaisseaux présents dans le derme pour l'apport de nutriments permettant leur survie.

Au sein de cet épiderme, on note la présence de terminaisons nerveuses impliquées dans la perception de stimuli sensoriels tels que le toucher, la pression, la température et la douleur (ceci sera détaillé dans la partie Chapitre 1, IV innervation cutanée).

I – 2 La jonction dermo-épidermique

Entre l'épiderme et le derme, la jonction dermo-épidermique (zone acellulaire) permet l'ancrage de l'épiderme au derme et évite la dissémination des kératinocytes dans le derme. Cette zone peut être divisée en 4 parties (Figure 3).

La zone la plus superficielle est constituée par la membrane plasmique des cellules de la couche basale de l'épiderme. Les kératinocytes présentent à ce niveau des structures d'attaches appelées hémidesmosomes. Les mélanocytes possèdent un système d'adhésion focal alors que pour les cellules de Merkel, on observe une densification de leur membrane plasmique au contact de la fibre nerveuse à laquelle elles sont associées.

Plus en profondeur, on distingue la *lamina lucida* qui a une épaisseur de 20 à 40 nm. Cette zone est traversée par des filaments d'ancrage de 5 à 7 nm de diamètre. Certains filaments sont riches en laminines 332 et 311 qui se lient à la partie extracellulaire de l'intégrine α6β4 présente à la surface des kératinocytes de la couche basale. Ces filaments d'ancrage forment alors un complexe avec les hémidesmosomes. Entre les hémidesmosomes, d'autres filaments d'ancrage moins nombreux présentent une composition différente. En effet, ils sont essentiellement constitués par les laminines 322, 311 ou 321 qui se lient à l'intégrine α3β1.

La *lamina densa* est une couche de 30 à 60 nm d'épaisseur. Elle est majoritairement composée de collagène de type IV mais également des laminines 511 et 321, de protéoglycanes et de nidogène. C'est une zone intermédiaire d'ancrage entre les filaments d'ancrage provenant de l'épiderme et ceux issus de la zone fibrillaire du derme.

La zone fibrillaire contient quant à elle des fibres d'ancrage de 20 à 60 nm d'épaisseur constituées majoritairement de collagène de type VII. Elle fait le lien entre la *lamina densa* et les plaques d'ancrage du derme superficiel.

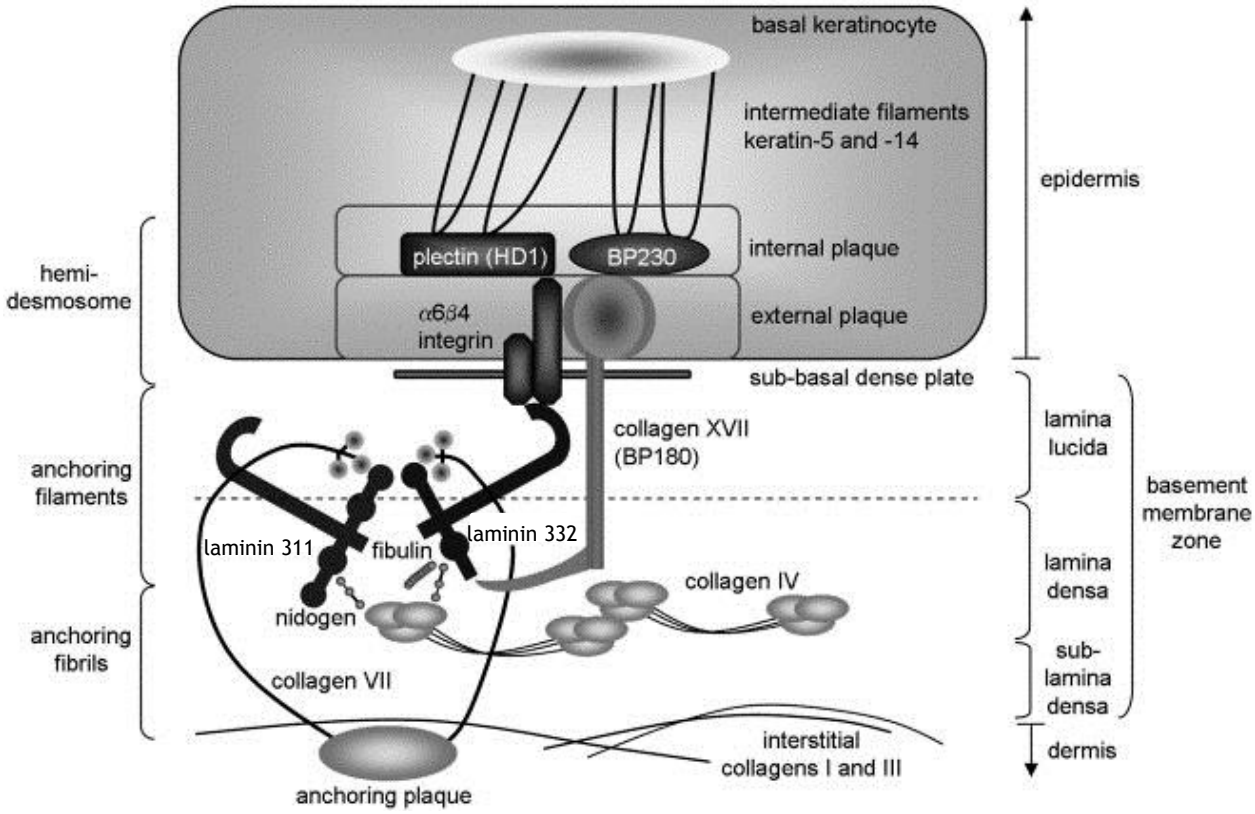


Figure 3 : Représentation de la jonction dermo-épidermique (adapté de Schneider et al., 2007).

I – 3 Le derme

Le derme est constitué majoritairement de fibroblastes et de matrice extracellulaire (MEC) qui apporte une résistance mécanique et élastique à la peau. Il apporte un soutien mécanique et nutritionnel à l'épiderme. Au sein du derme, on note la présence d'annexes cutanées et de plexus vasculaires. Le derme peut être divisé en deux zones : le derme papillaire, partie la plus superficielle du derme en contact avec l'épiderme et qui est constitué par les papilles dermiques localisées entre les crêtes épidermiques, et le derme réticulaire situé plus en profondeur. Ces deux zones se différencient par la morphologie des fibroblastes qu'elles contiennent mais aussi par la composition et l'organisation de leurs MEC.

Le derme papillaire, au contact de l'épiderme, est constitué de fibroblastes à forte capacité prolifératives et métabolique. La MEC du derme papillaire n'est pas organisée et présente des fibres disposées de façon aléatoire. Cette MEC est constituée de fibres de collagène de petit diamètre (collagène de type III et V) et ne présente pas de fibres d'élastine mature (Prost-Squarcioni et al., 2008). A ce niveau, on retrouve également de nombreux capillaires sanguins nécessaires pour la nutrition des cellules de l'épiderme et pour la régulation de la température corporelle. Des récepteurs sensoriels sont également présents et permettent notamment la détection de stimuli tels que la pression ou le toucher.

Le derme réticulaire quant à lui présente une MEC plus organisée : les fibres de collagène (essentiellement du collagène de type I) sont plus larges et on note la présence de fibres

élastiques matures. Ceci confère au derme réticulaire des propriétés de résistance mécanique supérieures au derme papillaire. A ce niveau, les fibres sont disposées parallèlement à la surface de la peau. Dans ce derme profond, on retrouve la présence de follicules pileux, de vaisseaux sanguins, de glandes sudoripares et sébacées mais également de nerfs et de récepteurs sensoriels.

La MEC est constituée majoritairement de collagène qui donne sa structure au derme et confère à la peau sa résistance mécanique. Le collagène représente 75% du poids sec de la peau. Il est majoritairement présent sous forme fibrillaire au niveau du derme. On distingue principalement 3 types de collagènes : le collagène de type I (60-80%), le collagène de type III (15-25%) et le collagène de type V (2-5%).

Des fibres élastiques sont également présentes dans le derme et apporte l'élasticité à la peau. En effet, leur structure leur permet d'être étirées jusqu'à 150% sans déformation résiduelle.

Au sein de cette matrice, on trouve également une matrice extrafibrillaire composée notamment de protéoglycanes et de glycosaminoglycanes. Ces molécules permettent notamment l'hydratation de la peau en permettant la diffusion de l'eau et lui apporte sa viscosité.

I – 4 L'hypoderme

Sous le derme, l'hypoderme permet de protéger le corps du froid et constitue une réserve d'énergie. Il est constitué majoritairement d'adipocytes mais aussi de cellules souches et contient de nombreux facteurs de croissance (Klein et al., 2007).

I – 5 Les annexes cutanées

I – 5.1 Les follicules pilo-sébacés

Les follicules pileux sont présents dans le derme à l'exception des zones glabres telles que la plante des pieds ou la paume des mains. La naissance du poil s'effectue dans le derme en profondeur à partir du bulbe pileux. Le poil ainsi que la gaine qui l'entoure dérivent d'une invagination de l'épiderme (Figure 4). Cette invagination forme la paroi de l'infundibulum puis la gaine épithéliale externe (Prost-Squarcioni, 2006).

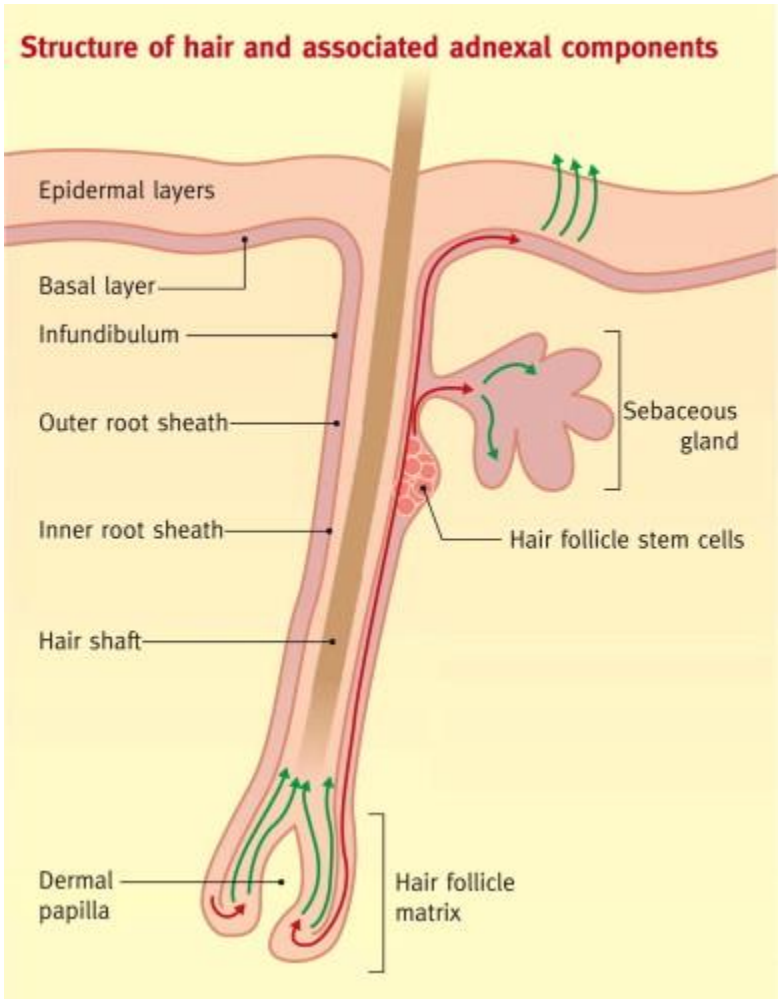


Figure 4 : Structure d'un follicule pileux (adapté de Lai-Cheong and McGrath, 2013).

Le cycle du poil s'effectue en 3 temps :

- la phase anagène : phase de croissance du poil ;
- la phase catagène : phase de résorption du poil ;
- la phase télogène : phase de repos.

Les glandes sébacées produisent du sébum et sont majoritairement associées aux follicules pileux permettant ainsi la sécrétion du sébum au niveau de l'isthme des follicules pilo-sébacés. Sous le muscle arrecteur du poil, un renflement appelé « bulge » contient des cellules souches kératinocytaires. Ces cellules souches sont importantes notamment lors du processus de cicatrisation permettant d'assurer une régénération de l'épiderme quand celui-ci est détruit.

I – 5.2 Autres annexes

En plus des follicules pileux, on trouve dans la peau les glandes sudoripares qui permettent la sécrétion de sueur et participe ainsi à la régulation de la température corporelle.

Des terminaisons nerveuses sont également présentes dans le derme et l'épiderme. Elles permettent de capter et de transmettre des stimuli tels que le toucher, la douleur, la pression,

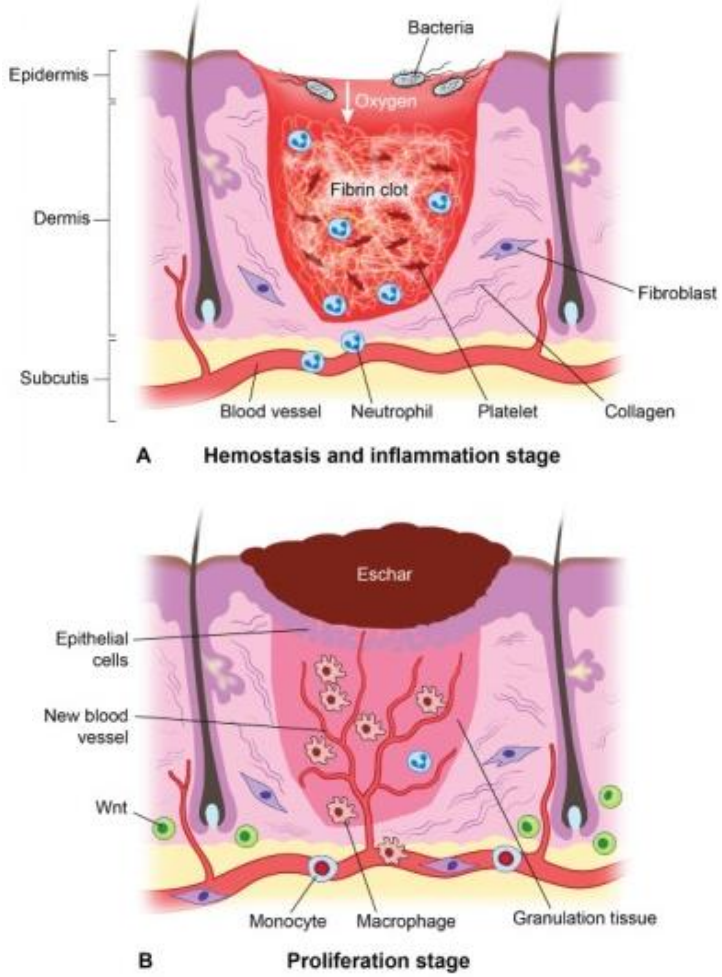
l'étirement, la température… L'ensemble de ces terminaisons nerveuses seront décrites en détails dans la partie IV.

II - Processus de cicatrisation et rôle de la matrice extracellulaire

Dans une peau saine, la MEC du derme est sécrétée par les fibroblastes. Cependant, après une lésion, la MEC détruite doit être remplacée lors du processus de cicatrisation. Cette nouvelle MEC est alors sécrétée par les myofibroblastes qui sont des acteurs essentiels lors du processus de cicatrisation.

Ces myofibroblastes représentent un stade final de différenciation des fibroblastes. Suite à une blessure, les fibroblastes évoluent d'abord en proto-myofibroblastes exprimant des fibres de stress contenant les actines β et γ cytoplasmiques. Ces proto-myofibroblastes peuvent se différencier en myofibroblastes avec l'apparition de l'expression de l'actine α-musculaire lisse (α-ML) qui est présente notamment dans les cellules musculaires lisses vasculaires contractiles. Ces myofibroblastes peuvent alors se contracter et sont ainsi impliqués dans les processus de cicatrisation.

Le processus de cicatrisation est classiquement décrit par 3 phases : la phase inflammatoire, la phase de prolifération et la phase de remodelage (Figure 5).



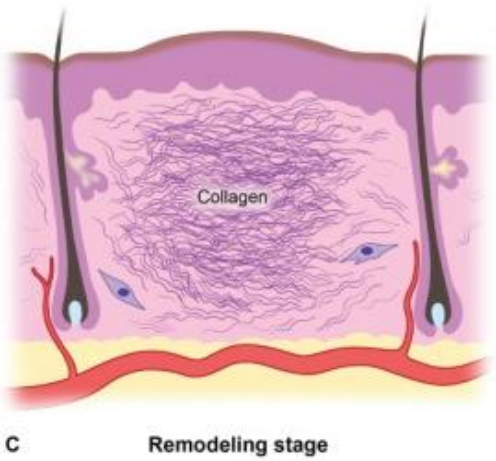


Figure 5 : Représentation des trois étapes du processus de cicatrisation (d'après Houschyar et al., 2015).

La phase inflammatoire débute dès l'apparition d'une lésion. Quelques minutes après la blessure, un caillot sanguin composé de fibrine et de fibronectine se développe pour stopper l'hémorragie et pour combler la perte de tissu générée par cette lésion. Au sein de cette matrice provisoire, de nombreux facteurs de croissance tels que le « Transforming Growth Factor-β1 » (TGF-β1), le « Epithelial Growth Factor » (EGF), le « Vascular Endothelial Growth Factor » (VEGF) sont libérés par les plaquettes et les macrophages. Ces facteurs permettent le recrutement des cellules de l'inflammation, des fibroblastes et des cellules endothéliales. Les premières cellules inflammatoires à rejoindre cette matrice provisoire sont les polynucléaires puis après 24h, les monocytes et les lymphocytes rejoignent la zone lésée (Kim et al., 2008). Cette inflammation se traduit par un érythème douloureux et chaud dû à la vasodilatation et à l'augmentation de la perméabilité vasculaire.

A la fin de cette phase inflammatoire, la phase proliférative se met en place et un tissu de granulation remplace progressivement la matrice provisoire. Au sein de ce tissu de granulation, une nouvelle MEC est synthétisée. De nouveaux capillaires se forment dans ce tissu de granulation pour permettre l'apport de nutriments et d'oxygène aux cellules présentes. Au cours de cette phase, les différentes cellules présentes vont s'activer. Les monocytes se différencient en macrophages permettant la phagocytose des déchets et la secrétions de nombreux facteurs (DiPietro, 1995). Les lymphocytes B s'activent en plasmocytes et sont ainsi capables de secréter des immunoglobulines. Les fibroblastes s'activent en myofibroblastes permettant, grâce à leur capacité contractile, de rapprocher les berges de la plaie. Ces myofibroblastes sont également responsables de la sécrétion de la nouvelle MEC déposée pour remplacer le tissu détruit.

La formation de ce néo-derme permet la mise en place du processus de ré-épithélialisation. Pour cela, les kératinocytes présents à proximité de la lésion migrent sur ce néo-derme à partir des berges de la plaie. La reconstitution de l'épiderme est nécessaire pour que la peau retrouve sa fonction de barrière vis-à-vis de l'extérieur (O'Toole, 2001).

La dernière phase du processus de cicatrisation est la phase de remodelage. Au cours de cette phase, la plupart des cellules présentes dans le tissu de granulation deviennent inutiles et

meurent donc par apoptose. En remplacement, de nouveaux fibroblastes envahissent la MEC. La composition de ce nouveau tissu conjonctif évolue également : le collagène de type III qui composé majoritairement le tissu de granulation est remplacé par du collagène de type I et l'élastine réapparait.

Au cours de ce processus de cicatrisation, des erreurs peuvent se produire conduisant à l'apparition d'une cicatrice pathologique. Ces anomalies sont en partie causées par les myofibroblastes. Par exemple, dans le cas d'une cicatrice hypertrophique (Figure 6), le tissu de granulation est toujours en croissance du à une sécrétion ininterrompue de MEC et/ou à un défaut de facteurs proapoptotiques. Cela se traduit par un épaississement de la couche dermique et cette situation est principalement rencontrée chez les jeunes patients.



Figure 6 : Photo d'une cicatrice hypertrophique 18 mois après une brûlure du 2ème degré (d'après Jain et al., 2014).

Par ailleurs, des problèmes lors de la cicatrisation cutanée se produisent également chez les personnes âgées. En effet, avec l'âge, la peau vieillit ce qui se traduit notamment par une diminution de son élasticité et une détérioration des fibres de collagène et d'élastine dermiques (Gould et al., 2015). Ces modifications cutanées sont notamment dues à des facteurs intrinsèques (senescence, stress oxydatif) mais aussi extrinsèques (tabac, UV).

Le vieillissement cutané entraine le plus souvent une augmentation du temps de cicatrisation due à une diminution de la formation du tissu de granulation, avec une réduction de l'angiogenèse et un nombre de myofibroblastes moins important et donc une synthèse de matrice extracellulaire diminuée. Du fait de ces caractéristiques de la peau âgée, les plaies cutanées présentes chez des patients âgés deviennent le plus souvent chroniques avec l'apparition d'ulcère ou d'escarre.

Le chapitre de livre « (Myo)fibroblasts/Extracellular Matrix in Skin Wound and Aging » qui suit décrit en détail le rôle des myofibroblastes et de la MEC dans la cicatrisation normale mais aussi les effets de l'âge sur la cicatrisation cutanée (Girard et al., 2016). La revue « Fibroblasts and myofibroblasts in wound healing » en annexe 1 apporte des précisions sur le rôle des fibroblastes dans le processus de cicatrisation.

2

CHAPTER

(Myo)fibroblasts/Extracellular Matrix in Skin Wound and Aging

Dorothée Girard,[1,2,a] *Betty Laverdet*[1,2,b] and *Alexis Desmoulière*[1,2,#,*]

❑ Introduction

The skin is the primary protection of the body against external injuries and is essential in the maintenance of general homeostasis. The dermis, located beneath the epidermis, represents the thickest compartment of the skin and is mainly composed of a dense collagen network supporting specific dermal apparatus such as hair follicles, sebaceous and sweat glands. Dermal (myo)fibroblasts play a major role in the synthesis and maintenance of the extracellular matrix (ECM) as well as in the wound healing process. During aging, the dermal ECM and associated resident cells undergo senescence affecting both the structure of the dermis and its role in skin repair. In addition, extrinsic factors such as ultraviolet (UV) irradiation and intrinsic factors such as diabetes or medicines can further accelerate this phenomenon. Thus, aging has direct consequences on the dermis via both the alteration of ECM components and the impairment of (myo)fibroblast function.

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❑ Skin Extracellular Matrix and Myofibroblastic Differentiation

Skin Extracellular Matrix

Dermal fibroblasts are responsible for the synthesis and maintenance of the ECM. The ECM is a complex network composed of macromolecules such as collagens, elastin and glycoproteins that strongly interact with each other and with resident cells to maintain the structural integrity and function of the skin (Table 1). The ECM of the dermal layer is composed mostly of fibrillar collagens with type I collagen being the most abundant followed by type III collagen. Type IV collagen which forms network of beaded filaments is also found (Kielty and Shuttleworth 1997) as well as fibril-associated collagens with interrupted triple helices (FACIT) collagens such as collagens XII and XIV (Gelse et al. 2003; Agarwal et al. 2012) and type V collagen to a lesser extent (Chanut-Delalande et al. 2004). Collagen fibers make up 70% of the dermis, giving it structural strength, tensile mechanical property and toughness. Another important component of the dermal ECM is elastin which maintains normal elasticity, stretching and flexibility. Proteoglycans and glycosaminoglycans represent another essential part of the ECM and provide viscosity and hydration to the skin (Mikesh et al. 2013). The dermis consists of two regions, the papillary dermis (or upper dermis) closer to the epidermis and the reticular dermis (or deep dermis), thicker than the papillary dermis, overlying the hypodermis. The papillary dermis is composed of thin elastin and collagen fibers mainly oriented perpendicularly to the skin surface, while the reticular dermis is composed of thicker and multidirectional fibers (Watt and Fujiwara 2011).

TABLE 1 Structure and roles of dermal extracellular matrix components. Molecules composing the extracellular matrix provide specific structural properties to the dermis such as elasticity, tensile strength and compressibility and are also involved in cell communication and signaling regulation via for example transforming growth factor-β and integrin signaling pathways (Schultz et al. 2005; Egbert et al. 2014).

Family	Name	Structure	Roles
Collagens	Types I, II, III, VI, V	Fibrillar/microfibrillar	Tensile strength
	Types XII, XIV, XVI	Fibril-associated collagen with interrupted triple helix	
Elastin		Elastic fiber	Stretching and resilience

Table 1 Contd.

Family	Name	Structure	Roles
Glycoproteins	Fibrillin	Glycoprotein	Maintenance of elastic fiber integrity
	Fibronectin	High molecular weight glycoprotein	Promotion of cell adhesion and communication
Glycosaminoglycans	Hyaluronic acid	Repetition of disaccharide units	Hydration and resistance to compression
Proteoglycans	Decorin, versican, heparan sulfate, dermatan sulfate, small leucine-rich proteoglycans	Glycosaminoglycan conjugated protein	Hydration and cell signaling regulation
Periostin		Matricellular protein	Regulation of collagen fibrillogenesis

Myofibroblastic Differentiation

Fibroblasts are spindle-shaped cells with long cytoplasmic prolongations and derive from multipotent mesenchymal cells. Fibroblasts form a heterogeneous cell population which plays a major role, as mentioned above, in the deposition of ECM components. Indeed, in the skin, fibroblasts are able to organize a complex ECM network responsible for dermal architecture and remarkable cutaneous biomechanical properties. Following internal or external injury signals, skin fibroblasts can acquire a myofibroblastic phenotype, presenting critical properties for wound healing, repair and "firmness" of aged and damaged skin.

Myofibroblastic differentiation of fibroblastic cells begins with the appearance of the proto-myofibroblast, whose stress fibers contain only β- and γ-cytoplasmic actin. Proto-myofibroblasts evolve, but not always necessarily, into the differentiated myofibroblast, the most common variant of this cell, with stress fibers containing α-smooth muscle (SM) actin (Hinz et al. 2012). Myofibroblasts can, according to the experimental or clinical situation, express other SM cell contractile proteins, such as SM-myosin heavy chains or desmin; however, the presence of α-SM actin represents the most reliable marker of the myofibroblastic phenotype (Desmoulière et al. 2005).

During the healing process, after the inflammatory and vascular phase, the granulation tissue develops with the appearance of myofibroblasts which synthesize and deposit ECM components which will replace the provisional matrix (Darby et al. 2014). These cells exhibit contractile properties, due to the expression of α-SM actin and play a major role in the contraction and the maturation of the granulation tissue (Hinz et al. 2001). During the resolution phase of healing, the cell number is dramatically reduced by apoptosis of vascular cells and myofibroblasts (Desmoulière et al. 1995) (Fig. 1).

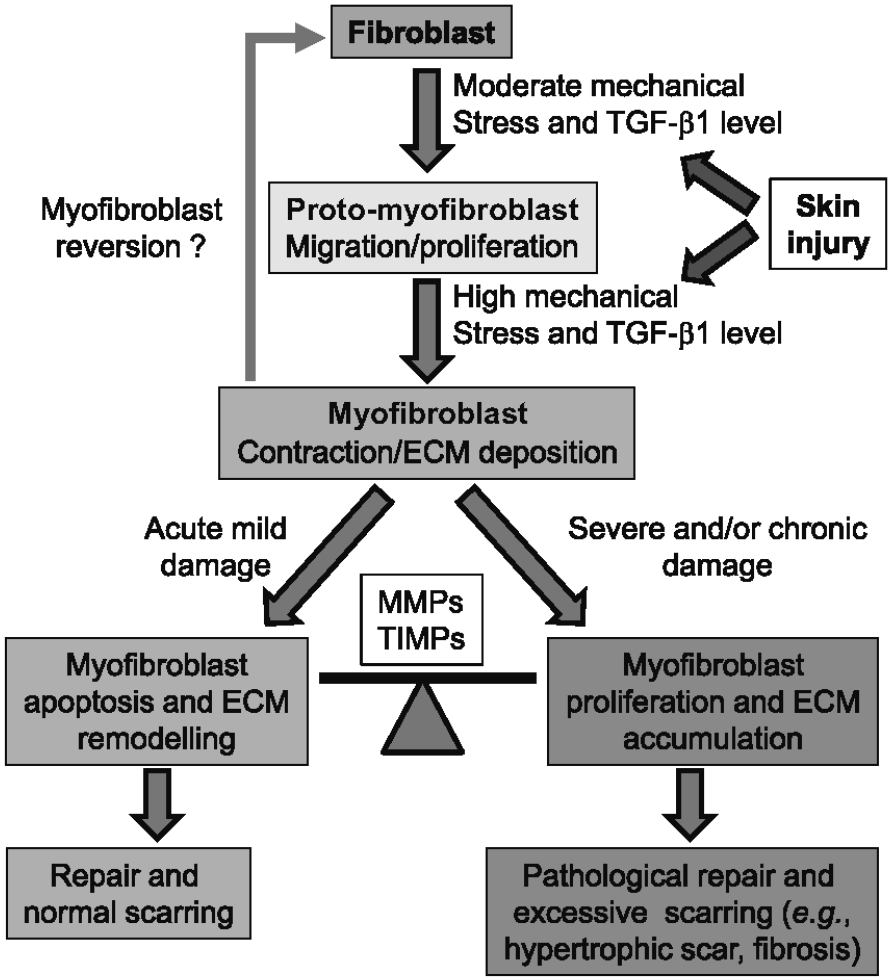


FIG. 1 Fibroblast-myofibroblast modulation during normal or pathological repair. *Proto-myofibroblasts express stress fibers containing cytoplasmic actins and develop focal adhesions. Myofibroblasts express stress fibers containing α-smooth muscle actin and develop supermature focal adhesions. Mechanical stress is a major inducer of myofibroblast differentiation via the extracellular matrix (ECM) and modifications of ECM are involved in transforming growth factor (TGF)-β1 activation leading to a vicious circle in pathological situations. In addition, the balance between matrix metalloproteinases (MMPs) and their inhibitors (TIMPs for tissue inhibitor of metalloproteinases) plays an essential role in ECM deposition/remodeling. During granulation tissue resolution, the reversion of the myofibroblast phenotype to the fibroblast phenotype remains to be demonstrated. (adapted from Desmoulière et al. 2003).*

In few cases, a pathological wound healing course of events can be encountered (Desmoulière et al. 2003). These abnormal repair processes are the result of an impaired remodeling of the granulation tissue leading for example to abnormal cutaneous repair in hypertrophic or keloid scars and to fibrosis in internal organs (Fig. 1). In cutaneous excessive scarring, the normal healing process is not achieved and the granulation tissue continues

to grow, due to an abnormal and excessive secretion of growth factors and/or to the lack of molecules inducing apoptosis or ECM remodeling. In internal organs, when the noxious stimulus responsible for a lesion persists, an excessive ECM deposition is observed leading to the development of organ fibrosis. As in pathological cutaneous wounding, the installation and persistence of fibrosis are the consequences of an imbalanced ECM synthesis and degradation by the myofibroblasts. In this situation, the balance between matrix metalloproteinases (MMPs) and their inhibitors (TIMPs for tissue inhibitor of metalloproteinases) plays an essential role.

❑ (Myo)fibroblasts and Extracellular Matrix During Normal Wound Repair

Main Pathways Controlling Extracellular Matrix Deposition and Myofibroblast Differentiation

Myofibroblasts are interconnected by gap junctions and are also connected to the ECM by a specialized structure called fibronexus, a transmembrane complex involving intracellular microfilaments in continuity with extracellular fibronectin fibers (Eyden 2008). More recently, the fibronexus has been assimilated to the mature or supermature focal contact, *i.e.* three-dimensional transcellular structure containing the fibronectin isoform ED-A and α-SM actin; these are organized by intracellularly and extracellularly originated forces and play a role in the establishment and modulation of the myofibroblastic phenotype (Dugina et al. 2001). It has also been shown that α-SM actin is crucial for focal adhesion maturation in myofibroblasts (Hinz et al. 2003).

Various cytokines and growth factors are involved in skin homeostasis and wound healing (Barrientos et al. 2008). Among all these soluble factors, some directly act on fibroblast activity and granulation tissue formation, especially the transforming growth factor (TGF)-β1, a potent inducer of myofibroblastic differentiation (Desmoulière et al. 1993). Beyond a specific effect on the induction of α-SM actin expression, TGF-β1 also promotes the deposition of large amounts of ECM; in fact, TGF-β1 not only induces synthesis of ECM but it also reduces MMP activity by promoting TIMP expression. It is interesting to underline that TGF-β1 action on myofibroblastic differentiation is only possible in the presence of ED-A fibronectin which underlines the fact that close relationships exist between ECM components, growth factor activation and cell function regulation.

Interestingly and less studied, it is now clear that innervation and neuropeptides play an important role for skin homeostasis and during wound healing. For example, substance P, which is released from nerve endings after injury, induces inflammation and mediates angiogenesis, keratinocyte proliferation and fibrogenesis. It is suggested that poorly healing wounds

such as diabetic wounds have insufficient substance P levels to promote a neuroinflammatory response necessary for normal wound repair, while, conversely, increased nerve numbers and neuropeptide levels could induce exuberant inflammation and excessive scarring (Scott et al. 2007). In aged patients, cutaneous repair processes are less efficient (Sgonc and Gruber 2013) and it could be partly due to a deterioration of the peripheral nervous system at the skin level (for review, see Laverdet et al. 2015). We have analyzed the effects of location and aging on intra-epidermal nerve fiber density (IENFD) (unpublished observations). Our results showed that hip (proximal) and ankle (distal) young skins are innervated with equivalent densities. Surprisingly, there was no difference between young and aged proximal skin biopsies in terms of IENFD. However, a significant location effect on IENFD was observed in aged patients. Actually, in aged patients, IENFD was significantly decreased in distal biopsies compared with proximal biopsies. This decrease of the IENFD, mainly involving type C and Aδ nerve fibers in skin biopsies performed in aged patients and far from the cell bodies of the sensory neurons (distal biopsies), can provide new insights into the skin breakdown appearing with aging and/or photo-aging, particularly in lower extremities where wounds are often difficult to repair.

Mechanical Forces

Fibroblasts and myofibroblasts, because of their contractile properties and privileged relationships with the ECM, can modify their activity depending on the mechanical environment. Myofibroblastic differentiation features, such as stress fibers, ED-A fibronectin or α-SM actin expression, appear earlier in granulation tissue subjected to an increase in mechanical tension by splinting a full-thickness wound with a plastic frame as compared to normally healing wounds (Hinz et al. 2001). Fibroblasts cultured on substrates of variable stiffness present different phenotypes (Achterberg et al. 2014). Cultured fibroblasts do not express stress fibers on soft surfaces; when the stiffness of the substrate increases, a sudden change in cell morphology occurs and stress fibers appear (Yeung et al. 2005). Shear forces exerted by fluid flow are able to induce TGF-β1 production and differentiation of fibroblasts cultured in collagen gels in the absence of other exterior stimuli such as cytokine treatment (Ng et al. 2005) and ECM pre-strain regulates the bioavailability of TGF-β1 (Klingberg et al. 2014). The role of mechanical stress in stimulating myofibroblast activity has also been shown in experiments where dermal wounds in mice are mechanically stressed by stretching or splinting the wound; this mechanical load increased myofibroblast activity resulting in increased scar formation and mimicking to some extent human hypertrophic scarring (Aarabi et al. 2007). In aged skin, it is suggested that old fibroblasts have an age-dependent reduction in the capacity for collagen synthesis and simultaneously experience a loss of mechanical stimulation

resulting from the decrease of intact collagen fibers (Fisher et al. 2008; Vedrenne et al. 2012).

❑ (Myo)fibroblasts and Extracellular Matrix Dysregulation in Aging

Intrinsic skin aging factors are typically non modifiable and include ethnicity, anatomical site differences and chronological changes such as the hormonal alterations following menopause; on the other side, extrinsic skin aging factors are largely modifiable with lifestyle choices (*e.g.*, smoking and alcohol consumption) but mainly UV light exposure accounting for up to 80% of visible skin aging (Farage et al. 2008; Vedrenne et al. 2012). Thus, aged human skin is characterized by a flattening of the dermal-epidermal junction, reduced mechanical tension and loss of elasticity mainly due to marked reduction of dermal collagen and elastin contents.

Senescence, Aging Fibroblasts and Extracellular Matrix

Like other organs in the body, skin undergoes alterations due to the passage of time commonly called senescence. The main phenomenon is based on the observation that diploid cells, such as fibroblasts, have a finite life-span and limited rounds of cell division also called replicative senescence. Although senescent cells remain viable, cellular senescence is associated with altered gene expression, genome instability and mitochondrial dysfunction. The progressive and inevitable decline of dermal fibroblast cellular integrity leads to functional impairment as well as degenerative changes in the dermis with a direct impact on ECM composition (Fisher et al. 2008; Tigges et al. 2014).

Senescent dermal fibroblasts are characterized by a decreased collagen-synthetic capacity leading to disorganized collagen fibers (Varani et al. 2006). In addition, aged fibroblasts switch from a matrix-producing to a matrix-degrading phenotype. It is translated by increased expression of MMPs (mainly MMP-1) associated with decreased expression of TIMPs (Hornebeck 2003; Fisher et al. 2009; Qin et al. 2014). This phenomenon further disrupts the organization of collagen fibrils as well as elastin network and has a deleterious impact on fibroblasts themselves. The loss of ECM structural integrity leads to decreased mechanical tension, reduced cellular interaction and impaired stimulation of fibroblasts.

Glycation and Oxidative Stress

Nonenzymatic protein glycation (or Maillard reaction) is a cascade of reactions yielding a heterogeneous class of compounds, collectively termed advanced glycation end-products (AGEs). The generation of AGEs is a complex process

that initially involves a condensation reaction between proteins and glucose, followed by Amadori rearrangement, cyclization, polymerization, cleavage and oxidation processes. AGEs have been implicated in the pathogenesis of diabetes where hyperglycemia is a main contributing factor, renal failure and aging (Brownlee 1995). Protein glycation contributes to skin aging as it deteriorates collagen and elastin network within the dermis by crosslinking and has consequently a deleterious impact on fibroblast function. Oxidative processes are part of the protein glycation mechanism and oxidative stress itself is another mechanism that contributes to skin aging. Skin is damaged due to free radicals (reactive oxygen species or ROS) that accumulate during the lifespan of an individual, especially when the skin is exposed to UV irradiation. This phenomenon is called the Free Radical theory and leads to irreversible cellular functional impairment. ROS can target proteins, as well as lipids and nucleic acids, leading to an accumulation of cellular oxidative damage and the formation of carbonyl groups which are well identified as biomarkers of oxidative stress. Thus, fibroblasts can be directly targeted as well as ECM components leading to further destruction and fragmentation of both collagen and elastin networks (Fisher et al. 2009).

Hormones/Menopause

It is now well admitted that many of the adverse effects associated with cutaneous aging are also inducible by glucocorticoids (GC) (Tiganescu et al. 2011). Systemically or after therapeutic administration, cortisol binds to the ubiquitously expressed GC receptor to modulate gene transcription. Within target tissues, cortisol concentrations are regulated at a pre-receptor level by isozymes of the 11β-hydroxysteroid dehydrogenase (11β-HSD) and the 11β-HSD type 1 activates cortisol from inactive cortisone (Tomlinson et al. 2004). 11β-HSD type 1 increases with age in human skins and in human dermal fibroblasts from both photo-protected and photo-exposed sites and 11β-HSD type 1 also increased with donor age (Tiganescu et al. 2011). Then, the age-associated increase in dermal 11β-HSD type 1 which induces an increased local GC activation, may contribute to the adverse changes in skin morphology and functions associated with chronological aging and photo-aging.

Interestingly, the 11β-HSD type 1 expression is increased in post-menopausal skin. The resulting increased level of GC induces the thinning of the dermis and epidermis, reduced proliferative capacities of keratinocytes and dermal fibroblasts and modifications of the ECM; these changes compromise barrier function, tensile strength and structural integrity resulting in a skin that is thinner, dryer and more prone to shearing. In addition, blockade of the age-related increase in 11β-HSD type 1 activity may promote improved structural and functional properties in aging skin (Tiganescu et al. 2013). It could be suggested that selective 11β-HSD type 1 inhibitors could be used to reverse

or prevent menopause related skin fragility. In addition, estrogen deficiency following menopause results in atrophic skin changes and acceleration of skin aging (Thornton 2013).

Aggravating Factors

Different factors are known to be implicated in the acceleration of skin aging. Among these are environmental factors such as tobacco smoking or sun exposure and medical factors related to specific diseases or their associated treatments.

Tobacco Smoking

Tobacco smoking is one of the most known environmental aggravating factors. Indeed, tobacco causes premature skin aging via different mechanisms. Tobacco acts directly on the dermis by inducing abnormal accumulation of elastic fibers and a decrease of collagen synthesis caused by an up regulation of MMP-1 and MMP-3 (Yin et al. 2000). Moreover, tobacco smoking has an effect on TGF-β1 expression which has a role in epidermal homeostasis and induces the synthesis of ECM proteins in the dermis. Tobacco smoking induces a non-functional latent form of TGF-β1 and down regulates TGF-β1 receptors (Yin et al. 2000). Thus, tobacco smokers display the so-called characteristic "smoker's face" that includes facial wrinkles, skin atrophy and grey appearance.

Ultra Violet/Environment

UV irradiation is also well known to induce premature skin aging. Abnormal skin exposure to UV has deleterious effects on fibroblasts and keratinocytes. The primary mechanism by which UV radiation induces molecular response is via the generation of ROS. Different studies have been realized to study the impact of UVB on fibroblast senescence *in vitro*. For example, a study has shown that exposure of dermal fibroblasts to sub-lethal doses of UVB induces premature senescence (Chainiaux et al. 2002). These cells expressed specific biomarkers of senescence such as decreased proliferation, senescence-associated β galactosidase (SA-βgal) activity and altered gene expression. These premature senescent fibroblasts also display increased TGF-β1, MMP-1 and MMP-2 mRNA expression (Debacq-Chainiaux et al. 2005). Concerning the effect of UVA on fibroblasts, it has been shown that this treatment induces growth arrest, increased expression of SA-βgal and alteration of cell morphology (Herrmann et al. 1998). UV irradiation acts also on collagen. Exposure to UV irradiation induces an increase of MMP levels (Fisher et al. 1996) which consequently promotes collagen degradation and a decrease of collagen production.

Diabetes

Diabetes is a chronic disease which is characterized by an inability to use insulin in response to hyperglycemia (type II diabetes). Diabetic patients can have different complications such as vasculopathy (microangiopathy and macroangiopathy) or neuropathy. This neuropathy induces a loss of sensitivity and generally begins on foot. When diabetic patients injure their foot, the wound is not painful and it can progress to an ulcer which may lead to amputation. As described above, diabetes complications can be attributed to reaction of glycation which can modify the mechanical properties of human skin. The AGEs induce a loss of skin plasticity and skin is thinner in diabetic patients compared to healthy patients (Hashmi et al. 2006). AGEs also stimulate fibroblast apoptosis (Alikhani et al. 2005) and promote senescence phenomenon (Ravelojaona et al. 2009). ECM is also modulated by AGEs: mRNA expression of TGF-β1 is increased and the MMP-1/TIMP-1 ratio is higher in diabetic skin tissues. ECM protein expression is also modified in diabetic skin tissues with an increased expression of MMP-9 and TGF-β1 (Ren et al. 2013).

❑ Effect of Age on Wound Repair

Definitively, age-related impairment in wound healing is a well-accepted fact. However, the mechanisms underlying this deficiency are not completely understood (Gould et al. 2015). Numerous alterations in aging skin can have an impact on wound repair, including in particular fibroblast senescence, the altered quality of the ECM and the decreased capacities of the fibroblasts to manage ECM deposition and remodeling leading to lower mechanical properties of the skin (see above and Quan and Fisher 2015). In this section, alterations in the repair process of aged skin and general factors associated with elderly that might impair cutaneous healing will be discussed.

Alterations in one of the steps which follow one another during the repair process can lead to a defect in healing. Aging alters the inflammatory and endothelial cell adhesion molecule profiles during human cutaneous wound healing (Ashcroft et al. 1998), the macrophage phagocytic function (Swift et al. 2001) and more generally decreases the inflammasome proteins (Stojadinovic et al. 2013). These alterations lead to delayed wound closure with diminished granulation tissue formation, decreased angiogenesis, decreased collagen and growth factor synthesis and reduced numbers of myofibroblasts (Mirza et al. 2009) (Fig. 2). In 2013, Sgonc and Gruber underlined that in young individuals, temporary hypoxia stimulates wound healing by inducing cytokine and growth factor production and by increasing granulation tissue formation while in elderlies, the response to hypoxia seems to be impaired. A reduced response to hypoxia linked to a defect in hypoxia inducible factor-1α signaling could affect angiogenesis through reduced vascular endothelial cell growth factor levels. In addition, aged fibroblasts present impaired migratory capacities with complete loss of responsiveness to hypoxia and deficits in the migratory and signal transduction responsiveness to TGF-β1

that may partly explain diminished healing capabilities often observed in aged patients (Mogford et al. 2002). An up-regulation of MMP-2 and MMP-9 in cutaneous wounds of healthy elderlies and a down-regulation of TIMP-1 and TIMP-2 are observed; in addition, the levels of TGF-β1 which favors collagen deposition are decreased (Ashcroft et al. 2002). Finally, this situation impairs ECM deposition resulting in impaired healing and certainly represents an essential factor leading to age-related delayed wound repair. However, it has been shown that the rate of scar maturation vary with age, with older subjects (>55 years) displaying accelerated maturation, whereas a prolonged high turnover state and a retarded rate of maturation are observed in younger subjects (<30 years) (Bond et al. 2008). Interestingly, it is observed that young patients easily develop hypertrophic scar after burns while it is rare in adult and elderly persons. We can suggest with others that in aging, even if early phases of wound healing are affected, the remodeling and maturation phases are better controlled leading to an improved scarring process. Obviously, it is true in healthy persons. If adverse situations are present, *i.e.* malnutrition, vascular or immune problems, kidney or liver pathologies, diabetes, non-healing wounds can develop usually secondary to chronic inflammation (Eming et al. 2007).

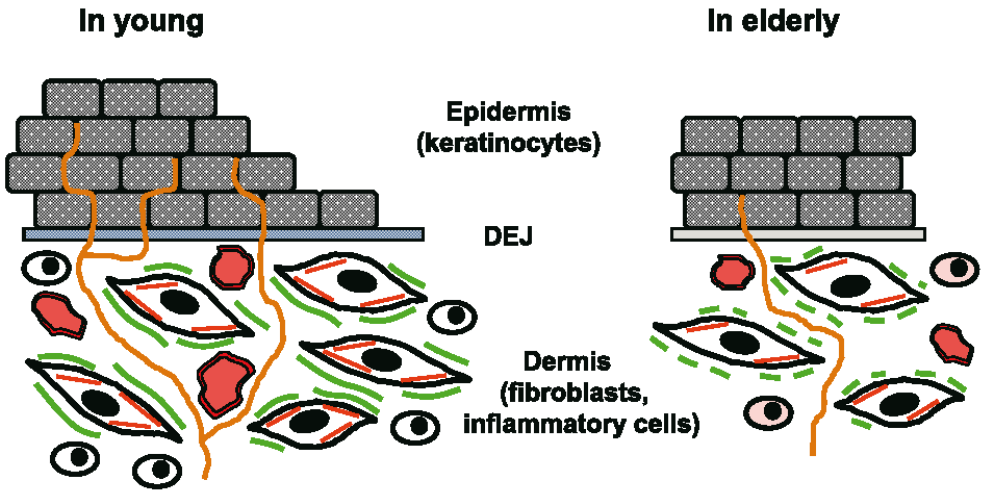


FIG. 2 Effects of aging on wound healing. *In elderly compared with young persons, an inflammation defect is present, reepithelialization (keratinocyte proliferation and migration), neoangiogenesis, reinnervation (brown lines), TGF-β1 levels and myofibroblastic differentiation (expression of α-smooth muscle actin; red bares in the fibroblast cytoplasm) are decreased and extracellular matrix (green lines) deposition is less effective with decreased secretion, increased matrix metalloproteinase levels and decreased tissue inhibitor of metalloproteinase levels. The quality of the dermal-epidermal junction (DEJ) is also affected.*

It is also important to underline that alterations in aging skin can have an impact on wound healing, but also modify the skin susceptibility to injury. As underlined above, for example, the skin fragility and the altered innervation increase the risk of skin lesion after mechanical stress or burn. In addition, the contribution of estrogen versus aging in age-associated delayed human

wound healing seems essential (Hardman and Ashcroft 2008) and knowing that estrogens have a role in all phases of wound healing by modifying the inflammatory response, accelerating re-epithelialisation, stimulating granulation tissue formation and regulating proteolysis (Emmerson and Hardman 2012), impaired wound healing associated with aging is coupled with estrogen deficiency (Thornton 2013). Moreover, elevated 11β-HSD type 1 activity in aging skin leading to increased local GC generation (see above), may account for adverse changes occurring in the elderly (Tiganescu et al. 2013).

❑ Models of Aged Skin

Investigating on how aging affects the skin as well as wound healing requires experimental models. Although studies on human biopsies and/or clinical trials remain the gold standard, several *in vivo* and *in vitro* models represent a precious alternative shedding lights on age-related mechanisms affecting (myo)fibroblast function and the ECM.

Animal Models

Research studies have been undertaken *in vivo* using animal models. Small mammals such as rat, mouse and rabbit models are the most currently used. They can be easily housed, are well-characterized and have the advantage to age relatively fast. However, the skin morphology and physiology of these models are quite different from the human skin and the extrapolation of conclusions from these studies to human condition has its limits. Large mammal models of aging and wound healing such as porcine models would be a more suitable alternative as their skin morphology is closer to human (Sullivan et al. 2001). Yet, limitations in the feasibility of using larger animals can be too detrimental to be overcome. Thus, many studies have used rat and mouse models and in a recent review article, Kim et al. (2015) have highlighted that these studies had assessed the deleterious effect of aging on wound healing and more particularly on dermal healing by investigating the granulation tissue remodeling process, ECM proteins content and/or tensile strength.

In vitro Reconstructed Skin

Studies performed *in vivo* have limitations and they can raise cultural and ethical issues. Therefore, *in vitro* models represent another valuable experimental asset. Human reconstructed skin or tissue-engineered skin, using either immortalized cell lines or primary cell culture, is now a well-established model that can recapitulate many morphological and molecular characteristics of normal human skin (Ali et al. 2015). Fibroblasts are cultured within a collagen matrix that can be supplemented with chitin to mimic the dermal layer. Different experimental strategies can be considered; cells can be isolated from patient skin biopsies and be exposed to stimuli promoting

aging and senescence such as UV irradiation, AGEs and oxidative stress (Marionnet et al. 2010; Pageon et al. 2014; Cadau et al. 2015).

❑ Therapeutic Options

Cosmetics

A wide range of cosmetics are available to try to limit visible effects of skin aging. Effects of several of these molecules are controversial but the efficiency of a few molecules on skin aging is confirmed by both *in vitro* and *in vivo* studies. Among these molecules, two types of products are mainly used in anti-aging cosmetics: retinoids and antioxidants. Sun blockers are also important to limit consequences of photo-aging. Actions on the dermal ECM of these different molecules are detailed in Table 2.

TABLE 2 Overview of the principal molecules used in anti-aging cosmetics.

Molecules	Effects on Dermal Cells and Extracellular Matrix	References
Retinoids	↗Production of collagen, elastin and glycosaminoglycans ↘ Expression of matrix metalloproteinase-1 No ultraviolet-protection	Ramos-e-Silva et al. 2001 Kligman 1996 Varani et al. 2000 Chapman 2012
Alpha hydroxy acids	Promote growth and cell differentiation ↗ Production of dermal glycosaminoglycan	Ramos-e-Silva et al. 2001 Smith 1996 Ramos-e-Silva et al. 2004
Alpha lipoic acid	Exfoliant No ultraviolet protection	Beitner 2003
L-ascorbic acid	↗ Synthesis of collagens types I and III Ultraviolet-protection	Farris 2005 Nusgens et al. 2001
Vitamin E	Ultraviolet-protection	Maalouf et al. 2002
Ubiquinone	↗Production of collagen, dermal extracellular matrix protection	Burke 2005
Vitamine B3	↗ Skin elasticity, skin color improvement	Bissett et al. 2005
Hormone replacement therapy	↗Production of collagen, dermal thickness improvement ↗ Skin elasticity	Brincat 2004 Sator et al. 2007
Hyaluronic acid injection	↗Skin hydration and fibroblast activation ↗Synthesis of collagen type I, matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1	Yoneda et al. 1988 Jäger et al. 2012

Wound Healing

Different therapeutic options exist in order to improve wound healing. The action of these treatments is mediated among others by an effect on (myo)fibroblasts. Thus, for chronic wounds such as ulcers, an option is to stimulate the activity of (myo)fibroblasts to induce wound contraction and promote wound healing. Today, only one treatment is available and can be used only on diabetic foot ulcers. Regranex ® (becaplermin) is a gel containing recombinant human platelet-derived growth factor-BB. This product increases ECM synthesis and improves wound healing (Pierce et al. 1992; Wu et al. 1997). Different trials have been performed on patients. For example, Wieman et al. (1998) have shown that wound healing of diabetic foot ulcers treated with Regranex ® is better than with placebo. Hindsight, Regranex ® commercialization was stopped in certain countries because there were a limited number of prescriptions and other alternatives were available.

Concerning hypertrophic scars, the opposite strategy is used: it is necessary to inhibit myofibroblast activities to stop ongoing fibrosis. Different factors are known to be suppressors of myofibroblast activities such as NF-κB (Mann et al. 2010), specific miRNAs such as miR-29 (Maurer et al. 2010) and miR-200a (Wang et al. 2011), interferon-γ (Pittet et al. 1994) and CXCL-10 (Jiang et al. 2010) for example. Likewise, pharmacological products can be used to reduce cellular division such as bleomycin or 5-fluorouracil (Aggarwal et al. 2008; Sadeghinia and Sadeghinia 2012). It is also possible to use corticosteroid injections into the scar to induce inhibition of fibroblast growth (Cruz and Korchin 1994) and to enhance collagen and fibroblast degeneration (Boyadjiev et al. 1995).

❑ Conclusion

An important aspect which remains to be clearly elucidated is the origin of myofibroblasts. It is well admitted that the major contribution for myofibroblast production originates from local recruitment of connective tissue fibroblasts. However, mesenchymal stem cells, circulating fibrocytes and bone marrow-derived cells, local pericytes, as well as resident epithelial cells through epithelial-to-mesenchymal transition may represent alternative sources of myofibroblasts when local fibroblasts are not able to comply with the request. Depending on the situation and the cellular microenvironment, these diverse cell types probably contribute to the appearance of (myo)fibroblast subpopulations whose phenotype can be modulated by their interactions with neighboring cells and the ECM. Moreover, research on skin progenitor cells which can differentiate in many different cell types and which are always present within the skin of elderly patients could represent an interesting source of cells able to promote, when correctly stimulated, the repair process of difficult-to-heal wounds. Finally, deleterious effects of exogenous factors such as UV exposure and AGEs on skin health during

aging, affecting particularly dermal fibroblasts and the ECM, must be underlined to prevent the appearance of skin problems which can become dramatic with age.

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III - La brûlure

De nombreuses agressions extérieures peuvent provoquer une lésion de la peau nécessitant la mise en place d'un processus de cicatrisation. Parmi celles-ci, la brûlure peut causer une atteinte de l'intégrité physique de la peau et peut, selon les cas, laisser de lourdes séquelles.

Les brûlures touchent de nombreuses personnes chaque année en France (environ 300 000) et les plus graves (10 000) nécessitent une hospitalisation. Les brûlures sont le plus souvent causées par une source de chaleur (11 millions de cas en 2004 dans le monde (Peck, 2011)) mais elles peuvent également être d'origine chimique (5%), électrique (10 %) ou encore radioactive (1%).

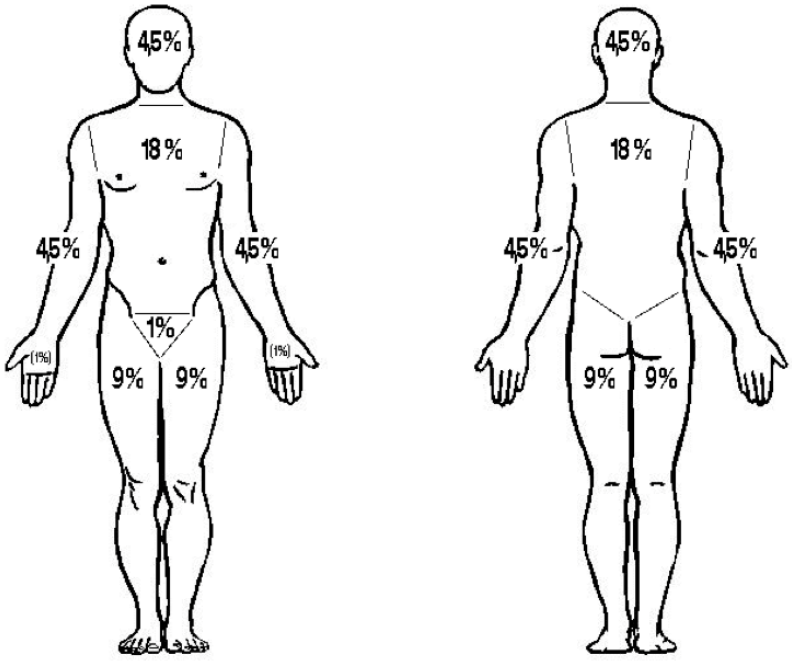
III - 1 Critères de gravité d'une brûlure

En plus de l'origine de la brûlure, différents critères existent pour classer les brûlures en fonctions de leur gravité et ainsi adapter le traitement.

Les différents critères sont :

- l'étendue de la brûlure (qui sera évaluée avec la règle des 9 de Wallace et la table de Lund et Browder (voir figure 7) ;
- la profondeur ;
- l'âge du patient et son état général.

A



B

%	0-12 mois	1-4 ans	5-9 ans	10-14 ans	15 ans	Adulte
Tête	19	17	13	11	9	7
Cou	2	2	2	2	2	2
Tronc Ant	13	13	13	13	13	13
Tronc Post	13	13	13	13	13	13
Fesse	2.5	2.5	2.5	2.5	2.5	2.5
Organe génitaux	1	1	1	1	1	1
Bras (X2)	4	4	4	4	4	4
Avant-bras (X2)	3	3	3	3	3	3
Main (X2)	2.5	2.5	2.5	2.5	2.5	2.5
Cuisse (X2)	5.5	6.5	8	8.5	9	9.5
Jambe (X2)	5	5	5.5	6	6.5	7
Pied (X2)	3.5	3.5	3.5	3.5	3.5	3.5

Figure 7 : Méthodes de calcul de la surface corporelle lésée. A. Représentation schématique de la règle des 9 de Wallace. B. Table de Lund et Browder permettant un calcul plus précis de la surface lésée notamment en fonction de l'âge du patient.

En clinique, 3 degrés de brûlures sont décrits (Figure 8) (Papini, 2004) :

Le premier degré correspond à une atteinte superficielle de l'épiderme. C'est ce type de brûlure que l'on observe lors d'un coup de soleil. La brûlure est douloureuse et on remarque un érythème. La guérison est spontanée et sans séquelle.

Le deuxième degré de brûlure correspond à une atteinte de la totalité de l'épiderme et d'une partie plus ou moins profonde du derme. Le deuxième degré est ainsi habituellement décomposé en deuxième degré superficiel ou deuxième degré profond en fonction de l'atteinte du derme.

Le deuxième degré superficiel concerne une atteinte de l'épiderme et des parties les plus superficielles des papilles dermiques. La brûlure est douloureuse à cause de la persistance de terminaisons nerveuses cutanées non lésées dans le derme plus profond. De plus, une phlyctène est observée. La cicatrisation d'une brûlure du deuxième degré superficiel est généralement spontanée et assez rapide.

Le deuxième degré profond quant à lui se caractérise par une destruction totale de l'épiderme et une atteinte profonde du derme. La peau brulée apparait blanche et le patient présente une hypoesthésie de la zone lésée due à une destruction de la majorité des terminaisons nerveuses cutanées. La cicatrisation est plus longue que dans les cas précédents et la cicatrisation

épidermique ne peut se faire qu'à partir des berges de la plaie. Une hospitalisation est le plus souvent nécessaire.

Le troisième degré de brûlure correspond à une destruction totale de l'épiderme et une destruction majeure ou totale du derme. La peau brulée apparait blanche ou noire et présente un aspect cartonné sans phlyctène. Le patient ne ressent aucune douleur due à la destruction totale des fibres nerveuses au niveau de la plaie. La cicatrisation spontanée est impossible et le patient doit être hospitalisé pour être généralement greffé et favoriser ainsi les chances de guérison.

Le quatrième degré de brûlure correspond à une carbonisation. Toute la peau est détruite avec une atteinte de l'hypoderme ou du muscle sous-jacent.

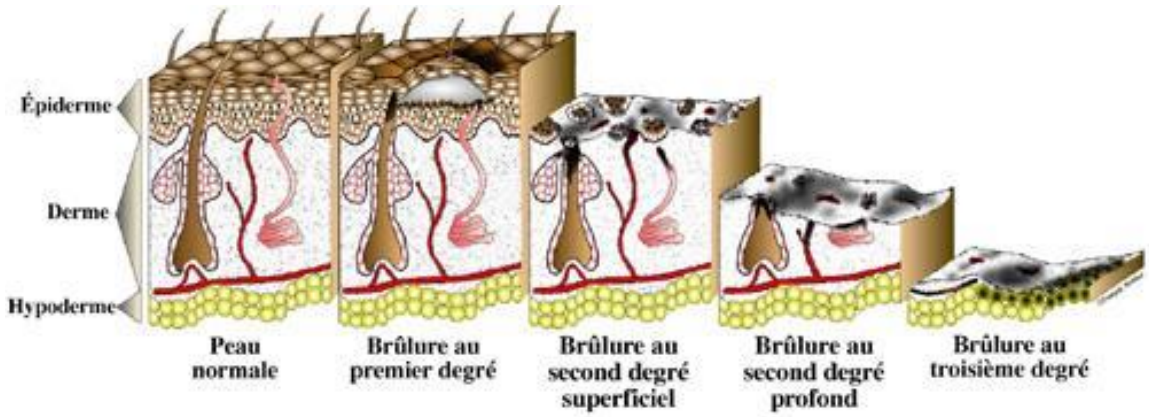


Figure 8 : Schéma représentant le degré de brûlure en fonction de la profondeur de la peau lésée (d'après Gagnon, 2005).

On considère qu'une hospitalisation est nécessaire lorsque la brûlure atteint plus de 10% de la surface corporelle, ou lorsqu'une brûlure du troisième degré affecte plus de 2 % de la surface corporelle ou des zones comme les mains, le visage ou des patients aux âges extrêmes de la vie. De plus, la présence de lésions associées telles qu'une atteinte respiratoire ou la présence de pathologies lourdes sont des motifs d'hospitalisation.

III – 2 Physiopathologie de la brûlure

A la suite d'une brûlure, le processus de cicatrisation débute. On observe une fuite d'eau, d'albumine et d'électrolytes importante vers le milieu interstitiel ce qui conduit à la formation d'un œdème. Ceci est associé à une hémoconcentration et une anoxie tissulaire. L'ensemble de ces modifications physiologiques entraine rapidement un choc hypovolémique chez le patient qu'il faut traiter dès que possible (Keck et al., 2009).

La réaction inflammatoire se met ensuite en place avec la libération massive de substances vaso-actives et la formation de radicaux libres toxiques pour l'organisme (Allgöwer et al., 2008).

A ces conséquences de la brûlure s'ajoutent des troubles de la régulation thermique et des troubles métaboliques. On observe notamment une hyperglycémie, une lyse des lipides et un hyper catabolisme protéique.

La brûlure entraine également une déficience immunitaire qu'il faudra surveiller pour éviter l'infection.

Une brûlure grave peut avoir des effets systémiques et entrainer une déficience multiviscérale. En effet, une défaillance cardio-circulatoire et une décompensation respiratoire peuvent survenir dans les premiers jours suivant la brûlure et aggraver ainsi le pronostic.

III – 3 Traitements des brûlures thermiques

III – 3.1 Traitements conventionnels

Le traitement de la brûlure est adapté au degré de brûlure préalablement déterminé (Alharbi et al., 2012).

Pour une brûlure superficielle, la cicatrisation étant spontanée et efficace, la prise en charge thérapeutique consiste seulement à hydrater la zone brulée.

Pour les brûlures plus profondes et/ ou plus étendue, la prise en charge à l'hôpital doit se faire le plus rapidement possible pour éviter les complications. En premier lieu, il faut traiter rapidement le choc hypovolémique par administration d'une solution composée de cristalloïdes associée ou non à des colloïdes. La moitié des volumes prévus pour 24h doit être administrée en moins de 8h (Dries, 2009).

Si besoin, une réanimation respiratoire peut être mise en place notamment en cas de brûlures de la face ou de suspicion de brûlures par inhalation.

Les patients gravement brûlés doivent être pris en charge en services spécialisés pour permettre leur survie. Le traitement de ces brûlures graves varie en fonction de la profondeur et de la surface de la brûlure. Ce traitement est le plus souvent suivi par une rééducation fonctionnelle.

Pour une brûlure du deuxième degré profond de petite surface, un traitement topique peut suffire. Cependant si la brûlure est étendue, il est nécessaire de retirer le tissu nécrosé qui prédispose au risque d'infection (Le Floch et al., 2015). La couverture de la lésion peut se faire par une autogreffe, une allogreffe ou encore un derme artificiel. Malgré ces traitements, après la fermeture de la plaie une cicatrice reste visible.

Pour une brûlure du troisième degré, il est également nécessaire de retirer le tissu nécrosé et de couvrir la plaie avec une autogreffe, une allogreffe ou un substitut de peau totale.

Le traitement conventionnel d'un patient brulé à moins de 40 % de la surface corporelle est l'autogreffe de peau mince sur les sites brulés. La technique consiste à prélever de la peau saine et à l'étirer pour couvrir un site lésé. Cette technique permet une bonne prise de greffe et une bonne cicatrisation du site donneur. Elle permet de couvrir de larges surfaces et sa mise en œuvre par les chirurgiens est relativement facile. Malheureusement, l'aspect esthétique final n'est pas satisfaisant et de nombreuses interventions chirurgicales sont le plus souvent nécessaires. Pour améliorer le résultat fonctionnel, il est possible de réaliser des greffes de peau épaisse.

Pour les patients brulés sur plus de 40 % de leur surface corporelle, le traitement conventionnel est impossible du fait de la faible quantité de sites donneurs de peau saine. Il faut alors faire appel à des substituts cutanés greffables obtenus par des techniques de bio-ingénierie tissulaire.

III – 3.2 Bio-ingénierie tissulaire appliquée au traitement de la brûlure

Pour couvrir les plaies des grands brulés, plusieurs options sont possibles. La bio-ingénierie tissulaire met à la disposition des chirurgiens des substituts cutanés permettant de remplacer uniquement l'épiderme ou le derme ou les 2 couches simultanément.

La majorité de ces substituts sont acellulaires (Tableau I). En effet, la présence de cellules dans un dispositif médical implique de nombreuses contraintes réglementaires, une technologie de haut niveau, et un coût élevé. Cependant, certains d'entre eux contiennent des cellules autologues ou allogéniques (Tableau II).

Origine	Nom/ Fabricant	Composition	Indications	Références
Bio-polymère	Hyalomatrix PA ® Fidia farmaceutici s.p.a	Couche supérieure : membrane de silicone Couche inferieure : dérivé de l'acide hyaluronique	Brûlure du 2nd degré	(Gravante et al., 2010; Perrot et al., 2011)
Polymère synthétique	Suprathel® PolyMedics Innovation GmbH	Tri-polymère de polylactide, trimethylene carbonate et ε-caprolactone	Couverture temporaire des sites donneurs et des brûlures superficielles et partiellement profondes	(Schwarze et al., 2008; Keck et al., 2012; Highton et al., 2013; Schiefer et al., 2014)
	Biobrane ® Smith and Nephew	Couche supérieure : membrane de silicone Couche inférieure : matrice tri-filamentaire en nylon sur laquelle sont fixés des peptides dérivés du collagène porcin de type I	Brûlure du 2nd degré et sites donneurs	(Lesher et al., 2011; Rahmanian-Schwarz et al., 2011)
	AWBAT ® Aubrey Inc	Couche supérieure : membrane de silicone Couche inférieure : matrice tri-filamentaire en nylon sur laquelle sont fixés des peptides dérivés du collagène de type I porcin (plus poreux que Biobrane ®)	Brûlure du 2nd degré et sites donneurs	(Woodroof, 2009; Vandenberg, 2010)
Animale	Epiflex ® DIZG	Derme humain décellularisé	Brûlures, cicatrices hypertrophiques	(Rössner et al., 2011)
	EZ Derm ® Mölnlycke Health Care	Derme porcin contenant du collagène réticulé aux aldéhydes	Brûlures	(Troy et al., 2013)
	Strattice Tissue Matrix ® LifeCell Corporation	Derme porcin acellulaire	Reconstruction abdominale, reconstruction mammaire, réparation des hernies	(Katerinaki et al., 2010; Freedman, 2012; Begum et al., 2016)
	Pelnac ® Gunze + bFGF	Couche supérieure : membrane de silicone Couche inférieure : éponge d'atélocollagène de tendon de porc	Brûlure du troisième degré, reconstruction cutanée après ablation de tumeur ou de nævus	(Wosgrau et al., 2015; Mitsukawa et al., 2016)
	Oasis ® Wound Matrix	Matrice extracellulaire dérivée de la muqueuse du petit intestin de porc	Brûlure du 2nd degré, ulcères	(Mostow et al., 2005; Shi and Ronfard, 2013)
	Matriderm ® MedSkin solutions	Membrane poreuse composée de collagène et d'un hydrolysat d'alpha-élastine d'origine bovine	Brûlures des 2ème et 3ème degrés, chirurgie reconstructrice	(Schneider et al., 2009; Lamy et al., 2013)

	Terudermis ® Olympus Terumo Biomaterials	Couche supérieure : membrane de silicone Couche inférieure : éponge de collagène bovin lyophilisé et réticulé	Brûlures, chirurgie reconstructrice, trauma	(Eo et al., 2011)
	Integra ® Integra LifeSciences Corporation	Couche supérieure : membrane de silicone Couche inferieure : matrice de collagène de tendon bovin de type 1 réticulé aux glycosaminoglycanes (chondroitine-6-sulfate)	Brûlures et chirurgie reconstructrice	(Lagus et al., 2013; Müller et al., 2013)
Humaine	Alloderm ® LifeCell Corporation	Derme cadavérique humain lyophilisé et ayant subi un processus pour éliminer les cellules	Brûlures, chirurgie reconstructrice	(Wainwright, 1995; Deneve et al., 2013; Wester et al., 2014; Agarwal et al., 2015)
	GammaGraft ® Promethean LifeSciences	Peau cadavérique humaine gamma irradiée	Brûlures, plaies chroniques	(Nyame et al., 2014)
	Glyaderm ® Euro Tissue Bank	Derme cadavérique humain glycérolé	Brûlures, chirurgie reconstructrice	(Verbelen et al., 2016)

Tableau I : Substituts acellulaires dermiques pour le traitement des brûlures.

Origine	Compartiment remplacé	Nom / Fabricant	Composition	Indications	Références
Cellules allogéniques	Derme	Dermagaft® Organogenesis	Fibroblastes néonataux de prépuce humain sur matrice de polyglactine	Ulcères	(Hart et al., 2012)
	Peau totale	Apligraf ® Organogenesis	Couche supérieure : kératinocytes néonataux de prépuce humain Couche inferieure : fibroblastes néonataux de prépuce humain dans une matrice de collagène bovin de type I	Ulcères	(Edmonds, 2009; Carlson et al., 2011)
		OrCel ™ Forticell Biosciences	Couche supérieure : kératinocytes néonataux humains Couche inferieure : fibroblastes néonataux humains dans une éponge de collagène bovin de type I	Epidermolyse bulleuses, sites donneurs	(Still et al., 2003)
Cellules autologues	Peau totale	PermaDerm ™ Regenicin	Couche supérieure : kératinocytes autologues Couche inferieure : fibroblastes autologues dans une matrice de collagène de type I	Brûlures du 3ème degré	(Woodroof et al., 2015)
		MyDerm ™ Cell Tissue Technology	Couche supérieure : kératinocytes autologues dans une couche de fibrine autologue Couche inferieure : fibroblastes dermiques autologues dans une couche de plasma autologue	Brûlures, ulcères, chirurgie reconstructrice	(Mazlyzam et al., 2007; Seet et al., 2012)
		ReCell ™ Avita Medical	Spray contenant les cellules de la peau : kératinocytes, mélanocytes, cellules de Langerhans, fibroblastes papillaires dermiques autologues	Brûlures, ulcères, vitiligo	(Wood et al., 2012; Hu et al., 2015; Sood et al., 2015)

Tableau II : Substituts cellulaires utilisés pour le traitement des brûlures.

Ces deux tableaux présentent une offre assez large de substituts cutanés contenant ou non des cellules. Les chirurgiens favoriseront toujours la greffe de peau saine en provenance du patient lui-même sur les zones brulées. Cependant, pour des brûlures étendues, le recours aux substituts cutanés est indispensable pour assurer la survie des patients.

On remarque que l'offre en substituts dermiques acellulaires est vaste contrairement aux substituts cellulaires. Cela s'explique en partie par le fait que la colonisation de ces substituts acellulaires par les cellules du patient se fait généralement rapidement après la greffe. Il n'est donc pas nécessaire d'utiliser des substituts dermiques cellulaires pour obtenir une cicatrisation correcte.

Néanmoins, les substituts cellulaires peuvent être utilisés. Les substituts dermiques contenant des fibroblastes allogéniques ou autologues peuvent être utilisés de manière définitive sans qu'il y ait de rejet. En effet, lors de l'utilisation de fibroblastes allogéniques, ceux-ci seront rapidement remplacés par les fibroblastes du patient receveur limitant ainsi le rejet. Cependant les substituts cutanés contenant des kératinocytes allogéniques sont quant à eux utilisés de façon temporaire. Ce type de substitut est alors principalement utilisé pour induire la cicatrisation par la sécrétion de facteurs de croissance épidermiques. Ils seront retirés après plusieurs semaines et pourront être remplacés si nécessaire par une culture de kératinocytes autologues.

De plus amples informations sur les substituts cutanés utilisés pour le traitement des brûlures sont décrites dans la revue « Biotechnological management of skin burn injuries : challenges and perspectives in wound healing and sensory recovery » (Chapitre 2, partie II – 2).

III - 4 Séquelles de la brûlure

Malgré la mise en place rapide de ces traitements, de nombreuses séquelles persistent après une brûlure (Chekaroua and Foyatier, 2005). Les plus fréquentes d'entre elles sont les cicatrices hypertrophiques et les cicatrices rétractiles (Buja et al., 2015).

Les cicatrices hypertrophiques surviennent dans les premiers mois suivant la brûlure. Elles sont dues à une prolifération non contrôlée des fibroblastes et des myofibroblastes conduisant à une hyperproduction de tissu fibreux. On observe alors une hyperproduction de pro-collagène et de TGF-β1 associée à une diminution de la production de « Matrix MetalloProteinase-1 » (MMP-1) (Chiang et al., 2016). Ces cicatrices hypertrophiques sont souvent provoquées par une ré-épithélialisation trop lente (> à 3 semaines) ce qui conduit à une inflammation prolongée. Pour prévenir le développement de ces cicatrices hypertrophiques et pour les traiter, il est possible d'utiliser des vêtements compressifs. Après plusieurs mois de port, ces vêtements permettent de réduire l'œdème et l'inflammation et induisent l'apoptose des fibroblastes/myofibroblastes provoquant ainsi la réduction de la production de collagène.

Les cicatrices rétractiles apparaissent dès les premières semaines après la brûlure. Comme les cicatrices précédentes, les cicatrices rétractiles sont liées aux myofibroblastes fortement sollicités. Ces cicatrices vont causer des brides conduisant à une perte de fonction au niveau de l'articulation concernée. Les traitements actuels sont la mise en place d'une kinésithérapie pour accompagner la mobilisation du membre et diminuer les tensions locales, voire des actes chirurgicaux pour les cas les plus graves.

En plus des séquelles fonctionnelles et esthétiques, de nombreuses affections cutanées peuvent survenir après une brûlure. La présence d'eczéma ou de prurits sont fréquemment décrites par les patients. On note également des troubles de la sensitivité cutanée et des troubles de la thermorégulation induisant un handicap chez le patient.

Ces troubles semblent dus à une repousse incorrecte des petites fibres nerveuses sensitives cutanées conduisant à des perceptions anormales.

IV – L'innervation cutanée

IV – 1 L'innervation cutanée sensitive

Chez l'homme, le système nerveux est composé du système nerveux central (SNC) et du système nerveux périphérique (SNP). Le SNC est constitué par l'encéphale et la moelle épinière. Le SNP quant à lui se situe à l'extérieur de la boite crânienne et du canal rachidien.

Le SNP est notamment constitué par les 31 paires de nerfs rachidiens naissant de la moelle épinière et des 12 paires de nerfs crâniens naissant des noyaux du tronc cérébral. Les ganglions nerveux et de très nombreux neurones présents dans l'organisme, en particulier au niveau du tube digestif, appartiennent également au SNP.

Au sein du SNP, on distingue des fibres afférentes et des fibres efférentes. Les fibres afférentes transmettent les informations captées par les récepteurs sensitifs de la périphérie vers le SNC. Les fibres efférentes partent du SNC pour acheminer des ordres aux organes ou aux muscles de la périphérie.

Les fibres afférentes peuvent être divisées en deux parties : les fibres somatiques reliées aux récepteurs sensitifs de la peau, des articulations et des muscles et les fibres viscérales qui sont rattachées aux viscères. La grande majorité des fibres sensorielles afférentes cutanées sont positives pour l'ubiquitine hydrolase « Proteine Gene Product » 9.5 (PGP 9.5) (Genç et al., 2015).

Parmi les fibres efférentes, on sépare les fibres somatiques, qui sont des fibres motrices capables d'activer les muscles squelettiques, des fibres du système nerveux autonome qui vont activer les glandes, le muscle cardiaque et les muscles lisses. Ce système nerveux autonome est divisé en systèmes nerveux sympathique et parasympathique qui ont des fonctions antagonistes.

Comme son nom l'indique, le système neveux autonome fonctionne de façon automatique sans intervention consciente. Il est entre autre impliqué dans la thermorégulation, dans la régulation cardiaque et vasomotrice ou encore dans la régulation respiratoire.

IV – 1.1 Structure du nerf périphérique

Un nerf périphérique est constitué de nombreuses fibres nerveuses (ou axone). Ces fibres nerveuses sont regroupées en fascicules et plusieurs fascicules forment un nerf. Au niveau des nerfs, on distingue trois types de tissus conjonctifs : l'endonèvre qui entoure l'axone, le périnèvre qui délimite les fascicules et l'épinèvre qui est la gaine regroupant l'ensemble des fascicules (Rigoard et al., 2009).

Une fibre nerveuse est constituée par l'axone d'un neurone, c'est-à-dire un prolongement cytoplasmique émis à partir du corps cellulaire du neurone, et par les cellules de Schwann. Ces corps cellulaires se situent dans ou à proximité de la moelle épinière. Les corps cellulaires des neurones moteurs se situent dans la corne antérieure de la moelle épinière alors que les corps cellulaires des neurones sensitifs se situent dans les ganglions dorso-rachidiens (GDRs).

Les cellules de Schwann sont des cellules gliales présentes au niveau du SNP. Elles sont essentielles dans le processus de myélinisation et les processus de réparation des fibres nerveuses.

IV – 1.1.1 L'axone

Un neurone se compose du corps cellulaire et de différents prolongements. Parmi ces prolongements, on distingue le ou les dendrites qui permettent de faire circuler l'influx nerveux de la partie la plus distale vers le corps cellulaire. Chaque neurone possède également un axone qui peut être ramifié. La longueur de cet axone est très variable, de quelques millimètres à 1 mètre de long. Le diamètre peut également varier de 1 à 25 µm ce qui influe sur la vitesse de conduction de l'influx nerveux. En effet, plus le diamètre de la fibre est important plus la conduction de l'influx sera rapide.

Il faut préciser que les neurones sensitifs présents dans les GDRs ne possèdent pas à proprement parlé de dendrite. Ces neurones sont dits unipolaires ou pseudo-unipolaires (ils possèdent un court prolongement qui se divisent rapidement en deux, un prolongement se dirigeant vers la périphérie et l'autre prolongement vers le système nerveux central ; ces deux prolongements constituent à eux deux, l'axone).

Le cytosquelette de l'axone est composé de 3 principaux groupes de filaments. Les microfilaments d'actine (diamètre de 5 nm) sont impliqués dans la mise en place de la synapse et dans la mobilité du cône axonal. Les neurofilaments (NF) (diamètre de 10 nm) sont un assemblage de 3 protéines : NF-light, NF-medium et NF-heavy. Ces neurofilaments jouent un rôle prépondérant dans la détermination du diamètre de l'axone qui aura une importance lors du processus de myélinisation. Les microtubules (diamètre de 20 nm) sont quant à eux impliqués dans la croissance et le flux axonal.

Le flux axonal se fait de façon antérograde (du corps cellulaire vers la périphérie) et rétrograde (de la périphérie vers le corps cellulaire) (Maday et al., 2014).

Un transport axonal antérograde rapide permet le renouvellement des protéines membranaires de l'axone et la migration des nouvelles mitochondries vers la périphérie pour remplacer

celles qui dégénèrent. La vitesse de ce transport antérograde rapide est de l'ordre de 100 à 400 mm / j.

Un transport axonal antérograde lent permet quant à lui le renouvellement des protéines du cytosquelette et des macromolécules. Sa vitesse est de 0,1 à 2 mm / j.

Le transport axonal rétrograde permet d'éliminer les déchets présents dans l'axone. Ces déchets sont ainsi acheminés vers le corps cellulaire du neurone pour y être dégradés ou recyclés. La vitesse de ce transport est rapide, de l'ordre de 300 mm / j.

IV – 1.1.2 La cellule de Schwann et la gaine de myéline

Ces cellules se trouvent en périphérie de l'axone et ont un rôle de soutien, de protection et de nutrition des neurones. Ces cellules sont également fortement impliquées dans la régulation de l'activité neuronale de par leur capacité à produire la gaine de myéline. En effet, la présence de cette gaine de myéline autour de l'axone permet d'accélérer la propagation de l'influx nerveux.

Il existe deux types de cellules de Schwann : les cellules myélinisantes et les non-myélinisantes. Les cellules de Schwann embryonnaires qui sont associées à des axones dont le diamètre est supérieur à 1 µm vont se différencier en cellules de Schwann myélinisantes. Celles qui sont associées à des axones de diamètre inférieur évolueront vers des cellules de Schwann non-myélinisantes (Armati and Mathey, 2013).

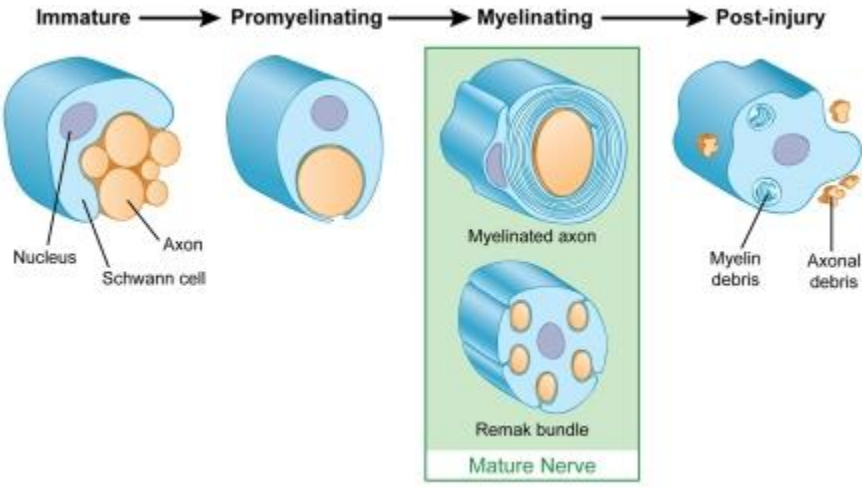


Figure 9 : Processus de différenciation d'une cellule de Schwann (adapté de Salzer, 2008).

Les fibres myélinisées sont constituées d'un seul axone associé à une succession de cellules de Schwann qui entourent l'axone et produisent la gaine de myéline (Figure 9). La gaine de myéline est interrompue régulièrement au niveau des nœuds de Ranvier. C'est au niveau de ces nœuds que la membrane de l'axone est excitable, la conduction nerveuse s'effectue donc de nœuds en nœuds, on parle de conduction saltatoire (Figure 10). La vitesse de conduction nerveuse est de l'ordre de 120 m/s.

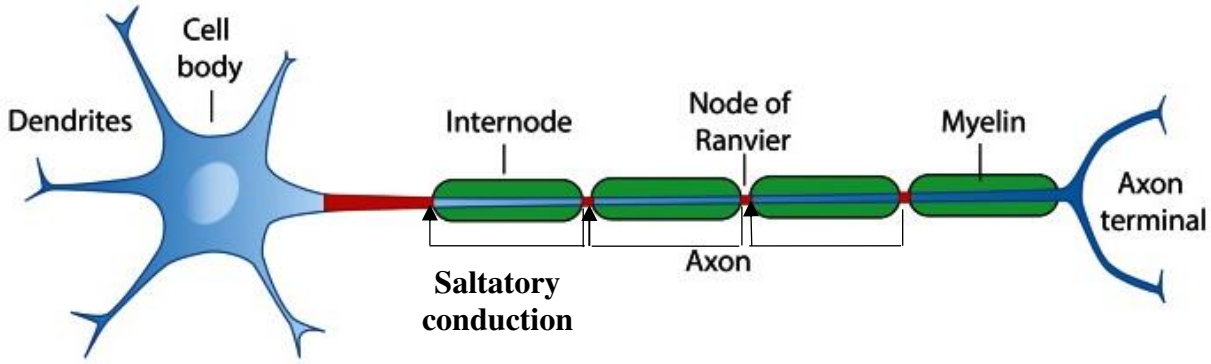


Figure 10 : Structure d'une fibre nerveuse myélinisée et conduction saltatoire (adapté de Freeman et al., 2016).

Les fibres amyéliniques sont quant à elles constituées de plusieurs axones regroupés au sein d'une cellule de Schwann. Chaque axone est alors logé dans une invagination cytoplasmique de la cellule de Schwann (Figure 9). La conduction nerveuse dans une fibre non myélinisée est continue avec une vitesse maximale de 15 m/s.

IV – 1.2 Structures nerveuses sensitives cutanées

Au sein de la peau, on retrouve différentes structures nerveuses qui sont impliquées dans la détection et la transmission de stimuli extérieurs vers le SNC.

En effet, la peau est riche en terminaisons nerveuses permettant de détecter le toucher, la pression, une vibration, une variation de température ou encore une douleur. Certaines de ces terminaisons nerveuses constituent de véritables récepteurs avec une organisation spécifique et sont situées aux extrémités des fibres nerveuses cutanées. On distingue trois types de fibres en fonction de leur diamètre :

- les fibres Aβ : ce sont des fibres myélinisées ayant un diamètre axonal de l'ordre de 10 µm. Elles représentent 20 % de la population des fibres sensitives cutanées. Ces fibres sont impliquées dans la transmission d'information concernant le toucher, la pression et les vibrations. Les récepteurs permettant de détecter ces stimuli sont notamment les corpuscules de Ruffini, de Pacini et de Meissner ainsi que les disques de Merkel (Figure 11).

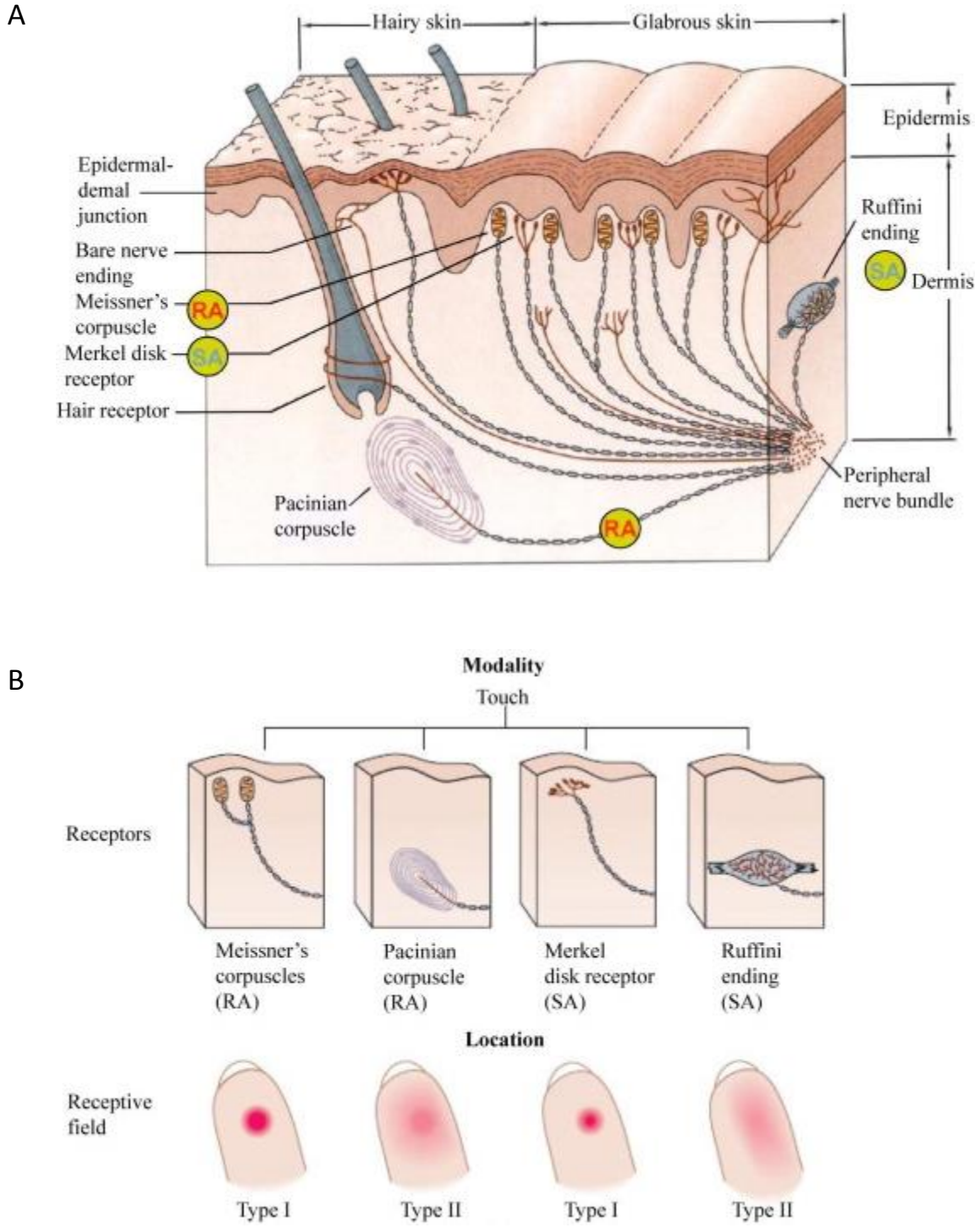


Figure 11 : Mécanorécepteurs cutanés. A. Représentation schématique de la peau et des différents mécanorécepteurs (RA : adaptation rapide, SA : adaptation lente). B Localisation des mécanorécepteurs dans le derme et précision de détection du stimulus (adapté de Ding and Bhushan, 2016).

- Les fibres Aδ : ce sont des fibres faiblement myélinisées dont le diamètre axonal et de l'ordre de 1 à 5 µm. Elles représentent 10 % des fibres sensitives cutanées. Les terminaisons nerveuses de ces fibres sont libres et se situent majoritairement dans le derme. Ces fibres sont impliquées dans la transmission de stimuli concernant une variation de température ou une douleur. La perception douloureuse est aiguë, très localisée, et on parle alors de douleur épicritique.

- Les fibres C : ce sont des fibres non myélinisées qui ont un diamètre axonal inférieur à 1 µm. Ce sont les fibres sensitives les plus nombreuses au niveau de la peau, environ 70 %. Ces fibres sont libres et leurs extrémités, situées principalement dans l'épiderme, ne présentent pas de récepteurs particulier. Les fibres C constituent avec les fibres Aδ les petites fibres sensitives cutanées. Comme les fibres Aδ, les fibres C sont également impliquées dans la transmission d'informations concernant des variations de température et la douleur. Cependant, la perception de la douleur par les fibres C est plus diffuse, type brûlure.

Différents récepteurs membranaires liés aux fibres Aδ et C ont été décrits comme permettant la détection du stimulus douloureux. Parmi ces familles de récepteurs, on peut citer la famille des récepteurs « Transiant Receptor Potential » (TRP), les récepteurs « Acid-Sensing Ion Channel » (ASIC), ou encore les récepteurs à l'ATP. La superfamille des récepteurs TRP regroupe des canaux perméables aux cations. Au sein de ce groupe, on distingue des canaux permettant la détection de stimuli thermiques douloureux : les récepteurs « Transiant Receptor Potential Vanilloid » (TRPV). Le récepteur le plus connu de cette famille est le récepteur TRPV1. Il est activé soit en cas de température supérieure à 43 °C, soit par un milieu extracellulaire acide ou par la fixation de vanilloïdes tels que la capsaïcine ou la résinifératoxine (RTX) (Figure 12) (Danigo et al., 2013).

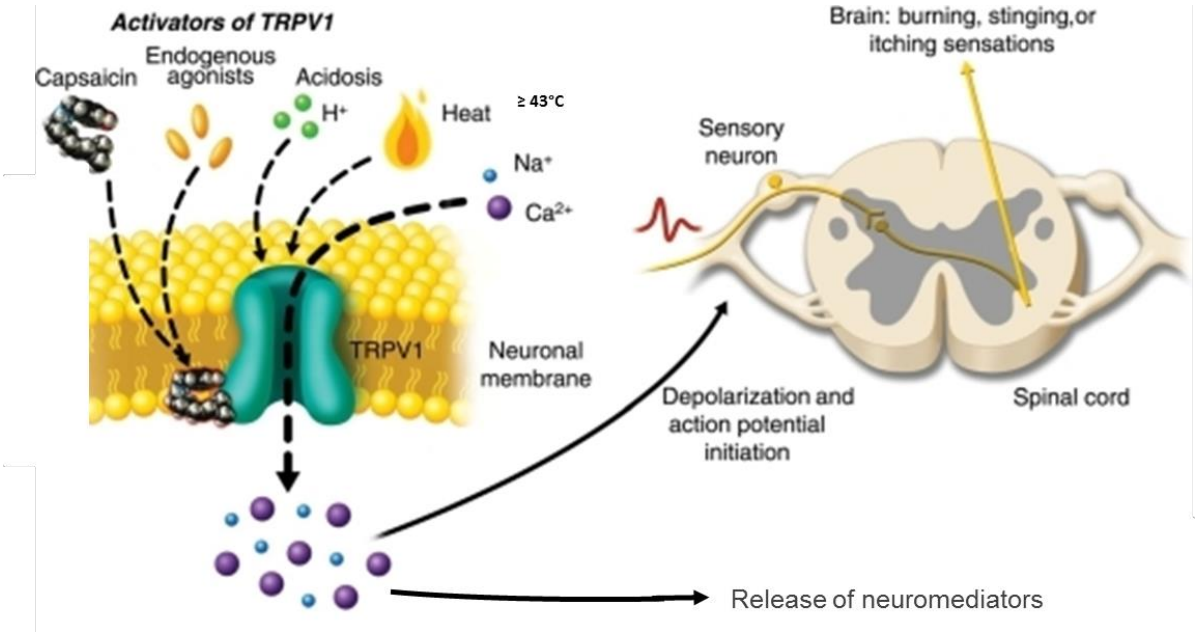


Figure 12 : Activateurs de TRPV1 et implication du récepteur dans la transmission du stimulus douloureux (adapté de Anand and Bley, 2011).

A la suite d'une lésion, de nombreuses substances sont libérées par les cellules lésées (ions H^+, K^+ et de l'ATP) et vont venir activer ces différents récepteurs. L'activation de ces récepteurs induit l'ouverture de canaux ioniques responsable du potentiel de terminaison nerveuse qui sera relayé via des canaux Na^+, situés le long de l'axone. Le potentiel d'action ainsi créé se propagera ensuite jusqu'aux corps cellulaires des neurones présents dans les ganglions rachidiens puis la moelle épinière. Du fait de la présence du canal ionique au sein de ces récepteurs, ces récepteurs sont dits ionotropes.

En parallèle de ce phénomène, la lésion induit l'activation des cellules de l'inflammation, ce qui a pour conséquence la libération de nombreuses substances (prostaglandines, interleukines, sérotonine, bradykinine, histamine…). Ces molécules vont alors se fixer sur des récepteurs présents à l'extrémité des terminaisons nerveuses libres. Cette fixation conduit *in fine* à la phosphorylation des récepteurs ionotropes abaissant ainsi leur seuil de réactivité. Les récepteurs seront alors plus sensibles, c'est-à-dire qu'ils vont déclencher un influx nociceptif pour des stimulations plus faibles, et on parle alors d'hyperalgie.

IV – 1.3 Classification des petites fibres nerveuses cutanées

Les fibres Aδ et C sont les plus nombreuses au niveau de la peau. En plus de leur classification en fonction de leur diamètre et de leur état de myélinisation, il est possible de les classer en fonction de leur rôle physiologique ou de leur composition biochimique.

IV – 1.3.1 Classification physiologique

Les fibres Aδ et C sont impliquées dans la détection et la transmission de la sensibilité thermique et douloureuse. Au niveau de ces fibres, on distingue des nocicepteurs purement thermiques, purement mécaniques ou polymodaux (détectant les deux types de stimuli). Ces

nocicepteurs possèdent néanmoins des caractéristiques communes : leur seuil de déclanchement est élevé et l'intensité de leur activité est proportionnelle à celle de la stimulation.

La majorité des nocicepteurs sont polymodaux et sont donc capables de répondre à toutes formes de stimulations. Les nocicepteurs mécaniques répondent aux stimulations telles que la torsion, le pincement ou une piqure de la peau. Ils sont majoritairement représentés par les fibres Aδ. Les nocicepteurs thermiques répondent à des stimuli thermiques bas (< 10 °C) ou élevé (> 45 °C) et sont essentiellement des fibres C.

Il a également été décrit des nocicepteurs polymodaux « silencieux ». Ils ne sont activés qu'en cas de situations pathologiques, notamment lors de processus inflammatoires chroniques.

IV – 1.3.2 Classification neuro-biochimique

En plus de la classification physiologique, il est possible d'établir une classification biochimique des nocicepteurs. En effet, les fibres C peuvent être divisées en deux sous types : les fibres peptidergiques et les fibres non peptidergiques. Au niveau anatomique, les fibres peptidergiques se projettent vers les couches I et II_{ext} de la corne dorsale de la moelle épinière alors que les non peptidergiques se projettent vers la couche II_{int}.

Les fibres C peptidergiques expriment la substance P (SP) et le « Calcitonin Gene-Related Peptide » (CGRP) qui seront décrits dans les paragraphes suivants et sont sensibles au « Nerve Growth Factor » (NGF). Les fibres non peptidergiques lient l'isolectine B4 (IB4). Ces fibres sont sensibles au « Glial cell-Derived Neurotrofic Factor » (GDNF) grâce à l'expression du récepteur RET associé au co-récepteur GDNFRα. Ces fibres non peptidergiques expriment également le récepteur à l'ATP P_2X_3.

IV – 1.4 Les neuropeptides SP et CGRP

A la suite d'une stimulation des fibres nerveuses peptidergiques cutanées, une des réponses est la libération en périphérie de petites molécules appelées neuropeptides. Les neuropeptides les plus connus sont la SP et le CGRP ainsi que le « Vasoactive Intestinal Peptide » (VIP) et la somatostatine (STT). Ces peptides sont produits dans le corps cellulaire du neurone dans le ganglion dorso-rachidien puis sont transportés le long de l'axone jusqu'à l'extrémité des terminaisons nerveuses libres (Figure 13).

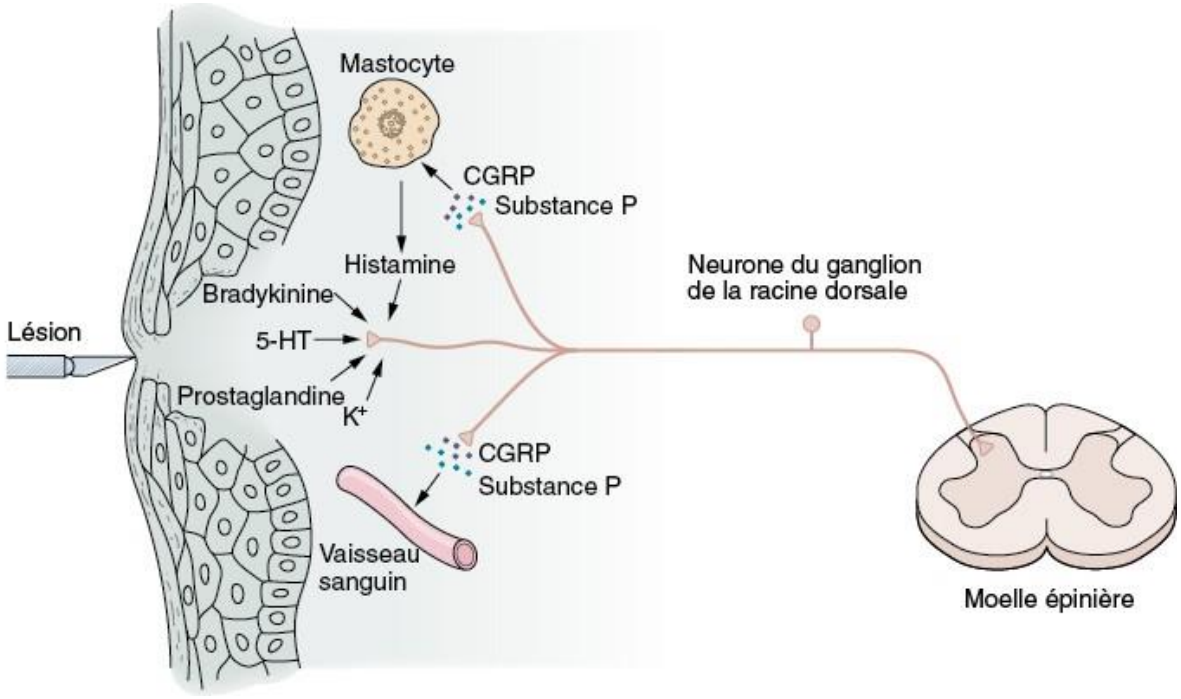


Figure 13 : Effets périphériques de la SP et de CGRP à la suite d'une lésion cutanée (Barman et al., 2012).

A la suite de la détection d'un stimulus par les terminaisons nerveuses, les neurones se dépolarisent, les canaux calciques de la cellule (notamment de type TRPV1) s'ouvrent permettant une entrée de calcium dans la cellule conduisant à l'exocytose des neuropeptides. Une fois libérés, ces neuropeptides se fixent sur leur récepteur ou seront dégradés rapidement par différentes enzymes. Nous ne décrirons ici que les deux principaux neuropeptides cutanés : la SP et le CGRP.

IV – 1.4.1 La substance P

La substance P est un des membres de la famille des tachykinines. En plus de la SP, cette famille comprend les neurokinines A et B et les neuropeptides K et Y. Les tachykinines agissent sur les récepteurs couplés aux protéines G NK1, NK2 et NK3 ayant respectivement une plus forte affinité pour la SP, la neurokinine A et la neurokinine B.

La substance P est un peptide de 11 acides aminés. Elle est majoritairement exprimée par les fibres nerveuses nociceptives mais on la retrouve dans d'autres cellules de la peau : les fibroblastes, les kératinocytes, les cellules endothéliales ainsi que dans de nombreux types cellulaires impliqués dans l'inflammation. Le récepteur NK1 est quant à lui exprimé par les fibroblastes, les kératinocytes, les cellules endothéliales et les cellules musculaires lisses vasculaires (Steinhoff et al., 2003). Une fois libérée, la SP induit une vasodilatation, une extravasation plasmatique et une hyperalgie. Elle est également capable d'induire la libération d'histamine par les mastocytes qui à son tour provoque la libération de SP. Un cercle vicieux se met donc en place provoquant une hyperalgie secondaire extensive. La SP est capable d'activer le système immunitaire en induisant la production d'anticorps par les lymphocytes B, la prolifération des lymphocytes T et la libération d'interleukines par les macrophages (Leal et al., 2015).

IV – 1.4.2 Le CGRP

Le CGRP est un peptide de 37 acides aminés exprimé par les fibres nerveuses peptidergiques cutanées où il colocalise avec la SP. Le récepteur du CGRP est un hétérorécepteur couplé aux protéines G. Il est composé d'une sous-unité liant le CGRP appelée « Calcitonin-Receptor Like Receptor » (CRLR) et d'une sous unité « Receptor Activity-Modifying Properties 1 » (RAMP1). Le CGRP est capable de stimuler la prolifération des fibroblastes et des kératinocytes et participe, comme la SP, à l'inflammation neurogène. Le CGRP est le plus puissant vasodilatateur connu (Peters et al., 2006).

Il est aujourd'hui bien décrit que l'ensemble de ces fibres, en plus de transmettre l'information sensitive au SNC, jouent un rôle important dans les processus de cicatrisation cutanée notamment via les neuropeptides qu'elles secrètent. Ce point est décrit dans la revue suivante : « Skin innervation : important roles during normal and pathological cutaneous repair ».

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Histology and Histopathology

Cellular and Molecular Biology

Review

Skin innervation: important roles during normal and pathological cutaneous repair

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Summary. The skin is a highly sensitive organ. It is densely innervated with different types of sensory nerve endings, which discriminate between pain, temperature and touch. Autonomic nerve fibres which completely derive from sympathetic (cholinergic) neurons are also present. During all the phases of skin wound healing (inflammatory, proliferative and remodelling phases), neuromediators are involved. Several clinical observations indicate that damage to the peripheral nervous system influences wound healing, resulting in chronic wounds within the affected area. Patients with cutaneous sensory defects due to lepromatous leprosy, spinal cord injury and diabetic neuropathy develop ulcers that fail to heal. In addition, numerous experimental observations suggest that neurogenic stimuli profoundly affect wound repair after injury and that delayed wound healing is observed in animal models after surgical resection of cutaneous nerves. All these observations clearly suggest that innervation and neuromediators play a major role in wound healing. Interactions between neuromediators and different skin cells are certainly crucial in the healing process and ultimately the restoration of pain, temperature, and touch perceptions is a major challenge to solve in order to improve patients' quality of life.

Key words: Skin healing, Cutaneous nerve fibres, Neuromediators, Peripheral neuropathy, Diabetes

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Skin innervation

Sensory as well as autonomic (essentially sympathetic) nerves are present within the skin and influence a variety of physiological and pathophysiological cutaneous functions. In unstimulated nerves, neuromediators are barely detectable within the skin tissues. Upon direct stimulation by physical or chemical means, or during pathological situations such as inflammation or trauma, a significant increase of neuromediators is observed. Thus, mediators derived from sensory or autonomic nerves may play an important regulatory role in the skin under many physiological and pathophysiological conditions, including particularly wound healing. It is important to underline that the perception of skin sensitivity is a basic need in daily life. Human beings can live while being deprived of other sensorial systems (blindness, deafness, anosmia or ageusia); in contrast, deprivation of skin sensory stimulation can cause major and irreversible disorders. For example, body thermoregulation involves the stimulation of temperature-sensitive nerve endings within the skin, and a rise of central body temperature, resulting in the reflex release of sympathetic vasoconstrictor/vasodilator tone in the skin of the extremities, is an essential process for the maintenance of body homeostasis.

In addition, as underlined by Roosterman et al. (2006), beside the peripheral nerves, a subtle complex communication network exists between the spinal cord, the central nervous system, and the immunoendocrine system. More precisely, an important brain-skin connection with local neuroimmunoendocrine circuitry exists, illustrated in the context of stress by Paus et al.

(2006). A modern concept of cutaneous neurobiology in which the central and peripheral nervous system, the endocrine and immune system, with the participation of numerous neuromediators (neuropeptides, neurotransmitters, neurotrophins, and neurohormones), and almost all skin cells are involved is now admitted (Steinhoff et al., 2003).

Cutaneous sensory nerve fibres are endings of dorsal root ganglia (DRG or spinal ganglia) neurons that carry signals from sensory organs toward the appropriate integration centre of the brain via the spinal cord (Fig. 1). In the skin, autonomic nerve fibres almost completely derive from sympathetic (cholinergic) neurons (in the face, in addition, rare autonomic nerve fibres derive from parasympathetic, also cholinergic, neurons) (Fig. 1). When deep skin damage occurs, cutaneous nerves (sensory and sympathetic nerves) and sensory receptors are destroyed, while the sensory and sympathetic neuron cell bodies persist in the ganglia along the spinal cord (respectively, dorsal root ganglia and paravertebral

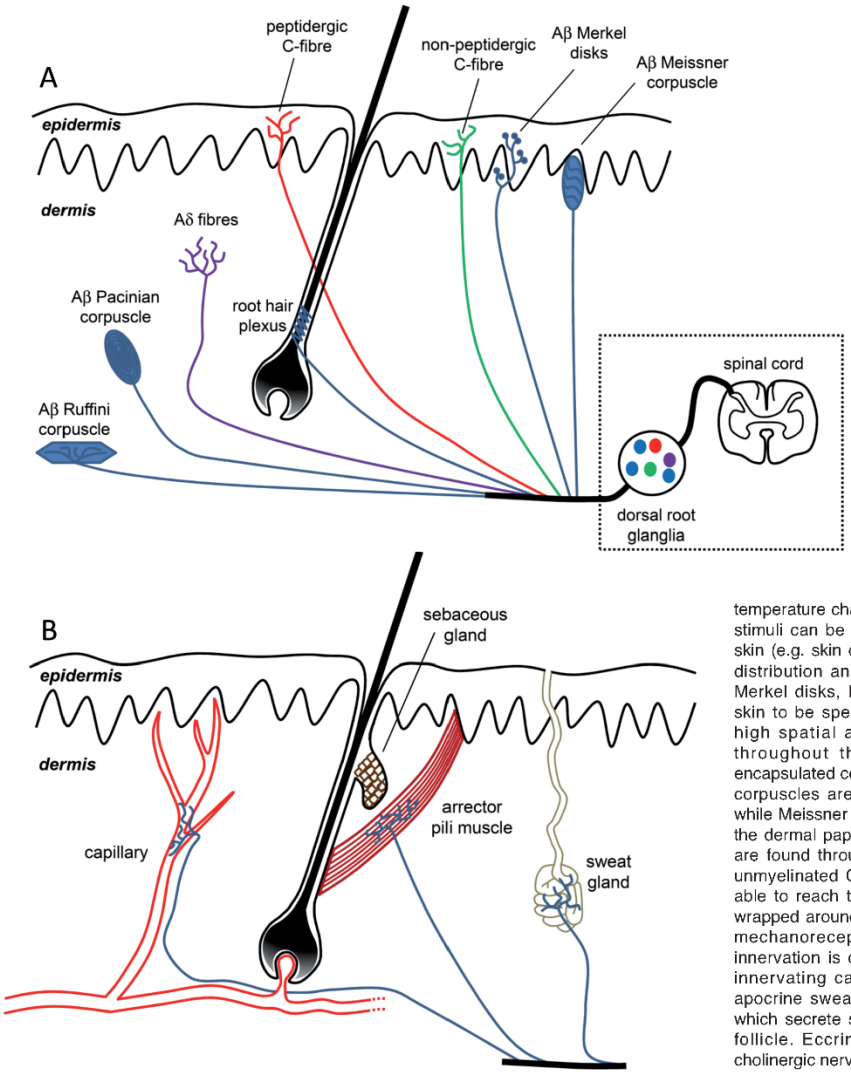


Fig. 1. Schematic diagram of skin innervation. A: Cutaneous sensory nerves arise from cell bodies located in the dorsal root ganglia and form different subtypes of nerve endings. These nerve endings mediate our sense of touch by detecting and encoding stimuli such as pain, itch, temperature changes, pressure or vibrations. Sensory stimuli can be elicited from both hairy and glabrous skin (e.g. skin of the palm); however, changes in the distribution and density of mechanoreceptors (e.g Merkel disks, Meissner corpuscles) allow glabrous skin to be specialized for discriminative touch with high spatial acuity. Nerve endings are located throughout the dermis and epidermis. Large encapsulated corpuscles such as Pacinian and Ruffini corpuscles are usually located deep in the dermis while Meissner corpuscles are preferably found within the dermal papillae. Moderately myelinated Aδ fibres are found throughout the dermis whereas only free unmyelinated C-fibre endings and Merkel disks are able to reach the epidermis. In hairy skin, plexuses wrapped around the hair follicles are also involved in mechanoreception. **B.** The cutaneous autonomic innervation is composed of adrenergic nerve fibres innervating capillaries, arrector pili muscles and apocrine sweat glands (not shown in the schema) which secrete sweat into the pilary canal of the hair follicle. Eccrine sweat glands are innervated by cholinergic nerve fibres.

sympathetic ganglia).

Sensory skin innervation

The skin is a highly sensitive organ which is densely innervated with different types of nerve endings, associated or not with specific receptors, which discriminate between pain, thermal and tactile sensations.

The different kinds of sensory stimuli that are picked up by sensory neurons are grouped into two categories: epicritic and protopathic. Epicritic neurons detect gentle touch such as caresses, light vibrations, the ability to recognize the shape of an object being held, and two-point discrimination which is the spacing of two points being touched simultaneously. Protopathic neurons are responsible for detecting pain, itch, tickle, and temperature. The different types of stimuli that are detected by a given receptor allow for a relative specificity between stimuli and receptor.

Sensory neurons arise from the dorsal root ganglia synapse in the dorsal horn of the spinal cord and transfer information about touch sensations (epicritic), or pain and temperature (protopathic). While both types of sensory neurons must first synapse in the dorsal horn of the spinal cord, the area of the dorsal horn where they synapse is different. Their pathways to reach the thalamus are also different (for more information, see Kandle et al., 2013).

Sensory cutaneous receptors are found in the dermis and the epidermis. They are part of the somatosensory system. Sensory receptors in the skin are mechanoreceptors (Ruffini, Meissner and Pacinian corpuscles, Merkel disk and free nerve endings), thermoreceptors and nociceptors (Paré et al., 2002; Lewin and Moshourab, 2004; McGlone and Reilly, 2010) (Fig. 2).

In both the epidermis and the dermis, thermoreceptors and nociceptors are found. Mechanoreceptors observed in the epidermis are Merkel disk and free nerve endings. Mechanoreceptors present in the dermis are Ruffini, Meissner, and Pacinian corpuscles, and free nerve endings which are slight axon expansions. In mucous membranes (as in the conjunctiva or genitals), bulboid corpuscles (or end-bulbs of Krause) functioning as sensory cold receptors are also found. In non-glabrous skin, root hair plexus are nerve endings which form a network around hair follicles and serve as receptors for touch sensation.

Merkel disks and Meissner corpuscles (also known as tactile corpuscles or type I mechanoreceptors) are the most superficial and are closely affiliated with the pattern of fingerprints in monkeys and humans. Merkel disks are nerve endings flattened against specialized Merkel cells clustered in the basal lamina at the base of the thickened intermediate epidermal ridges that underlie the raised fingerprints (Smith, 1970). Meissner corpuscles are located in dermal papillae that bud off dermal papillary ridges between the intermediate epidermal ridges. They are supplied by several axons that terminate between lamellar Schwann cells (Cauna, 1956). Pacinian and Ruffini corpuscles (also known as type II mechanoreceptors) are located deeper in the dermis. Pacinian corpuscles (also known as lamellar corpuscles) are ellipsoid structures consisting of an axon terminal wrapped by several layers of lamellar cells (Chouchkov, 1971). Ruffini corpuscles (also known as bulbous corpuscles) have been described as long

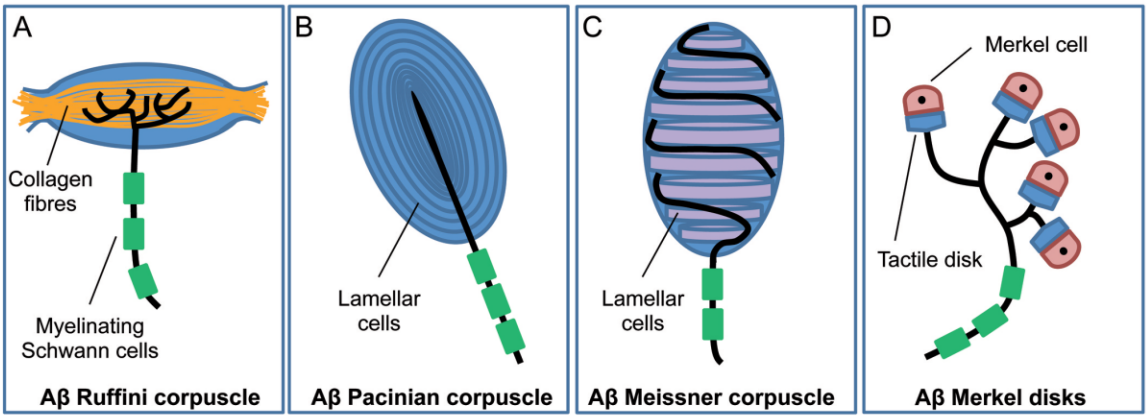


Fig. 2. Schematic diagrams of main sensory cutaneous receptors. **A.** The Ruffini corpuscle is a slowly adapting spindle-shaped receptor and is sensitive to skin stretch, pressure and distorsion. **B.** The Pacinian corpuscle is a rapidly adapting oval-shaped receptor constituted of layered lamellar cells. It can detect deep pressure and high-frequency vibration. **C.** The Meissner corpuscle is a rapidly adapting ellipsoid receptor constituted of stacked lamellar cells. It is involved in fine touch and detects pressure and low frequency vibration. **D.** Merkel receptors are slowly adapting receptors composed of tactile disks and Merkel cells involved in fine touch.

fusiform encapsulated structures within which a large-calibre axon terminates as numerous branches intertwined among collagen bundles (Chambers et al., 1972).

With the above mentioned receptor types, the skin can sense touch, pressure, vibration, temperature and pain modalities. These modalities and their receptors are partly overlapping, and are innervated by different types of fibres (Table 1).

Cutaneous sensory nerves are broadly classified according to their diameter and speed of impulse as Aβ, Aδ, and C nerve fibres, from the biggest and fastest to the smallest and slowest, respectively (Table 2). All kinds of fibres are accompanied by Schwann cells in the dermis but not in the epidermis. The larger nerve fibres, Aβ and free nerve endings Aδ, are associated with Schwann cells, which secrete a basal lamina around them and also produce myelin sheaths. C free nerve endings are the termination of unmyelinated fibres and are associated in the dermis with Schwann cells which do not produce myelin (Fig. 3).

Mechanical stimuli are detected via mechano-receptors associated with sensory corpuscles through Aβ fibres or with Aδ free nerve endings, temperature via the thermo-receptors through Aδ and C fibres, and pain via the nociceptors through Aδ and C fibres (Table 1) (for more details, see the review by Roosterman et al., 2006). Aδ fibres constitute 80% of primary sensory nerves sprouting from dorsal root ganglia, whereas C fibres make up 20% of the primary afferents (Alvarez and Fyffe, 2000; Lawson, 2002). Moreover, the activation threshold of Aδ fibres is higher than C fibres. In addition to mechanical, thermal and pain stimuli, both C and Aδ free nerve endings respond to a variable range of stimuli such as physical (trauma, heat, osmotic changes, ultraviolet light) as well as chemical (toxic agents, allergens, proteases, microbes) agents (reviewed in Steinhoff et al., 2003).

Table 1. Overview of different types of fibres and receptors activated according to stimulus modalities.

Modality	Type	Fibre type
Touch	Rapidly adapting cutaneous mechanoreceptors (Meissner and Pacinian corpuscles, hair follicle receptors, some free nerve endings)	Aβ fibres
Touch & pressure	Slowly adapting cutaneous mechanoreceptors (Merkel disk and Ruffini corpuscles, some free nerve endings)	Aβ fibres (Merkel disk and Ruffini corpuscles), Aδ fibres (free nerve endings)
Vibration	Meissner and Pacinian corpuscles	Aβ fibres
Temperature	Free nerve ending thermoreceptors	Aδ fibres (cold receptors) C fibres (warmth receptors)
Pain	Free nerve ending nociceptors	Aδ fibres (cold receptors) C fibres (warmth receptors)

Nociceptors are particular receptors generally divided into 4 classes: mechanoreceptors, thermal receptors, chemoreceptors, and polymodal receptors (these respond to all 3 stimuli). Nociceptors are similar to other receptor types but generally respond to higher levels of stimulus, e.g. general thermal receptors respond to temperatures <45°C, whereas nociceptive thermal receptors respond to temperatures >45°C. Nociceptors have either free nerve endings or non-encapsulated end organs.

In human peripheral nerves, 45% of the cutaneous afferent nerves belong to a subtype of sensory nerves that are mechano-heat responsive C fibres. However, only 13% of these nerves were found to be only mechano-sensitive, 6% were heat sensitive, 24% were neither heat nor mechano-responsive, and 12% were of sympathetic origin (Schmidt et al., 1995).

Autonomic skin innervation

Although very effective, autonomic (essentially

Table 2. Description of different types of cutaneous nerve fibres.

Type of fibres	Myelinated	Axonal diameter (µm)	Conduction velocity (m/s)
Aβ	moderately	6-12	80
Aδ	thin myelin sheath	1-5	4-30
C	unmyelinated	0.2-1.5	0.5-2

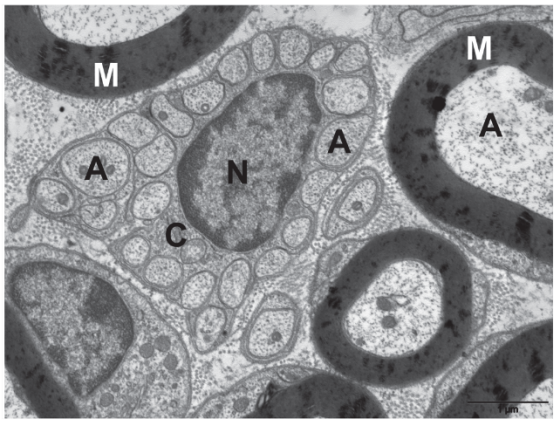


Fig. 3. Transmission electron microscopy of a rat sciatic nerve cross section. Aβ fibres are surrounded by a thick layer of myelin while C fibres are unmyelinated. Unmyelinated axons are surrounded by cytoplasmic invagination of non-myelinating Schwann cells, forming Remak bundles. Aδ fibres (not visible on this image) like Aβ fibres are myelinated but the layer of myelin is thinner and the diameter of the fibres is smaller. C: cytoplasm of a Schwann cell; N: nucleus of a Schwann cell; A: axoplasm; M: myelin sheath.

sympathetic) nerve fibres constitute only a minority of cutaneous nerve fibres compared with sensory nerves. They are restricted to the dermis, innervating blood vessels, arteriovenous anastomoses, lymphatic vessels, erector pili muscles, eccrine glands, apocrine glands, and hair follicles (Vetrugno et al., 2003). The cutaneous autonomic nervous system plays a crucial part in regulating sweat gland function, vasomotricity, skin blood flow and thereby body temperature homeostasis. Acetylcholine is an important regulator of sweating but adult human sweat gland innervation also co-expresses all of the proteins required for full noradrenergic function.

Various neuropeptides are produced and released by a subpopulation of unmyelinated afferent neurons (C fibres) defined as C-polymodal nociceptors, which, as mentioned above, represent 70% of all cutaneous C fibres in the skin. To a lesser extent, small myelinated Aδ fibres and autonomic nerve fibres are also capable of releasing a number of neuropeptides that also act on neuronal and non-neuronal target cells. In addition, recently, cutaneous cells themselves such as keratinocytes, microvascular endothelial cells, Merkel cells, fibroblasts, or leukocytes were found to be capable of releasing neuropeptides under physiological circumstances.

General mechanisms involved in skin repair and neuromediators associated with the different phases of healing

In our daily life, the skin may be damaged by chemical, physical or mechanical injuries. A rapid and efficient repair process is essential to restore skin integrity and re-establish its main function as protective barrier for the body against the environment.

Immediately after wounding, the healing process begins and consists of a coordinated succession of cellular and biochemical events that can be classified in three time-dependent phases: the inflammatory phase, the proliferative phase and finally the remodelling phase. Each phase can be distinguished by specific morphological changes ultimately leading to the formation of a scar (Martin, 1997; Gurtner et al., 2008; Profyris et al., 2012). Neuropeptides, short chains of amino acids secreted by neurons, are implicated in various phenomena involved in skin wound healing, such as induction of vasodilatation and angiogenesis, promotion of cell chemotaxis by mast cell degranulation, modulation of immune response and stimulation of migration and proliferation of keratinocytes and fibroblasts (Brain, 1997; Toda et al., 2008; Amadesi et al., 2012).

Inflammatory phase

The first stage of wound healing is immediately initiated after injury and is completed after several hours or days depending on the extent of the wound. The damage of blood vessels triggers the extravasation of blood constituents leading to the formation of a clot and of a provisional wound matrix mainly composed of fibrin and fibronectin (Robson et al., 2001). In the meantime, platelets involved in the blood clot and the matrix formation release cytokines, growth and differentiation factors contributing to the recruitment of leukocytes and the initiation of the inflammatory response. Neutrophils quickly migrate to the wound followed by monocytes and lymphocytes (24 hours after injury). These additional immune cells secrete growth factors and cytokines attracting more cells implicated in wound healing and stimulating their proliferation and survival. Immune cells secrete antibacterial products (reactive oxygen species, proteinases) and cells with phagocytic activities such as macrophages help to prevent infection. Platelets also contribute to the recruitment of fibroblasts and endothelial cells via the release of chemokines (Steed, 1997; Gawaz and Vogel, 2013). Mast cells are also involved in the inflammatory phase via the secretion of histamine and pro-inflammatory mediators acting on vascular permeability and cell recruitment (Wulff and Wilgus, 2013). Due to the injury, nerve endings may be crushed or sectioned; however cutaneous nerve fibres are important actors in the wound healing process. It has been shown that sensory nerves and to a lesser extent autonomic nerves can produce and release neuropeptides involved in the inflammatory, proliferative and remodelling phases which allow cross-talks with different cell populations participating in tissue repair (see Table 3). Neuropeptides such as tachykinins (SP, neurokinin A, neuropeptide Y), calcitonin gene related-protein (CGRP), vasoactive intestinal peptide (VIP), play a crucial role in the inflammatory phase (Lotti et al., 1995; Scholzen et al., 1998; Roosterman et al., 2006; Chéret et al., 2013). SP released on the site of injury allows vasodilatation and vascular permeability promoting plasma extravasation (Hughes et al., 1990; Holzer, 1998). Nitric oxide (NO) and histamine mediated-vasodilatation is induced by SP via the neurokinin (NK)-1 receptor present on both endothelial cells and mast cells (Ansel et al., 1993; Columbo et al., 1996). SP also stimulate the amplification of the inflammatory response by inducing the degranulation of mast cells and release of tumour necrosis factor (TNF)-α and histamine, the synthesis and release of interleukin (IL)-1β and transforming growth factor (TGF)-β by keratinocytes, the production of TNF-α, IL-2, IL-6 and IL-8 by leukocytes (Ansel et al., 1993; Delgado et al., 2003; Wei et al., 2012). CGRP is implicated in vasodilatation and the formation of an inflammatory oedema (Brain et al., 1986). CGRP also stimulates mast cells and keratinocytes to secrete respectively TNF-α and IL-1α (Niizeki et al., 1997). VIP participates in the inflammatory phase by inducing histamine release by mast cells and acts on NO-mediated vasodilatation (Gonzalez et al., 1997).

Inflammatory mediators secreted by platelets, immune cells and sensory fibres elicit pain. Thus, bradykinin, serotonin (5-HT), prostaglandins, cytokines

and H^+ ions can directly or indirectly stimulate nociceptors present on unmyelinated C-nerve fibres (Julius and Basbaum, 2001; Ständer et al., 2003). Another well studied signal transducer is the transient receptor potential cation channel subfamily V member 1 (TRPV1) also known as the capsaicin receptor or the vanilloid receptor 1. Sensitization of TRPV1, especially after a burn injury, causes the depolarization of nociceptors and transduction of pain signals (O'Neill et al., 2012).

Proliferative phase

During the proliferative phase, leukocytes and cells from the connective tissue such as fibroblasts, mast cells, macrophages and endothelial cells are recruited to the injury site and create an inflammatory granuloma called granulation tissue. The cell composition of the granulation tissue evolves over time. Initially, neutrophil, the major immune cell population, is progressively replaced by monocytes and activated macrophages. Monocytes and macrophages secrete different factors, cytokines and chemokines to recruit B and T lymphocytes in order to eliminate necrotic tissue and apoptotic cells (Koh and DiPietro, 2011). Meanwhile, fibroblasts and endothelial cells migrate and proliferate within the granulation tissue and new blood vessels are formed. Angiogenesis is essential to restore vascular perfusion in the wound and to deliver nutrients and oxygen to the cells involved in tissue repair (Singer

Table 3. Neuropeptides derived from cutaneous sensory nerves involved in wound healing.

Neuropeptides					Involvement in wound healing phases				
Family	Name	Acronym	Receptor	Targets	Inflammatory	Proliferative	Remodelling	Actions	References
Tachykinin	Substance P	SP	NK-1R	mast cell, keratinocyte, endothelial cell, fibroblast	+	+	+	↗pro-inflammatory cytokines, histamine, prostaglandins, NGF and EGF. Vasodilatation, plasma extravasation, angiogenesis, neurite outgrowth, proliferation and migration of fibroblasts and keratinocytes	Paus et al., 1995; Ansel et al., 1996; Wiederman et al., 1996; Holzer, 1998; Altun et al., 2001; Burbach et al., 2001; Pennefather et al., 2004; Chéret et al., 2014
	Neurokinin A	NKA	NK-2R				ND*		
	Neuropeptide Y§	NPY	Y1 to Y6	endothelial cell				vasoconstriction, angiogenesis	Kellogg, 1985; Movafagh, 2006
Calcitonin	Calcitonin gene-related peptide§	CGRP	CL-R/RAMP1	mast cell, endothelial cell, keratinocyte, melanocyte, immune cell	+	+	+	vasodilatation, immunomodulation, ↗NGF, keratinocyte proliferation, angiogenesis	Sung et al., 1992; Brain and Grant, 2004; Dallos et al., 2006a; Chéret et al., 2014
Secretin	Vasoactive intestinal peptide§	VIP	VPAC1, VPAC2	keratinocyte, Merkel cell, immune cell, endothelial cell	+	+	+	vasodilatation, anti-inflammatory immunomodulation, ↗VEGF, NGF angiogenesis	Schulze et al., 1997; Dallos et al., 2006a; Kakurai et al., 2009; Yang et al., 2009; Chéret et al., 2014
	Pituitary adenylate cyclase-activating peptide	PACAP		endothelial cell, keratinocyte	+	+	ND	vasodilatation, plasma extravasation, keratinocyte proliferation	Granoth et al., 2000
	Peptide histidine methionine/ peptide histidine isoleucine	PHM/PHI		keratinocyte	ND	+	ND	keratinocyte proliferation	Takahashi et al., 1993
Somatostatin		SST	SSTR1 to R5	keratinocyte, fibroblast, Merkel cell, Langerhans cell, endothelial cell, immune cell	+	+	ND	anti-inflammatory, anti-proliferative effect on keratinocyte, inhibit neurotransmitter release	Gaudillère et al., 1997; Hagströmer et al., 2006; Wang et al., 2009; Vockel et al., 2011
Galanin		GAL	GALR1 to R3	endothelial cell, keratinocyte	+	+	ND	vasoconstriction, axonal regeneration,↗NGF, keratinocyte proliferation	Holmes et al., 2005; Dallos et al., 2006b; Schmidhuber et al., 2007; Hill et al., 2010

* Not determined. § Neuropeptides shown to be released by cutaneous autonomic nerves.

and Clark, 1999; Profyris et al., 2012). Fibroblasts produce a large amount of extracellular matrix (ECM), mainly composed of type III collagen, and are activated into myofibroblasts expressing α-smooth muscle actin (Darby et al., 1990). Myofibroblasts display contractile properties and induce the progressive contraction and maturation of the granulation tissue (Hinz et al., 2012; Vedrenne et al., 2012). Keratinocytes are also key players in the proliferative phase. They migrate from the edges of the damaged site and proliferate to promote the reepithelialisation of the wound (O'Toole, 2001; Gurtner et al., 2008). During the proliferation phase, growth factors such as transforming growth factors (TGF-α, β1, β2 and β3), fibroblast growth factors (FGF-2, 7 and 10), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) are released and amplify the cellular response and modulate granulation tissue morphology (Singer and Clark, 1999; Barrientos et al., 2008). EGF stimulates keratinocyte migration in the reepithelialisation process (Morris and Chan, 2007; Peplow and Chatterjee, 2013).

Neuropeptides secreted by sensory and autonomic nerves are implicated in the proliferative phase (see Table 3). They contribute to cell stimulation with CGRP participating in angiogenesis by enhancing endothelial cell proliferation (Haegerstrand et al., 1990). CGRP and VIP are also implicated in reepithelialisation by stimulating keratinocyte proliferation (Hara et al., 1996). NGF acts on B lymphocytes by increasing their proliferation and differentiation and promoting fibroblast migration in the granulation tissue (Church and Clough, 1999; Chen et al., 2014). Neurotrophins such as NGF, neurotophin-3 (NT-3), brain-derived neurotrophic factor (BDNF) and their receptors (high affinity TrkA, B, C and low affinity p75NTR) are expressed by keratinocytes, fibroblasts, myofibroblasts and melanocytes and promote their proliferation and differentiation (Marconi et al., 2003; Botchkarev et al., 2006; Palazzo et al., 2012). Neurotrophins also have an impact on sensory nerve fibres themselves by stimulating neurite outgrowth and promoting nerve regeneration (Lentz et al., 1999; Cao and Shoichet, 2003; Gölz et al., 2006) (see Table 4).

Remodelling phase

The third phase consists of the remodelling of the granulation tissue and in scar tissue formation. Matrix metalloproteases (MMPs) secreted by keratinocytes, (myo)fibroblasts, endothelial cells as well as immune cells play a major role in this process by modulating the ECM and promoting cell migration (Martins et al., 2013). Meanwhile, the synthesis of ECM by (myo)fibroblasts is reduced and progressively type III collagen is replaced by type I collagen which is the main component of intact uninjured dermis. As the remodelling process progresses, the major part of the cell population within the ECM undergo apoptosis and the skin tends to recover its normal composition with a type I collagen matrix forming the scar tissue (Desmoulière et al., 1995). Although little is known about the role of sensory and autonomic fibres in the remodelling phase, a recent study has shown that SP, CGRP and VIP can modulate MMP-2 and MMP-9 activities. These neuropeptides also affect collagen I and collagen III production during skin wound healing (Chéret et al., 2014).

Peripheral neuropathies and wound healing delay

Prominent medical factors contributing to the incidence of cutaneous wounds include multifactorial pathologies such as diabetes, obesity and aging. In these situations, patients tend to develop spontaneous skin injury (pressure sores, diabetic foot ulcers, chronic venous ulcer) and fail to heal properly. Wound healing deficiency can be attributable, in the case of these pathologies, to vascular, neuronal and/or immune factors. Impaired or delayed wound healing is also observed in neurologic disorders like spinal cord injury or hereditary sensory neuropathy, suggesting that cutaneous nerve terminals are crucial in skin repair. Increased incidence of skin injury in sensory small fibre neuropathy (SSFN) as in the rare syndrome of congenital insensitivity to pain may be explained by lack of sensation. Patients are not aware of pain, and lose protective withdrawal reflex to avoid tissue harm.

Table 4. Neurotrophins involved in cutaneous sensory nerve regeneration during wound healing.

Neurotrophins					Involvement in wound healing phases			
Name	Acronym	Receptor	Sources	Targets	Inflammatory	Proliferative	Remodelling	Actions
Nerve growth factor	NGF	p75NTR, TrkA	keratinocyte, fibroblast, myofibroblast, melanocyte	keratinocyte, fibroblast, myofibroblast, sensory fibre	ND*	+	ND	keratinocyte proliferation; myofibroblast differentiation, migration and contraction; neurite outgrowth, neuropeptides release and nerve regeneration
Neurotrophin-3	NT-3	p75NTR, TrkC						
Neurotrophin-4/5	NT-4/5	p75NTR, TrkB						
Brain-derived neurotrophic factor	BDNF	p75NTR, TrkB						

* Not determined.

However, several studies also demonstrate specific involvement of cutaneous innervation in pathological wound healing.

Diabetes mellitus

Diabetes is a particularly important risk factor for the development of chronic wounds because it is often associated with vasculopathy and neuropathy. Diabetic neuropathy (DN) is a common complication of diabetes mellitus. Approximately 60-70% of diabetic patients develop a DN (World Health Organization, 2011), most commonly seen as distal symmetrical sensorimotor polyneuropathy. DN impairs sensory, autonomic and motor nerves. Sensory nerve impairment diminishes the perception of pain that is protective when tissue injury has occurred. Autonomic nerve impairment causes the skin to become dry and susceptible to skin fissures, tearing and infection due to a loss of sweat and sebaceous gland function. Motor nerve impairment induces muscle atrophy particularly in the feet, resulting in altered biomechanical properties and thereby increased risks for the development of neuropathic foot ulcers in combination with other neuropathic complications. However, motor impairment when it occurs is only seen in the most advanced cases of DN. Polyneuropathy found in diabetes is a main risk factor for non-healing wound and lower-extremity amputation (Adler et al., 1999). Diabetic patients fail to heal because of cutaneous homeostasis, which is compromised not only by nerve dysfunctions, but also by vascular dysfunctions. These complications are intimately involved in skin repair and it is difficult to distinguish underlying nervous mechanisms that occur in chronic wounds of diabetic patients.

Diabetic neuropathy biology

Hyperglycemia is a leading causative factor for DN, but the pathogenesis of neuropathy remains a subject of debate. Several hypotheses have been proposed. The "vascular" concept proposes that microangiopathy is a key player in the development of DN, implying that endoneurium hypoxia induced by endothelium dysfunction induces nerve degeneration (Ogawa et al., 2006). Another concept is "metabolic" and based on the molecular alterations of the peripheral nervous system induced by hyperglycemia. Protein fixation and accumulation of advanced glycation end-products in nerves can produce structural and functional alterations of peripheral nerves (Singh et al., 2014). Peroxynitrite, a product of superoxide anion radical reaction with nitric oxide, is a major oxidant in pathological conditions associated with oxidative-nitrosative stress, including diabetes, and is involved in DN (Stavniichuk et al., 2014). Hyperglycemia activates the polyol pathway activity, leading to the production of sorbitol and depletion of *myo*-inositol. Intracellular accumulation of sorbitol in Schwann cells and nerve fibres results in osmotic stress and then in nerve degeneration; depletion of *myo*-inositol reduces ATPase Na^+/K^+ activity, necessary for nerve depolarization (Zychowska et al., 2013). Both vascular and metabolic disorders which occur in diabetes contribute to DN. It has been shown for example that in patients with mild to moderate diabetic neuropathy, elevated triglycerides correlated with myelinated fibre density loss independently of disease duration, age, diabetes control, or other variables, supporting the evolving concept that hyperlipidemia is instrumental in the progression of diabetic neuropathy (Wiggin et al., 2009).

Wound healing with diabetic neuropathy

Early symptoms of DN manifest by the symmetrical loss of distal skin innervation resulting from degeneration of small cutaneous nerve fibres. Multiple studies confirm a marked decrease in intra-epidermal nerve fibre (IENF) density in diabetic patients as well as in subjects with impaired glucose tolerance (Beiswenger et al., 2008). Sensory nerve impairment is marked by the degeneration of nerve fibres expressing SP and CGRP in skin of patients with early diabetic neuropathy (Lindberger et al., 1989). The content of neuropeptide Y, a marker of sympathetic neurons, is reduced in diabetic skin (Wallengren et al., 1995). Cutaneous autonomic nerve degeneration is highlighted by a significant loss of pilomotor nerve fibres (Nolano et al., 2010) and reduced sweat gland nerve fibre density (Gibbons et al., 2009), both correlated with sweating impairment. The reduction of Meissner corpuscle density and their myelinated afferent nerve fibres, associated with myelin abnormalities, was demonstrated in glabrous skin of diabetic patients (Peltier et al., 2013). In addition to skin denervation, a decrease of NGF level within the skin and in serum, usually correlated with nerve conduction velocity, is seen in diabetic patients (Blakytny and Jude, 2006). Finally, a reduced rate of IENF regeneration is found in diabetic patients (Polydefkis et al., 2004).

In healthy subjects, cutaneous wound repair progresses linearly through different phases of wound healing. In contrast, all phases of the wound healing process are affected in diabetic wounds. The impaired healing of cutaneous wounds in diabetic patients involves multiple pathophysiological mechanisms, including the presence of peripheral neuropathy. NGF, CGRP and SP levels, relevant to wound healing, decrease in the skin of patients with DN. The vasodilatation phase that occurs in response to injury is a neurovascular phenomenon and is impaired in diabetic patients (Schramm et al., 2006). Normal skin of diabetic patients displays an increase of inflammation and of blood vessel density (Tellechea et al., 2013). A study reports that pre-existing increased serum level of inflammatory cytokines and growth factors as well as an increase in pro-inflammatory factors at the skin level are associated with failed diabetic foot ulcer healing (Dinh et al., 2012). In contrast, Galkowska et al. (2006)

described low inflammatory cell accumulation in diabetic wound which can be correlated with a reduction of foot skin innervation and neurogenic factor expression, and they hypothesized that low inflammation may lead to chronicity of diabetic foot ulcer healing process. Diabetes affects leukocyte and neutrophil function, impairs host resistance and ultimately leads to wounds more prone to infection (Galkowska et al., 2006; Alavi et al., 2014). Moreover, mast cells play an important role in leukocyte recruitment, but this function is impaired in denervated skin (Siebenhaar et al., 2008). Angiogenesis decreases during wound healing in diabetes leading to a reduced entry of inflammatory cells into the wound, which could be associated with CGRP and SP content depletions (Galkowska et al., 2006). Thus, inflammation in diabetes is clearly impaired, with a chronic inflammatory state associated with hyperglycemia and an inflammatory response to injury in skin, which is disrupted and associated, in part, to the neuropathy. Another fundamental aspect of tissue repair is reinnervation. Polydefkis et al. (2004) show that the presence of neuropathy is associated with a decreased rate of cutaneous nerve regeneration. Similarly, diabetic patients developing or not a neuropathy, exhibit reduced regenerative rate. In a mouse model, diabetic animals exhibited a failure to send axons into newly reconstituted wound tissue (Cheng et al., 2013). Although diabetes is a complex disease with diverse consequences, much evidence highlights the specific and crucial involvement of nerve during cutaneous wound healing in this pathology.

Obesity and metabolic syndrome

Obesity is characterized by abnormal or excessive fat accumulation that may impair health. The World Health Organization considers that a body mass index (BMI, kg/m^2) greater than or equal to 30 signs a situation of obesity. In 2008, more than 10% of the world's adult population was obese. The prevalence of obesity continues to increase with more than 40 million children under the age of 5 who were overweight or obese in 2012. The common consequences of obesity are increased risks of coronary disease, hypertension, dyslipidemia, type 2 diabetes, musculoskeletal disorders, cancers, stroke and sleep apnea. The metabolic syndrome represents a cluster of metabolic abnormalities that occur together and increase the risk for coronary disease, stroke and type 2 diabetes. All risk factors for metabolic syndrome are related to obesity and include abdominal obesity, dyslipidemia (high levels of total and low-density lipoprotein cholesterol and triglycerides and low levels of high-density lipoprotein cholesterol), high blood pressure and elevated fasting plasma glucose (FPG) (Alberti et al., 2009). Obesity and metabolic syndrome have a major detrimental impact on the wound healing process. Obesity and metabolic syndrome greatly increase the risk of wound infections, pressure and venous ulcers and delayed wound healing after major surgeries (Guo and Dipietro, 2010; Pierpont et al., 2014). Growing evidence suggests that metabolic syndrome is associated with increased risk for the development of microvascular complications (retinopathy, nephropathy) and, most commonly, peripheral painful neuropathy, both crucial for the wound healing process (Smith and Singleton, 2013). Studies on humans and animals demonstrate that obesity and metabolic syndrome with or without hyperinsulinemia or hyperglycemia are associated with somatic small nerve fibre neuropathy (Pittenger et al., 2005; Herman et al., 2007). SSFN implicates impairment of small diameter cutaneous nerve fibres composed of both Aδ- and C-nociceptors and is a risk for wound occurrence and impaired wound healing (Illigens and Gibbons, 2013). SSFN in obese patients without evidence of hyperglycemia was confirmed by measure of pain and flare response to topical capsaicin application (Herman et al., 2007) or by assessment of IENF density in skin biopsy (Pittenger et al., 2005; Zhou et al., 2011; Smith and Singleton, 2013).

Metabolically-induced nerve injury

A 2008 study showed that the prevalence of dyslipidemia, but not hypertension, was higher in patients with neuropathy than in diabetic patients without neuropathy (Smith et al., 2008). Diabetic sensory polyneuropathy is often diagnosed after years of hyperglycemia in type 1 diabetes. In type 2 diabetes, SSFN is often detected before diagnosis, when the patient is in a pre-diabetic state (metabolic syndrome). Several clinical studies showed that glycemic control prevents development of clinical neuropathy in type 1 diabetes whereas it is not enough in type 2 diabetes (Callaghan et al., 2012). This observation allows to hypothesize that sensory neuropathy could appear in the absence of outright diabetes and could be a consequence of other factors than prolonged hyperglycemia. The aetiology of neuropathy developing prior to overt hyperglycemia is not well understood, and a number of clinical and experimental studies implicate obesity, elevated triglyceride, cholesterol, and non-esterified fatty acids, as well as oxidative-nitrosative stress (Obrosova et al., 2007; Lupachyk et al., 2012; Smith and Singleton, 2013). In a recent review, Callaghan and Feldman (2013) have recently provided a broad overview of the mechanisms underlying metabolically induced nerve injury and therapeutics. The authors describe a feed-forward cycle of cellular damage caused by hyperlipidemia that induces nerve injury. To simplify, hyperlipidemia causes fatty deposition in the nerve and extracellular protein glycation and oxidation. Excess nutrients lead to cellular damage with production of reactive oxygen species and mitochondrial dysfunction. Mitochondrial dysfunction leads to oxidative and endoplasmic reticulum stress which was associated with pre-diabetic neuropathy (Lupachyk et al., 2013). Moreover, chronic metabolic inflammation encountered

in obesity and metabolic syndrome (Kalupahana et al., 2012) maintains a vicious cycle intensifying nerve injury, with the recruitment of macrophages to stressed nerve and hypertension resulting in nerve ischemia.

Wound healing with obesity and metabolic syndrome

Wound healing impairment occurring in obesity and metabolic syndrome resulted from numerous factors which are summarized in Table 5. In addition, impairment of cutaneous wound healing in obese patients is an important factor of complication after various bariatric surgeries such as gastric bypass.

Aging

The term aging refers to the biological process of growing older in a deleterious sense, what several authors call "senescence". Aging is one of the most complex biological processes. Briefly, aging leads to a progressive loss of physiological integrity, leading to impaired function and increased vulnerability to death (López-Otín et al., 2013). Age-related diseases include arthritis, osteoporosis, cardiovascular disease, cancer, diabetes, and various neurodegenerative diseases such as dementia and Alzheimer's disease. According to the World Health Organization, the proportion of the world population over 60 years will double between 2000 and 2050. This population is expected to increase from 605 million to 2 billion over the same period. As the aging population is growing, age-related diseases including chronic and non-healing wound are increasing. A correlation between aging and wound healing impairment was first described in 1916 (Du Noüy, 1916), and since then many studies have examined age-related impairment of wound healing in human, in animal and in *in vitro* models. The conclusions of these studies are that aging (>60 years) is an independent risk factor for delayed wound healing but does not impair the quality of wound healing (Gosain and DiPietro, 2004). Among age-related skin changes that could be involved in delayed wound healing, a reduction in nerve endings can be taken into account (Sgonc and Gruber, 2013). Aging markedly influences morphological and functional features of the peripheral nervous system, leading to peripheral neuropathy. An assessment performed in subjects with different ages demonstrated that aging was inversely correlated with nerve conduction velocity and amplitudes of both sensory and motor responses (Rivner et al., 2001). Structural changes include loss of myelinated and unmyelinated fibres and decreased production of the major myelin proteins with subsequent myelin deterioration. This loss is not compensated because of the decrease in regenerative and re-innervating capacities of nerve fibres in the elderly (Verdú et al., 2000). The prevalence of idiopathic peripheral neuropathy in the elderly that is not associated with some underlying disease process such as diabetes, has been reported to 26% in persons aged 65-74 years and to 54% of people aged 85 and older (Mold et al., 2004). Loss of lower extremity sensory input is associated with impaired balance, falls and unintentional injury in the elderly (Richardson, 2002). As in diabetes, aging is associated with multiple pathophysiological pathways that impair the immune system, vascular functions, and central and peripheral nervous system. Alterations in the aging skin have been described and can be associated with a deleterious impact on wound healing. Peripheral neuropathy occurring in the elderly could be involved in these changes and in age-related delayed wound healing.

A review of the age-related impairments of wound healing reveals that all phases of cutaneous healing undergo characteristic age-related changes, including enhanced platelet aggregation, increased secretion of inflammatory mediators, delayed infiltration of macrophages and lymphocytes, impaired macrophage function, decreased secretion of growth factors, delayed reepithelialisation, delayed angiogenesis and collagen deposition, reduced collagen remodelling and decreased muscle strength (Gosain and DiPietro, 2004). Some of these changes could be attributed, in part, to age-related peripheral neuropathy. Sensory denervation in skin results in delayed protein extravasation and cell migration during the inflammatory phase, delayed wound contraction and epithelialisation, delayed angiogenesis and re-innervation into the wound (Chéret

Table 5. Summary of factors related to wound impairment in obesity (from Guo and Dipietro, 2010).

Local wound conditions	Associated diseases and conditions	Factors altering immune and inflammatory response
1. Decreased vascularity in adipose tissue	1. Hard to reposition	1. Adipokines: leptin, adiponectin, resistin
2. Skin folds harbor micro-organisms	2. Coronary heart disease	2. Cytokines: TNF-α, IL-1, IL-6, IL-8, IL-10
3. Increased wound tension	3. Atherosclerosis	3. Chemokines: IL-8, MCP-1, IP-10
4. Increased tissue pressure	4. Type 2 diabetes	4. Impaired release of neuropeptide: CGRP, SP, neuropeptide Y
5. Hematoma and seroma formation	5. Cancer	
6. Venous hypertension	6. Hypertension	
7. Decreased innervation in skin	7. Dyslipidemia	
	8. Stroke	
	9. Respiratory problem	
	10. Small fibre neuropathy	

et al., 2013; Ishikawa et al., 2014).

Spinal cord injury

Spinal cord injury (SCI) designates damage to any level of the spinal cord resulting from a trauma (e.g. road accident) or a disease (e.g. tumour, spinal cord infarction, ankylosing spondylitis). According to the World Health Organization (2011), the global annual incidence is estimated from 40 to 80 cases per million population and 90% of cases would be from traumatic origin. SCI causes permanent changes of body functions below the site of injury and the severity of these changes obviously depend on the extent of injury. SCI leads to blockage of nerve impulses resulting in paralysis and/or tactile disturbances in the body parts governed by the damaged nerves. The area of skin supplied by a single spinal nerve is called a dermatome, and similarly, a myotome is a group of muscles innervated by a single spinal nerve. The higher the injury on the spinal cord occurs, the more dysfunction will arise. Symptoms may include partial or complete loss of sensory function and/or motor control below the affected area. The severity of sensory, autonomic and motor loss also depends on the level of injury to the spinal cord, described as "complete" or "incomplete" lesion. Complete SCI at high cervical level causes paralysis of the four limbs (tetraplegia) and affects the systems that regulate bowel or bladder control, breathing, heart rate and blood pressure. Complete thoracic SCI commonly causes sensory and/or motor loss in trunk and legs (paraplegia). Incomplete injury defines a situation where sensory and/or motor functions are preserved below the lowest sacral segments. Chronic pain occurs in all forms of SCI (Kirshblum et al., 2011). A common occurrence in SCI is the frequency of skin breakdown, particularly on the sitting surface. Ulcers developed in patients with SCI are difficult to heal as in diabetic neuropathy. Paraplegic and tetraplegic patients present impairment of wound closure below the level of the spinal cord lesion (Basson and Burney, 1982). Tissue changes induced by denervation due to SCI impact cutaneous homeostasis and lead to chronic wounds (Rappl, 2008).

Pathophysiology of spinal cord injury

Traumatic SCI symptoms result from two separate mechanisms: primary injury and secondary injury. Primary injury corresponds to neurological damage in the spinal cord that begins after the initial mechanical damage (compression, laceration, contusion). The initial impact induces bleeding and cell death at the impact site leading to a cascade of biological events described as "secondary injury" (minutes or weeks after injury). Secondary injury leads to further neurological damages and is followed by the "chronic phase" (days to years after injury). Secondary impairment may lead to inflammation, neuronal cell death, loss of oligodendrocytes and activation of microglia and astrocytes, which results in glial scarring (Silva et al., 2014). The astroglial scar forms a barrier impeding axonal growth and regenerative processes. Functional neuronal connections between peripheral nerves and spinal cord are disrupted below the injury causing dormant body areas. Loss of peripheral nerve to spinal cord connection induces tissue changes in target areas.

Wound healing in spinal cord injury

Physiological changes occurring in trunk dermatomes contribute to the development of pressure ulcers and to the slow healing rate encountered in SCI patients. The consequences of denervation in skin are hypothesized to interrupt the natural wound healing cycle (Rappl, 2008). The skin of individuals with SCI is thinner and less distensible that in healthy subjects. Denervated tissues exhibit abnormal vascular reaction and impaired immune function (Rappl, 2008). Histopathology studies report that SCI patients present dermal fibrosis, progressive skin thickening and nail hypertrophy on lower limbs (Stover et al., 1994). This study shows that the severity of the phenotypes is progressive and correlates with the degree of injury, suggesting a role of the peripheral nervous system in the maintenance of skin and nail. Changes of skin associated with SCI result from deficient vascular reactivity, decreased fibroblast activity and higher collagen catabolism (Gefen, 2014). Metabolic changes associated with denervation tend to render the tissue insensate and thus increase the risk of developing ulcers and affecting the healing rate once an ulcer develops.

Hereditary sensory (and autonomic) neuropathy type 1

Hereditary sensory and autonomic neuropathy (HSAN) or hereditary sensory neuropathy (HSN), are clinically and genetically heterogeneous disorders of the peripheral nervous system that affect predominantly the sensory and autonomic neurons. HSN/HSANs are currently classified into five types (HSN/HSAN 1-5). Typically, HSN/HSANs are described as slowly progressive neurological disorders characterized by primarily prominent distal sensory loss, and variable autonomic and motor disturbances. HSN1 is an axonal form of hereditary sensory neuropathy distinguished by prominent early sensory loss and later positive sensory phenomena including dysesthesia and characteristic "lightning" or "shooting" pains. Negative and positive sensory symptoms are mainly distributed to the distal parts of the upper and lower limbs. HSN1 affects dorsal root ganglia neurons and motor neurons of the spinal cord. A study shows distal loss of unmyelinated and small myelinated nerve fibres before degeneration of large myelinated axons in nerve biopsy (Houlden et al., 2006). Loss of sensation can lead to painless injuries, which, if unrecognized, result in slow wound healing and subsequent osteomyelitis requiring distal amputations (Houlden et al., 2006; Auer-Grumbach,

2008). Ulcero-mutilating lesions developed in HSN1 are the most serious complication of this pathology. Although foot ulcerations in HSN1 are compared with "diabetic foot syndrome", no microvascular impairment is described in this disease. These observations suggest and reinforce the crucial role of innervation during wound healing.

Others cases of impaired or delayed wound healing

Leprosy

In 2009, the World Health Organization reported that leprosy is detected in 17 countries (including India and Brazil) with a prevalence of the disease less than 1 per 10,000 people (except for Brazil where prevalence is 2 per 10,000 people). Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*, predominantly affecting peripheral nerves and skin. Leprosy causes a complex peripheral neuropathy of immunological origin that results in autonomic, sensory and motor nerve disorders. The presence of *Mycobacterium leprae* in peripheral nerves elicits an inflammatory response that is composed of epithelioid granuloma with lymphocytes or acid fast bacilli-loaded macrophages, leading to nerve degeneration. The most prevalent clinical presentations are mononeuropathy, multiple mononeuropathy and polyneuropathy (Cabalar et al., 2014). Leprosy is usually not recognized until a cutaneous manifestation occurs, and most patients first present numbness, sometimes years before the skin lesions appear. Temperature sensitivity by the skin is lost in the initial stages of the disease, followed by vision loss and pain. Sensory symptoms are especially focused on body extremities; hands and feet (Nascimento, 2013). Skin injury in patients with leprosy is defined as a chronic ulceration of the anaesthetic area, resistant to local or systemic therapy and characterized by recurrence (Price, 1959). Ulcers in patients with leprosy remain the most common consequence of leprosy. Leprosy-induced anaesthesia is the central factor in the pathogenesis of hand and foot ulcers. Un-protective behaviours and poor quality of scar, resulting from previous ulceration, are the main reasons for recurrence of neuropathic ulcer, and can lead to deformity and/or amputation of the affected limb.

Wound healing in patients with cancer

Payne et al. review physiological changes that patients with cancer undergo and how these changes impact on the wound healing process (Payne et al., 2008). Cancer therapy such as chemotherapy directly impairs acute wound healing. In animal studies, alkylating agents such as cisplatin have been demonstrated to reduce wound tensile strength, to decrease fibroblast proliferation, to reduce connective tissue proliferation and to inhibit neovascularisation. Vincristine, a plant alkaloid, appears to transiently decrease wound tensile strength during the first days after surgery, without inducing wound complication. The effect of cancer therapies on acute wound healing is multifactorial. Chemotherapy weakens the immune function of the patients, induces neutropenia, anemia and thrombocytopenia, making wounds more prone to infections. In addition to immune impairment, chemotherapy affects the nervous system. Chemotherapy-induced peripheral neuropathy (CIPN) is a common and potential dose-limiting complication of cancer therapy. CIPN is usually present at the point most distal from the trunk first, i.e. fingertips and toes. According to the substance used, CIPN may be in the form of purely sensory and painful neuropathy (cisplatin and carboplatin) or mixed sensory-motor neuropathy which may be accompanied by dysfunction of the autonomic nervous system (vincristine and taxanes) (Quasthoff and Hartung, 2002). Structural changes in peripheral nerves include loss of IENF (Han and Smith, 2013). The literature describes the involvement of different chemotherapy drugs in wound healing impairment and in nervous system damage but the relationship between innervation and wound healing delay is not however obvious in this context.

Proof of concept in animal models

Relationships between impaired wound healing and peripheral neuropathy are highlighted in several animal experiments, described in Tables 6 and 7. Two types of experiments have been performed in order to further study the link between sensory nerve dysfunction and wound healing; either using animal models of skin denervation (Table 6) or stimulating cutaneous nerve (Table 7) during wound healing.

Skin denervation can be induced surgically, chemically or genetically. Studies have shown that surgical denervation slows down skin wound healing with decreased wound contraction, delayed reepithelialisation and reduced inflammatory cell infiltration (Richards et al., 1999; Buckley et al., 2012). Capsaicin is a potent agonist of TRPV1, a cation channel expressed by both sensory Aδ- and C-fibres. Capsaicin induces the degeneration of TRPV1-expressing nerve fibres and leads to reduced and delayed wound healing, mostly associated with poor reepithelialisation (Smith and Liu, 2002; Toda et al., 2008; Martínez-Martínez et al., 2012). Oxidopamine (6-OHDA)-induced sympathectomy also causes impaired wound healing which was associated with a decrease of neurogenic inflammation (Kim et al., 1998; Souza et al., 2005). Streptozotocin (STZ) is a chemical compound particularly toxic for insulin-producing β cells located in the pancreas. Therefore, injection of STZ promotes diabetes in animal models and is used to study the impact of diabetic neuropathy on wound healing. Skin wounds of STZ-treated mice and of db/db mice (genetically-induced diabetes) show delayed healing compared to non-diabetic animals, and are associated with impaired re-innervation of the wound (Gibran et al.,

2002; Cheng et al., 2013). These experiments further confirm that the functional integrity of the cutaneous nervous system is necessary for optimal wound healing (Table 6).

Gürgen et al. (2014) have set up a daily transcutaneous electrical nerve stimulation (TENS) treatment on rats during cutaneous wound healing and have shown that nerve stimulation shortened the healing time and increased wound repair efficacy. TENS is a method based on electrical stimulation which primarily aims to provide a degree of symptomatic pain relief by exciting sensory nerves. Recently, Kant et al. (2013) have shown that daily topic application of SP on cutaneous wound of healthy rats increases wound closure and induces faster reepithelialisation (Table 7).

Animal experiments highlight the crucial role of sensory nerve in wound healing and the ability of sensory peptides, SP and CGRP, in modulating wound

Table 6. Animal models of impaired wound healing associated with denervation.

Species	Disease	Wound type	Results	Reference
Male mice, 8 weeks old	Knockout for CGRP Capsaicin-mediated local skin denervation	Full-thickness skin wounds of 8 mm diameter (dorsal midline)	Reduced and delayed wound healing, reduction of VEGF expression in granulation tissue, suppressed neovascularization and angiogenesis Delayed wound healing	Toda et al., 2008
Sprague Dawley rat, 12 days old (developing rats)	Capsaicin mediated systemic denervation (50 mg/kg, s.c. injection at postnatal day 2 and 9)	Full-thickness circular skin wound (below the scapulae)	Capsaicin treatment led to reduced CGRP-immunoreactive sensory innervation which was associated with impaired healing; increased wound area and volume, prolonged scab retention and delayed re-epithelialisation. Denervated tissue exhibited increase of granulation cell proliferation without proportionate increase in apoptosis.	Smith and Liu, 2002
Wistar rat, 8-10 weeks old	Chemical sympathetic denervation with 6-OHDA (hydrobromide salt)	Skin linear incision or full-thickness excisional wound (4 cm^2)	6-OHDA lesion delayed cutaneous wound healing; increased wound contraction, reduced mast cell migration, delayed re-epithelialisation. Authors hypothesize that sympathectomy induced a decrease of neurogenic inflammation.	Kim et al., 1998 ; Souza et al., 2005
CD-1 mice, 3 month old	Streptozotocine-induced diabetic neuropathy	Dorsal skin wound of 3 mm diameter with punch biopsy	Mild decline of DIENF in hairy dorsal skin. Diabetes was associated with slower decline of wound size, failure of plasticity to send axons into newly reconstituted tissue.	Cheng et al., 2013
Male CD57L mice, 8 to 12 week old	Genetically-induced diabetes, db/db	Dorsal full-thickness wound of 1.5*1.5 cm.	Db/db mice had reduced number of epidermal nerve fibre and healed more slowly than non-diabetic mice.	Gibran et al., 2002
Wistar male rat, 8 week old	Neonatal capsaicin treatment-induced sensory denervation	Full thickness wound of 6 mm diameter (dermal punch biopsy)	Sensory denervation: reduced activation of keratinocyte proliferation, impaired follicle cell migration, impaired bulge stem cell progeny migration to the epidermis, slowed down reepithelialisation.	Martínez-Martínez et al., 2012
C57BL/6 female mice, 7 to 12 week old	Surgical denervation of the ear	2 mm punch biopsy wound at the centre of the ear	Surgical denervation affected wound healing and was associated with increased wound area, extensive necrosis and inability to reepithelialise the distal wound margin of the ear hole.	Buckley et al., 2012
Adult female Sprague-Dawley rat	Surgical denervation by section of the inferior epigastric nerve	Full thickness wound of 1 cm diameter on the groin	Healing of denervated wound was delayed with decrease in wound contraction, and reduced count of macrophages and T-lymphocytes in the granulation tissue.	Richards et al., 1999

Table 7. Animal models of improved wound healing associated with nerve stimulation.

Species	Wound type	Treatment	Results	Reference
Adult male Wistar rats (140-160 g)	Full-thickness excisional wound (4 cm^2) on the back	Daily topic application of SP (10^{-7} M, 400 μL) on wound during 14 days	SP stimulated inflammatory cell recruitment and angiogenesis by increased TGF-β1 and VEGF expression. Il-10 was increased in SP treated wound, which led to stop inflammatory phase. SP increased wound closure and induced faster reepithelialisation phase.	Kant et al., 2013
Albinos male and female rats	Dorsal full-thickness incision wound of 1 cm	Daily TENS (transcutaneous nerve stimulation) for 5 days after wound	TENS improved wound healing by accelerating closure of wound edges and reepithelialisation, and by decreasing pro-inflammatory cytokines levels (IL-1β, IL-6 and TNF-α)	Gürgen et al., 2014
Genetically diabetic male mice, 8 to 12 week old	Dorsal full-thickness wound of 1.5*1.5 cm	Topical application of SP dissolved in polyethylene glycol (5%)	Diabetic wound treated with SP healed faster than untreated wound	Gibran et al., 2002

healing.

Conclusion

The skin is a major component of the human body and is the first barrier and protection against outside aggressions. Thus, deciphering cellular and molecular mechanisms involved in wound healing is of paramount importance as skin integrity is crucial in the maintenance of general homeostasis. The skin is a complex tissue, which is highly vascularised and very richly innervated as highlighted in this review. Although skin/nerve interactions are far from being completely understood, much evidence suggests that a proper cutaneous innervation is essential in the process of skin maintenance, protection and healing. Clinical observations in patients with peripheral and central nervous system disorders have greatly helped to understand the roles of the nervous system in skin protection. Moreover, animal models allow dissecting molecular and cellular mechanisms involved in skin protection and repair that are highly regulated and are partly dependent upon the nervous system to be properly orchestrated. A better understanding of these mechanisms is crucial to develop new therapeutic tools and protective strategies that will be useful in the improvement of cutaneous wound healing.

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Comme cela est décrit dans cette revue, l'innervation cutanée est importante lors d'une lésion et participe activement au processus de cicatrisation. Il est donc important d'avoir une innervation cutanée fonctionnelle pour informer le SNC en cas de stimulation tactile ou de pression, en cas de variation de température au niveau de la peau ou de douleur mais également pour pouvoir répondre de façon adéquate et optimale à une lésion.

Objectifs de la thèse

A la suite d'une brûlure sévère du deuxième ou du troisième degré, la peau et ses annexes, y compris les fibres nerveuses sensitives, sont détruites. La cicatrisation de ce type de brûlure n'est pas spontanée et nécessite le plus souvent la mise en place d'une autogreffe ou d'une allogreffe et l'utilisation de produits issus de l'ingénierie tissulaire. Ces protocoles permettent de couvrir la lésion et de favoriser la cicatrisation. Les fibres nerveuses sensitives cutanées doivent également retrouver leur place dans la peau et permettre ainsi aux patients brulés de retrouver une sensibilité cutanée correcte. Malheureusement cela est rarement le cas et les patients vont garder à vie des séquelles suite à cette innervation défectueuse.

Il est donc important de comprendre pourquoi l'innervation cutanée n'est pas correctement rétablie dans le tissu cicatriciel après brûlure. Cela permettra d'envisager de nouvelles thérapies favorisant une repousse des fibres nerveuses cutanées satisfaisante, limitant ainsi les séquelles de la brûlure.

Ce projet de thèse s'intègre dans une étude plus globale financée par la Direction Générale de l'Armement : projet NERVAL (RéiNERVAtion après brûLures) (DGA, No 2013 94 0903). Cette étude impliquant 3 autres équipes de recherche françaises a pour but :

- d'étudier la cicatrisation et la réinnervation du site lésé après une brûlure thermique du deuxième ou du troisième degrés (modèle *in vivo* chez l'animal et recueil de biopsie chez des patients brûlés).
- d'explorer les interactions cellulaires qui peuvent s'établir entre les 3 populations cellulaires de la peau à savoir les kératinocytes, les fibroblastes et les cellules nerveuses.
- de proposer un modèle de peau reconstruite innervée pouvant être greffée.

Mon projet de thèse s'intéresse plus spécifiquement aux deux premiers points ci-dessus. Pour cela, un modèle de brûlure thermique a été développé chez le rat pour suivre la cicatrisation et la réinnervation. De plus, des études *in vitro* ont été menées pour mettre en évidence les interactions cellulaires qui pouvaient s'établir entre les fibroblastes et les cellules neuronales.

Chapitre 2 : Etude expérimentale

I – Brûlures et innervation

Pour étudier le rôle de l'innervation lors du processus de cicatrisation après brûlure, nous avons mis au point un modèle de brûlure chez des rats présentant ou non une neuropathie.

I – 1 Modèle de neuropathie et RTX

La RTX est une substance extraite de l'*Euphorbia Resinifera*. C'est un analogue naturel de la capsaïcine qui elle est extraite du piment. Ces deux substances sont des agonistes du récepteur TRPV1 mais la RTX possède une plus forte affinité que la capsaïcine pour ce récepteur (Szallasi and Blumberg, 1989).

Après la fixation de ces agonistes sur le récepteur TRPV1 présent sur les petites fibres sensitives peptidergiques, on observe un relargage massif de neuropeptides (notamment la SP et le CGRP) en périphérie. La neuropathie s'installe donc par une désensibilisation des petites fibres nerveuses (Hsieh et al., 2012a). En effet, les stocks de SP et de CGRP sont en grande partie déversé en périphérie et ces molécules ne seront donc plus présentent dans la fibre nerveuse en cas de stimulation ultérieure. La désensibilisation est transitoire, elle persiste le temps de la néo-synthèse de neuropeptides au niveau du corps cellulaire et de leur migration à l'extrémité de l'axone. A dose élevée cependant, il est possible d'obtenir une destruction des petites fibres nerveuses sensitives, et la neuropathie est alors définitive (Jancsó et al., 1985).

Différentes études utilisant la RTX pour créer un modèle de neuropathie ont été réalisées. Hsieh Y. et al (Hsieh et al., 2012b) ont par exemple utilisé cette substance sur des souris à une dose de 50 µg/kg pour induire une neuropathie et ensuite pouvoir tester un antagoniste de P2X3 (récepteur à l'ATP présents sur les fibres sensitives et impliqué dans la transmission de stimuli douloureux). L'utilisation de cet antagoniste a permis d'inhiber de manière dose-dépendante l'induction de la neuropathie par la RTX. En plus de ces capacités à induire une neuropathie dans le but de tester différentes molécules à potentiel thérapeutique, la RTX peut également être utilisée chez des animaux pour des études sur le traitement des douleurs neuropathiques notamment celles provoquées lors d'un cancer des os au stade avancé (Menéndez et al., 2006; Brown et al., 2015).

I – 2 Méthodologie générale

L'induction de la neuropathie chez les animaux a été réalisée par injection intrapéritonéale de RTX à 185 µg/kg 1 semaine avant la brûlure. Les animaux du groupe contrôle ont été injectés avec une solution à 10 % de dimethylsulfoxide (véhicule dans lequel est dissout la RTX). La mise en place de cette neuropathie et son maintien dans le temps ont été suivis notamment en réalisant différents tests sensitifs nociceptifs : le Randall-Selitto et le test de la plaque chauffante (matériels Bioseb, Vitrolles, France). Ces tests ont été réalisés avant l'injection puis toutes les semaines jusqu'à l'euthanasie des animaux.

Le test du Randall-Selitto permet l'étude de la nociception mécanique par l'application d'une pression d'augmentation linéaire sur le dessus de la patte de l'animal jusqu'au retrait de celle-ci pour cause de douleur (Figure 14).



Figure 14 : Dispositif expérimental du Randall-Selitto (Bioseb) pour l'étude de la nociception mécanique.

La plaque chauffante permet quant à elle l'étude de la nociception thermique. L'animal est alors placé sur une plaque chauffée à 52 °C et le temps de latence avant l'apparition des premiers signes de douleur est mesuré (Figure 15).



Figure 15 : Dispositif expérimental du test de la plaque chauffante (Bioseb) pour l'étude de la nociception thermique.

Ces tests nociceptifs nous permettent d'avoir une réponse sur la fonctionnalité des fibres nerveuses impliquées dans la détection et la transmission du stimulus douloureux.

Par ailleurs, pour comprendre le mécanisme d'induction de la neuropathie par la RTX, différentes études ont été réalisées. La structure des nerfs sciatiques a été analysée 6 jours

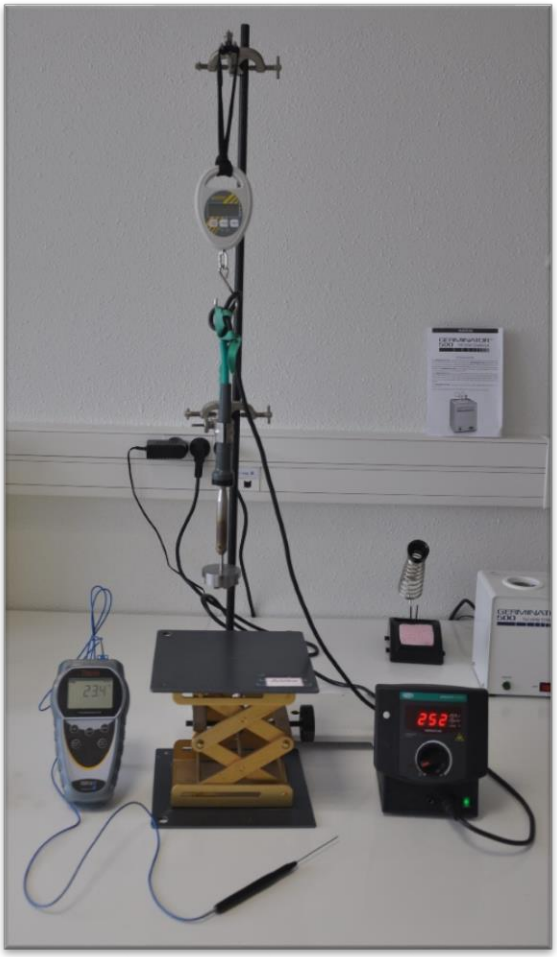
après l'injection de RTX par microscopie électronique à transmission. De plus, une étude immunohistochimique a été effectuée pour étudier l'impact de l'injection de la RTX sur l'expression des neuropeptides. Notamment ; les immunomarquages pour le CGRP et la PGP 9.5 ont été effectués sur les GDRs et la peau des pattes chaque semaine après l'injection lors de l'euthanasie des animaux.

Pour l'analyse des GDRs, les cellules positives pour chaque anticorps ont été comptées. La densité des neurones positifs pour PGP 9.5 est exprimée en nombre de neurone / mm^2. La densité des neurones peptidergiques est exprimée par le ratio nombre de neurones CGRP+/ nombre de neurones PGP 9.5+.

Pour l'analyse des fibres nerveuses intraépidermiques (FNIEs) dans la peau des pattes, leur nombre a été évalué par le comptage des fibres PGP 9.5+ traversant la jonction dermo-épidermique (selon la méthode de Lauria et al., 2005).

Une semaine après l'injection de RTX ou du véhicule, deux brûlures ont été réalisées sur le dos des animaux de part et d'autre de la moelle épinière.

Une pièce de métal (7 mm x 12 mm) chauffée à 70°C ou 90°C a été appliquée sur le dos de l'animal pendant 45 secondes avec une force de 0,6 N (Figure 16). A la suite de la brûlure, un pansement était appliqué sur la plaie et changé tous les 2 jours. Une semaine après la brûlure, la plaie était détergée et un nouveau pansement laissant respirer la plaie était appliqué. Ce pansement était également changé tous les 2 jours jusqu'à l'euthanasie des animaux.

A. 

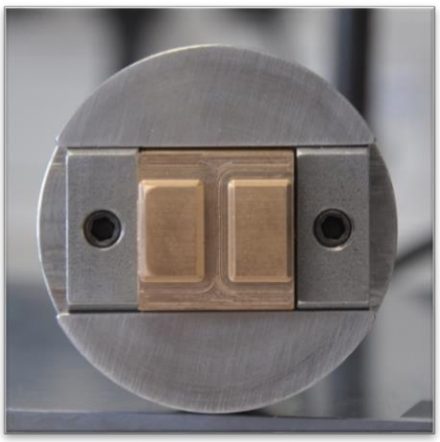
B. 

Figure 16 : Dispositif permettant de réaliser la brûlure. A. Dispositif général avec contrôle de la température et de la force appliquée sur la peau. B. Photo de la pièce de métal appliquée sur le dos des animaux.

Pour comprendre pourquoi l'innervation n'est pas correctement rétablie dans le tissu cicatriciel après une brûlure, nous avons suivi la cicatrisation et l'état de l'innervation lors des différentes étapes du processus de cicatrisation. Pour cela des animaux des 2 groupes étaient euthanasiés chaque semaine après réalisation de la brûlure.

L'analyse histologique de la cicatrisation a été effectuée par une coloration au trichrome de Masson et une coloration avec une solution d'hématéine-éosine-safran. Une étude immunohistochimique pour analyser l'expression de l'actine α-ML a également été réalisée.

La réinnervation de la plaie a quant à elle était analysée par western blot et par immunofluorescence. L'étude par western blot a recherché l'expression de deux marqueurs neuronaux dans la plaie : la PGP 9.5 et la périphérine (marqueur spécifique des petites fibres sensitives). L'étude par immunofluorescence a également permis de mettre en évidence l'expression de la « Growth Associated Protein 43 » (GAP-43) (marqueur de la plasticité neuronale) dans la plaie au cours du processus de cicatrisation.

I – 3 Principaux résultats

Les résultats concernant la validation du modèle de neuropathie induite par la RTX et l'analyse de la cicatrisation et de la réinnervation de la plaie après une brûlure à 90°C sont présentés dans le manuscrit ci-après accepté dans le journal Burn.

La première partie de ce manuscrit confirme la mise en place d'une neuropathie chez les animaux ayant reçu la RTX à une dose de 185 µg/kg. En effet, on observe une hypoalgésie thermique et mécanique chez les animaux traités comparés aux animaux contrôles. Cette hypoalgésie est réversible puisqu'un retour à une sensibilité normale est obtenu 14 jours après l'injection de RTX pour la nociception mécanique et 28 jours après l'injection de RTX pour la nociception thermique (Figure 2).

De plus, cette neuropathie n'est pas provoquée par une atteinte de la structure des fibres nerveuses puisque les images en microscopie électronique à transmission des fibres nerveuses du nerf sciatique montrent des morphologies similaires entre les animaux ayant reçu la RTX et les animaux contrôles (Figure 3).

Pour compléter l'étude du mécanisme de l'induction de la neuropathie, une analyse de l'expression du CGRP en périphérie (FNIEs dans la peau de la patte) et au niveau des corps cellulaires (GDRs) a été réalisée. On a pu observer en immunofluorescence une diminution de l'expression du CGRP au niveau des GDRs et au niveau de la peau des pattes chez les animaux ayant reçu la RTX. Cette réduction d'expression du CGRP est obtenue rapidement après l'injection (différence significative dès 6 jours après l'injection). Un retour aux taux normaux est retrouvé 35 jours après injection de RTX pour l'expression ganglionnaire et 42 jours après l'injection pour l'expression en périphérie. Cette différence de 7 jours entre le retour à la normale dans les GDRs et en périphérie peut s'expliquer par le fait que les neuropeptides qui sont synthétisés dans les corps cellulaires des neurones présents dans les GDRs doivent ensuite migrer le long de l'axone pour atteindre son extrémité (Figure 4).

La neuropathie induite par la RTX se met donc en place rapidement après l'injection et elle est réversible dans le temps.

La seconde partie de ce manuscrit s'intéresse à l'étude de la cicatrisation et de la réinnervation chez les 2 groupes d'animaux après une brûlure à 90°C.

Cette brûlure thermique induite par l'application d'une pièce de métal chauffée à 90°C et appliqué sur le dos de l'animal pendant 45 secondes avec une force de 0,6 N induit une brûlure du troisième degré (destruction de l'épiderme et du derme jusqu'à la couche musculaire appelée *panniculus carnosus*) (Figure 5A).

Lorsque l'on mesure macroscopiquement le taux de fermeture de la plaie au cours du temps, aucune différence significative n'est observée entre les animaux ayant reçu la RTX et les animaux contrôles (Figure 5B). Cependant, après une coloration au trichrome de Masson de coupes de plaies à 14 jours après la brûlure, on se rend compte que microscopiquement, le processus de cicatrisation est retardé chez les animaux ayant reçu la RTX (Figure 6). En effet,

chez ces animaux, on observe une plus forte cellularité du tissu de granulation comparée aux animaux contrôles ainsi qu'une plus grande expression d'actine α-ML dans le tissu de granulation ce qui traduit un retard dans la succession des phases qui caractérisent la cicatrisation. De plus, le remodelage du collagène est plus avancé chez les animaux contrôles. Enfin, le processus de réépithélialisation est également retardé chez les animaux ayant reçu la RTX par rapport aux animaux contrôles.

Pour l'étude de la réinnervation de la plaie par western blot, on observe une importante augmentation de l'expression de la PGP 9.5 (marqueur de l'ensemble des fibres nerveuses) et de la périphérine (marqueur des petites fibres sensitives) 14 jours après brûlure chez les animaux contrôles puis un retour aux taux avant brûlure dans les semaines suivantes. Cette augmentation d'expression de la PGP 9.5 14 jours après la brûlure est également observée chez les animaux ayant reçu la RTX. Cependant, l'augmentation de la périphérine est plus faible dans la plaie des animaux ayant reçu la RTX comparés aux animaux contrôles (Figure 7).

Enfin, l'étude de la plasticité et de la repousse axonale a été réalisée grâce à un marquage en immunofluorescence pour GAP-43 sur les coupes de plaies après brûlure à 90°C. Chez les deux groupes d'animaux, on observe une faible expression de ce marqueur 14 jours après la brûlure mais une forte augmentation de son expression 28 jours après la brûlure (Figure 8).

Ce travail *in vivo* nous aura donc permis, après avoir validé notre modèle de neuropathie induite par la RTX, d'étudier la cicatrisation des rats après une brûlure du troisième degré. Même si la cinétique de fermeture des plaies est identique entre les deux groupes, l'avancée de la cicatrisation est moins rapide chez les animaux présentant cette neuropathie. Cela conforte l'idée que l'innervation et les neuropeptides (absents à la suite de l'induction de la neuropathie) sont essentiels pour obtenir un processus de cicatrisation correct. Bien que la plasticité nerveuse dans la cicatrice soit semblable entre les animaux présentant ou non une neuropathie, la quantité de petites fibres sensitives (périphérine positives) dans la plaie 14 jours après la brûlure est réduite chez les animaux présentant la neuropathie en comparaison avec les animaux contrôles.

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Highlights

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Effects of small-fiber neuropathy induced by resiniferatoxin on skin healing and axonal regrowth after burn

Betty Laverdet, Dorothée Girard, Audrey Bayout, Nelly Bordeau, Claire Demiot, Alexis Desmoulière*

- Resiniferatioxin promoted transient hypoalgesia without inducing nerve degeneration.
- Neuropeptides were depleted in cell bodies and endings of primary sensory neurons.
- Resiniferatoxin-mediated neuropathy delayed granulation tissue remodeling after burn.
- The proportion of small fibers expressing peripherin was reduced during healing.
- After burn healing, nerve regrowth and plasticity within the wound were not modify.

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Effects of small-fiber neuropathy induced by resiniferatoxin on skin healing and axonal regrowth after burn

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ABSTRACT

Background: Damage to the peripheral nervous system influences wound healing and, after a deep burn, imperfect cutaneous nerve regeneration occurs. A third-degree burn model was developed in rats combined with the use of resiniferatoxin (RTX), known to promote sensory neuropathy.

Methods: Rats were injected intraperitoneally either with RTX or vehicle. A mechanical sensory assay and the hot plate thermal sensory test were performed. The structural integrity of the sciatic nerve was assessed using transmission electron microcopy. After RTX injection, third-degree thermal burns were performed. Wound closure was monitored and samples were collected for histological analysis, immunohistochemistry and immunoblotting for neuronal markers.

Results: RTX promoted both mechanical and thermal hypoalgesia. This transient RTX-mediated sensory deficit occurred without damaging the integrity of nerve fibers and induced a significant depletion of neuropeptides in both neuronal bodies and intraepidermal nerve fibers. Although wound closure rates were similar in both groups, the kinetic of granulation tissue remodeling was delayed in the RTX group compared with control group. A significant reduction of the peripherin expression in the RTX group was observed indicating impaired axonal regrowth of small fibers within the wound.

Conclusion: Our study confirms the important roles of innervation during skin healing and the defect of nerve regeneration after burn.

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Abbreviations: α-SMA, α-smooth muscle actin; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion; GAP43, growth associated protein 43; PGP 9.5, protein gene product 9.5; RTX, resiniferatoxin; TRPV1, transient receptor potential vanilloid-1.

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1. Introduction

Representing a protecting interface between our body and the external environment, the skin is a highly sensitive organ innervated by the peripheral nervous system and where specific structures express sensors permitting the perception of a wide range of signals [1]. In particular, the skin is densely innervated by different sensory nerve fiber subtypes which react to temperature variation, pain and tactile stimuli. It is also known that sensory nerve fibers are tightly implicated in a variety of cutaneous physiological and pathological processes [2]. Several clinical observations indicate that damages to the peripheral nervous system influence wound healing [3].

The local destruction of the cutaneous nerve fiber network during a burn leads to an immediate neuropathy, which is obviously more serious in the context of a full-thickness burn. Although nerve fibers may regenerate after spontaneous wound healing and/or skin grafting, abnormal nerve fiber density is often observed, resulting in the alteration of skin sensation [4]. These complications which include sensibility losses, itch, paresthesia and pain are common in the first months following the burn but often gradually decrease with time. However, depending on the anatomic site of the scar or on the injury severity, they can impact the patient's quality of life and even delay their overall rehabilitation [5].

The diabetic model induced by streptozotocin treatment is a commonly used model to study peripheral neuropathy. However, in this model, diabetic neuropathy impairs sensory, autonomic and motor nerves. Small fiber neuropathy is caused by selective damages affecting C and Aδ fibers which are the main peripheral transmitters involved in nociception. In this study, we have used our model developed in mice by administration of resiniferatoxin (RTX) [6,7]. RTX is an ultra-potent capsaicin analog that acts on transient receptor potential vanilloid type 1 (TRPV1) which is a nonselective cation channel mainly expressed in sensory C and Aδ fibers. In this study, we have first completed the description of this model using rats. In addition, we have developed a model of burn in which parameters of temperature, pressure, duration and surface were strictly monitored. By combining these two models, we have analyzed the effects of a small-fiber neuropathy induced by RTX on skin healing and axonal regrowth after burn. After RTX injection, a third-degree thermal burn was performed, wound closure was monitored and samples were collected for histological analysis, immunohistochemistry and immunoblotting for neuronal markers.

2. Material and methods

2.1. *Animals and resiniferatoxin treatment*

Experiments were performed on male Sprague Dawley rats (7–8 weeks old, 320–330 g). All animal manipulations were realized according to recommendations of the European Directive of 22 September 2010 (2010/63/EU) on the protection of animals used for scientific purposes, and the protocols of "Establishment of a model of peripheral sensitive neuropathy" and "Establishment of a model of thermal burns in the rodent" were approved by the Regional Ethical Committee (CREEAL n° 1-2013-1 and n° 09-2014-09 respectively). Animals were randomized into two groups: vehicle ($n = 22$) and resiniferatoxin (RTX) (Sigma–Aldrich, Lyon, France) ($n = 21$). RTX (185 μg/kg) and vehicle (10% DMSO) were injected intraperitoneally under anesthesia with isoflurane 7 days before burn (D-7) (Fig. 1).

2.2. *Resiniferatoxin effect assessment*

2.2.1. *Evaluation of nociceptive behaviors*

To confirm the small fiber neuropathy induced by RTX and to follow it, two sensitive tests were realized at D-8 (24 h before RTX injection), D-7 (just before RTX injection), D-6, D-1, D7, D14, D21, D28, D35 and D42 after burn (Fig. 1).

2.2.1.1. Mechanical nociception. A paw pressure analgesia meter (Bioseb, Vitrolles, France) was used for performing the Randall–Selitto test in order to determine the animal threshold response to pain induced on the paw by the application of a uniformly increasing pressure. After 10 min of acclimatization, a linear increasing pressure was applied on the right hind foot with a cut off at 300 g to avoid tissue injury. The mechanical nociception threshold corresponds to the

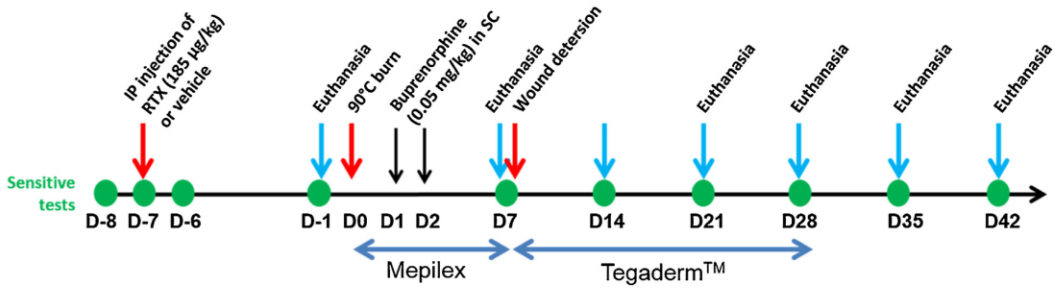


Fig. 1 – Design of the experiments. To assess neuropathy induced by resiniferatoxin (RTX), two sensitive tests were realized at D-8 (24 h before RTX injection), D-7 (just before RTX injection), D-6, D-1, D7, D14, D21, D28, D35 and D42 after burn. Euthanasia was realized at D-1 for transmission electron microscopy analysis of sciatic nerves and at D7, D14, D21, D28, D35 et D42 for dorsal root ganglia (DRGs), hind footpad, and back skin (normal or burned) sampling.

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pressure at which the animal withdraws its foot from the device. Three tests separated by 10 min were performed and mean value of these tests was calculated to represent the mechanical nociception threshold of each animal.

2.2.1.2. *Thermal nociception.* Thermal withdrawal latencies were measured with a 52 °C hot plate (Bioseb, Vitrolles, France). After 10 min of acclimatization, the animal was placed on hot plate until it shows nociceptive behaviors like hind paw shaking or jumping. The cut off was fixed at 30 s to avoid tissue damage. Three tests separated by 10 min were performed and mean value of these tests was calculated to represent the thermal nociception threshold of each animal.

2.2.2. *Transmission electron microscopy*

At day 6 after injection of RTX or vehicle, sciatic nerves of 3 rats of each group were collected and fixed in 2.5% glutaraldehyde diluted in Sorensen buffer for 30 min at room temperature, rinsed and incubated for 30 min at room temperature with a 2% OsO_4 solution. Then, samples were dehydrated in successive alcohol baths and embedded in Epon 812 resin (Euromedex, Souffelweyersheim, France). After polymerization (for 48 h at 60 °C), capsules were removed. Semi-thin sections were stained with toluidine blue. Ultrathin sections (60–100 μm thickness) were made with a diamond knife. Sections were collected on a 200 mesh rhodium grids and incubated with 0.5% uranyl acetate and lead citrate solutions. Sections were examined on a JEM-1011 transmission electron microscope (JEOL, Croissy-sur-Seine, France).

2.2.3. *Immunofluorescence staining*

Dorsal root ganglia (DRGs) were removed, fixed 6 h in 4% paraformaldehyde, cryoprotected in 30% sucrose, embedded in OCT (CellPath, Newtown, Wales, UK) and then frozen at −80 °C. Hind footpad skin samples were also removed with a punch biopsy (3 mm) and similarly treated. Cryostat tissue sections (30 μm-thick sections for footpad skin samples and 10 μm-thick sections for DRGs) were incubated overnight at 4 °C with the primary antibodies recognizing protein gene product (PGP) 9.5 and calcitonin gene-related peptide (CGRP) (Table 1) diluted in a solution composed with phosphate buffered saline (PBS), 2% goat serum, 2% bovine serum albumin (BSA) and 0.3% Triton X-100. Sections were then incubated with the appropriate secondary antibody diluted in PBS during 2 h at room temperature (Table 1). After washing, nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI) during 5 min at room temperature. Sections were examined with a DM IRB inverted fluorescence microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) equipped with specific filters. Images were acquired using NIS-Elements microscope imaging software (Nikon, Champigny-sur-Marne, France).

DRG neurons positive for each antibody were counted. The density of PGP 9.5+ neurons was expressed as the number of neurons per mm^2. The density of peptidergic neurons was expressed as the number of CGRP+ neurons per PGP 9.5 expressing neurons.

The number of intraepidermal nerve fibers (IENFs) was quantified using PGP 9.5 immunostaining of footpad skin sections, by determining the number of fibers crossing over the basement membrane of the epidermis per mm of dermal-epidermal junction analyzed (according to [8]). Among these IENFs, those expressing CGRP were determined.

2.3. *Burn achievement*

At D-1 before burn, the back of animals was shaven and depilated with depilatory cream under isoflurane anesthesia. Just before burn, animals were anesthetized by an intraperitoneal injection of a solution containing ketamine (Mérial, Lyon, France) (100 mg/kg) and xylazine (Bayer, Lyon, France) (4 mg/kg). The burn was realized by applying on the back of animal, on one side of the spine, a metal piece (7 mm × 12 mm) heated at 90 °C during 45 s with a force of 0.6 N. Just after burn, animals received subcutaneous injection of buprenorphine (0.05 mg/kg) and during two days after burn (two injections/day). After burn, animals were individually

Table 1 – Antibodies used for immunofluorescence staining, immunohistochemistry and Western blotting.

Primary antibodies* used for immunofluorescence staining[a], immunohistochemistry[b] and Western blotting[c]	Dilution	Reference
Rabbit polyclonal anti-protein gene product (PGP) 9.5 [a,c]	1/500	UltraClone Limited (Island of Wight, UK): RA95101
Mouse monoclonal anti-calcitonin gene-related peptide (CGRP) [a]	1/200	Abcam (Cambridge, UK): ab81887
Rabbit polyclonal anti-growth associated protein 43 (GAP43) [a]	1/500	Millipore (Molsheim, France): AB5220
Mouse monoclonal anti-α-smooth muscle (SM) actin [b]	Ready to use	Cell Marque (Rocklin, CA, USA): CMC 28330020
Mouse monoclonal anti-peripherin [c]	1/5000	Abcam: ab129007
Mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [c]	1/10000	Sigma-Aldrich (Lyon, France): G8795
Secondary antibodies used for immunofluorescence staining		
Goat polyclonal anti-mouse (AF594)	1/1000	Invitrogen (Cergy-Pontoise, France): A11005
Goat polyclonal anti-rabbit (AF488)	1/1000	Invitrogen: A11008
Secondary antibodies used for immunoblotting		
Goat polyclonal anti-rabbit IgG HRP	1/1000	Dako (Glostrup, Denmark): P0448
Goat polyclonal anti-mouse IgG HRP	1/1000	Dako: P0447

* All these primary antibodies have been extensively used and their specificity has been clearly documented.

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housed and were treated with paracetamol (1 mg/ml) in the drinking water.

To avoid injury of the wound by animals, a dressing was applied. It first consisted in the application of Flammazine® cream (Sinclair Pharma, Paris, France) on the burn. Then, the wound was covered by Mepilex transfer™ (Mölnlycke health care, Wasquehal, France) which was maintained by Nylex fix™ (Urgo, Chenove, France) overcovered by Urgoderm™ (Urgo) and Urgo strapping (Urgo). This dressing was changed every two days.

After one week, wounds were cleansed under isoflurane anesthesia and a new dressing was used: after Flammazine® cream application on wound, Tegaderm film™ (3M, Cergy-Pontoise, France) was applied and maintained by Urgo strapping (Urgo). This dressing was changed every two days until euthanasia. Animals were euthanized at day 7, 14, 21, 28, 35, and 42 after burn (Fig. 1). For euthanasia, animals were anesthetized as above by an intraperitoneal injection of a solution containing ketamine (Mérial) and xylazine (Bayer) and an intracardiac perfusion of PBS was realized. For each animal, burned and normal skin, DRGs and footpad skin were collected.

2.4. *Burn wounding analysis*

2.4.1. *Histological analysis*

Samples of burned and normal skin were collected and fixed overnight in 10% formalin solution and embedded in paraffin. Sections of 4 μm were stained with Masson's trichrome and hematein-eosin-safran solutions. Sections were scanned using the NanoZoomer 2.0-RS device (Hamamatsu Photonics, Hamamatsu City, Japan).

2.4.2. *Immunohistochemistry*

Four μm-thick formalin fixed paraffin embedded tissue sections were realized. A mouse monoclonal antibody recognizing α-smooth (SM) actin (Table 1) was used. Immunohistochemistry was performed on a BenchMark XT instrument (Ventana Medical Systems, Tucson, Arizona) using iView/DAB detection system (Ventana Medical Systems). Slides were counterstained with hematoxylin and bluing reagent (Ventana Medical Systems) and scanned as above.

2.4.3. *Immunofluorescence staining*

Burned and normal skin samples were collected, fixed 6 h in 4% paraformaldehyde, cryoprotected in 30% sucrose, embedded in OCT (CellPath) and then frozen at −80 °C. Then, immunostaining was performed on 30 μm-thick cryostat sections as described above (Section 2.2.3) using a primary antibody recognizing growth associated protein 43 (GAP43) (Table 1).

2.4.4. *Western blotting*

Biopsies of normal and burned skin were collected and grinded in RIPA lysis and extraction buffer (150 mM NaCl, 1% triton, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8) with stainless steel balls using MM300 TissueLyser (Retsch France, Verder SARL, Eragny-sur-Oise, France) (2 cycles of 2 min). Samples were centrifuged at 10,000 rpm during 20 min at 4 °C. Supernatants were collected and stored at −80 °C. Quantification of total protein concentration was performed using Bradford method. The samples (20 μg of proteins/well) was applied to 12% acrylamide gel, proteins were separated (125 V for 2 h) and then transferred to polyvinylidene fluoride membrane (Bio-Rad, Marnes-la-Coquette, France) for 10 min at 1.3 A using Trans-Blot® Turbo™ Transfert Blotting System (Bio-Rad). Membranes were blocked in solution composed by 5% milk in tris-buffered saline with Tween 20 (TBS-T) at 0.1% for 1 h at room temperature. Membranes were then incubated with primary antibodies recognizing PGP 9.5, peripherin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1) diluted in 5% milk in TBS-T overnight stirring at 4 °C. GAPDH was used as a loading control. After incubation, membranes were washed three times for 10 min with TBS-T and incubated with secondary antibodies (Table 1) diluted in 5% milk in TBS-T during two hours at room temperature. Subsequently, membranes were washed three times for 10 min with TBS-T. Detection was performed with Clarity™ Western ECL Substrate (Bio-Rad).

2.5. *Statistical analysis*

Data were presented as means ± SD and significant level was set at $p < 0.05$. Depending of experiments, data were compared using an unpaired t test or two ways repeated measures ANOVAs followed by Bonferroni's *post hoc* test by means of the GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, USA). Microscope pictures were analyzed using image J software (NIH, USA) and scanned tissue sections were analyzed using the NDP.view 2.5 software (Hamamatsu Photonics, Hamamatsu City, Japan).

3. Results

3.1. *Effect of resiniferatoxin treatment on thermal and mechanical sensitivity and on nerve morphology*

The effect of RTX injection on peripheral small nerve fibers was investigated overtime using nociceptive tests. Mechanical nociception was assessed by measuring Randall–Selitto paw pressure withdrawal latencies. Results highlighted transient hypoalgesia in the RTX-treated animals compared with the control animals from day 1 after RTX injection up to day 14 after RTX injection (Fig. 2A). Similarly, results of the hot plate test showed thermal hypoalgesia in RTX-treated animals compared with control animals. The thermal hypoalgesia was more pronounced and also reversible as it started from day 1 after RTX administration and lasted up to day 28 after RTX injection (Fig. 2B). The ultrastructural examination of sciatic nerve sections was assessed by transmission electron microscopy at day 6 after RTX injection. Our results showed that unmyelinated fiber morphology was unchanged after RTX injection when comparing control rats (Fig. 3A) with RTX-treated rats (Fig. 3B). The structure and integrity of non-myelinating Schwann cells protecting small fibers bundles was also unaffected by the administration of RTX.

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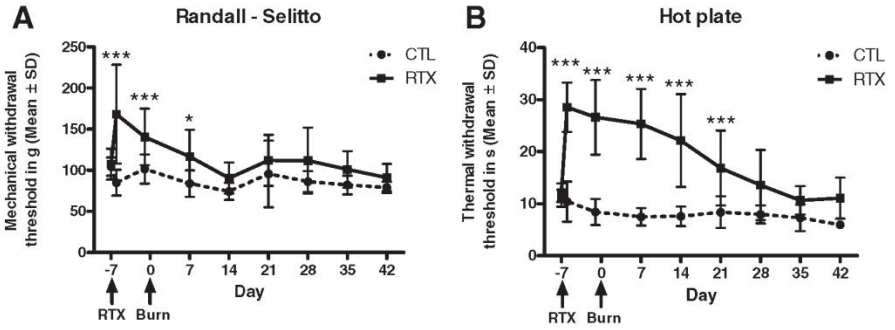


Fig. 2 – Effect of resiniferatoxin (RTX) on mechanical and thermal nociception. (A) Randall–Selitto mechanical nociception; (B) Hot plate thermal nociception. Resiniferatoxin (RTX) induces significant mechanical and thermal hypoalgesia, which is reversible. With time, mechanical (day 14 after RTX injection) and thermal (day 28 after RTX injection) nociception are restored. $^{***}p < 0.001$, $^{*}p < 0.05$.

3.2. *Effect of resiniferatoxin treatment on calcitonin gene-related peptide expression in dorsal root ganglia neurons and intraepidermal nerve fibers*

At D14 after burn, immunohistochemistry analysis of neuronal markers was performed on DRGs and highly innervated footpad glabrous skin in order to further confirm if the small fibers neuropathy induced by RTX was still ongoing. Indeed, cutaneous sensory nerve fibers are endings of DRGs neurons that carry signals from sensory organs toward the appropriate integration center of the brain or of the spinal cord. The density of DRG neuronal bodies positive for PGP 9.5 was not affected by the RTX treatment (Fig. 4A) confirming that the integrity of neuronal cell bodies was preserved. However, when looking at the CGRP neuropeptide, compared with control animals, at D-1 and D14, a significant reduction of its expression was observed in RTX-treated animals and was translated by a significant reduction of the CGRP+ neurons/PGP 9.5+ ratio in the RTX group (Fig. 4A). Thereafter, at D28, no difference was observed between RTX-treated animals and control animals.

We then examined IENFs located in the footpad skin and our results showed no difference in PGP 9.5+ IENFs/mm when comparing both groups (data not shown). However, at D-1, D14 and D28, a significant reduction of CGRP+ IENFs/mm was observed in RTX-treated rats compared with control animals (Fig. 4B). Thereafter, at D35, no difference was observed between RTX-treated animals and control animals.

These results confirmed a partial depletion of CGRP in both cell bodies and axons of small fibers and the lasting establishment of the RTX mediated neuropathy.

3.3. *Effect of resiniferatoxin-mediated small fibers neuropathy on wound closure kinetics and healing processes*

Seven days after RTX injection and while the RTX-mediated small fibers neuropathy is well established, a third-degree thermal burn was performed on the dorsal skin of both

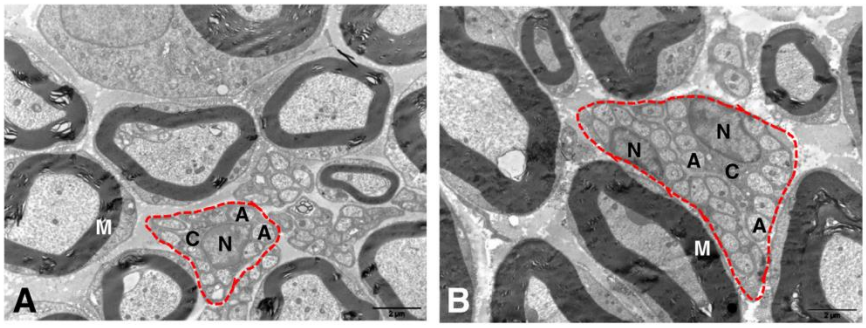


Fig. 3 – Sciatic nerve transmission electron microscopy 6 days after resiniferatoxin (RTX) injection. (A) Control rat; (B) RTX-treated rat. The large myelinated nerve fibers and the non-myelinating Schwann cells (red dotted lines) which surround several small diameter axons, ensheathing each axon in a pocket of its cytoplasm, forming a Remak bundle, are preserved. A, axoplasm; C, non-myelinating Schwann cell cytoplasm; M, myelin; N, non-myelinating Schwann cell nucleus. $n = 3$ in each group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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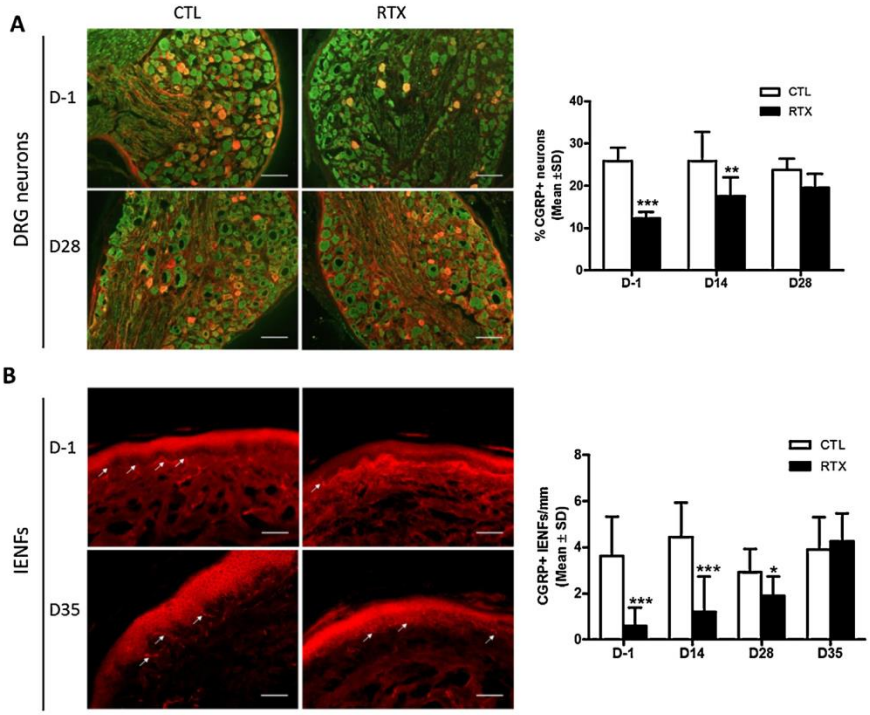


Fig. 4 – Effect of resiniferatoxin (RTX) on dorsal root ganglia (DRGs) neurons and intraepidermis nerve fibers (IENFs) expressing calcitonin gene-related peptide (CGRP). (A) DRGs of control (CTL) and RTX-treated groups are immunostained for PGP 9.5 (green) and CGRP (red). The % of CGRP+ neurons is quantified. A significant reduction of CGRP+ neuronal cell bodies in RTX-treated animals is observed at day 6 and 21 after injection. At day 35 after injection, the CGRP expression in DRG neurons is restored. (B) Footpad skin samples of CTL and RTX-treated groups are immunostained for CGRP (red). The number of CGRP+ IENFs/mm is quantified. A significant reduction of CGRP+ IEFNs in RTX-treated animals is observed at day 6, 21 and 35 after injection. At day 42, the CGRP expression in IENFs is restored. $^{***}p < 0.001$, $^{**}p < 0.01$,$^{*}p < 0.05$. Scale bar = 500 μm. $n = 3$ in each group and at each time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

RTX-treated and control rats using a 90 °C heated template. First, the histology analysis of wounded skin tissues confirmed the establishment of a third-degree burn reaching the *panniculus carnosus* underneath the dermis. (Fig. 5A). The cutaneous healing process in rodents mainly involves a strong contraction of the wound due to the contractile activity of activated myofibroblasts expressing α-smooth muscle (SM) actin [9]. Wound closure kinetics was followed by measuring the wounded area overtime but no macroscopic difference was observed when comparing control animals and the RTX-treated animals (Fig. 5B).

We then examined microscopically wounded tissue samples at specific time points and using trichrome staining and immunohistochemistry for α-SM actin in order to investigate if the different healing phases were affected by the RTX treatment. Importantly, the detailed examination of the wound at D14 after burn highlighted delayed granulation tissue remodeling in RTX-treated rats (Fig. 6B) compared with control animals (Fig. 6A). The extant and high cellularity of the granulation tissue was more pronounced in RTX-treated rats and the remodeling of the collagen composing the dermal extracellular matrix were more advanced in control animals (Fig. 6A and B). At D14 after burn, the expression of α-SM actin was always important in RTX-treated rats (Fig. 6D) compared with control animals (Fig. 6C), again underlining the delay in the remodeling of the granulation tissue observed in RTX-treated rats. To finish, at D14 after burn, the re-epithelialization process was also delayed in RTX-treated animals (Fig. 6B) compared with control rats (Fig. 6A).

3.4. *Effect of resiniferatoxin-mediated small fiber neuropathy on peripherin expressing small fibers within the wound*

We analyzed the expression of PGP 9.5 and of peripherin in the healing tissue by Western blot. In control animals, our results showed an important increase of PGP 9.5 and peripherin

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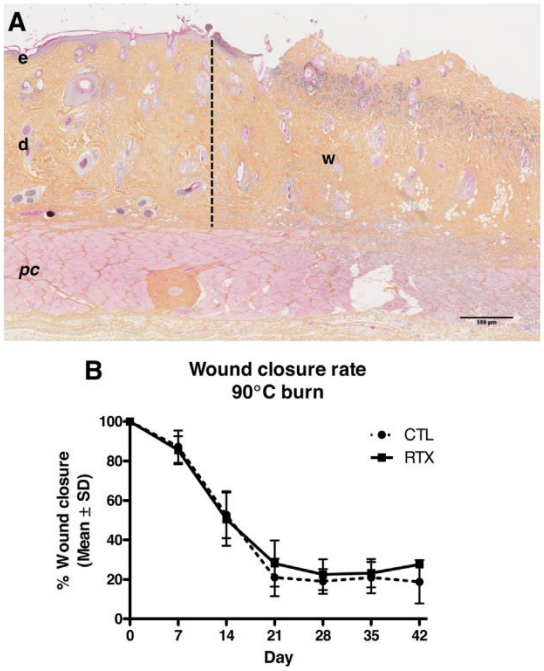


Fig. 5 – Third-degree burn and subsequent wound closure kinetic. (A) Representative photo of the third-degree burn establishment (arrow) using hematein-eosin-safran staining. e, epidermis; d, dermis; pc, *panniculus carnosus*; w, wound. (B) Wound closure rate followed macroscopically. No macroscopic difference is observed when comparing control (CTL) animals and the resiniferatoxin (RTX)-treated animals.

expression between D-1 and D14 ($p < 0.001$ for both PGP 9.5 and peripherin) with a decrease thereafter between D14 and D28 ($p < 0.001$ and $p < 0.01$ respectively) (Fig. 7A and B). At all times point studied, a similar pattern for the expression of PGP 9.5 was observed in RTX-treated animals with an important increase between D-1 and D14 ($p < 0.05$) and a decrease thereafter between D14 and D28 ($p < 0.05$) (Fig. 7A). More interestingly, a significant reduction ($p < 0.001$) of the expression of peripherin was observed in RTX-treated animals compared with control rats at D14 after burn (Fig. 7B).

3.5. *Effect of resiniferatoxin-mediated small fiber neuropathy on nerve regrowth and plasticity within the wound*

Nerve regrowth and plasticity was studied by immunofluorescence using the expression of GAP43 which is known to be expressed during axonal regeneration and synaptic plasticity modulation [10,11]. At D14 after burn, the expression of GAP43 was very low in both control (Fig. 8A) and RTX-treated animals (Fig. 8B). At D28 after burn, there was an important increase of GAP 43 expression in control animals (Fig. 8C) which was not affected by RTX treatment (Fig. 8D).

4. Discussion

In this study, a third-degree burn model was developed on rats combined with the use of RTX, a potent TRPV1 agonist known to promote sensory neuropathy affecting small fibers.

We confirmed that RTX has a direct impact on small fibers and induced reversible mechanical and thermal hypoalgesia. RTX induced a systemic but transient small fibers neuropathy and the reversibility of this effect was a first indication that the integrity of the nerve fibers was preserved. Indeed, using transmission electron microscopy, we confirmed that the integrity of the sciatic nerve morphology was preserved after RTX-treatment. In addition, the percentage of PGP 9.5 expressing neurons in DRGs and the number of IENFs in footpad were not modified. In contrast, an important decrease of CGRP expression was observed both in DRG neurons and in footpad IENFs indicating a depletion of this neuropeptide in these structures after RTX-treatment. Interestingly, using CGRP expression, the reversibility was observed 35 days after RTX injection in DRG neurons but 42 days after RTX treatment in IENFs, underlining that, to restore CGRP content, there is a shift between deep structures such as DRG neuronal cell bodies and peripheral structures such as IENFs. However, this CGRP depletion does not seem to affect permanently the skin sensitivity because using the mechanical Randall–Selitto sensory assay and the hot plate thermal sensory test, the reversibility of the RTX effect was observed at day 14 and day 28 respectively after RTX treatment. In contrast, CGRP depletion could definitively affect wound healing.

To analyze the effect of a neuropathy on skin wound healing processes, we have set up a third-degree burn model. In contrast with humans in which a third-degree burn is not usually able to heal without grafting, third-degree burned rats heal spontaneously (obviously depending on the surface of burned skin). Indeed, after burn, we have observed that RTX treatment induced a delay in the sequence of the different phases classically described during wound healing, *i.e.* (i) inflammatory and vascular phase with the deposition of a provisional matrix, (ii) proliferation phase with the development of the granulation tissue and the reepidermization, and (iii) remodeling phase with scar formation [12]. The evolution of the granulation tissue is a good index to appreciate the quality of the wound healing process. Indeed, in normal condition, during granulation tissue remodeling, myofibroblasts expressing α-SM actin disappear by apoptosis and the extracellular matrix is reorganized to progressively become almost similar to the extracellular matrix present in normal dermis [13]. Fourteen days after RTX treatment, myofibroblasts had disappeared in control animals but were still observed in RTX-treated animals. In parallel, we have shown that RTX induced a depletion in CGRP present in skin nerves. We can suggest that the decrease of neurogenic inflammation materialized by CGRP depletion and neuropathy could induce this delay in the progress of healing. Without the presence of the neuroinflammatory "soup" released by nerve endings in which different neuropeptides such as CGRP are present [14],

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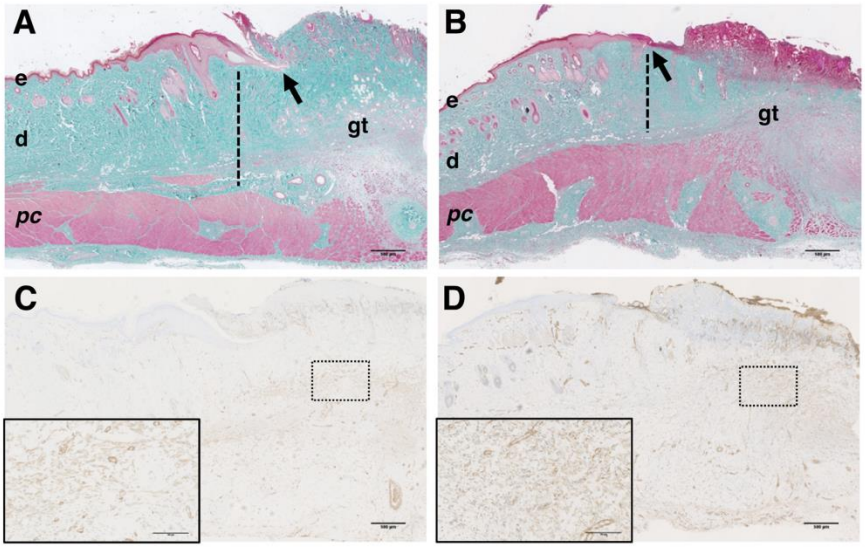


Fig. 6 – Wound histology during healing after burn. Representative microscopic structure of wound samples at D14 after burn using Masson's trichrome staining (A, B) and α-smooth muscle (SM) actin immunohistochemistry (C, D) in control (A, C) and resiniferatoxin (RTX)-treated (B, D) animals. e, epidermis; d, dermis; pc, *panniculus carnosus*; gt, granulation tissue. A high cellularity with less collagen in the granulation tissue is observed in RTX-treated rats (B) compared with control animals (A). The expression of α-SM actin is always important in RTX-treated rats (D) compared with control animals (C). This underlines the delay in the remodeling of the granulation tissue observed in RTX-treated rats. In addition, the re-epithelialization process (arrows) is delayed in RTX-treated animals (B) compared with control rats (A).

the inflammatory phase is less efficient and the development/remodeling of the granulation tissue is delayed. Indeed, it has been observed that delayed wound healing occurred in animal models after surgical resection of cutaneous nerves and patients with cutaneous sensory defects due to lepromatous leprosy, spinal cord injury, and diabetic neuropathy develop ulcers that fail to heal because inflammatory phase persists and do not allow the development of the granulation tissue [15].

Concerning the regeneration of cutaneous nerve endings, it is classically admitted that it can occur with the migration of new nerve fibers from the wound bed or from the collateral sprouting of nerve fibers from the adjacent uninjured area. It has been shown that, during development, nerve fibers provide a template to pattern arteriogenesis [16] and we can then suggest that, in skin healing, the nerve regeneration occur in association with regeneration of blood vessels at the wound

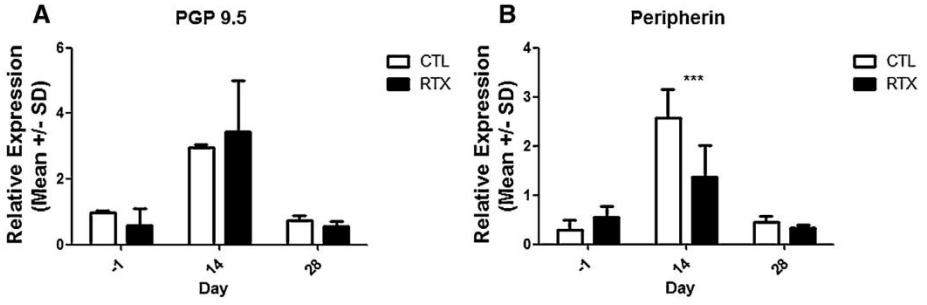


Fig. 7 – Western blotting for PGP 9.5 and peripherin in wound tissue. PGP 9.5 (A) and peripherin (B) expression was studied 24 h before burn and at D14 and D28 after burn in control (CTL) and resiniferatoxin (RTX)-treated animals. In CTL animals, an important increase of PGP 9.5 (A) and peripherin (B) expression between D-1 and D14 and a decrease thereafter between D14 and D28 are observed. At all times point studied, a similar pattern for the expression of PGP 9.5 was observed in RTX-treated animals with an important increase between D-1 and D14 and a decrease thereafter between D14 and D28 ($p < 0.05$) (A). A significant reduction (*$p < 0.001$) of the expression of peripherin was observed in RTX-treated animals compared with control rats at D14 after burn (B).**

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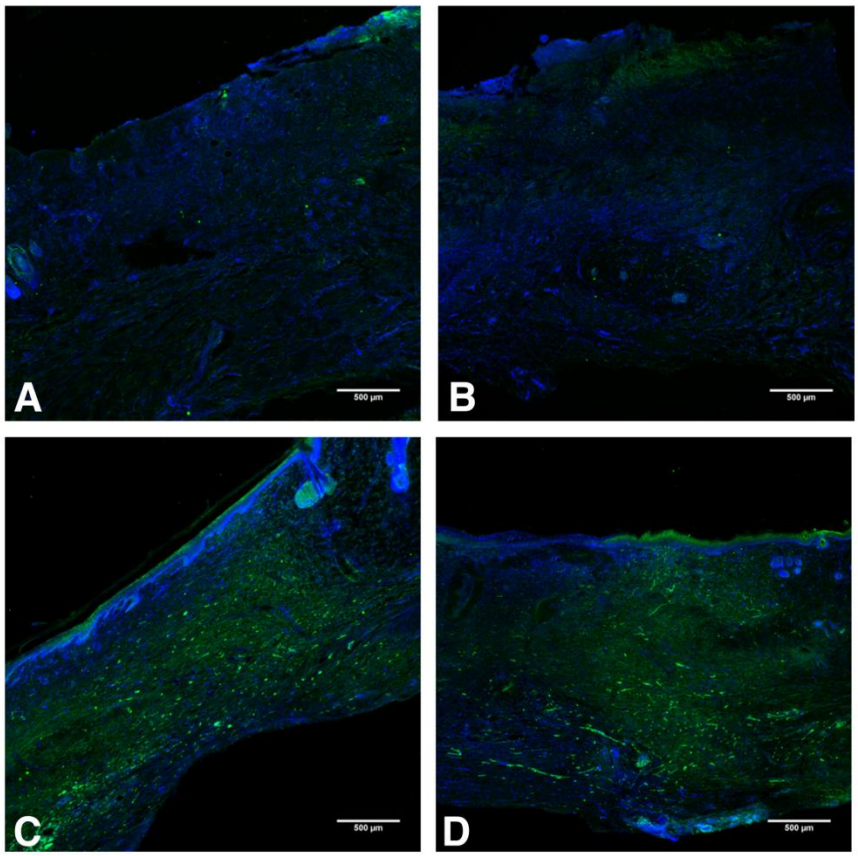


Fig. 8 – GAP43 immunofluorescence in wound tissue. Representative photo of GAP43 (green) expression in the wound at D14 (A, B) and D28 (C, D) after burn in control animals (A, C) and resiniferatoxin (RTX) treated animals (B, D). Nuclei are stained with DAPI (blue). At D14 after burn, the expression of GAP43 is very low in both control (A) and RTX-treated animals (B). At D28 after burn, an important increase of GAP 43 expression in control animals (C) which was not affected by RTX treatment (D) is observed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

margins. However, this nerve regeneration process is imperfect, resulting in the alteration of skin sensation and sometimes chronic cutaneous pain. Analysis of nerve regrowth after burn has showed that the total nerve density in a normotrophic burn scar never reaches pre-burn levels [17]. In a study using guinea pigs, Kishimoto showed that following a burn, the number of the substance P-containing nerve fibers decreased acutely after the burn; then, the density gradually increased to peak at D14 after burn. Following that peak period, the density of the fibers gradually decreased to end up lower than controls [18]. This leads to a variety of sensory deficits and abnormal sensations, as outlined in various clinical and histological studies [15]. Interestingly, in hypertrophic scars where the granulation tissue is not remodeled, even if data in the literature are not coherent, it is usually admitted that the number of nerve fibers is increased [19,20] as if the decrease observed by Kishimoto [18] was not observed in hypertrophic scars. This decrease in the number of nerve fibers takes place in parallel with the remodeling of the granulation tissue, remodeling which does not develop in hypertrophic scars.

GAP43 expression was studied to investigate nerve fiber regrowth and plasticity. GAP43, also known as neuromodulin, is a neural-specific calmodulin binding protein and a major protein kinase C substrate found in developing and regenerating neurons [21]. Interestingly, at D14 after burn, when the expression of PGP 9.5 and of peripherin was important, the expression of GAP43 was very low, illustrating that nerve fiber growth during the 14 days after damage does not require the involvement of GAP43. We can assume that this innervation rapidly established allows the delivery of neuropeptides (neuroinflammatory "soup") important for the development of the inflammatory phase. However, this innervation is not functional (from a sensitive point of view) and it is certainly the reason why it disappears between D14 and D28 after burn

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as shown by the study of Kishimoto [18] and by our study. In contrast, at D28 after burn, the expression of GAP 43 is important while the expression of PGP 9.5 and peripherin drops. It might illustrate the rearrangement of a new nerve fiber tree during the granulation tissue remodeling. During the early phase of formation of the granulation tissue, the disordered extracellular matrix rapidly deposited appears favorable for small fiber growth. However, later, during granulation tissue remodeling and maturation, an important decrease of the nerve fiber density is observed. And, in parallel, the GAP 43 expression develops suggesting that the progressive development of a more physiological extracellular matrix involves the remodeling and plasticity of the nerve fiber network within the wound.

5. Conclusions

he standardization and reproducibility of an experimental model of peripheral neuropathy was necessary to study the effects of this neuropathy on wound healing as peripheral neuropathy induced in animals treated with streptozotocin is not specific to small fibers. We have developed in rats a reversible model of small fiber neuropathy in which nerve morphology was preserved but a neuropeptide deletion was observed in cell bodies and nerve endings of dorsal root neurons. This model specifically affected small fibers. Using a model of cutaneous burn, we have seen that during inflammatory phase and granulation tissue development, an important innervation developed within the wound tissue but disappeared thereafter when the granulation tissue remodeling progressively occurred. This innervation could participate to the delivery of neuropeptides important for the healing process and it is probably the reason why, after RTX-treatment which induced a neuropeptide depletion, the sequence of the healing process was delayed. Then, during granulation tissue remodeling, innervation is reorganized, process certainly facilitated by the modification of the extracellular matrix. A more normal organization is progressively established in order to reacquire the best physiological skin structure. All these observations suggest that innervation is involved in the different processes occurring during wound healing.

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Author's contribution

BL, DG and AD designed the experiment. BL, DG, AB and NB conducted the experiment. BL, DG, AB and AD analyzed data. AD wrote manuscript. BL, DG, CD, and AD revised manuscript. All authors approved the final manuscript.

Conflict of interest

None declared.

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I – 4 Résultats complémentaires : analyse de la brûlure à 70°C

Les mêmes analyses de cicatrisation et de réinnervation ont été réalisées sur les plaies des brûlures à 70°C.

I – 4.1 Détermination du degré de la brûlure à 70°C.

L'analyse des plaies réalisées par l'application d'une pièce de métal chauffée à 70°C pendant 45 secondes avec une force de 0,6 N sur le dos des animaux nous a permis de déterminer le degré de brûlure obtenu avec ces paramètres. Comme on peut le voir sur la figure 17, on observe une destruction de l'épiderme et d'une partie du derme sans atteinte du *panniculus carnosus*. Nous sommes donc en présence d'une brûlure du second degré profond.

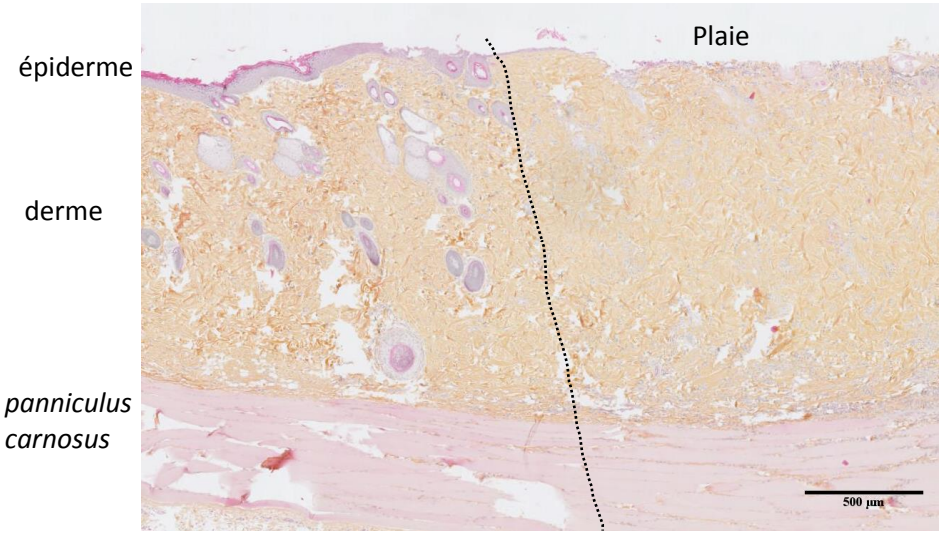


Figure 17 : Coloration HES d'une coupe de plaie à 70°C 7 jours après réalisation de la brûlure.

I – 4.2 Analyses de la fermeture de la plaie à 70°C

La cinétique de fermeture de la plaie à 70°C a été suivie chez les animaux présentant ou non une neuropathie. Macroscopiquement, la vitesse de cicatrisation est semblable dans les deux groupes d'animaux (Figure 18).

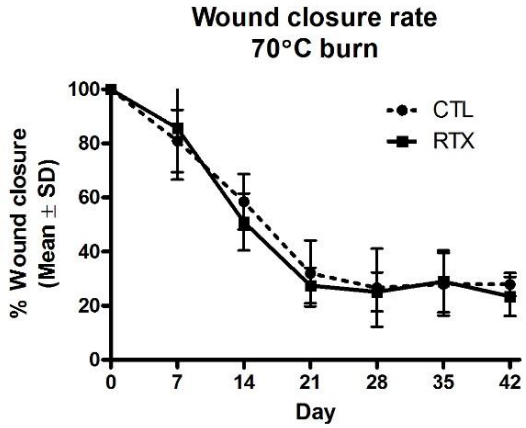


Figure 18 : Cinétique de fermeture des plaies à 70°C chez les animaux présentant ou non une neuropathie.

Pour approfondir l'analyse de la cicatrisation, une étude microscopique des plaies à 70°C a été réalisée à 14 jours après la brûlure (Figure 19). Les figures 19 A et B représentent une coloration au trichrome de Masson des coupes de plaies. On observe ici un retard de cicatrisation chez les animaux présentant une neuropathie (Figure 19B). En effet, comme pour la brûlure du troisième degré, on remarque, chez ces animaux, un tissu de granulation plus important et contenant davantage de cellules. De plus, on note également un retard dans le processus de réépithélialisation chez les animaux ayant reçu la RTX.

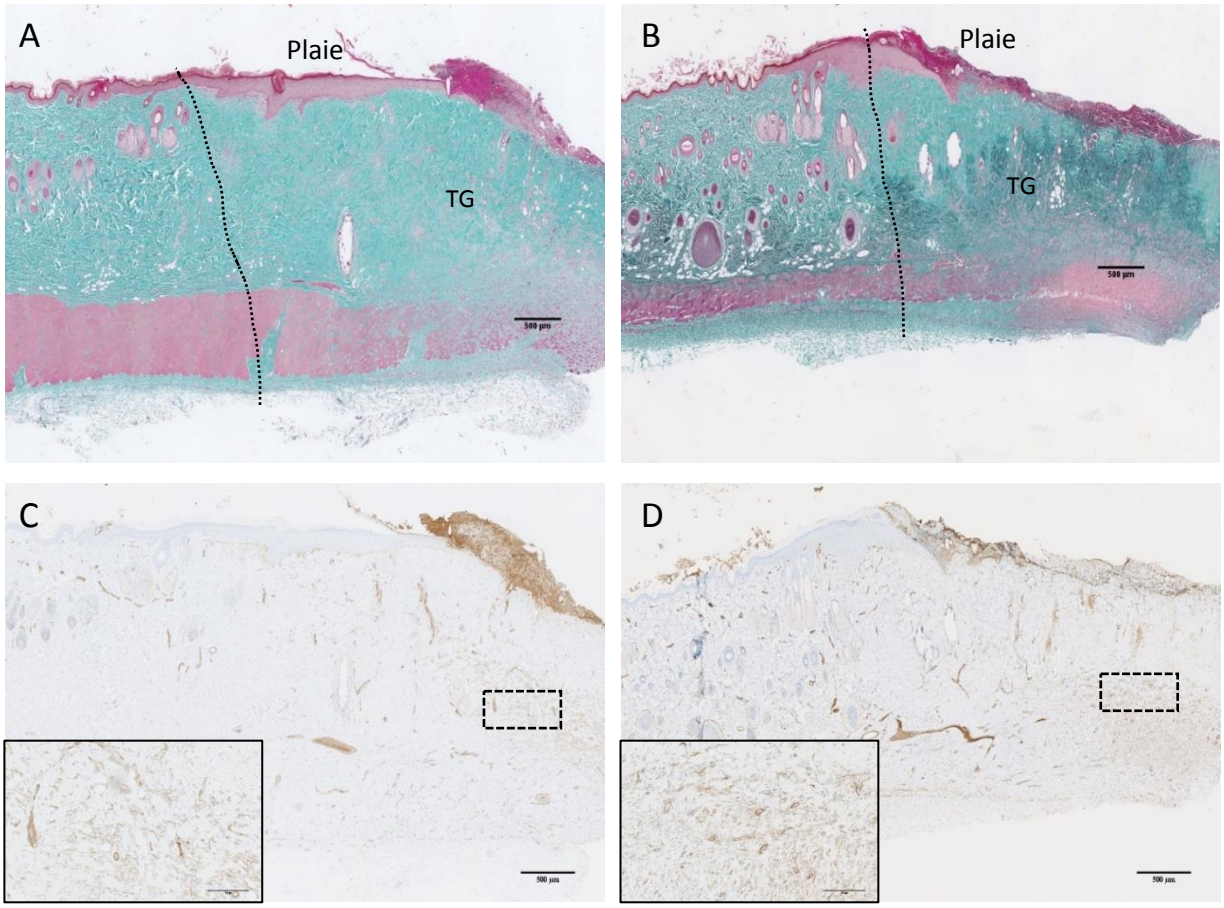


Figure 19 : Histologie des plaies à 70°C 14 jours après la brûlure. Analyse microscopique de la structure de la plaie après coloration au trichrome de Masson (A, B) et immunohistochimie dirigée contre l'actine α-ML (C, D) chez les animaux contrôles (A, C) et chez les animaux ayant reçu la RTX (B, D). TG : tissu de granulation.

Pour compléter cette étude, un immunomarquage dirigé contre l'actine α-ML a été réalisé (Figure 19 C et D). On note une plus forte expression de l'actine α-ML dans le tissu de granulation des animaux ayant reçu la RTX (Figure 19 D) par rapport aux animaux contrôles. Ceci confirme un retard dans le remodelage du tissu de granulation chez les animaux présentant une neuropathie.

Ces premières analyses montrent donc que malgré le fait que la cinétique de fermeture de la plaie soit semblable dans les 2 groupes, l'aspect microscopique des plaies permet de distinguer un retard dans l'évolution des différentes phases impliquées dans la cicatrisation chez les animaux présentant une neuropathie.

I – 4.3 Etude de la réinnervation lors de la cicatrisation après une brûlure du deuxième degré

Pour compléter cette étude sur la cicatrisation, une évaluation de la réinnervation au sein de la plaie a été effectuée par western blot (Figure 20). Comme on peut le voir sur cette figure, la quantité totale d'innervation (évaluée grâce à l'expression de la PGP 9.5) au sein de la peau avant brûlure (J-1) ou au cours du processus de cicatrisation est identique dans les deux groupes au cours du temps (Figure 20 A). Lorsque l'on s'intéresse spécifiquement aux petites fibres sensitives (périphérine +), on observe une forte augmentation de l'expression de ce marqueur dans la plaie des animaux contrôles 14 jours après la brûlure. Cette augmentation est transitoire puisque 28 jours après la brûlure, l'expression de la périphérine est identique au taux avant brûlure chez les animaux contrôles. Cette augmentation d'expression de la périphérine dans la plaie n'est pas observée chez les animaux présentant une neuropathie (traités avec la RTX) (Figure 20 B).

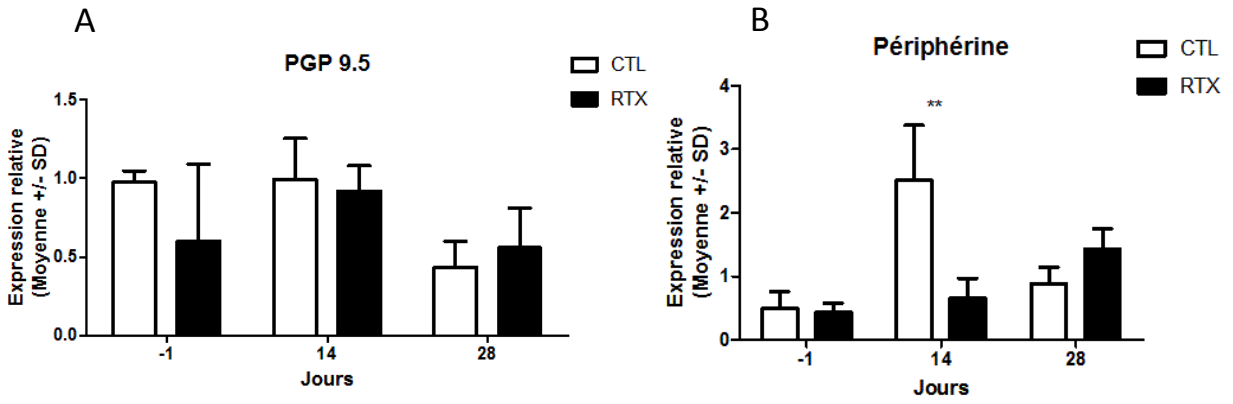


Figure 20 : Analyse de l'innervation au cours du processus de cicatrisation après une brûlure du 2ème degré, par western blot. Expression relative de l'expression de la PGP 9.5 (A) et de la périphérine (B) dans les plaies des animaux contrôles et traités avec la RTX au cours du temps.

En plus de cette étude par Western Blot, une analyse par immunofluorescence dirigée contre GAP-43 a été réalisée (Figure 21).

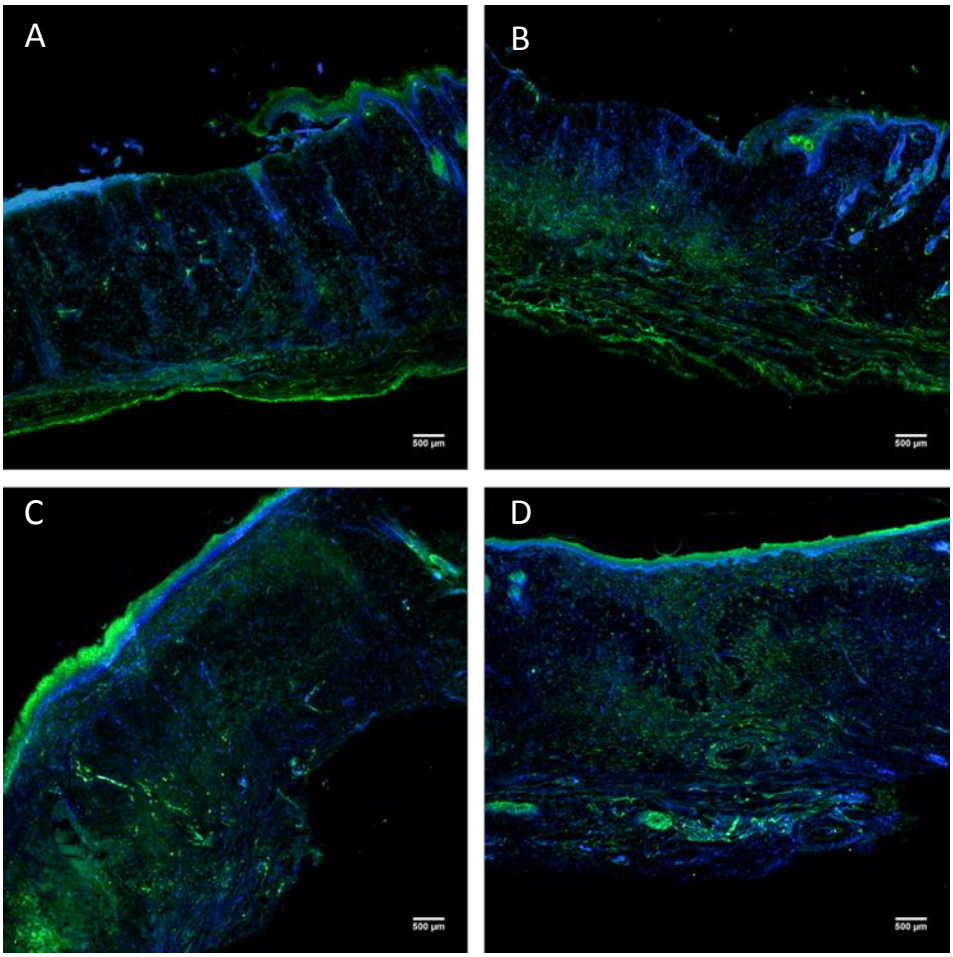


Figure 21 : Immunofluorescence dirigée contre GAP-43 (vert) dans la plaie de la brûlure à 70°C chez les animaux contrôles (A, C) et les animaux traités à la RTX (B, D). L'analyse a été effectuée à 14 jours (A, B) et à 28 jours (C, D) après la brûlure.

L'expression de GAP-43, dans le tissu cicatriciel après une brûlure du 2ème degré, est présente dès 14 jours après la brûlure chez les animaux présentant ou non une neuropathie. Cette expression est augmentée à 28 jours après la brûlure sans que l'on ne puisse observer de réelles différences entre les animaux contrôles et les animaux ayant reçus la RTX.

I – 5 Discussion

L'objectif de ce travail était de s'intéresser à la cicatrisation cutanée après une brûlure thermique et de déterminer le rôle de l'innervation dans ce processus de réparation. En effet, de nombreuses brûlures graves ont lieu chaque année chez les civils et les militaires. Même si la médecine a fait beaucoup de progrès dans le traitement de ces brûlures et permet aujourd'hui de sauver de nombreux patients, la qualité de la réinnervation de la plaie n'est pas satisfaisante conduisant le plus souvent à des handicaps importants. Il est donc important de comprendre pourquoi la réinnervation est défectueuse après une brûlure thermique profonde afin de faire progresser les traitements actuels pour ainsi améliorer la qualité de vie des patients brulés.

Pour déterminer le rôle de l'innervation dans la cicatrisation, nous avons développé un modèle de neuropathie chez le rat. Ces animaux étaient par la suite brulés et nous avons suivi la qualité de la cicatrisation et de la réinnervation après la brûlure.

La neuropathie a été induite par l'injection de RTX en intrapéritonéale à une dose unique de 185 µg/kg. Les résultats obtenus avec ce modèle montrent que la neuropathie se développe rapidement après l'injection et que celle-ci est transitoire. La RTX en se fixant sur son récepteur TRPV1, induit une entrée massive de calcium dans la cellule responsable de la libération de neuromédiateurs par les terminaisons nerveuses. C'est ce que l'on a pu observer dans notre modèle puisque l'expression de CGRP dans les fibres intraépidermiques et dans les GDRs est fortement diminuée après l'injection de RTX. De plus, dans notre modèle, aucune dégénérescence des fibres nerveuses n'a été observée.

Après avoir établi notre modèle de neuropathie, les animaux ont été brulés thermiquement par l'application d'une pièce de métal sur le dos de l'animal. Les paramètres de température, de surface, de temps et de pression ont été bien déterminés et respectées à chaque brûlure pour obtenir des brûlures similaires entre chaque animal. D'autres modèles de brûlures thermiques existent, notamment les brûlures réalisées à l'eau chaude (Silva et al., 2013; Fantinati et al., 2016). L'application d'une pièce de métal pour réaliser la brûlure permet de contrôler strictement la surface de brûlure ce qui n'est pas toujours le cas lors des brûlures à l'eau chaude. Il existe également d'autres types de brûlures, notamment les brûlures chimiques (par exemple à l'ypérite) que subissent souvent les militaires. Pour reproduire ces brûlures chez les animaux et permettre ainsi de les étudier, des locaux spécifiques sont nécessaire pour éviter toute atteinte de l'expérimentateur.

Les paramètres que nous avons choisis pour la réalisation des deux brûlures nous ont permis de réaliser des brûlures du deuxième degré (70°C) ou du troisième degré (90°C). La cicatrisation et la réinnervation de la plaie ont ensuite été suivies au cours du temps. Le but était de comprendre le rôle de l'innervation dans la cicatrisation. En effet, il est maintenant admis que le défaut d'une innervation fonctionnelle perturbe le processus de cicatrisation. C'est par exemple le cas chez les diabétiques qui présentent après plusieurs années de maladie une neuropathie périphérique. La présence de cette neuropathie est en partie responsable du défaut de cicatrisation observé lors d'un ulcère du pied ou de toute autre plaie diabétique (Gibran et al., 2002) et conduit très souvent à une amputation.

Dans le cas de notre modèle de brûlure, les animaux présentant une neuropathie ont un processus de cicatrisation qui est retardé par rapport aux animaux contrôles (pour les deux types de brûlures). En effet, 14 jours après la brûlure, le tissu de granulation des animaux contrôles est en partie remodelé alors qu'il est encore présent chez les animaux ayant reçu la RTX.

Cela est également visible lorsque l'on étudie l'expression d'actine α-ML dans ce tissu. Lors d'une cicatrisation normale, les fibroblastes s'activent en myofibroblastes (et expriment donc l'actine α-ML) lors de la phase proliférative. Ces cellules sont capables de secréter une grande quantité de matrice extracellulaire permettant de remplacer celle détruite lors de la lésion. Les myofibroblastes sont également capables de se contracter, ils permettent ainsi de rapprocher les berges de la plaie. Dans la phase de remodelage, ces myofibroblastes ne sont plus nécessaires et vont mourir par apoptose (Desmoulière et al., 1995). L'expression de l'actine α-ML diminue donc fortement. Dans notre étude, les animaux présentant la neuropathie possèdent une expression d'actine α-ML plus importante que les animaux contrôles 14 jours après la brûlure. Cela signifie donc que la cicatrisation est à une étape plus précoce que les animaux contrôles. Cela confirme l'importance d'une innervation fonctionnelle pour obtenir un timing correct dans la succession des différentes phases qui se succèdent lors du processus de cicatrisation.

Pour terminer cette étude *in vivo,* nous avons analysé la réinnervation au sein de la cicatrice. Chez les animaux contrôles, on note une augmentation de l'expression de la périphérine 14 jours après la brûlure puis ce taux diminue progressivement pour revenir à la normale. Cette augmentation d'expression de la périphérine n'est cependant pas retrouvée dans les plaies des animaux ayant reçu la RTX. Peu d'études se sont intéressées à cet aspect de la cicatrisation après brûlure. Kishimoto a montré la présence d'un pic d'expression des fibres substance P positives 14 jours après la brûlure chez le cochon d'Inde puis une diminution de cette expression pour atteindre un niveau inférieur aux animaux contrôles lorsque la cicatrice est formée (Kishimoto, 1984). Pour compléter cette analyse de la réinnervation, un immunomarquage dirigé contre GAP-43 a été réalisé. L'expression de GAP-43 dans la plaie est très faible 14 jours après la brûlure. Cependant, 28 jours après la brûlure, on observe une forte augmentation de son expression dans la cicatrice. Toutefois, il n'y a pas de différence d'expression de ce marqueur entre les animaux présentant ou non une neuropathie. Cela peut s'expliquer par le fait qu'un mois après la brûlure, l'effet de la RTX sur l'innervation est faible. En effet, à ce temps, les sensibilités mécaniques et thermiques, et les taux d'expression de CGRP sont semblables entre les 2 groupes.

II – Peaux reconstruites innervées

Pour étudier la cicatrisation et créer de nouveaux modèles de peau, des peaux reconstruites *in vitro* ont été élaborées. Les plus simples d'entre elles consistent en un gel de collagène colonisé par des fibroblastes, on parle alors uniquement de derme équivalent. Ce gel de collagène peut être recouvert par un épithélium, on parle alors réellement de peau reconstruite (Bell et al., 1983). Ces peaux reconstruites peuvent être complétées par des cellules endothéliales, des cellules souches, des cellules nerveuses (Black et al., 1998; Blais et al., 2009)…

Ces peaux reconstruites sont utilisées par l'industrie cosmétique pour tester de nouveaux produits en limitant l'utilisation d'animaux. Elles peuvent également servir au screening de molécules potentiellement intéressantes pour le traitement d'affections peu profondes (Pageon et al., 2008).

Ces peaux reconstruites peuvent également être greffées pour favoriser la cicatrisation de plaies cutanées profondes et/ou étendues. Après plusieurs semaines de greffe, on observe le développement d'une structure comparable à celle de la peau normale (Kempf et al., 2011). Cette approche pourrait donc être une nouvelle stratégie pour le traitement des plaies dont la guérison n'est pas spontanée.

II – 1 Peaux reconstruites *in vitro*

Le travail suivant « Use of mesenchymal stem cells for cutaneous repair and skin substitute elaboration » présente le potentiel des cellules souches mésenchymateuses dans l'élaboration d'une peau reconstruite.

Dans un premier temps, après avoir rappelé les différentes étapes observées lors du processus de réparation cutanée et les mécanismes mis en jeu, les différentes sources possibles de cellules souches mésenchymateuses (CSMs) pouvant être utilisées pour l'élaboration de peaux reconstruites ont été décrites. Ces sources sont très variées : la moelle osseuse, le tissu adipeux, les tissus extra-fœtaux, le tissu gingival ou encore le derme.

L'objectif de cette étude était de comparer la capacité des CSMs de la moelle osseuse comparée à celle des fibroblastes dermiques dans l'élaboration d'une peau reconstruite. Les différents résultats montrent que lorsque ces CSMs sont utilisées pour l'élaboration d'un derme équivalent à la place des fibroblastes, elles sont capables d'induire très efficacement la croissance et la différenciation de l'épiderme. Ces cellules souches pourraient donc être une alternative à l'utilisation des fibroblastes et pourraient servir à l'élaboration de substituts cutanés.

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Use of mesenchymal stem cells for cutaneous repair and skin substitute elaboration

Utilisation des cellules souches mésenchymateuses pour la réparation cutanée et l'élaboration de substituts de peau

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ABSTRACT

Human mesenchymal stem cells (MSCs) are a heterogeneous population of fibroblast-like cells, which are present in different locations, including bone marrow, adipose tissue, extra-foetal tissues, gingiva and dermis. MSCs, which present multipotency capacities, important expansive potential and immunotolerance properties, remain an attractive tool for tissue repair and regenerative medicine. Currently, several studies and clinical trials highlight the use of MSCs in cutaneous repair underlining that their effects are essentially due to the numerous factors that they release. MSCs are also used in skin substitute development. In this study, we will first discuss the different sources of MSCs actually available. We will then present results showing that bone marrow-derived MSCs prepared according to Good Manufacturing Practices and included in a dermal equivalent are able to promote appropriate epidermis growth and differentiation. These data demonstrate that bone marrow-derived MSCs represent a satisfactory alternative to dermal fibroblasts in order to develop skin substitute. In addition, MSCs could provide a useful alternative to sustain epidermis development and to promote wound healing.

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Mots clés :
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RÉSUMÉ

Les cellules souches mésenchymateuses (CSMs) représentent une population hétérogène de cellules ressemblant à des fibroblastes et qui sont présentent dans différentes localisations, notamment la moelle osseuse, le tissue adipeux, les tissus extra-fœtaux, la gencive et le derme. Les propriétés des CSMs (multipotence, prolifération *in vitro* et immunotolérance) sont intéressantes en vue de leur utilisation en réparation tissulaire et en médecine régénératrice. Aujourd'hui, différents travaux et études cliniques soulignent l'intérêt des CSMs pour aider la réparation cutanée, soulignant que leurs effets bénéfiques sont essentiellement dus aux nombreux facteurs que ces cellules sécrètent. Les CSMs sont aussi utilisées pour développer des substituts cutanés. Dans cette étude, nous discuterons d'abord les différentes sources de CSMs disponibles. Nous présenterons ensuite des résultats montrant que des CSMs issues de la moelle osseuse et préparées en accord avec les bonnes pratiques de production généralement définies, sont capables, lorsqu'elles sont utilisées pour construire un derme équivalent, de promouvoir de façon

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tout à fait satisfaisante, la croissance et la différenciation épidermique. Ces résultats démontrent que les CSMs issues de la moelle osseuse représentent une alternative intéressante à l'utilisation des fibroblastes dermiques. De plus, les CSMs contribuent au développement de l'épiderme et peuvent ainsi faciliter la réparation des plaies.

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1. Introduction

1.1. Reminders on tissue repair mechanisms

Tissue damages and particularly skin wounds usually heal in a highly regulated sequence of overlapping processes that require coordinated activities of different cell types [1,2]. Wound healing phases include:

- a vascular and inflammatory phase during which vascular repair, fibrin clot formation and invasion of inflammatory cells into the wound with release of pro-inflammatory cytokines and growth factors are observed;
- a proliferative phase characterized by granulation tissue formation and re-epithelialization;
- a remodelling phase during which the extracellular matrix is reorganized with wound contraction followed by apoptotic death of inflammatory, vascular and fibroblastic cells.

In normal wound healing, platelets are a major source of pro-inflammatory cytokines and growth factors. Then, macrophages, endothelial and fibroblastic cells invade the wound space and the provisional fibrin extracellular matrix rich in plasma and inflammatory cell-derived cytokines and growth factors. The predominant factors are cytokines of the fibroblast growth factor (FGF) family, platelet-derived growth factor (PDGF), tumour necrosis factor (TNF)-α, epidermal growth factor (EGF), hepatocyte growth factor (HGF), transforming growth factor (TGF)-β1, and vascular endothelial growth factor (VEGF) [3,4]. Macrophages increase inflammatory responses and additionally secrete VEGF and FGF, which promote angiogenesis [5]. The formation of numerous new capillaries confers to the granulation of tissue, giving it a typical granular appearance. Neovascularization is essential for the synthesis, deposition, and organization of a new extracellular matrix. Moreover, FGF, TGF-β1, and PDGF cause fibroblast infiltration and activation into contractile myofibroblasts, which dominate the proliferation and remodelling phases of wound healing. Initially, fibroblastic cells use the fibrin matrix to migrate across the wound, subsequently adhering to fibronectin. Then, fibroblastic cells progressively transform in myofibroblasts [6], which deposit collagen (mainly collagen type III) into the wound bed, leading to the formation of a sophisticated extracellular matrix network to which they can adhere, permitting their migration, and allowing the final formation of the granulation tissue. The extracellular matrix not only regulates cellular functions via cell adhesion and migration, but also provides a storage and diffusion system for signalling molecules, ions, hormones, nutrients and waste products. When normal healing wounds are closed and re-epithelialized, cellularity within the granulation tissue decreases due in part to myofibroblast and endothelial cell apoptosis [7]. For wounds to heal successfully, all phases must occur in the proper sequence. Any factors interfering with one or several phases of this highly regulated process can cause impaired (chronic wounds) or overly (fibrosis or hypertrophic scarring) wound healing [8].

1.2. Definition of a MSC

Mesenchymal stem cells (MSCs) are described to be multipotent progenitor cells with ability to self-renewal and to differentiate into multiple cell lineages, such as adipocytes, chondrocytes, osteoblasts [9].

The term "mesenchymal stem cell" has been applied to these cultured cells because:

- they meet the criteria customarily accepted for stem cell definition, namely, a high capacity for self-renewal and the ability to differentiate into a number of different tissues;
- tissues into which they may develop are from mesenchymal origin [10].

These cells can also acquire a fibroblastic phenotype in classical two-dimensional culture [11]. Today, the term MSC has been revisited and defines adult "fibroblast-like" cells that can mature along multiple differential pathways according to their trophic activity [12,13]. Officially, MSCs have been defined by the International Society of Cellular Therapy as multipotent stromal cells on the basis of three main characteristics:

- their adhesion to a plastic support;
- their expression of a specific set of membrane molecules (CD73, CD90 and CD105), combined with a lack of expression of hematopoietic markers (CD14, CD34, and CD45) and human leukocyte antigen-DR;
- their ability to differentiate along osteoblastic, adipocytic and chondrocytic pathways [14].

2. Different sources of MSCs

Even if the first isolation of MSCs has been made in the bone marrow stroma, some researchers have now described MSCs in many others organs, such as adipose tissue, extra-foetal tissues, gingival tissue and dermal tissue (Fig. 1). In some specific situations (see below), these cells have been described to be involved during skin repair processes; in addition, the interest to use these cells to improve wound healing has been studied.

2.1. Bone marrow-derived MSCs

The bone marrow stroma is the first location where MSCs were described in 1966 by Friedenstein et al. [15]. Additional studies by Owen and Friedenstein [16] demonstrated the ability of these cells to differentiate into adipocyte and osteocyte lineages. These plastic-adherent cells, fibroblast-like cells are able to give rise to colonies of cells termed colony forming unit-fibroblasts. To isolate these cells, iliac crest aspiration is generally realized and MSCs are selected *in vitro*. This method is invasive and the number of MSCs present in the bone marrow swab is limited (from 0.001 to 0.01% of bone marrow nucleated cells). Conventional procedure to select bone marrow-derived MSCs is relied on the expansion of unselected bone marrow cells based on their capacity to adhere on the plastic surface of

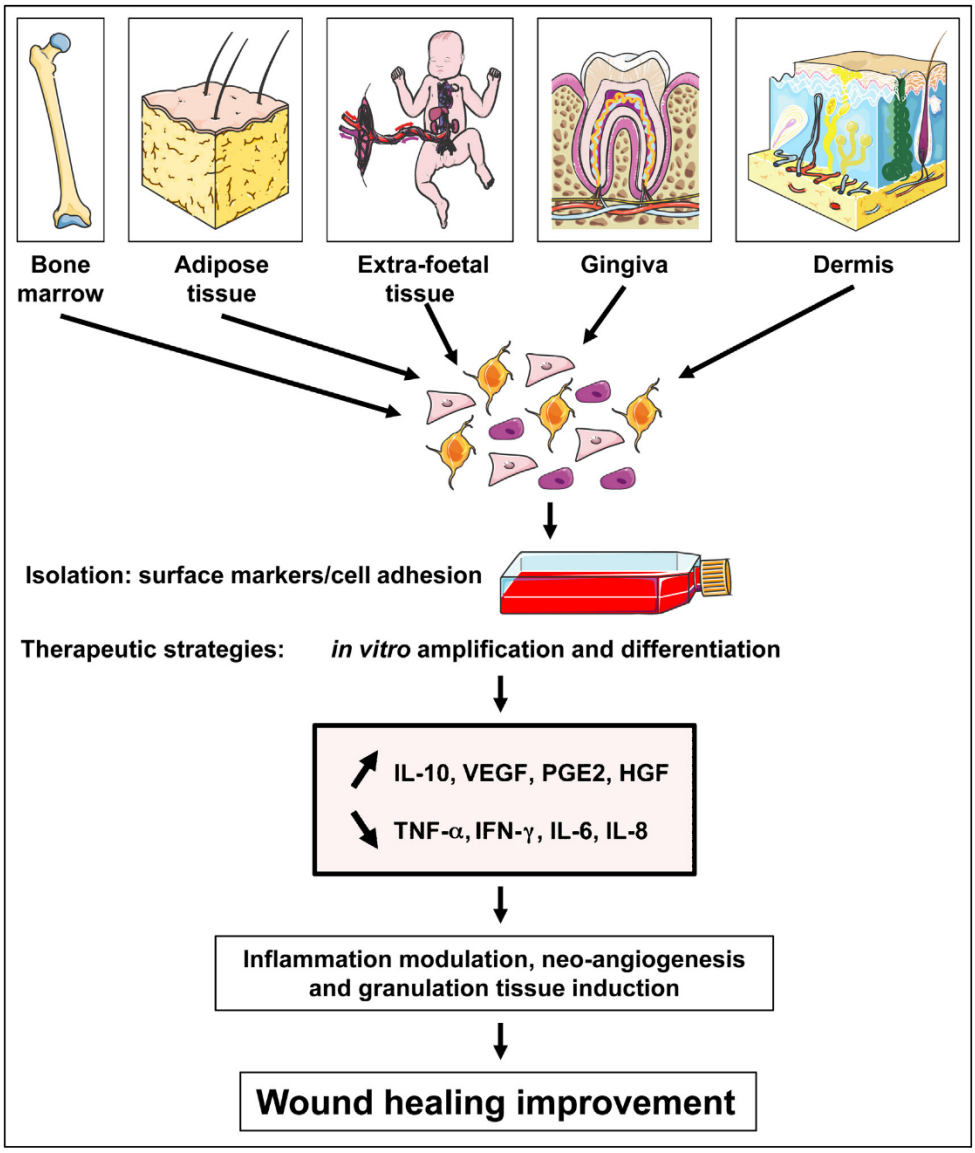


Fig. 1. Different sources for the preparation of mesenchymal stem cells. Mesenchymal stem cells can be isolated *in vitro*. For therapeutic strategies, they can be differentiated or grafted on artificial support. Usually, they do not induce immunological reactions. In addition, except for MSCs derived from extra-foetal tissues, autologous MSCs can be used. However, by secreting numerous and various factors, mesenchymal stem cells modulate inflammation and induce neo-angiogenesis, improving wound healing. Illustrated with Servier Medical Art, http://www.servier.fr/servier-medical-art

culture dishes. After this functional isolation, MSCs are expanded in media containing pooled human platelet lysate which is the most suitable animal serum substitute in clinical scale bone marrow-derived MSC expansion [17,18]. After 15 days of culture, the number of bone marrow-derived MSCs obtained is several million. Bone marrow-derived MSC have been successfully used in the treatment of irradiated burn patients (see below).

2.2. Adipose tissue-derived MSCs

Adipose tissue-derived MSCs present some advantages compared with bone marrow-derived MSCs. Indeed, they are easier to isolate, abundant and they show an important potential to expand *in vitro* [19]. To isolate adipose tissue-derived MSCs, an enzymatic digestion of the tissue is necessary, using for example collagenase. After, adipose cell suspension is plated on culture dishes and adherent cells are considered adipose tissue-derived MSCs [20].

2.3. Extra-foetal tissue-derived MSCs

Extra-foetal tissue-derived MSCs are isolated from different sources, such as amniotic fluid, Wharton's Jelly, placenta and amnion [21]. Amniotic fluid-derived MSCs isolated from 2 mL of amniotic fluid are able to give more than 180 million cells within 4 weeks. These cells express CD90 and CD105, which are mesenchymal cell surface markers but also octamer-binding transcription factor 4 (Oct-4), which is associated with pluripotent cells. Stem cells isolated from amniotic fluid are able to differentiate into adipocytes

and osteoblasts *in vitro*. For adipocyte differentiation, cells are cultured in the presence of insulin for 3 weeks whereas osteogenic differentiation is permitted by a treatment with ascorbic acid and glycerol-phosphate for 3 weeks [22]. Interestingly, when stem cells of amniotic fluid are transplanted into normal or ischemic brain, these cells survive and differentiate into neural lineages, predominantly astrocytes [23].

MSCs extracted from Wharton's jelly are able to differentiate into fat, bone and skeletal muscle. Wharton's jelly is the connective tissue protecting the umbilical cord. Wharton's jelly is isolated from umbilical cord after delivering. After fragment dissociation with collagenase and trypsin, cells are cultured in culture dishes. Adherent cells are considered as MSCs. The isolated cells express CD29 and CD54, consistent with a mesenchymal cell phenotype. They also express the stem cell markers c-Kit, Nanog, Oct-4 and Sox2. Wharton's jelly-derived MSCs are capable to develop a chondrogenic differentiation [24]. Wharton's jelly-derived MSCs may be the first candidate for future cartilage repair. Moreover, they also present interesting characteristics for skin repair [25].

Another source of extra-foetal tissue-derived MSCs is the placenta. After delivery, the placenta is treated with collagenase followed by mechanical dissociation. MSCs are isolated by their adherent plastic properties after one week culture. These MSCs are highly proliferative and multipotent. They express mesenchymal surface markers, such as CD29 and CD44 and pluripotency markers, including Oct-4 and Rex1. They can differentiate *in vitro* into osteoblasts and adipocytes but also into mesodermal chondrocyte-like and ectodermal neuron-like derivatives. Osteoblast or adipocyte differentiation is realized with the same technic as that of the amniotic fluid-derived MSCs i.e. a treatment with insulin for adipocyte differentiation and a treatment with ascorbic acid and glycerol-phosphate for osteogenic differentiation. Neurogenic differentiation is induced with retinoic acid in serum free culture medium [26,27]. In addition to their use in skin repair (see below), these stem cells are also employed in artificial construction of heart valves [28].

The amnion or amniotic membrane is also a source of extra-foetal derived stem cells. The amnion is an avascular structure, which delineates the gestational sac where foetus is developing during gestation. The amnion is composed of three layers: an inner epithelial layer, an acellular intermediate layer and an outer mesenchymal cell layer. From this tissue, several populations of stem cells have been isolated, including amniotic epithelial cells (AECs), amniotic mesenchymal cells (AMCs) and amnion-derived stem cells (ADSCs). AECs derive from amnion epithelial layer, which is derived from ectoderm, whereas AMCs and ADSCs derive from outer mesenchymal layer, which is derived from mesoderm [29]. AECs are epithelial cells whereas AMCs and ADSCs are mesenchymal cells [30]. The AECs are the best characterized multipotent stem cells from the amnion and able to differentiate in vitro into all three germ layers, endoderm (such as hepatocyte), mesoderm (cardiomyocyte), and ectoderm (neural cells) [31]. Concerning AMCs, they have *in vitro* a fibroblastic morphology similar to that described for MSCs derived from bone marrow. AMCs are able to differentiate into neuron-like cells [32], but also into cardiomyocyte-like and hepatocyte-like cells [33]. ADSCs have been isolated from the rat amnion using a method, which inhibits the growth of AECs. Depending on the environment in which these cells were placed, they are able to present osteoblast-, adipocyte-, hepatocyte-, and neuron-like cells differentiation [34]. However, further researches are needed to demonstrate that human homologues of ADSCs exist.

2.4. Gingival tissue-derived MSCs

Gingiva is also a source of MSCs. Gingival tissue-derived MSCs are easy to isolate (without general anaesthesia) and proliferate faster than bone marrow-derived MSCs [35]. About 3% of cells present in a gingival fibroblast culture are able to form colonies and express similar membrane markers than bone marrow-derived MSCs [36]. Under different defined culture conditions, they are able to differentiate into osteogenic, adipogenic, chondrogenic lineages [35–37] and have been shown to keep a stable phenotype when using culture medium enriched with platelet lysate [38]. In 2009, Zhang et al. [39] showed that infusion of gingival tissue-derived MSCs attenuated colitis and restored normal digestive function in mouse inflammatory bowel disease model. In 2012, the same researchers showed that spheroid gingival tissue-derived MSCs (obtained in 3D culture) enhance the reduction of oral mucositis in a murine model of chemotherapy-induced oral mucositis [40]. Interestingly, gingival tissue-derived MSCs inhibit the fragmentation of elastic network in an *ex vivo* culture model of artery segments by inhibiting MMP-9 secretion and its activity through the formation of TIMP-1/MMP-9 complexes [41]. Furthermore, in an *in vivo* model in rabbit, gingival tissue-derived MSCs placed 15 min in the lumen of an experimental aneurism region migrate within the arterial wall and reduce the size of the aneurisms. This efficiency is linked to the elastic network repair and gingival tissue-derived MSCs can still be detected in the arterial wall 6 months after cell therapy [42]. There is also evidence that gingival tissue-derived MSCs inhibit apoptosis of arterial wall smooth muscle cells and contribute to regulate the differentiation of dendritic cells [43].

2.5. Dermal tissue-derived MSCs

Different stem cell populations have been described in adult skin. The most studied from long time are epidermal stem cells [44,45]. The advantage for MSC isolation from skin versus bone marrow (or gingiva and even adipose tissue) is that they are easy to access. These skin-derived MSCs can be isolated from skin biopsy within or outside of hair follicles. Hair follicle MSCs are located in bulge region located in the outer root sheath at the insertion point of the arrector pili muscle. These MSCs are implicated in hair growth during the three phases of each cycle (*i.e.* anagen or growth phase, catagen or transitional phase, and telogen or resting phase) but also in wound healing (see below). Hair follicule MSCs express nestin [46]. *In vitro*, these cells are able to differentiate into adipocytes and osteogenic lineages [47]. Amoh et al. [48] showed that transplantation of hair follicule MSCs in immunocompetent mice enhances regrowth of sciatic nerves. Different researchers have reported the identification of multipotent cells from adult dermis, outside of hair follicles [49,50]. These MSCs are able to differentiate into neural cells, adipocytes, chondrocytes and osteocytes. Toma et al. [49,51] described skin-derived precursors cells (SKPs). *In vitro*, these cells are able to form spheres [52]. When they are treated with EGF or FGF, differentiation of SKPs into neurons, Schwann cells, adipocytes, osteoblasts, chondrocytes, melanocytes, pericytes and smooth muscle cells is obtained. In the absence of growth factors, SKPs differentiation is oriented to hepatocytes and insulin producing cells [49,51].

3. MSCs and skin repair

It is generally accepted that the major source of (myo)fibroblasts involved in skin repair are local connective tissue fibroblasts [53]. It has also been suggested, even if this process is still discussed, that differentiated (or malignant) epithelial and endothelial cells can undergo a phenotypic conversion that gives rise to the matrix-producing fibroblasts and myofibroblasts (epithelial- and endothelial-to-mesenchymal transition process) [54]. In addition, it is now well admitted that, in some situations, stem cells, particularly MSCs, can be recruited to play a role during

skin repair. The local MSCs described in the dermal sheath that surrounds the outside of the hair follicle facing the epithelial stem cells are involved in the regeneration of the dermal papilla and they can also became wound healing (myo)fibroblasts after a lesion [47,55]. Moreover, MSCs originating from bone marrow have been suggested to participate in tissue repair. These cells, which are bone marrow-derived non-hematopoietic precursor cells [56], contribute to the maintenance and regeneration of connective tissues through engraftment [57]. Indeed, they have the capacity to seed into several organs and to differentiate into wound healing myofibroblasts. It seems well admitted that this engraftment in injured organs is regulated by the severity of the damage and these cells are recruited after severe acute damage or in chronic diseases when the local sources of mesenchymal cells able to be involved in the repair process are depleted [58].

We will discuss more in this section, cell therapy strategies using exogenous MSCs delivered at the site of a wound in order to improve skin repair. Indeed, the use of MSCs in cutaneous repair and eventually regeneration begins to be well documented [59]. Globally, in wound bed, MSCs contribute to the generation of high quality and well-vascularized granulation tissue, enhance re-epithelialization and attenuate formation of scar tissue.

Various studies have shown the capability of MSCs in accelerating cutaneous wound repair [60–62]. Several clinical trials with the application of MSCs in wound therapies have demonstrated that grafts of MSCs support and promote skin repair both in chronic and acute wounds. Several groups showed this effect with bone marrow-derived MSCs [63–65] but adipose tissue-derived MSCs [66] and Wharton's jelly-derived MSCs [67] were also used. Patients presenting a severe radiation burn were treated with several local autologous bone marrow-derived MSC administrations performed in combination with skin autograft [68,69]. MSCs were expanded according to a clinical grade protocol using platelet-derived growth factors [17]. The clinical evolution of most of the patients was favourable and no recurrence of radiation inflammatory waves occurred during more than patient's 3-years follow-up. It was proposed that the benefit of this local cell therapy is linked to the "drug cell" activity of MSCs by modulating the radiation inflammatory processes, as suggested by the decrease in the C-reactive protein level observed after each MSC administration [69]. These results demonstrate a real benefit for these patients [70], avoiding amputation and/or death when associated with a dosimetry-guided surgery [71]. Interestingly, bone marrow-derived MSCs can also be used as a feeder layer to promote autologous keratinocyte expansion and preparation of epidermal sheets for burns (Lataillade et al, unpublished observations).

Clinical trials with MSCs included in an artificial dermis have already been done. Indeed, an appropriate biomaterial-based scaffold is important to trigger stem cell differentiation, proliferation, paracrine activity as well as regulation of extracellular matrix deposition [72]. Yoshikawa et al. [64] described the recovery of 18 out of 20 patients with non-progressive skin lesions; they used bone marrow-derived MSCs placed after culture, in an artificial dermis made of collagen sponge. Nie et al. [73] tested the effect of adipose tissue-derived MSC local delivery on wound healing using an acellular dermal matrix (ADM) scaffold. ADM, which is a human skin allograft obtained from volunteer human donors through tissue banks, is extensively used but inadequate revascularization is often observed. They showed that the adipose tissue-derived MSCs delivered in ADM scaffold could differentiate into endothelial and epithelial lineages under appropriate conditions; moreover, adipose tissue-derived MSCs engrafted in ischemic injury models release angiogenic factors, which may be partially responsible for adipose tissue-derived MSC-mediated enhanced angiogenesis. Recently, Sahin et al. [74] showed in rat full-thickness dorsal skin defects that the combination of bone marrow-derived MSCs delivered in ADM with negative pressure wound therapy has synergic effects for neo-angiogenesis, resulting in an improvement of skin repair.

Indeed, the benefit of MSCs in wound repair could be explained either by the differentiation of these cells in skin cells acting on repair processes or by the large quantity of growth factors that these cells produce and which are able to improve skin repair [75,76]. Current data suggest that the contribution of MSC differentiation when delivered into the wound is limited due to a poor survival of MSCs in the site of injury. Then, MSC paracrine signalling is likely the primary mechanism explaining the beneficial effects of MSCs in wound repair, even if it is not excluded that MSCs which persist within the grafted tissue could also play an important role as suggested by gingival tissue-derived MSC observations [42].

MSCs can develop interesting effects in the different phases of wound healing (vascular and inflammatory, proliferative and remodelling phases). Yang et al. [77] showed that MSCs secrete soluble mediators, which induce interleukin (IL)-10 production by splenocytes suppressing T-cell proliferation. They produce prostaglandin E2, which induces a reduction of T-cell migration and NK cell proliferation [78]. Aggarwal and Pittenger [79] showed that the addition of MSCs to purified subpopulations of immune cells decreases the secretion of TNF-α and interferon-γ (pro-inflammatory cytokines) and increases production of IL-10 and IL-35 (anti-inflammatory cytokines), showing the immunomodulatory functions of MSCs, which could be therapeutic for reduction of graft-versus-host disease, rejection, and modulation of inflammation. Taken together, these effects contribute to reduce inflammation in wound bed and are beneficial particularly in chronic wounds where chronic inflammation interferes with the development of granulation tissue. Interestingly, it was also shown that MSCs have antimicrobial activity. This effect is mediated via the secretion of antimicrobial factors, such as LL-37 [80] but also via the secretion of immunomodulative factors, promoting bacterial phagocytosis by immune cells [81].

As underlined above, MSCs are also important for helping neo-angiogenesis development, which is an important step for facilitating granulation tissue development. Indeed, in chronic wounds, an anti-angiogenic environment is present with a reduction in growth factor efficacy and an increase in matrix metalloproteinase production [82]. In this situation, first, it seems that MSCs can differentiate into endothelial cells [83]. MSCs can also differentiate into angiogenic progenitors [84] and can allow the recruitment of endothelial cells [85]. Second, MSCs are able to produce pro-angiogenic factors, such as VEGF, which stimulates angiogenesis [86]. These pro-angiogenic factors secreted by MSCs induce endothelial cells proliferation and tube formation [87,88]. Lu et al. [89] showed that adipose tissue-derived MSCs enhance angiogenesis through the differentiation into endothelial cells and production of angiogenic growth factors. In addition, bone marrow-derived MSCs provide an important early signal for dermal fibroblast responses to cutaneous injury [90]. The later step of wound repair is, after re-epithelialization, the remodelling of granulation tissue finally resulting in scar formation. Wu et al. [91] showed that MSCs are able to reduce scar formation in mouse. As mentioned above, MSCs favour the secretion of anti-inflammatory cytokines and particularly IL-10 by T-cells and macrophages [92]; IL-10 induces a downregulation of IL-6 and -8 and consequently a reduction of collagen production [93]. MSCs also produce HGF, which is implicated in the downregulation of fibrosis [94]. HGF, which is also involved in cell recruitment to repopulate wound bed [95], causes a downregulation of TGF-β but also a reduction in collagen formation by fibroblasts [96].

4. Use of MSCs for skin substitute elaboration

The concept of *in vitro* organogenesis had led to the development of a two-layered living skin equivalent, composed of both fibroblasts and keratinocytes, respectively dermal and epidermal cells [97]. These two cell types are essential to obtain a functional skin substitute after grafting in humans [98]. For example, dermal fibroblasts interact with keratinocytes, notably by reorganizing the "dermal" matrix, which serves as adhesion substrate for epidermal cells [99]. The development of skin equivalent models provides a system for studying cell–cell and cell–matrix interactions, and cell responses to pharmacological agents [100–102]. It is also of interest for skin damage covering after important injuries or severe burns [98,103]. However, in some situations, there is a limit to the amount of dermal fibroblasts available for skin equivalent construction. Then, the integration of MSCs in skin substitutes could provide an alternative approach to construct skin equivalents efficient to promote wound healing.

A translational research program (Cultivated Adult Stem Cell Alternative for Damaged tissuE or CASCADE project) has been initiated by the European Community. The objective of CASCADE was to provide the European Community with unique expertise which focuses on the Good Manufacturing Practice (GMP) production of MSCs and their use in treating skin wounds, so that quality and safety criteria are paramount and match therapeutic expectations. The outcome was a consortium forming a comprehensive network of university hospitals, research laboratories, and platforms that encompasses all the technologies and skills required to efficiently develop innovative technologies for the production of clinical grade MSCs and to translate this production into a cell drug to repair wounds.

The aim of the study described here was to investigate the efficiency of human bone marrow-derived MSCs isolated during the CASCADE project to replace dermal fibroblasts in a skin equivalent model *in vitro*. Human dermal fibroblast (DF) cultures, used as reference, were classically derived from human skin explants which were obtained from patients undergoing mammoplasty and after giving their written consent (Hôpital de la Mère et de l'Enfant, CHU Dupuytren, Limoges, France). Human bone marrow-derived MSCs were obtained from Dr Schrezenmeier (Institute for Transfusion Medicine, University of Ulm, Germany) in the framework of the European research project CASCADE. Cells were extracted from bone marrow, cultured and cryo-preserved in accordance with the GMP [104]. To expend the cells, platelet lysate pool (Institute for Transfusion Medicine, University of Ulm, Germany) and non-animal recombinant trypsin solution were used. The bone marrow-derived MSCs analysed in this study were obtained from male donors and were characterized in the CASCADE consortium, according to the criteria defined by the International Society of Cellular Therapy [14].

In a first time, dermal equivalent containing either DFs or MSCs were constructed [105] and compared. Cell counting, dermal equivalent retraction and matrix metalloproteinase (MMP)-2 and MMP-9 quantification, were performed. In a second time, epidermal outgrowth and differentiation from thin calibrated skin biopsies placed on dermal equivalents [99,106] containing either DFs or MSCs, were analysed.

4.1. Compared with dermal fibroblasts, the collagen matrix better controls MSC density and MMP secretion

After a slight decrease observed just after seeding in collagen lattices, an increase in cell number was observed in dermal equivalents for both DFs and MSCs, but the number of MSCs did not increase as much as the number of DFs (compared to the number of seeded cells on day 0, after 15 days, the number of DFs was ×6 while this number was only ×2 for MSCs) (Fig. 2). The dermal equivalents retracted in a similar way whatever the cell type (DFs or MSCs) included in the collagen lattice; after an important decrease of the dermal equivalent diameter during the first 5 days (–50% of the initial diameter), a soft and gradual decrease was then observed to reach about 25% of the initial diameter at day 14. Zymography allowed the detection in dermal equivalent supernatants of the pro-MMP-9 (92 kD), pro-MMP-2 (72 kDa) and active MMP-2 (64 kDa) (Fig. 3). Pro-MMP-9 (92 kDa) was present in both cell type supernatants from day 1 to day 14. Pro-MMP-9 activity remained relatively constant with MSCs whereas a slight increase with time of culture was observed with DFs. Concerning MMP-2, both pro (72 kDa) and active (64 kDa) forms increased with time of culture in the 2 cell types. These data using GMP produced MSCs globally confirm the results already obtained by Fioretti et al. [107].

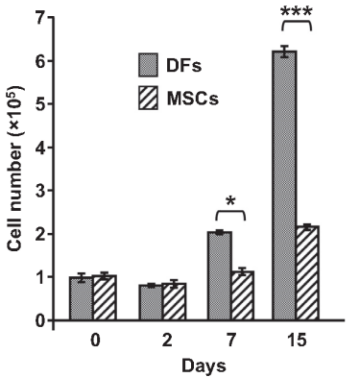


Fig. 2. Cell counting of dermal fibroblasts (DFs) and of bone marrow-derived mesenchymal stem cells (MSCs) incorporated within collagen lattices. Both cell types were seeded at the same density (1×10^5 cells per 5 mL collagen lattice). The number of cells observed during culture was assessed by Trypan Blue dye exclusion and/or Coulter Counter methods for 15 days after collagenase digestion. Values represent mean ± SD of three independent cell culture experiments. $^{*}P < 0.05$; $^{***}P < 0.001$.

4.2. Similarly to dermal fibroblasts, MSCs were able to promote an epidermal outgrowth

In each experiment, a control, which consisted of a collagen lattice without cells, was prepared. In these conditions, after the

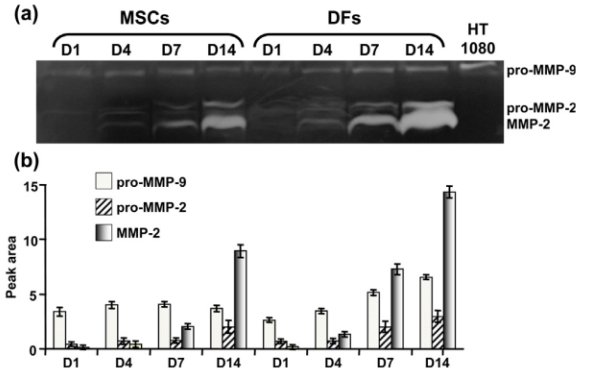


Fig. 3. Analysis of gelatinolytic activity in supernatants of dermal equivalents containing either dermal fibroblasts (DFs) or bone marrow-derived mesenchymal stem cells (MSCs). Gelatin zymography was performed according to Fioretti et al. [107]. a: a representative zymography is shown. Supernatant of the fibrosarcoma cell line HT1080 was used as a reference; b: the enzymatic activity was quantified using image J software (NIH, USA) as described previously [108]. Values represent mean ± SD of three independent cell culture experiments.

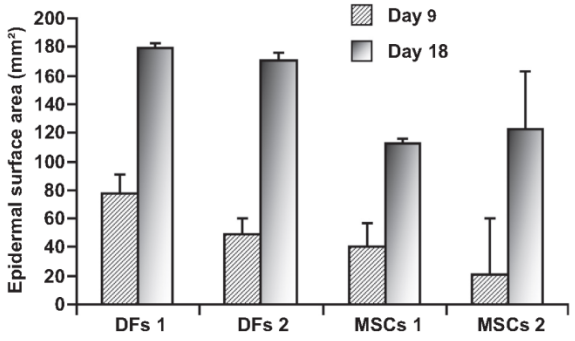


Fig. 4. Measurement of the epidermal outgrowth after Nile blue sulfate staining of reconstituted skin. The skin equivalents were prepared according to Coulomb et al. [99] by using small skin biopsies from which keratinocytes will migrate on the surface of dermal equivalent. To visualize the epidermal outgrowth over the surface of the dermal equivalent, Nile Blue sulphate was added to the culture medium at a final concentration of 1:10,000 [106]. The epidermal outgrowth was measured after 9 and 18 days of culture. The Optimas 6.2 (Imasys, Suresnes, France) image analysis software was used to measure the epidermal outgrowth. Graph depicts the epidermal surface area of the neo-epidermis present on dermal equivalent containing either dermal fibroblasts (DFs) or bone marrow-derived mesenchymal stem cells (MSCs). Two different strains of DFs and of MSCs are analysed. Values represent mean ± SD of three independent cell culture experiments.

implantation of the skin biopsy, neither collagen lattice retraction nor epidermal outgrowth, were observed during the culture period. An epidermal outgrowth was obtained with both cell types (Fig. 4). However, whatever the time of culture, the epidermal surface area observed on dermal equivalents containing DFs remained larger (between 50 and 80 mm^2 at day 9, before reaching 180 mm^2 at day 18) in comparison with the epidermal surface area observed on dermal equivalents containing MSCs (between 20 and 40 mm^2 at day 9, before reaching about 120 mm^2 at day 18).

Interestingly, bone marrow-derived MSCs were obtained from male donors and biopsies used for epidermalization from female donors. Fluorescent in situ hybridization using X and Y chromosome specific probes was performed; only female cells were detected in the neo-epidermis, underlining that MSCs did not participate to the neo-epidermis formation.

4.3. Compared with dermal fibroblasts, MSCs promote epidermal differentiation

Whatever the cell type (DFs or MSCs) present in the dermal equivalent, an increase in the neo-epidermis thickness was observed along the culture time (Fig. 5). However, at day 9, the neo-epidermis obtained at the surface of dermal equivalent containing MSCs was thicker. At day 18, the total thickness was similar with both cell types (DFs or MSCs), but a stratum corneum was only obtained on the neo-epidermis obtained in the presence of MSCs.

Cytokeratin 10 was observed at days 9 and 18, in the neo-epidermis obtained at the surface of dermal equivalent containing MSCs; at day 9, cytokeratin 10 expression was mainly observed in the upper layers of the neo-epidermis. At day 18, cytokeratin 10 was clearly localized under the stratum corneum. There was no expression of cytokeratin 10 neither at day 9, nor at day 18, in the presence of DFs.

Whatever the cell type (DFs or MSCs), an important involucrin expression was observable at days 9 and 18. Involucrin expression was uniformly observed in all the layers of the neo-epidermis at day 9. At day 18, although involucrin was detected from the first suprabasal layers of the neo-epidermis, its expression appeared to be more pronounced ascending towards the neo-epidermis surface. Then, together with the neo-epidermis thickening, involucrin expression increased with culture time but remained more important in the upper layers of the neo-epidermis.

Whatever the cell type (DFs or MSCs), a slight filaggrin expression was detected at day 9 in the upper layers of the neo-epidermis. At day 18, compared to day 9, filaggrin expression was more pronounced and was found in all the layers of the neo-epidermis, although it appeared to be more expressed in the upper layers. However, at day 18, filaggrin expression in the neo-epidermis obtained at the surface of dermal equivalent containing MSCs was stronger compared with filaggrin expression observed in the presence of DFs.

Then, an increase in the epidermal differentiation was observed with time in culture in the neo-epidermis developing on dermal equivalents containing DFs or MSCs. This increase was demonstrated, on the one hand, by the thickness increase of the

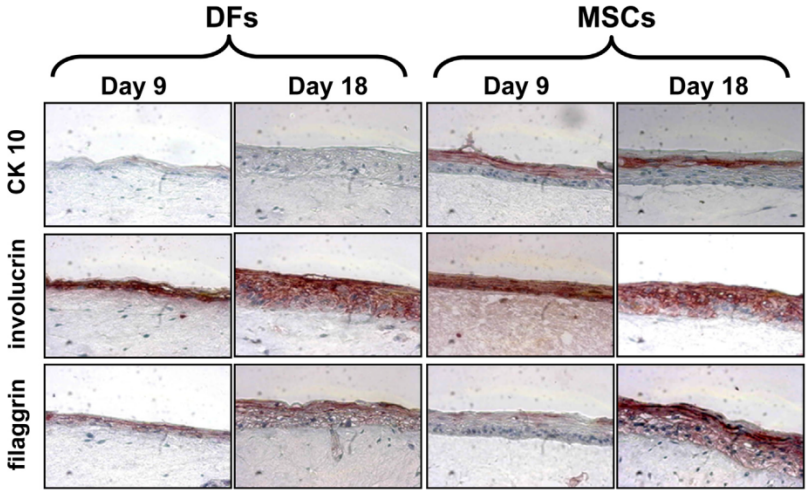


Fig. 5. Immunostaining of epidermalized dermal equivalents using antibodies recognizing cytokeratin 10, involucrin and filaggrin. The epidermal differentiation was characterized after 9 and 18 days of culture by immunohistochemical staining of formaldehyde fixed and paraffin embedded skin equivalents. Primary antibodies directed against markers of keratinocyte differentiation were used: mouse monoclonal IgG antibodies against cytokeratin 10 (Tebu-bio, Le Perray en Yvelines, France), involucrin (Sigma Aldrich, Lyon, France) and filaggrin (Sigma Aldrich). In this representative experiment, skin biopsies, used as a source of epidermalization, are from the same donor and placed on dermal equivalents containing either DFs or MSCs.

neo-epidermis and, on the other hand, by the stronger expression of the differentiation markers between day 9 and day 18. Nevertheless, the neo-epidermis differentiation in the presence of MSCs appeared sooner and was more pronounced that in the presence of DFs as shown by the development of a stratum corneum and the expression of cytokeratin 10.

5. Conclusion/perspectives

To conclude, it is important to remind that in the skin, different epithelial stem cells and MSCs are present and can be involved in repair process. Interestingly, some niches, which represent special localisations where specific interactions between different stem cells and their microenvironment have been described, for example around vessels [109] and hair follicles [110]. These niches, which are easily accessible, could be useful in regenerative medicine. In addition, when exogenous MSCs are used to favour healing, they could differentiate in skin cells but, especially, they release various cytokines and growth factors, which interact favourably with healing processes [111]. Finally, MSCs can be delivered at the wound site associated with an extracellular matrix scaffold or used to construct skin equivalents. By using the dermal equivalent model initially described by Bell et al., which is known to promote DF differentiation [97,112,113], similar behaviours between MSCs and DFs have already been reported [107]. In this study where we used bone marrow-derived MSCs produced under GMP, we confirmed that MSCs can constitute an attractive source to populate dermal substitutes. Like DFs, MSCs were able to retract collagen lattices and secrete essential MMPs for extracellular matrix remodelling. In addition, as observed by Laco et al. [114], MSCs were also able to promote an epidermal development. However, as in dermal equivalents, the cell density of the MSCs was reduced compared with DFs, then, for all these parameters, collagen matrix retraction, gelatinolytic activity and epidermal outgrowth, MSCs appeared more efficient than DFs. Furthermore, the neo-epidermis differentiation in the presence of MSCs is more rapid and more pronounced. As underlined above [111], we can suggest that the efficiency of MSCs as compared with DFs is linked with their ability to release cytokines and growth factors, which are able to promote neo-epidermis growth and differentiation. Some preliminary observations using adipose tissue-derived MSCs prepared in accordance with the GMP criteria of the CASCADE project indicated that these MSCs could also represent an interesting source to populate dermal equivalents used to develop reconstructed skin *in vitro*. These data confirmed the interest of such cells in the development of skin substitutes, promoting not only dermal remodelling, but also growth and differentiation of the epidermis.

In conclusion, in addition to MSC therapy strategies already accessible in humans, such as the combination of autologous MSC injection and surgery for the treatment of patients presenting severe radiation burn, the development of skin substitutes using MSCs not only permits to propose complementary cell therapy strategies for patients, but also represents interesting tool for MSC biology analysis.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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II – 2 Peaux reconstruites pour le traitement des brûlures

Après une brûlure sévère, la greffe de peau est souvent nécessaire pour faciliter la cicatrisation. Cette technique a permis d'améliorer la survie des patients mais de nombreux handicaps liés à un défaut d'innervation fonctionnelle se développent à la suite de cette cicatrisation. Les patients peuvent se plaindre d'une perte de sensibilité, d'un prurit ou de douleurs au niveau de la zone brulée. Il est donc important de proposer une nouvelle stratégie thérapeutique pour améliorer la réinnervation lors de la cicatrisation de ces brûlures.

La stratégie que nous avons développée porte sur le développement de peaux reconstruites innervées. A ces peaux reconstruites, il est possible d'ajouter des biomatériaux ou des cellules souches qui pourront, une fois implantés *in vivo*, favoriser la cicatrisation et une repousse nerveuse correcte.

Un point sur les connaissances actuelles concernant ces nouvelles approches pour la cicatrisation après brûlures est réalisé dans la revue suivante « Biotechnological management of skin burn injuries : challenges and perspectives in wound healing and sensory recovery » acceptée dans le journal Tissue Engineering part B.

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REVIEW ARTICLE

AU1 ▶ Biotechnological Management of Skin Burn Injuries: Challenges and Perspectives in Wound Healing and Sensory Recovery

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Many wound management protocols have been developed to improve wound healing after burn with the primordial aim to restore the barrier function of the skin and also provide a better esthetic outcome. Autologous skin grafts remain the gold standard in the treatment of skin burn, but this treatment has its limitation especially for patients presenting limited donor sites due to extensive burn areas. Deep burn injuries also alter the integrity of skin-sensitive innervation and have an impact on patient's quality of life by compromising perceptions of touch, temperature, and pain. Thus, patients can suffer from long-term disabilities ranging from cutaneous sensibility loss to chronic pain. The cellular mechanisms involved in skin reinnervation following injury are not elucidated yet. Depending on the depth of the burn, nerve sprouting can occur from the wound bed or the surrounding healthy tissue, but somehow this process fails to provide correct reinnervation of the wound during scarring. In addition, several clinical observations indicate that damage to the peripheral nervous system influences wound healing, resulting in delayed wound healing or chronic wounds, underlining the role of innervation and neuromediators for normal cutaneous tissue repair development. Promising tissue engineering strategies, including the use of biomaterials, skin substitutes, and stem cells, could provide novel alternative treatments in wound healing and help in improving patient's sensory recovery.

Keywords: burn injury, cutaneous wound healing, skin engineering, nerve fiber regrowth

Introduction

A BURN INJURY may be induced not only by thermal agents but also by radiations, radioactivity, chemicals, or friction. The most common causes of burns are fire for adults and scald for children.[1,2] In France, burns requiring medical attention affect ~500,000 people per year. Ten thousands need hospitalization and among those patients, 10% die.[3] In Europe, the rate of death in hospitalized patients ranges from 1.4% to 18% across countries.[4] In 2004, the World Health Organization revealed that fire burn affects 11 million people and accounts for more than 300,000 deaths per year.[5] However, it unequally affects populations since low- and middle-income countries have the highest mortality rates.

Whatever the cause of the burn, the severity of an injury mainly depends on its depth and extent. Besides, the assessment of these two features is crucial to providing proper treatment without delay, especially for extensive burns.[6] Then, when an injury extends over more than 10–15% of the total body surface area (TBSA), admission to critical care units is required. In that case, the first hours following the injury are dedicated to prompt fluid resuscitation to prevent hypovolemic shock, which occurs secondary to persistent

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edema and outflow of osmotically active molecules such as proteins.[7] After these potential vital treatments, burn wound management is performed using occlusive dressing or, if necessary, wound excision and skin grafting.

The postburn mortality rate, which is highly correlated with age, the TBSA, and the inhalation injury, has been decreasing over the past decades.[4,8,9] This is likely due to improvement of resuscitation procedures, treatment of infection, and wound healing management. Currently, the challenge is to improve the patients' rehabilitation and hence their quality of life. Indeed, in addition to any post-traumatic stress disorder, burn patients may suffer from their scars. At best, they are unesthetic because of depigmentation, hyperpigmentation, or skin thickening (hypertrophic scar). For some, scars become very disabling when scar contractures occur.[10] Overall, itching and pain are frequent *sequelae* that may disturb daily life.[11,12] The occurrence of these symptoms suggests that injured sensory nerve fibers regenerate improperly or insufficiently. This demonstrates that efforts in wound healing management should still be made to improve nerve fiber regeneration. In addition to improvement of sensory perceptions, it would allow significant progress in the wound healing process since nerve fibers are known to be involved in skin repair and cutaneous homeostasis.[13,14]

In this review, we will first overview the skin repair process and regeneration of nerve fibers. After describing the current deep wound management and the possible postburn *sequelae*, we will address the question of innovative strategies to improve wound healing and nerve fiber regeneration.

Skin Repair Process and Peripheral Innervation

Overview of wound healing

Wound healing consists in the restoration of the integrity of the damaged tissue. In a similar manner, the cutaneous healing process relies on complex cellular dialogs and can be divided into three sequential and intercorrelated phases. The inflammatory and vascular phase starts as soon as the damage occurs. The skin is richly vascularized and the disruption of blood vessels in the dermis, and in the hypodermis if the injury is more severe, leads to formation of a blot clot and of a provisional matrix mainly comprising fibrin and fibronectin. Platelets involved in the blood clot have also a major role in the recruitment of inflammatory cells such as neutrophils, macrophages, and mast cells to the wound due to the local release of cytokines and chemokines. Fibroblasts and endothelial cells are also drawn to the wound by chemotaxis and will be major actors in the second stage of wound healing, the proliferation phase. The hallmark of the proliferation phase is the formation of granulation tissue in which fibroblasts are stimulated to proliferate and undergo major cellular changes characterized by the expression of α-smooth muscle actin. They are consequently called myofibroblasts and display contractile properties that are essential in the maturation of granulation tissue overtime.[15] They also secrete and deposit extracellular matrix, mainly collagen type III that will progressively replace the provisional matrix. Most myofibroblasts derive from resident fibroblasts, but it is important to note that different subpopulations of fibroblasts presenting their own proper capacities of differentiation are present in the dermis.[16] Other sources of myofibroblasts have been highlighted such as local stromal stem cells, blood circulating progenitors, and bone marrow (BM)-derived stem cells.[17] To support the strong cellular activities occurring during the proliferation phase, endothelial cells recruited to the wounded area also proliferate and contribute to the angiogenesis process. A dense network of capillaries can then deliver all the necessary nutrients to the healing area.[15] The third and last phase of skin wound healing, the remodeling phase, leads to progressive formation of the scar. The scarring process involves two major phenomena: reepithelialization and final maturation of the granulation tissue. At the edges of the wound, keratinocytes display a migratory phenotype. They express specific integrins, allowing reepithelialization and wound closure.[18] Upon wound closure, maturation of the granulation tissue is marked by the synthesis of collagen type I and disappearance of the myofibroblast population by apoptosis.[19] The persistence of myofibroblasts in the granulation tissue is a major cause of well-documented pathological conditions involving hypertrophic scarring and tissue deformation.[20] Both myofibroblast differentiation and apoptosis are driven by specific signals such as the release of the cytokine transforming growth factor (TGF)-β1, which is the major inductor of myofibroblast differentiation, intercellular and/or matrix interactions, and finally mechanical stress.[21,22] It is known that a stiffer environment leading to a lower rate of myofibroblast apoptosis is a cause of hypertrophic scar.[23] In addition, keratinocytes and the epithelium certainly play a role in the normal evolution of the granulation tissue and in myofibroblast apoptosis. Indeed, it has been shown that perturbation of dermal–epidermal interactions can lead to excessive scarring. Interestingly, in such pathological situations, neurogenic inflammation seems to be involved.[24]

Cutaneous innervation and role of sensory receptors in skin perceptions

In the skin, different nerve endings are implicated in the detection and transmission of sensitive information to the central nervous system.

Nerve fibers express neuromediators. Without stimulation, there is a basal expression of these neuromediators, whereas after chemical injury, physical damage, or inflammation, the quantity of neuromediators dramatically increases. These mediators have been described to be involved in different physiological and pathological situations, including wound healing (for review, see Roosterman *et al.*[25]). Autonomic nerve fibers present in the skin play a major role in body thermoregulation by acting on smooth muscles in arterioles, on erector pili muscles, and on sweat glands (Fig. 1). Sensory information is detected by specific receptors present on sensory nerve fibers (Fig. 1). This information is transmitted to the cell body located in dorsal root ganglia (close to the spinal cord) and finally to the central nervous system for integration. ◀F1

Cutaneous nerve fibers can detect stimuli such as thermal and tactile sensation or pain.[26,27] After skin lesion, these nerve fibers and their receptor are damaged or sometimes destroyed, but neuronal cell bodies are still present in the dorsal root ganglia (Fig. 2). ◀F2

Mechanoreceptors present on Aβ and Aδ fibers can detect mechanical stimuli, while temperature and pain are detected, respectively, by thermoreceptors and nociceptors present on

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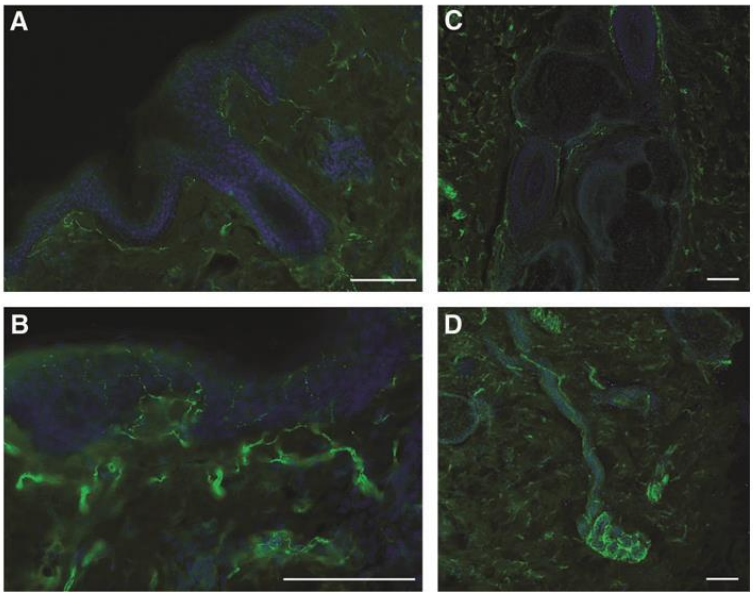


FIG. 1. Skin innervation. In these pictures, cutaneous nerve fibers are labeled using an anti-PGP 9.5 antibody revealed by a secondary FITC-conjugated antibody (the cell nuclei are colored with DAPI). The superficial nerve plexus follows the dermal–epidermal junction in the dermis and the small sensory nerve fibers, Aδ and C, sprout into the epidermis reaching its upper layers (**A**, **B**). Autonomic nerve fibers are the main fibers innervating the skin appendages, that is, hair follicles (**C**) and sweat glands (**D**). Scale bars: 100 μm. ◀AU9 ◀4C

Aδ and C free nerve endings, also called small fibers (for review, see Roosterman *et al.*[25]).

Role of innervation in skin healing and therapeutic options

It has been recently shown that cutaneous innervation plays important roles in normal and pathological repair processes.[14,28] However, the precise roles of sensory and autonomic innervation during wound healing remain to be clearly established. Not only keratinocytes and melanocytes but also fibroblasts and myofibroblasts express different neurotrophins such as nerve growth factor (NGF), neurotophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), and their receptors, which promote their proliferation and differentiation.[29,30] Neuropeptides such as calcitonin gene-related peptide (CGRP), substance P, and vasoactive intestinal peptide can modulate the activity of matrix metalloproteinase (MMP)-2 and MMP-9, which are major actors involved in granulation tissue remodeling and scar formation. In addition, these neuropeptides also act on collagen type I and type III production during skin wound healing and promote the adhesion of dermal fibroblasts and their differentiation

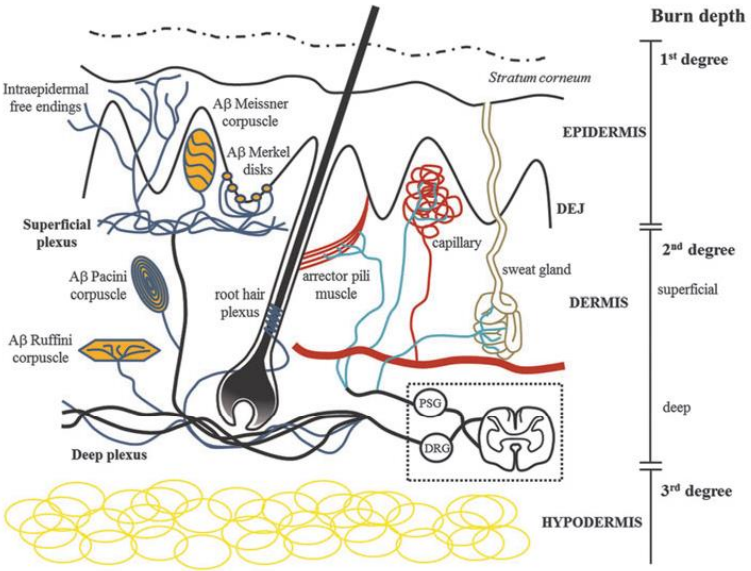


FIG. 2. Schematic representation of skin innervation. Skin nervous structures are endings of sensory and sympathetic neurons, which have their cell bodies located, respectively, in DRG and PSG along the spinal cord. Sensory and sympathetic skin structures are located, respectively, on the *left* and on the *right* part of the schema. Concerning sensory intraepidermal free endings, they are constituted of Aδ fibers and of peptidergic and non-peptidergic C fibers. In addition, axonal free endings are also present in the dermis. DEJ, dermal–epidermal junction; DRG, dorsal root ganglia; PSG, paravertebral sympathetic ganglia. ◀4C

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into myofibroblasts.[31] The effects of these neuropeptides on the extracellular matrix composition and arrangement are certainly essential as it is well established that the mechanical microenvironment organized by the extracellular matrix could interfere with fibroblast-to-myofibroblast differentiation.[14] In addition, the modulation of MMPs acts on subsequent MMP activation of latent TGF-β1.[32]

Skin damages induce the release by immune cells and sensory nerve endings of inflammatory mediators, including interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), bradykinin, substance P, CGRP, NGF, and prostaglandins, contributing to the inflammatory soup.[33] It has been shown that altered substance P levels could be involved in impaired cutaneous healing responses observed in diabetes mellitus[34] or during hypertrophic scar formation.[35] It has also been shown *in vitro* that direct contact of fibroblasts with neurites is able to induce myofibroblastic differentiation, increasing collagen gel retraction, which is an important process during wound healing.[36]

In keloid, the density of nerve fibers is significantly higher than in normal skin samples[37] and symptoms such as itch and pain and abnormal thermosensory thresholds to warmth, as well as cold and heat pain, are present, suggesting that small nerve fibers are involved in the pathogenesis of this disease.[38] In hypertrophic scar, data in the literature are not coherent with either a decrease[39] or an increase[40] of the number of observed nerve fibers. Nevertheless, in burn patients with chronic pain, abnormal cutaneous innervation is reported.[41] Recently, in a mouse model of hypertrophic scarring induced by mechanical loading, Li *et al.* suggest that both inflammation and the cutaneous nervous system contribute to hypertrophic scar formation.[42]

Animal models of skin denervation have helped in investigating a possible role of sensory innervation in skin wound healing. Skin denervation models have been designed using surgery, chemicals, or genetically engineered murine strains. Thus, studies have shown that surgical denervation induces delayed wound healing with reduced inflammatory cell infiltration, altered wound contraction, and delayed reepithelialization.[43,44] Another skin denervation model using chemical sympathectomy induced by intraperitoneal administration of 6-hydroxydopamine (6-OHDA) also interferes with wound healing. 6-OHDA-induced sympathectomy modifies wound healing with an increase in wound contraction, reduction of mast cell migration, and delayed reepithelialization. These modifications are associated with a decrease in neurogenic inflammation.[45,46] Capsaicin, a potent agonist of TRPV1, has also been used to induce depletion of neuropeptides (substance P and CGRP) from Aδ and C fibers. When administered to neonatal rats, capsaicin can provoke total sensory denervation, while in adults, it can be used to promote transient sensory neuropathy. Studies have shown that capsaicin induces delayed wound healing and further highlight that neuropeptides released by sensory fibers play a major role in this process.[47–49] Moreover, Toda *et al.* have also shown that angiogenesis and wound closure were significantly suppressed in a CGRP knockout mouse model.[48]

Topical application of sensory neuropeptides following cutaneous wounding has also been investigated. Several studies have highlighted a beneficial therapeutic effect of substance P on wound closure and angiogenesis.[34,50,51] The intraperitoneal or intradermal injection of CGRP has also been investigated with positive outcomes on wound contraction.[52]Altogether, these studies indicate that sensory innervation and neuropeptides such as substance P and CGRP can modulate the overall cutaneous wound healing process (for review, see Cheret *et al.*[28]) and could offer promising therapeutic options.

Mechanisms of nerve regeneration during cutaneous healing

Following skin damage, the mechanisms involved in nerve regeneration are not fully elucidated. Nevertheless, during wound healing, remodeling of regenerating nerve fibers is observed and nerve fiber density is modified. During healing of a burn injury in guinea pigs, it has been shown that the number of substance P-containing nerve fibers acutely decreases after the burn and then gradually increases with a maximum on day 14 postburn. Following that peak, the fiber density gradually decreases to end up lower than controls.[53]

Interactions during wound healing between myofibroblastic differentiation necessary for granulation tissue formation and innervation certainly play a major role. Indeed, myofibroblasts possess neurotrophic properties and are able to regulate innervation during healing. They synthesize and secrete all neurotrophins and express neurotrophin receptors[30] being certainly involved in high levels of neurotrophins such as NGF observed in the wound site.[54] Myofibroblasts also produce extracellular matrix components[55] such as laminin, which are known to promote neurite outgrowth.[56]

Relationships between nerves and myofibroblasts during cutaneous wound healing in the developing rat have been studied by Liu *et al.*[57] Indeed, it is well known that changes in wound healing capacities occur with age with a delay in wound healing observed in elderly. Liu *et al.*[57] show that in neonatal animals, rapid wound closure is associated with important myofibroblast proliferation and a marked increase in innervation density; in contrast, in adult rats where a delayed wound closure occurs compared with neonatal animals, the appearance of both myofibroblasts and nerves is reduced compared with younger rats. The early regeneration of nerves associated with proliferation of myofibroblasts could, at least in part, be responsible for the rapid and efficient healing process observed in neonate animals. In mature rats, altered nerve–myofibroblast relationships may contribute to reduce healing.

In the skin, it seems clear that nerve fibers are located close to the vascular tree and relationships could exist between these structures.[58] Interesting studies have been performed using MRL/MpJ mice, which present an accelerated ability to heal ear punch wounds without scar formation, whereas wounds on the dorsal surface of the trunk heal with scar formation. Indeed, during dorsal skin healing (leading to scar formation), the wounded area becomes rapidly hypervascularized by as early as day 7 postwounding, while at that time, peripheral nerve regeneration is only found in the outer regions of the wound where nerve fibers have begun to sprout into the wound area from surrounding healthy tissue. In contrast, in the ear wound (which heals without scar formation), nerve regeneration precedes vascularization, recapitulating early mammalian development.[59] In addition, denervation of the ear obliterates the regenerative capacity of

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the MRL/MpJ mice and also has a severe negative effect on the ear wound repair mechanisms of the C57BL/6 strain (a control strain known to have poorer regenerative capacity).[44] It suggests that innervation may be important not only for regeneration but also for normal wound repair processes.

Interestingly, it has been shown that the human intervertebral disc aggrecan inhibits both endothelial cell adhesion and neurite extension, repelling sensory neurite growth.[60,61] These studies underline once more the role of extracellular matrix components in angiogenesis and nerve fiber regeneration.

In utero, fetal wounds heal in a regenerative manner without a scar.[62] Antony *et al.* suggest that during development, neurotrophins regulate peripheral innervation formation and, after injury, these factors promote the survival and regeneration of peripheral neurons.[63] Identification of this pattern of neurotrophin and neurotrophin receptor expression in fetal skin, which could be different in adult skin, could provide new insights into understanding the fetal scarless repair mechanisms in response to injury.

In damaged skin, at the level of the nerve fiber, the classical Wallerian degeneration process cannot be involved as far as the distal part of the nerve ending is destroyed. However, we can imagine that at the edges of the lesion, a similar process can develop. It is well admitted that macrophages and Schwann cells are actors in the clearance of debris. Surprisingly, it has been shown in Zebrafish skin that epidermal cells also phagocytose debris generated after injury to peripheral axons.[64] Schwann cells that surround the axon of the fiber ending certainly play a major role to promote and to guide axon sprout. The growth of these sprouts is supported by growth factors produced by Schwann cells, particularly neurotrophic factors, including neurotrophins.[65] In addition, mesenchymal stem/stromal cells (MSCs) such as skin-derived precursors (SKPs) present in the dermis (e.g., SKPs located within the dermal papillae at the base of the hair follicle) (see below) can certainly release factors able to act on nerve regeneration.[66]

Deep Burn Wound Management

Skin damages may have multiple causes, including genetic disorders, acute trauma, chronic wounds, or surgical interventions. Among them, burn trauma represents a type of injury that can be caused by heat, freezing, electricity, chemicals, radiation, or friction. In 2004, fire burn injuries affected 11 million people around the world, including superficial and severe cases.[67] Despite significant improvements in terms of mortality, severe burns cause considerable functional, cosmetic, and psychological *sequelae* and represent a major public health concern.[4]

Classification of burn depths and gravity

Severity of burn wound and prognosis depend on injury depth and extent of the affected surface area.[68,69] The depth of burn wound varies over time and patient needs to be evaluated for depth of the wound regularly (Table 1). A first-degree burn involves only the superficial layer of the epidermis without affecting the basal layer. A second-degree burn affects all the epidermis and part of the dermis from a superficial to a deep degree. A third-degree burn or full-thickness burn involves the destruction of all the epidermis and dermis and may extend to deeper tissues (forth-degree burn affects fat layer, muscle, or bones). The severity of the

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TABLE 1. DESCRIPTION OF CLINICAL CHARACTERISTICS OF BURN WOUNDS OF VARIOUS DEPTHS AND TREATMENTS

Degree	*Injured skin layer*	*Wound aspect*	*Healing time*	*Treatment*	*Prognosis*
Superficial or first degree	Suprabasal epidermis	Red, no blister, dry	3–7 days	Topical treatment	Good
Superficial partial thickness or second degree	Epidermis and superficial or papillary dermis	Red, blister, moist, blanches with pressure	1–3 weeks	Topical antimicrobial agents and occlusive dressings	Good
Deep partial thickness or second degree	Epidermis and dermis (papillary and reticular)	White, nonblanching, dry	3–6 weeks, with scars	Topical antimicrobial agents and occlusive dressings for small surface or eschar excision and autograft application	Scar
Full thickness (third degree)	Full thickness of skin including subcutaneous fat or deeper	Hard texture, white, dry	Does not heal by primary intention	Eschar excision and autograft application if burn TBSA <50% or dermal allograft and skin substitute or autograft highly expanded if burn TBSA >50%	Scar, weak skin

Adapted from Mukouyama *et al.*[58]
TBSA, total body surface area.

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damage is also evaluated by the extent of the surface affected and is expressed as a percentage of the whole body. For a rapid estimation of the extent of burn wounds, the rule of nine is used.[70] However, the Lund–Browder chart provides accurate estimation of the extent of burn wounds in pediatric patients.[68] A calculation program can also be used for a better estimation.[71] Other criteria are also important during the assessment of the burn severity, including patient's age, smoke inhalation, location of burns, and medical state of the patient.

Burn pathophysiology

Skin burns produce a significant imbalance in tissue homeostasis and result in both local and systemic responses. The local tissue damage may be divided into three zones.[72] At the center of the injury, protein coagulation results in irreversible tissue loss called coagulation zone. This area of necrosis can extend to the adjacent zone of stasis characterized by decreased tissue perfusion. Indeed, the central zone may damage the adjacent tissue by the release of inflammatory factors and reactive oxygen or nitrogen species.[73] The external zone of burn wound is called zone of hyperemia, which is characterized by vasodilation and inflammatory changes without structural damage. If the tissue in the zone of hyperemia could almost always recover, the evolution of the zone of stasis depends on the resuscitation technique necessary to rapidly revascularize the tissue.[74]

Beyond 10% of burned area, the local damage may become systemic and induce hypovolemia due to the destruction of the skin barrier function, increased vasopermeability, and plasma exudation.[75] The burned tissue is highly toxic. Indeed, between 100°C and 500°C, melting lipids and membrane proteins create toxic lipid–protein complexes responsible for serious systemic problems.[73,76] These lipid–protein complexes may, in part, be responsible for low survival rate of severely burned patients given that administration of antilipid–protein complex serum in burned mice greatly increases their survival.[76]

Necrosis also triggers the release of inflammatory mediators that generate local inflammation. The inflammatory response accompanied by eventual infections may contribute to systemic effects inducing a systemic inflammatory response syndrome and organ dysfunction, with a threshold around 20–30% of TBSA burned.[77] Systemic disease may cause pulmonary edema and severe organ failure, requiring specific care in burn treatment centers. The inflammatory response is complex and characterized by early secretion of proinflammatory factors such as TNF-α and IL-6, followed by prolonged anti-inflammatory response linked to IL-4, IL-10, and TGF-β production,[78] leading to temporary immune suppression. Therefore, patients become more susceptible to pathogenic microorganism contaminations.[76]

Current management of burn injuries

Management of deep burn injuries depends on both depth and surface area of burn wounds (Table 1). In the particular case of burns, reepithelialization of injuries of first or superficial second degree remains possible by the migration of keratinocytes from the edges of the wound, from hair follicles, and sweat glands, followed by their proliferation, stratification, and redifferentiation to form an intact epithelium.[79] Antimicrobial creams and occlusive dressings are applied on the wound to avoid infection, to limit wound progression, and to improve epithelialization progression.[80]

In contrast, in more severe skin burns such as deep partial-thickness or full-thickness burn, epithelial regenerative elements residing in the basal layer of the epidermis and in the dermis (i.e., epidermal appendages such as hair follicles) are fully destroyed. In these cases, only reepithelialization from the edges of the wound is possible.[81] Full-thickness wounds larger than 1 cm in diameter need special treatment to prevent delayed reepithelialization and extensive scar formation that reduces mobility and induces cosmetic deformities.[79] To date, standard medical treatment for severe skin burns consists in rapid eschar excision and split-thickness skin autograft taken from healthy skin of the same patient. The grafts are usually taken several times from the same area once the donor site has had sufficient time to regenerate.[79,82] Skin grafts are meshed to stretch the graft and so that they can cover a larger area.

Besides being slow to heal and painful, skin autograft is very difficult to perform in patients with burns affecting over 50–60% of the TBSA because of the poor availability of healthy tissue. Different techniques are currently available over the different burn treatment units around the world. The main objective is to reconstitute permanently the dermis and epidermis in the injured area. The first, and faster, alternative is grafting of allogeneic skin, coming from cadaveric skin that can be obtained from skin banks. However, allografts cannot cover the patient wounds permanently because the epidermis is rapidly rejected even if burned patients are immune suppressed.[83] Sandwich techniques can be applied for more permanent covering alternatives where widely meshed split-thickness skin autografts are covered with narrowly meshed allografts[84] or where widely expanded postage stamp autografts regularly distributed over the wound bed (Meek technique)[85] are combined with an overlay glycerol-preserved allograft (modified Meek technique).[86]

In 1975, Rheinwald and Green described for the first time the culture of epidermal sheets (cultured epidermal autograft [CEA]) produced with human autologous keratinocytes derived from a small sample of uninjured skin.[87] Several burn treatment units used the technique of Cuono, which consists of early debridement of all burned tissue in the wound and coverage of it with meshed, expanded cryopreserved allografts coming from cadaveric skin. Later, allografts are abraded to remove mechanically allogeneic epidermis and CEAs are applied directly to the allogeneic dermal bed, taking benefit of a prerevascularized matrix.[88] Since then, several combined procedures have been developed to overcome the lack of donor sites. Among these methods, the combination between the Meek technique and sprayed autologous cultured keratinocytes[89] has given interesting outcomes.

Other alternative methods such as the combined technique can also be used.[90] The first steps of Cuono technique can be applied until epidermis abrasion. Then, widely meshed autografts are grafted, followed by the application of CEA. Keratinocytes from CEA will colonize mesh autograft and play a trophic role for epidermal regeneration. If this technique seems interesting in terms of percentage of engraftment, it needs enough available healthy skin to collect autografts. That is why the Cuono technology remains widely used despite a varying degree of graft take.

Grafting efficiency of CEA is highly variable and depends mainly on the metabolic status of the patient. However,

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nowadays, there is no other option to enhance patient survival and to provide enough surface for the epidermal barrier.[91,92] However, several drawbacks with the use of CEA have been noticed such as poor dermo-epidermal junction maturation, their high cost, their fragility, the use of animal proteins and/or cells in the culture process, and variable grafting efficiency.[93] Several kinds of acellular biomaterials can be used in combination or not with CEA grafting to improve grafting efficiency (Table 2).

For example, to overcome these weaknesses, researchers have cultured CEA on fibrin matrices first obtained from purified fibrinogen[127,128] and more recently on fibrin matrices obtained from clotted human plasma (human plasma-based epidermal substitute)[129,130] (Fig. 3).

Chronic Sensory Disabilities Following Deep Burn Injuries

The local destruction of the cutaneous nerve fiber network during a burn injury leads to immediate neuropathy, which is obviously more serious in the context of a full-thickness burn (see above). Although nerve fibers may regenerate after a skin grafting and subsequent wound healing, their density often remains lower than before the injury.[39,131–133] The persistence of neuropathy is shown to be associated with some risk factors. For instance, the electrical cause is more deleterious.[134,135] Especially, a low-voltage electrical burn induces more frequent *sequelae* than a high-voltage injury and correlates with the occurrence of mononeuropathy.[136,137] Other additional factors promote neuropathy. For example, the prevalence is higher in adults and in people displaying a large TBSA (over 20%), a full-thickness burn, or a hypertrophic scar.[39,134,138]

As cutaneous innervation is crucial for wound healing, the persistence of neuropathy delays it.[14] Furthermore, neurological symptoms such as sensibility losses, itch, paresthesia, and pain may occur. These complications are common in the first months following the burn injury, but often gradually decrease with time. However, depending on the anatomic site of the scar or on injury severity, they can impact the patient's quality of life and even delay their overall rehabilitation.[139–141]

Sensibility losses

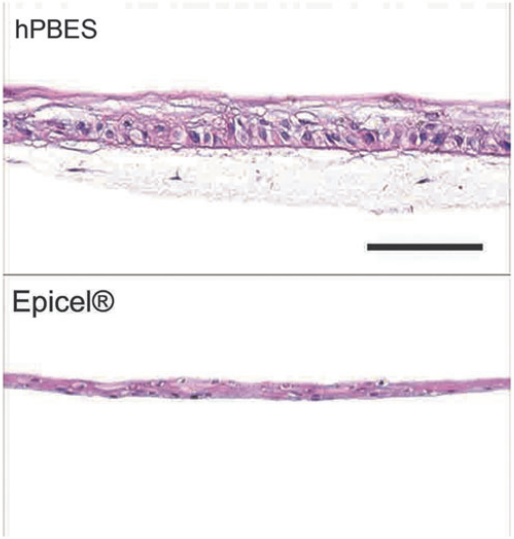
A lot of burned patients complain of a transient or permanent loss of sensibility, which affects their perception of temperature, pressure, or touch and is very often associated with painful sensations and paresthesia.[142] Hermanson *et al.* were among the first to assess the sensibility in burned patients using quantitative sensory measurements.[143] They demonstrated that in years following burn, the touch threshold is increased at the scar site compared with the uninjured contralateral side skin. Such an abnormality is noticed in spontaneously healed scars and in early and late excised grafted scars. These findings suggest that treatments fail to improve the touch sensibility and that the severity of the burn injury does not influence the occurrence of a sensibility loss. A more recent study that enrolled a larger number of patients confirmed that the touch threshold is increased in scars compared with uninjured skin from healthy volunteers. However, deep burns requiring skin graft displayed significantly higher touch threshold than superficial burns.[142] It was the same for the heat pain threshold and the two-point discrimination, which measures the spatial tactile acuity.

TABLE 2. ACELLULAR BIOMATERIALS COMMERCIALLY AVAILABLE AND/OR USED IN CLINICS FOR BURN TREATMENT (DERMAL REPLACEMENT AND/OR SKIN REPAIR)

Source of biomaterial	*Product/Company*	*References*
Human skin or dermis		
Cadaveric skin (cryopreserved, glycerolized, lyophilized, or acellularized)	Tissue Bank, Alloderm® Life cell Corporation, Gammagraft® Promethean Life Science, Glyaderm® Euroskinbank	77,94–102
Animal dermis		
Porcine acellularized dermis	Strattice Tissue Matrix® Life cell Corporation, Epiflex® DIZG, EZ Derm® Mönlycke Healthcare	103–106
Lyophilized porcine intestinal mucosa with growth factor	Oasis Wound Matrix® Johnson and Johnson	105
Porcine tendon-derived atelocollagen type I+bFGF	Pelnac+bFGF	106
Lyophilized bovine dermis	Matriderm® MedSkin Solutions Dr.Suwelack, Terudermis® Olympus Terumo Biomaterials	107–111
Bovine collagen and chondroitin 6-sulfate		112
Bovine collagen and glycosaminoglycans	Integra® Integra Lifescience	113–115
Synthetic polymer		
Polylactide, trimethylene carbonate, and e-caprolactone copolymer	Suprathel® Polymedics Innovations GmbH	116–118
Nylon coated with porcine peptides	Biobrane® Smith and Nephew, AWBAT® Aubrey, Inc.	119–121
Biopolymer		
Derivatives from hyaluronic acid	Hyalomatrix PA® Fidia Advanced Biopolymers	122–125
Allogenic fibrin	Engineered skin substitute	126,127

bFGF, basic fibroblast growth factor.

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FIG. 3. Hematoxylin–phloxine–saffron staining of hPBES and of a cultured epithelial autograft, Epicel®. For the hPBES substitute, a well-organized basal layer of cuboidal or columnar keratinocytes is observed, similar to healthy skin. Scale bar: 100 μm. hPBES, human plasma-based epidermal substitute.

The assessment of the cold sensibility revealed that this threshold is significantly lower in deep burns than in superficial burns, still confirming that the scar sensibility loss does depend on the severity of the injury. Other studies focusing on grafted patients also reported impaired sensory thresholds in their scars and demonstrated that the sensibility loss was correlated with the amount of neuronal structures within the burned area.[132,133,144] However, local deficiency of these structures is insufficient to explain a sensibility loss since a lot of patients also exhibit slightly impaired sensory thresholds at their uninjured contralateral side.[142,143,145] These data highlight that a sensibility loss also results from an altered processing of the afferent or the efferent information by the central nervous system.

Itching and paresthesia

Paresthesia is an abnormal perception that may be long-term *sequelae* after burn. The severity, frequency of this symptom, and impact on the quality of life are assessed using questionnaires. They revealed that more than two-thirds of patients suffer from paresthesia, of which the most frequent are tingling, stiffness, numbness, or pinpricks.[146,147] Itching is also a common postburn paresthesia and affects at least 70% of burn patients at 1 or 2 years postburn, and still around 40% in the following decade.[12,148,149] The itching prevalence also depends on the injured anatomic sites. Contrary to the face and the neck, legs are typically affected, especially in the first months.[150,151] Furthermore, itching is generally more intense during the first 3 months postburn and its severity significantly decreases between the 3rd and 12th month postburn, a time frame consistent with improvement of the scar quality.[148,151,152] Sometimes, however, it delays healing because of frequent scratching and alters the quality of life.[153] Moreover, numerous risk factors promote itching persistence and severity. Willebrand *et al.* demonstrated that it is positively associated with the total burned skin area, while Kuipers *et al.* rather showed that it is stronger as the number of itchy body surface areas is high.[151,154] This apparent discrepancy between the two studies likely stems from the fact that the postburn time was around few years in the first one and only around few months in the second one. Furthermore, participants were slightly older and had average TBSA and the percentage of full-thickness burn was twice higher in Willebrand's study. Overall, itching appears to be related to the severity of the burn injury since other data highlighted its positive association with the time required for wound healing and the number of surgical interventions.[150,155] Moreover, grafted burn scars are itchier than nongrafted scars, especially in the first months.[151] These findings support previous outcomes showing more substance P-immunoreactive fibers in grafted skin in the first years postburn, although the number of total nerve fibers was decreased.[132] Interestingly, substance P was shown to trigger a release of histamine, promoting itching.[156,157] This corroborates outcomes showing that this neuropeptide is especially elevated in case of hypertrophic scars, a complication tightly correlated with thermal injury and highly associated with itching.[158–160] All of these data support many neurophysiological studies demonstrating that itching results from neuropathic mechanisms.[161] Finally, the postburn itching mechanism is close to that observed in numerous peripheral neuropathic diseases.[162]

Pain

Postburn pain characteristics differ depending on the stage after the injury. Postburn pain is first acute pain, but becomes chronic pain in the rehabilitation phase. Three main subtypes of acute pain are distinguished.[163] The procedural pain occurs during treatments, whereas the background pain continuously affects patients even when immobile. Afterward, with the decrease in analgesic medications and the increasing ability to move, patients may complain of upsurge in pain called breakthrough pain. The mechanism of acute pain is directly related to the tissue lesions, which lead to inflammation and damaged nerve structures. Inflammation is mediated by cytokines such as IL-6, which promote hyperalgesia.[164] For their part, injured nerve fibers exacerbate this inflammation by releasing neuropeptides such as substance P and CGRP, well known to mediate neuropathic pain. The chronic pain arises later during the recovery phase and may persist for a long time. Indeed, it affects at least one-third of patients in the first years postburn.[146,147] A survey among 336 burned patients reported that 52% of them complained of pain, although their injury happened 10 years before.[11] For those with ongoing pain, at least half declared that it impeded their daily life and even delayed their rehabilitation. Chronic pain especially affects older patients and those displaying higher grafted burned skin areas.[165] In addition, pain is exacerbated by factors such as temperature changes, light touch, and also positions, especially when injuries affect extremities.[165,166] It is worth noting that psychological aspects should also be

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addressed. For instance, anxiety and depression are associated with greater pain.[167] Conversely, pain raises the level of anxiety and depression.[167] This highlights that emotional distress needs to be considered to minimize pain, even if pain often significantly decreases between the 3rd and the 12th month postburn.[152,165] As well as for itching, this positive trend corresponds to the improvement of the scar quality. The understanding of the mechanism of chronic pain mainly focuses on substance P and CGRP. Although pain has been related to the release of these two neuropeptides, chronic neuropathic pain was rather found to be related to the release of CGRP.[41,168]

Strategies to Improve Wound Healing and Nerve Regrowth

Biomaterials

The quality of wound healing relies also on the capacity to recover the sensitivity of repaired areas, contributing also to promote tissue repair. To support nerve regrowth and therefore improve the recolonization of wounded regions by neuronal extensions, various biomaterials have been studied. These biomaterials can be separated into two families: materials of biological origin and synthetic materials. In both cases, the addition of specific molecules was tested to improve the adhesion of the nerve fiber endings onto the material and to enhance their growth.

The biomaterials aimed at the fabrication of a nerve support must have specific properties such as biocompatibility, biodegradability, and mechanical strength. Several bioengineered conduits have already been commercialized for clinical applications to replace a sectioned or crushed nerve,[169] yet none of these products present a full functional recovery. Moreover, these kinds of tridimensional materials do not address directly the problem of skin reinnervation and some modifications in the structure or the shape of the biomaterials are needed to be used to this specific aim.

Material properties. Biomaterials aimed at guiding axonal regrowth need to present various properties. Their biocompatibility is linked to the interactions between the material and its biological environment. Tissue–material interactions should not provoke irritation or create significant inflammatory response.

Moreover, the material needs to be flexible to react to the movements of the skin without breaking or creating rigidity of the wounding.[170] Ideally, electrical conductivity could help nerve regeneration by stimulating axon regrowth and orientation due to the charged membrane surface. To date, most of the materials described in the literature are nondegradable.[171] The disadvantages of the nonbiodegradable materials or nonfully absorbable materials reside in the risk to provoke a reaction of the immune system, which can lead to scarring or prolonged inflammatory responses. Additionally, a second surgical intervention is often required to remove the material.

In the specific case of wound healing, a biomaterial must tolerate modifications in physico-chemistry during the various phases of cell proliferation, reepithelialization, and extracellular matrix reorganization. Mechanical properties (traction force, elongation at rupture, tenacity) of the materials need to be tested together with the other cell types from the area (mainly fibroblasts and keratinocytes) to verify the efficacy of the biomaterial and its potential interactions with other cells from the healing area.

Natural materials. Because of their enhanced biocompatibility and specific structural motifs, natural polymers have been commonly used.[172] Chitosan, derived from chitin, is an amino polysaccharide significantly studied in the literature. This material is considered nontoxic and biocompatible with many applications in tissue engineering and particularly for wound healing.[173] It is used to create matrices presenting adjusted degrees of porosity. In addition, chitosan has been described to interact with laminin, fibronectin, and collagen type IV, molecules from the extracellular matrix able to promote adhesion, migration, and differentiation of cells from the nervous system.[174] Another promising candidate, collagen, the main structural protein in the body, is often employed as a scaffold supporting cells.[175] The use of collagen to make nerve conduits restores partially the nerve functionality.[176,177] Nevertheless, mechanical properties and biodegradation rates of chitosan and collagen are not optimal.[173] So, the studies are directed to other natural materials such as hyaluronic acid, keratin, or silk fibroin.

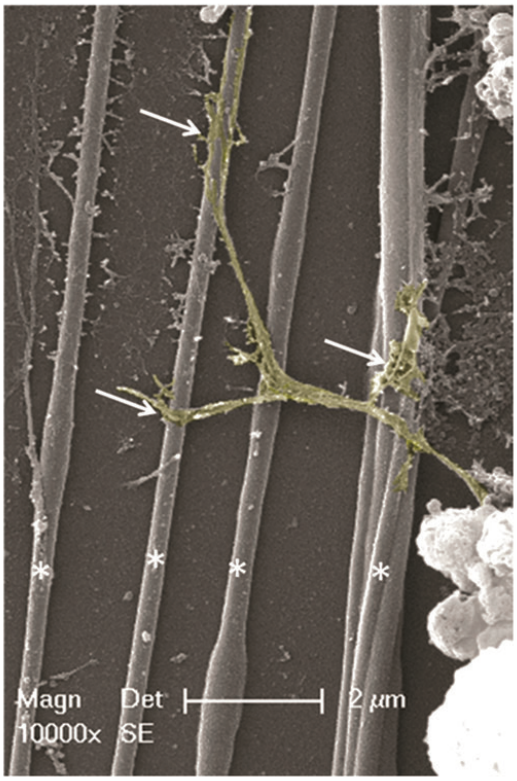
The ability of hyaluronic acid to augment keratinocyte proliferation, fibroblast migration, and endothelial cell angiogenic responses in the wound makes it a useful biopolymer for wound healing.[178] Hyaluronic acid can limit scar tissue and can facilitate functional recovery of neoformed tissues.[179] It is interesting to underline that fetal skin, which is rich in hyaluronic acid, heals without scarring.[180] Moreover, this molecule can accelerate nerve regeneration.[178,181]

As for hyaluronic acid, mouse fibroblasts proliferate well on keratin-covered surfaces, demonstrating the biocompatibility of this molecule.[182] Furthermore, tridimensional materials made of a scaffold of keratin have been used for specific bioapplications, such as wound dressings or hydrogels or scaffold, guiding the growth of neural tissues.[183–185] *In vivo* study showed that keratin hydrogel stimulated Schwann cells' migration and dedifferentiation from the proximal nerve ending. Moreover, these materials could block the infiltration of macrophages described during the Wallerian degeneration of the distal nerve part.[186]

Silk fibroin, another natural polymer, has been used for various applications such as cosmetics or food additives. In recent literature, silk proteins have also been described as having vast promise in biomedical and engineering fields because of its specific biological properties, such as biocompatibility, biodegradability, and induced limited inflammatory responses *in vivo*.[187–190] These promising properties have encouraged development of silk fibroin-based nerve conduits. Indeed, the use of silk fibroin allows high structural integrity and nervous tissue colonization[191] (Fig. 4). Moreover, silk has robust mechanical properties, no toxicity toward neurons, and can be biofunctionalized, permitting the acquisition of new physico-chemical properties.[192–194] ◀F4

Synthetic materials. Synthetic materials also can be used in tissue engineering: they are structurally stable for implantation, are biomimetic, and able to support repair and regeneration. Moreover, these materials are not toxic for cells of the original tissues or organs.

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4C ▶ **FIG. 4.** Scanning electron microscopy observation of neuron cells on electrospun fibroin nanofibers. Primary cell culture of dorsal root ganglion cells obtained from young male Sprague-Dawley rats (1–3 months old) seeded on electrospun fibroin nanofibers (*). Close interactions between axonal growth cones and fibroin nanofibers are visualized (*arrows*).

Poly(ε-caprolactone) (PCL) is a synthetic polymer presenting good mechanical properties while being biocompatible and biodegradable.[195] The nanofibrous PCL is a dependable substrate supporting the growth and differentiation of a variety of cell types.[196] PCL is also used in the development of tubular nerve guidance systems.[197] Polylactic acid (PLA) is another example. Gautier *et al.* have demonstrated the qualities of resorption and biocompatibility of this material specifically using Schwann cells and neurons from the spinal cord.[198] Despite some concerns about the structural stability of the material, PLA scaffolds loaded with Schwann cells and surgically inserted in transected rat spinal cord allowed the regrowth of neural tissues and their revascularization, proving the high interest in this material.[199]

Poly(d, l-lactic-co-glycolic acid) (PLGA), a copolymer from lactic acid and glycolic acid, has also been used as a therapy vector for the release of active molecules or cells. This copolymer typically offers a higher primary stability and is more amenable to macro/microstructure formation than natural biomaterials. Among the various uses of this material, nanospheres or microspheres made of PLGA have gained popularity, mainly because of their tissue compatibility and biodegradability.[200] Chang *et al.* showed that animals implanted with conduits made of PLGA and supporting cultured Schwann cells presented a higher number of myelinated axons.[201]

Other polymers are also used, such as polyvinyl chloride (PVC), polyethylene glycol (PEG), and polyamidoamine (PAA), with some success. Indeed, an improvement in the density and size of the axons, as well as greater myelin thickness, was observed following the use of PAA nerve conduits.[202] Other teams have shown that the use of PVC improves myelination and high structural integrity.[203] Koob *et al.* have shown greater improvement in exploratory behavior of injured PEG-treated rats.[204]

Biofunctionalization of biomaterials. Various strategies have been tested to give specific functions to biomaterials either based on structural modifications of the material to enhance cell adhesion[205] or to stimulate cell growth at its contact.[169] Specifically for neuronal-related application, the option of grafting or adding a neurotrophic factor to the biomaterial has been widely studied. The most common factor inserted is NGF, followed by glial cell line-derived neurotrophic factor, BDNF, NT-3, and neurotrophin-4/5, as these molecules have been demonstrated to improve peripheral nerve regeneration. These proteins have been added either in microspheres or in microgels[206–208] to diffuse in the microenvironment or in regenerative conduits[209,210] aimed at guiding peripheral nerve regeneration.

Nevertheless, if neurotrophic factors seem in fact the most accepted candidates to biofunctionalize materials aimed at helping reinnervation, other molecules have been shown to have interesting potentials. For example, bone morphogenetic protein-2 was demonstrated as able to increase the number of axons and their diameter.[211]

No study has been found in the literature where growth factors specific to the epidermal layer were added to biomaterials to help skin reinnervation, although fibroblast growth factors were described as allowing faster rehabilitation after peripheral nerve injury.[212]

Skin substitutes

As mentioned above, to allow the coverage of deep and extensive burns over a TBSA of more than 50% to 95%, tissue-engineered epithelial sheets made of patient's own keratinocytes were developed in the 70s by Rheinwald and Green.[87,213–215] These CEAs were successfully grafted on wounds promoting efficient epidermal healing, with esthetical and functional results not as good as split-thickness skin grafts, but efficient to cover burns.[216] The technique was improved over the years, allowing to prepare the sheets in about 2 weeks in sufficient amounts.[217] The production of CEAs was manufactured as Epicel® in United States under the Humanitarian Device Exemption regulations by Genzyme (which sold this division to Aastrom Biosciences in 2014). The main advantage of using CEAs is the reduction of the delay to achieve complete coverage of patient's extensive burns, leading to better survival and shorter stay in the burn unit.[218] Its main drawback is the high cost of

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treatment (that may exceed 100,000 U.S. $ per patient) that could be compensated by the reduced cost of the shorter hospitalization and the lower need for subsequent reconstructive surgeries.

For a better healing quality of the wound, the combination of the epidermal autograft with a dermal compartment would be desirable.[219] However, since the dermis is a three-dimensional (3D) tissue, its *in vitro* reconstruction proved to be much more complex than the epidermis. Beside the development of acellular dermal substitutes,[112] the first attempt to produce a living dermal substitute was performed by Bell in 1979 by culturing fibroblasts embedded in a collagen gel[220] (Table 2). This dermal tissue was then seeded with keratinocytes to produce a tissue-engineered skin.[221] This living skin equivalent permitted to demonstrate the importance of the presence of dermal fibroblasts in skin substitutes to rapidly promote the formation of a functional neodermis in humans[222] (Table 3). T3 ▶

This skin substitute was then manufactured by Organogenesis as Apligraf®, made of human fibroblasts and keratinocytes. Since it is a heterologous tissue, it is only intended to treat venous leg and diabetic foot ulcers as temporary biological dressing, but not burns, which require autologous epidermal graft for permanent coverage.[238] Some attempts were made to apply Apligraf over meshed split-thickness autografts transplanted on burn wounds and showed cosmetic and functional advantages, but the cost/benefit ratio of this approach is questionable.[239] Moreover, whereas Apligraf was shown to efficiently improve ulcer healing, it is rarely used in the clinic because of its high cost and the availability of much cheaper dressings with nearly similar efficacy and much easier handling.[240] Several other dermal substitutes were developed to produce tissue-engineered skin based on the culture of fibroblasts in a de-epidermized dermis,[241,242] a collagen sponge,[231,243] a biodegradable mesh,[244] or a self-assembled fibroblast sheet,[245] to name a few. Most of these models were transplanted in mice and showed good results in terms of take or dermal and epidermal remodeling.[245,246] One aspect has recently been given more attention, the delay of complete vascularization of the graft. Indeed, it was shown that even if skin substitutes were rather thin, a compromised survival of the epidermis could be feared in dermal compartments thicker than 100 µm, exceeding the maximal distance for diffusion of oxygen and nutrients from the wound bed.[247] These skin substitutes would then require specific strategies to enhance vascularization of the dermis, through the incorporation of endothelial cells to promote capillary formation in the tissue before graft.[248–250] A complete vascularization of the graft was observed only 4 days after transplantation, instead of 2 weeks in the control without capillaries, through inosculation of the network of capillaries from the endothelialized skin substitute with the vascular network of the wound bed.[250]

Another important aspect of the application of an autologous skin substitute to cover deep and extensive burns is to what extent it may improve nerve regeneration and sense of touch recovery. It was shown that transplantation of skin substitutes on mice promoted nerve migration into the graft after 3–4 months.[251,252] However, the major advantage of reconstructing skin *in vitro* is that it is possible to incorporate into it molecules or cells that could specifically enhance nerve regeneration.[253] Moreover, it is possible to investigate the potential benefit of these approaches *in vitro* through the design of an innervated reconstructed skin. This model was developed by the incorporation of sensory neurons extracted from mouse embryo dorsal root ganglia. They were seeded on the fibroblast-populated sponge 1 week before keratinocytes to form a nerve network (Fig. 5). The *in vitro* impact ◀F5 on nerve migration of any molecule or cells incorporated into the model can be analyzed by quantification of the number of sensory neurites.[254] These neurons, whereas they were of mouse origin, were shown to release neuropeptides (substance P) efficiently modulating the human keratinocyte behavior.[255] Thanks to the high versatility of these tissue-engineered skin models, it was possible to perform a wound in the epidermis to analyze the effect of innervation on re-epithelialization *in vitro*, compared with a control without nerves. Wound closure was shown to be twice faster in the presence of nerves because of their release of substance P. Indeed, this effect was completely abolished after blocking

Table 3. Cellularized Biomaterials Commercially Available and/or Used in Clinics for Burn Treatment (Skin Repair)

Source of biomaterial	*Cells*	*Product/Company*	*References*
Synthetic polymer			
PGA/PLA	Neonatal foreskin fibroblasts	Dermagraft® Organogenesis	223,224
Animal			
Bovine collagen	Neonatal fibroblasts	Apligraf® Organogenesis	225–228
Bovine collagen	Autologous fibroblast		
Bovine collagen	Neonatal fibroblasts and keratinocytes	Orcel® Forticell Bioscience	229
Bovine collagen	Autologous cultured keratinocytes and fibroblasts	Tissue-cultured skin autografts	230
Bovine collagen+GAG	Autologous cultured keratinocytes and fibroblasts	Engineered skin substitute Amarantus	231–233
Human			
Autologous fibrin	Autologous cultured keratinocytes and fibroblasts	MyDerm® Cell Tissue Technology	234,235
Autologous plasma	Autologous cultured keratinocytes and allogenic fibroblasts	Engineered skin substitute	236,237

AU8 ▶ GAG; PGA; PLA, polylactic acid.

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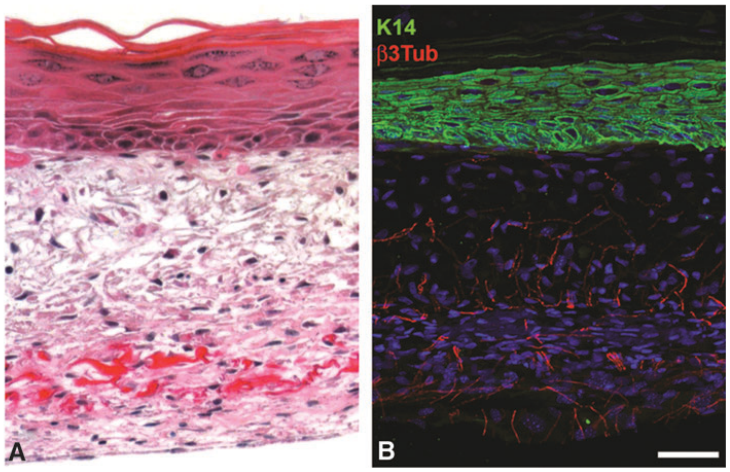


FIG. 5. *In vitro* characterization of an innervated reconstructed skin. (**A**) The tissue-engineered skin is made of human keratinocytes and fibroblasts cultured in a collagen–chitosan sponge biomaterial for 42 days, in which mouse sensory neurons are incorporated at day 14 (1 week before keratinocytes) on the opposite side compared with epidermis. Keratinocytes form a well-differentiated epidermis over the fibroblast-populated dermis as seen on hematoxylin–eosin histological cross section. (**B**) Keratinocytes express keratin 14 (stained in *green*) and sensory axons express anti-β III tubulin, a neuronal marker (stained in *red*), and generate a homogeneous network of neurites migrating from the bottom of the dermis (where neurons are located) up to the epidermis. Cell nuclei are stained with DAPI. Scale bar: 50 μm.

4C ▶

the NK1 receptor for substance P with an antagonist.[255] This experiment showed that nerves promote direct enhancement of reepithelialization, independently of their induction of neurogenic inflammation *in vivo*, which is well known to improve wound healing.[31]

To enhance *in vivo* nerve regeneration of skin substitutes after graft, different approaches were investigated. Laminin, a natural component secreted by Schwann cells and known to facilitate axon migration, was added into a tissue-engineered skin and induced a major increase in nerve migration after graft. It allowed complete functional recovery of all the three types of cutaneous nerve fibers (i.e., Aβ, Aδ, and C fibers).[256] Since laminin is a stable and large molecule, it could be easily incorporated in skin substitutes. The addition of Schwann cells in the tissue-engineered skin also demonstrated an enhancement of nerve regeneration and pain and temperature perception recoveries, but should be more complex and expensive to use for a clinical application.[254] Target cells for sensory nerves, such as Merkel touch domes[251] or immature hair follicles,[257] could increase the speed of nerve regeneration and promote guided nerve migration and a potential sense of touch recovery through the connection of nerves with a sensory unit, but are not yet feasible in a human context for clinical application. Even if some of these techniques have been proved to be efficient to increase nerve regeneration, the question of the quality and functionality of this neoinnervation remains to be clearly demonstrated in clinical studies.

These encouraging results point out the potential of skin substitutes to markedly improve sensory recovery. However, split-thickness skin also contains Schwann cells and Merkel touch domes, but its graft does not always promote good sense of touch recovery. The main reason for that might be the anarchic structure of the wound bed, which may compromise efficient nerve regeneration.[142,145] Thus, the time required to prepare these skin substitutes could become an important limitation in their use since a delay to cover burns could induce an unfavorable remodeling of the wound bed, preventing further nerve migration.

In addition, all these exciting improvements of skin substitutes with more sophisticated characteristics and enhanced potential for tissue function recovery face the challenge of their manufacturing, which emerged as a bottleneck to translate these skin substitutes to the clinic. As observed with the CEA technology, whereas it was beneficial to patients, its high cost has always limited its application. Moreover, this complex manufacturing process has even probably never been profitable for the company itself. The reason is the need to use patient's own cells for each treatment, and one can easily see how the extraction of fibroblasts in addition to keratinocytes and reconstruction of the dermal compartment may dramatically increase the cost and the time of tissue production that may not be affordable to most burn units. An alternative could be to develop a local nonprofit unit of production of skin substitutes linked with regional burn units, but that would require highly qualified personnel and regulatory approval, such as those established in Europe and Canada.

Finally, these autologous skin substitutes are clearly highly beneficial to the burn patients and may not be that expensive on a long-term perspective. This is why it is still so important to continue developing an ideal tissue-engineered skin, easy to manufacture, and as efficient as possible to achieve complete cutaneous recovery of function, including tactile and pain perception.

Mesenchymal and induced pluripotent stem cells

Biomaterials and skin substitutes can be associated with stem cells as another strategy to promote nerve sprouting from the surrounding healthy tissue and guide axonal regrowth within the forming scar. MSCs have the capacity to generate different cell lineages and offer a wide range of future therapeutic approaches in skin healing and sensory recovery.[258] Because of their multipotency, large *ex vivo* expansive potential, and immunotolerance properties, autologous MSCs represent an attractive source of stem cells that could be included in a wound management protocol.[259]

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Another major drawback in the study of skin reinnervation is the limited sources of human mature sensory neurons that can be used in *in vitro* and *in vivo* experimental models. Using MSC-derived neurons or induced pluripotent stem cells (iPSCs) could help overcoming this issue in future experimental investigations.

Therapeutic potential of adult MSCs. The skin and more precisely the dermal compartment is a source of adult MSCs named SKPs. These SKPs possess capacities of self-renewal and multipotency and they can differentiate into both mesodermal and neural progeny.[260] Neural crest stem cells have a similar broad potential and contribute to development of the dermis and, in this regard, SKPs form a neural crest-related stem cell niche that arises in the skin during embryogenesis and persists in lower numbers into adulthood.[261] SKPs are present in several locations in the dermis, hence translating cellular heterogeneity. The largest and most studied source is located within the dermal papillae at the base of the hair follicle.[262,263] Other sources of dermal SKPs include the hair bulge, sebaceous gland, and sweat gland, as well as a perivascular niche recently described.[262 264] After isolation, SKPs are maintained in culture as spheroids and express specific markers such as nestin, vimentin, and fibronectin.[265,266] Neuronal differentiation is achieved using AMPc and a cocktail of neurotrophins such as BDNF, NT-3, and NGF, while glial differentiation into Schwann cell is promoted by the addition of forskolin and heregulin 1β to the culture medium[267 270] (Fig. 6). Little is known regarding the role of SKPs in skin wound healing or a potential involvement in sensory nerve regrowth, but several studies have shown that SKP-derived Schwann cells help in promoting sciatic nerve regeneration in rodents.[271 273] It suggests that SKP-derived Schwann cells are fully functional in supporting axonal regrowth following injury. Recently, Ke *et al.* have shown that collagen sponges seeded with SKPs facilitate skin wound healing in diabetic mice by promoting local vascular regeneration.[274] Another study has also shown that intradermal injections of SKPs around full-thickness excisional cutaneous wounds in diabetic mice mediate faster wound closure and reepithelialization, earlier angiogenesis, and might promote wound reinnervation.[275] Interestingly, another *in vivo* study has highlighted that SKP transplantation in denervated cutaneous wounds on nude mice promotes wound closure and local secretion of neuromediators such as substance P and CGRP, as well as NGF.[276] More studies have to be performed to determine if local or transplanted SKPs can either differentiate into Schwann cells following skin injury or if they somehow help mediating the migration of local Schwann cells and/or axonal regrowth of nerve fibers during scarring. The study of SKP secretome could shed new light into factors contributing to this phenomenon. Thus, the isolation of SKP-derived autologous precursors from adult human skin represents an accessible and very promising source of neurons and Schwann cells to help restore normal innervation after skin damage.

The adipose tissue represents another valuable and abundant source of adult MSCs. It has the advantage of being accessible using liposuction procedures. Adipose-derived stem cells (ASCs) can be easily expanded *ex vivo* by isolating the stromal vascular fraction from the adipocytes using enzymatic digestion. Like SKPs, autologous ASCs can be driven toward neurogenic or glial differentiation.[277,278] Many studies have shown the ability of ASC-derived Schwann cells in promoting peripheral nerve regeneration and wound healing, but again, little is known about their potential in mediating cutaneous sensory recovery following skin damage. The subcutaneous adipose tissue could then be of interest as a close by reservoir of ASCs following skin injury. Recently, Tomita *et al.* have shown that in rats, Schwann cell-like cells differentiated from ASCs could improve cutaneous nerve regeneration in skin flaps by producing NGF and BDNF.[279]

BM-derived MSCs have also been used in the treatment of skin wounds.[280] BM-derived MSCs are isolated using BM aspirate and selected *in vitro*. The BM aspirate is an invasive method and the number of MSCs present in the BM swab is limited (0.001–0.01% of total BM nucleated cells). The selection of BM-derived MSCs relies on their ability to adhere to plastic before expansion. Interestingly, BM-derived MSCs have been suggested to participate in tissue repair. They are able to migrate to the damaged tissue and differentiate into wound healing (myo)fibroblasts.[281] BM-derived MSCs have also been shown to differentiate into neurons and Schwann cells.[282,283]

In addition, extrafetal tissues are a source of great interest. In extrafetal tissues, MSCs have been described in the amniotic fluid and in different layers of placenta, principally the amnion and chorion. They have also been described in Wharton's jelly around cord vessels. These cells have particularly interesting immunological features and hepatocyte-like differentiative capacities.[284] It has also been shown that progenitor cells are present in gingival connective tissue.[285] Based on their ability to differentiate into several lineages, to proliferate from single cells, to induce calcium deposits, and to secrete collagen *in vivo* after transfer on hydroxyapatite

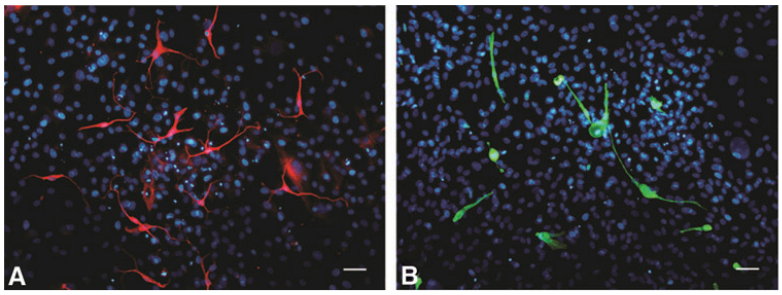


FIG. 6. Neuronal and glial differentiation of human SKPs. (**A**) Neuron-like SKPs express β III tubulin (*red*). (**B**) Schwann cell-like SKPs express S100β (*green*). DAPI is used for nuclear staining (*blue*). Scale bars: 100 μm. SKP, skin-derived precursor.

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carriers, these cells correspond to gingival multipotent progenitor cells. The exceptional healing capacity of the gum can be correlated with the presence of these progenitor cells, which also represent a new safe therapeutic strategy for wound healing.

It gradually became apparent that MSC ability to change a pathological environment and enhance wound healing is not only related to their capacity of differentiation but also to their ability to modulate the behavior of other cell types. Their activities mainly go through secretion of different kinds of bioactive molecules (e.g., growth factors, cytokines, and chemokines).[286] They are also able to realize mitochondrial transfer and to produce microvesicles and exosomes containing protein, mRNA, miRNA, or mitochondrial fragments.[287,288] Thereby, Zhang *et al.* have shown that exosomes derived from perinatal MSCs are able to accelerate the healing of skin burns by increasing the reepithelialization and angiogenesis process through Wnt and PI3K/AKT signaling pathways.[289] Finally, it is possible to optimize MSC efficiency by modulating their culture environment with various kinds of stimulations, called priming or licensing.[288] This optimization has two objectives: (1) to prepare them for the environment in which they will be injected to and (2) to modulate their behavior to counterbalance or promote a physiological reaction. For example, pretreatment with hypoxia[290] or with cytokines such as TGF-β1[291] or TNF-α[292] can enhance wound healing. Studying the paracrine communication of MSCs in both their differentiated and naive states could also be of foremost interest.

Induced pluripotent stem cells. iPSCs were first generated in 2006 using both embryonic and adult mouse fibroblasts.[293,294] The experimental protocol consists in the genetic reprogramming of somatic cells into pluripotent stem cells by targeting four specific genes: Oct4, Sox2, Klf4, and c-Myc. Thus, human iPSCs display characteristics of embryonic stem cells and can generate a wide range of cell types, including neurons.[294–296] The generation of iPSC-derived Schwann cells has not been reported so far. The major advantage of iPSCs is the availability of the source material, a simple skin biopsy being necessary to collect dermal fibroblasts. However, the genetic reprogramming of fibroblasts, maintenance, and differentiation of iPSCs is technically challenging and time-consuming. Moreover, to reduce safety concerns associated with viral vectors, protocols using plasmids or recombinant proteins channeled into the cells have been developed.[297,298]

As an alternative to iPSCs, the direct conversion of fibroblasts into neurons using small molecules has recently been described. Using a cocktail of chemicals and neurotrophic factors such as forskolin and CHIR99021, a selective inhibitor of glycogen synthase kinase 3, researchers were able to generate functional neurons in 21 days.[299,300] This method represents a new advantageous tool to generate mature human neurons that could be used in future experimental approaches.

Bioprinting

Since several years, printing technology has rapidly progressed from two-dimensional (2D) to 3D printing where different kinds of materials can be used. Therefore, the field of tissue engineering has benefited from this technology to improve the seeding of a wide range of cells onto solid and biodegradable scaffolds. It allows reproducing the complex 3D structure of extracellular matrix components and designing tissues by adding biomolecules.

Several 3D bioprinting techniques exist such as inkjet bioprinting, microextrusion bioprinting, and laser-assisted bioprinting.[301–303] Laser-assisted printing is the most favorable technique to maintain cell viability and print good quality vertical structures with high resolution. Microextrusion is the best technique to apply ink with high viscosity and inkjet bioprinters are used when low cell density is needed.

Materials or bioinks must be easily printable to facilitate handling and deposition. They must be biocompatible for long-term transplantation and must degrade at rates that match the ability of cells to produce their own extracellular matrix while displaying short-term stability.

Several tissues and organs can be printed efficiently. For example, a proof of concept for skin bioprinting has been demonstrated by several teams with good cell viability and architecture of the tissue[304] and also with bioprinted vascularization.[305] Moreover, Skardal *et al.* show that it was possible to bioprint dermal substitutes combined with MSCs directly *in situ*, inducing faster wound closure.[306] However, functional vascularization that needs to be fully addressed to allow engineered tissue to survive could be improved with the use of Pluronic F127 as a sacrificial bioink that can form open lumens concurrently with the printing of encapsulated cells around the vessels.[307] Innervated bioprinted skin has not yet been produced, but fabrication of a synthetic nerve graft by printing cell-dense tubes of Schwann cells and MSCs has been shown to be a promising approach for nerve regeneration.[308] While bioprinting technology is promising in wound healing, several improvements have to be made in terms of rapidity of printing and of bioengineering complex hollow structures.

Conclusions and Perspectives

The skin is not only a protective barrier but also serves as an interface between our body and the external environment. It is indeed a highly sensitive organ. In addition to different cell types expressing many sensory receptors,[309] skin comprises several sensory nerve fiber subtypes that perceive and convey various external stimuli, such as temperature variations, pain, or tactile stimuli.

In addition to their sensory role, cutaneous nerve fibers are known to be tightly involved in a variety of physiological and pathological processes.[25] It has been shown in several clinical observations that injury to the peripheral nervous system impairs wound healing, sometimes leading to development within the affected area, of chronic wounds. Wound healing may be delayed, as demonstrated by studies using *in vivo* models of peripheral neuropathies, by denervation or chemical impairment of nerve fibers.[47] Likewise, patients with peripheral neuropathies due to lepromatous leprosy, spinal cord injury, or diabetes mellitus develop ulcers that fail to heal.[14] In elderly, cutaneous repair processes are also less efficient,[310] partly due to degeneration of the nerve fibers within the skin.[311] Moreover, defective

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innervation and/or inadequate levels of neuropeptides can negatively influence healing processes, underlining that innervation and neuropeptides are major players for normal cutaneous repair. Promoting normal reinnervation and adequate levels of neuropeptides during the healing process is certainly crucial to improve skin healing and to avoid the appearance of pathological situations.

When a major skin injury occurs such as a deep burn, sensory nerve endings are destroyed while cell bodies in the dorsal root ganglia along the spinal cord are maintained. Cutaneous nerve regeneration and progressive reinnervation of the scar are possible and may result either from regeneration of injured nerve fibers present in the wound bed or from sprouting of nerve fibers located in the adjacent uninjured area. However, the nerve regeneration process is imperfect, as suggested by frequent impairment of skin perceptions or the occurrence of chronic pain and disabilities. After wound healing, itching and pain tend to decrease.[152] However, cutaneous nerve fiber populations have been shown to be modified in scars compared with matched uninjured skin. Interestingly, the density of C fibers, which are involved in pain perception, is higher in scars.[132] Not surprisingly, this density is also increased in scars from patients with chronic pain compared with scars from patients without pain.[41] These outcomes suggest that unmyelinated small C fibers involved in pain detection regenerate faster than Aδ and Aβ myelinated fibers. Overall, it becomes clear that regeneration of the destroyed nerve fibers needs to be improved during skin healing management and medical treatment.

Until now, various techniques have been used in wound care. It includes occlusive dressings, autograft application, dermal allograft and skin substitute, or highly expanded autograft, depending on the size of the lesion (see Table 1). Currently, the development of more sophisticated skin substitutes is in progress and aims to improve a patient's rehabilitation. New designs of skin substitutes, innovative biomaterials, and stem cells represent promising therapeutic strategies that could promote both correct wound healing and sensory recovery. In these innovative products, the presence of neuronal cells, Schwann cells, and/or the addition of neurotrophins could favor the development of a more physiological innervation in the repaired skin and minimize *sequelae* often associated with burn scar. The 3D bioprinting technology could especially offer new opportunities. This recent approach in which cells and materials are directly deposited on or in a patient[312] could be particularly interesting after extensive burns. However, these new biotechnological approaches are still challenging to apply in burn wound management. Limitations such as cost, ethical issues for stem cells, and complex designs of skin substitutes still need to be addressed. Moreover, technical limitations related to incorporation and/or selection of appropriate innervation structures, especially tactile corpuscles, have to be overcome.

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Disclosure Statement

No competing financial interests exist.

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Cette revue montre que l'utilisation de peaux reconstruites *in vitro* plus ou moins complexes pourrait être une stratégie thérapeutique dans le traitement des brûlures. Il semble donc important de comprendre les interactions cellulaires qui peuvent s'établir au niveau de ces peaux reconstruites pour perfectionner ce modèle et permettre une éventuelle utilisation thérapeutique en routine après brûlure sévère.

Compte tenu du rôle de l'innervation dans la cicatrisation, nous nous sommes particulièrement intéressés aux interactions cellulaires entre les fibroblastes dermiques et les cellules neuronales.

II – 3 Matériel et méthodes

II – 3.1 Isolement et culture des fibroblastes dermiques

Des échantillons de peau prélevés après plasties mammaires chez des femmes âgées de 18 à 55 ans ont été récupérés à l'Hôpital de la Mère et de l'Enfant de Limoges. Ces échantillons sont des déchets opératoires et sont collectés avec le consentement des patientes. Ces prélèvements sont dégraissés puis l'épiderme et le derme sont séparés par traitement enzymatique permettant d'extraire les kératinocytes d'une part et les fibroblastes d'autre part. Des explants de derme sont déposés dans une boîte de Pétri de 100 mm de diamètre et sont chacun recouverts par 3 µL de DMEM (Life Technologies, Carlsbad, CA, USA 61965-059) contenant 20 % sérum de veau fœtal (SVF, Life Technologies, lot n°41Q4740K), 1% pénicilline-streptomycine (P/S) (Life Technologies 15140-122), 1% fungizone (Life Technologies, 1590-026) et sont mis minimum 2h à l'incubateur à 37°C et 5% de CO_2 pour favoriser l'adhésion des explants à la boîte de Pétri. Une fois les explants adhérés, 8 ml de milieu DMEM contenant 20% SVF sont ajoutés dans chaque boîte. Les boîtes de Pétri sont remises à l'incubateur pendant 3 semaines et le milieu est changé tous les 3 jours. Au bout de 3 semaines, les fibroblastes ont colonisé la boîte de Pétri. Les morceaux de derme sont retirés puis les cellules sont trypsinées. Pour cela, après un lavage au « Phosphate Buffer Saline » (PBS), 4 ml de trypsine (Life Technologies, 25300-054) sont ajoutés par boîte. Après 5 minutes à 37°C, les cellules se sont décollées. Elles sont alors récoltées et un volume équivalent de DMEM contenant 10 % SVF est ajouté à cette suspension cellulaire pour arrêter l'action de la trypsine. Les cellules sont alors centrifugées pendant 10 minutes à 1200 rpm. Le culot cellulaire obtenu est resuspendu dans du milieu DMEM contenant 10% SVF et les cellules sont ensemencées dans ce même milieu.

II – 3.2 Culture des cellules neuronales

La lignée cellulaire SH-SY5Y (cellules issues d'un neuroblastome humain, ATCC CRL-2266 ™) a été utilisée comme modèle de cellules neuronales. Ces cellules sont cultivées dans du milieu DMEM contenant 10 % SVF, 1% P/S et 1% fungizone. Elles peuvent être activées par l'utilisation du NGF (PeproTech Inc., Rocky Hill, NJ, USA, 450-01) à une concentration de 100 ng/ml.

II – 3.3 Coculture 2D directe

La coculture 2D directe est réalisée dans des plaques 24 puits. Au fond de chaque puits, une lamelle de verre (Dutcher, Brumath, France, 100040N) est déposée puis recouverte avec 300 µL de poly-L-lysine (Sigma-Aldrich, Saint-Quentin Fallavier, France, P9155) à une concentration de 100 µg/ml. Les plaques sont alors mises 2h à 37°C. Une fois que ce « coating » est réalisé, un lavage au PBS est effectué dans chaque puits et les cellules sont ensemencées : 14 600 cellules SH-SY5Y et 5500 fibroblastes par puits. Le temps de doublement des cellules SH-SY5Y est plus long que celui des fibroblastes ce qui explique les proportions choisies. Les différentes conditions pour cette expérience sont :

- fibroblastes seuls ;
- fibroblastes + TGF-β1 (BioLegend, San Diego, CA, USA, 580702) à 5 ng/ml ;
- fibroblastes + cellules SH-SY5Y ;
- cellules SH-SY5Y seules.

Les cellules sont ensemencées dans du milieu DMEM contenant 10% SVF, 1% P/S et 1% fungizone. Après adhésion, les cellules sont cultivées dans du milieu DMEM sans sérum pendant la nuit. Le lendemain, le traitement au TGF-β1 est réalisé. Les cellules sont alors cultivées dans 300 µL de DMEM contenant 1% SVF, 1% P/S et 1% fungizone avec ou non du TGF-β1 pendant 72h. Après 3 jours de coculture, les différents surnageants de culture (ou milieux conditionnés) sont récupérés, centrifugés pendant 10 minutes à 1200 rpm, aliquotés puis congelés à -20°C. Un lavage au PBS est effectué dans chaque puits afin de mettre en œuvre une immunofluorescence.

II – 3.4 Immunofluorescence

Pour mettre en évidence une éventuelle activation des fibroblastes ou des cellules SH-SY5Y, une immunofluorescence dirigée contre l'actine α-ML (marqueur de l'activation des fibroblastes) et NF-M (marqueur des cellules neuronales permettant de mettre en évidence les neurites) a été effectuée. Pour cela, les cellules sont fixées avec de l'acétone à 4°C pendant 10 minutes. L'acétone est ensuite retirée et trois lavages au PBS sont effectués. La saturation et la perméabilisation sont effectuées grâce à un mélange de PBS contenant 2% BSA (Sigma-Aldrich, A9647), 2% sérum de chèvre (Vector Laboratories, Burlingame, CA, USA, S-1000), 0,1% de Tween 20 et 0,5% de Triton X-100 mis en contact des cellules pendant 30 minutes.

La solution de dilution des anticorps primaires est composée de PBS contenant 2% BSA, 2% sérum de chèvre et 0,3% Triton X-100. L'anticorps primaire monoclonal de souris dirigé contre l'actine α-ML (Dako, Les Ulis, France, M0851) est utilisé au 1/200^{ième} et l'anticorps primaire polyclonal de lapin dirigé contre NF-M (Merck Millipore, Molsheim, France, AB1987) est utilisé au 1/100^{ième}. 200 µl de cette solution contenant les anticorps primaires sont mis au contact des cellules toute la nuit à 4°C.

Le lendemain, la solution est retirée puis 3 lavages de 10 minutes au PBS sont effectués. Les anticorps secondaires sont alors ajoutés au 1/1000^{ième} (Invitrogen, Carlsbad, CA, USA, 11005 et 11008) dans une solution de PBS contenant 0,5 µg/ml de DAPI (Sigma-Aldrich, D9542).

100 µl de cette solution sont ajoutés dans chaque puits pendant 2h à température ambiante, à l'abri de la lumière. 3 lavages au PBS sont ensuite effectués, et les lamelles au fond des puits sont retirées et déposées sur une lamelle de verre avec du milieu de montage (Dako, S3023). Les photos ont ensuite été prises avec un microscope à fluorescence inversé DM IRB (Leica Microsystems, Wetzlar, Allemagne). Les images ont été acquises avec le logiciel NIS-Elements (Nikon, Minoto-ku, Tokyo, Japon).

II – 3.5 Analyse du milieu conditionné par le « Proteome Profiler ™ Human XL Cytokine Array Kit »

Afin d'étudier l'effet de la coculture fibroblastes / cellules SH-SY5Y sur les secrétions cellulaires par rapport aux cellules seules activées ou non, une analyse des cytokines sécrétées a été réalisée sur les milieux conditionnés récoltés à la fin de la coculture. Pour cela le kit « Proteome Profiler ™ Human XL Cytokine Array Kit » (R&D systems, Minneapolis, MN, USA, ARY022) a été utilisé. Brièvement, les membranes contenues dans le kit sont immergées dans du « Array buffer 6 » sous agitation pendant 1h pour bloquer les sites aspécifiques. Pendant ce temps, les échantillons sont préparés. Pour cela, 400 µL de milieu conditionné de chaque condition sont mélangés à 1,1 ml de « Array Buffer 6 ». Après une heure d'incubation, le tampon présent sur les membranes est retiré et 1,5 ml du tampon contenant les échantillons est déposé sur chaque membrane (1 membrane / échantillon). L'incubation se fait sous agitation toute la nuit à 4°C. Le lendemain, les solutions contenant les échantillons sont éliminées et 3 lavages de 10 minutes avec le « Wash buffer » sont réalisés sous agitation à température ambiante. La solution contenant les anticorps de détection liés à l'avidine est ensuite ajoutée sur les membranes. Pour cela, une solution contenant un mélange de 1,5 ml de « Array Buffer 4/6 » et 30 µl de de « Detection Antibody Cocktail » est mis en contact avec chaque membrane sous agitation pendant 1h. Le milieu est ensuite retiré et 3 lavages de 10 minutes sont effectués. La Streptavidine-HRP diluée dans le « Array buffer 6 » est ensuite ajoutée sur les membranes pendant 30 minutes. Pour finir, 3 lavages de 10 minutes sont effectués et les membranes sont révélées par chimiluminescence grâce à la GBox (Syngene, Cambridge, United Kingdom) en utilisant un mélange des « Chemi Reagent » 1 et 2.

II – 3.6 Coculture 2D indirecte

Les cocultures indirectes des fibroblastes et des cellules SH-SY5Y ont été réalisées dans des plaques 12 puits à l'aide d'inserts « transwell » (Corning Life Sciences,Tewksbury, MA, 353181). Les inserts « transwell » utilisés ont une porosité de 0,3 µm ce qui permet de ne laisser passer que les molécules solubles. Une lamelle de verre est déposée au fond de chaque puits et elle est recouverte avec de la poly-L-lysine à 100 µg/ml pendant 2h à 37°C. Les cellules sont ensuite ensemencées soit sur la lamelle de verre au fond du puits soit sur la membrane de l'insert. Sur la lamelle de verre, 30 000 fibroblastes ou 40 000 cellules SH-SY5Y sont ensemencés dans un volume de 100 µl. Sur la membrane de l'insert, 7000 fibroblastes ou 9400 cellules SH-SY5Y sont ensemencés dans un volume de 50 µl. Une fois ensemencée, la plaque 12 puits et les inserts de culture sont mis à l'incubateur jusqu'à adhésion des cellules. Après adhésion, du milieu de culture DMEM contenant 1% SVF, 1%

P/S et 1% fungizone est ajouté : 1 ml au fond du puits et 500 µl dans l'insert de culture. Les traitements au TGF-β1 (5 ng/ml) et au NGF (100 ng/ml) sont alors effectués (Tableau III).

puits	Cellules au fond du puits	Cellules sur la membrane de l'insert
1	Fibroblastes	SH-SY5Y
2	Fibroblastes + TGF-β1	SH-SY5Y
3	Fibroblastes	SH-SY5Y +NGF
4	SH-SY5Y	Fibroblastes
5	SH-SY5Y + NGF	Fibroblastes
6	SH-SY5Y	Fibroblastes + TGF-β1
7	Fibroblastes	
8	Fibroblastes + TGF-β1	
9	SH-SY5Y	
10	SH-SY5Y +NGF	

Tableau III : Conditions de culture pour la coculture indirecte des fibroblastes et des cellules SH-SY5Y.

Les cellules sont cultivées pendant 72h puis une analyse immunocytochimique est réalisée (protocole section III – 3.4) sur les lamelles de verre et les membranes des inserts.

II – 3.7 Fabrication de la matrice de collagène

Pour étudier les interactions cellulaires entre les fibroblastes et les cellules SH-SY5Y en conditions plus physiologiques, ces cellules ont été cultivées en 3D grâce à une matrice de collagène-chitosan.

Pour cela, le collagène bovin purifié de type I (Symatese, Chaponost, France, CBPE2US050) est dissout dans du PBS pour obtenir une solution à 9 mg/ml. Le chitosan (Glentham Life Sciences, Corsham, UK, GP7325) est dilué dans de l'acide acétique 0,1% pour obtenir une concentration de 2,5 mg/ml.

Pour la réalisation de la matrice, une solution composée de 670 µl de collagène bovin purifié à 9 mg/ml et de 180 µl de la solution de chitosan à 2,5 mg/ml est réalisée sur glace puis versée dans chaque puits d'une plaque 12 puits. Immédiatement après cette étape, la plaque est mise à - 80°C pendant 1h. Après la congélation, les matrices sont lyophilisées pendant 24h en utilisant le Cryotec (Saint Gély du Fesc, France, COSMOS 2). Une fois lyophilisées, les matrices sont conservées à - 20°C jusqu'à leur utilisation. Lors de leur utilisation en culture cellulaire, chaque matrice est placée dans un puits d'une plaque 6 puits, mise 1h dans de l'éthanol à 70% puis une nuit dans du milieu DMEM brut avant d'être ensemencée.

II – 3.8 Microscopie électronique à balayage

A la suite de la lyophilisation, une étude de la structure de la matrice par microscopie à balayage a été effectuée. Cette analyse a été effectuée avec un microscope électronique à balayage Quanta FEG 250 (FEI, Hillsboro, OR, USA).

II – 3.9 Microscopie électronique à transmission

Une étude de la structure de la matrice a également été réalisée par microscopie électronique à transmission. Pour cela, les matrices lyophilisées ont été fixées dans une solution de glutaraldéhyde 2,5% dilué dans du tampon Sorensen pendant 10 minutes à température ambiante. Les matrices sont ensuite rincées et incubées dans une solution de tétroxyde d'osmium pendant 30 minutes à température ambiante. Les échantillons sont déshydratés dans des bains successifs d'alcool puis inclus dans de la résine Epon 812 (Euromedex, Souffelweyersheim, France). Des section semi-fines sont effectuées et sont examinées au microscope électronique à transmission JEM-1011 (JEOL, Croissy-sur-Seine, France).

II – 3.10 Coculture 3D directe

Les matrices, après réhydratation dans du milieu DMEM brut, sont ensemencées en déposant 600 000 fibroblastes goutte à goutte sur le sommet. Les matrices ensemencées sont alors mises à l'incubateur jusqu'à adhésion des cellules. Du milieu DMEM contenant 5% SVF et de l'acide ascorbique (Sigma-Aldrich, A4544) à 100 µg/ml est ensuite ajouté dans chaque puits (2 ml/puits). Ce milieu est changé tous les 3 jours. Après une semaine, le milieu est retiré et 600 000 cellules SH-SY5Y sont ensemencées goutte à goutte sur la matrice. Après adhésion des cellules à 37°C, 1,5 ml de milieu DMEM contenant 1% SVF et de l'acide ascorbique à 100 µg/ml sont ajoutés dans chaque puits. Des conditions contenant seulement des fibroblastes ou des fibroblastes en présence de TGF-β1 à 5 ng/ml sont également réalisées. Après trois jours de culture, les surnageants de culture (ou milieux conditionnés) sont récoltés, centrifugés pendant 10 minutes à 12 000 rpm, aliquotés puis congélés à - 20°C pour une analyse avec le « Proteome Profiler ™ Human Protease/Protease Inhibitor Array Kit ». Les matrices contenant les cellules sont quant à elles rincées au PBS puis fixées pendant 10 minutes au paraformaldéhyde (PFA) 4%. Après un rinçage au PBS, les matrices sont incluses dans de l'OCT (Cell Path, Wales, UK, KMA-0100-00A) et congelées à - 80 °C.

II – 3.11 Analyse du milieu conditionné par le « Proteome Profiler ™ Human Protease/Protease Inhibitor Array Kit »

Afin d'étudier l'effet de la coculture en 3D des fibroblastes et des cellules SH-SY5Y, l'analyse des molécules sécrétées lors de cette coculture a été réalisée en utilisant le « Proteome Profiler ™ Human Protease/Protease Inhibitor Array Kit » (R&D systems, ARY025). Brièvement, les membranes présentes dans le kit sont mises en présence de 2 ml de « Array Buffer 6 » pendant 1h sous agitation pour bloquer les sites aspécifiques. Pour chaque condition de culture, 2 solutions d'échantillons sont préparées et incubées pendant 1h :

- une pour la membrane permettant de détecter les protéases : 400 µl de milieu conditionné issu de la culture 3D ajoutés à 15 µl du « Protease Detection Antibody Cocktail » et complétés avec de « l'Array buffer 6 » pour un volume final de 1,5 ml ;
- une pour la membrane permettant de détecter les inhibiteurs de protéases : 400 µl de milieu conditionné issu de la culture 3D ajoutés à 15 µl du « Protease Inhibitor

Detection Antibody Cocktail » et complétés avec de « l'Array buffer 6 » pour un volume final de 1,5 ml.

Une fois les temps d'incubation écoulés, les solutions d'échantillons sont placées sur les membranes correspondantes (membrane protéases ou membrane inhibiteurs de protéases) à 4°C sous agitation pendant toute la nuit.
Le lendemain, comme mentionné à la section II – 3.5, les membranes sont lavées puis mises en contact avec la solution de Streptavidine-HRP. Les membranes sont ensuite rincées puis révélées à la GBox grâce à une solution de « Chemi reagent » 1 et 2.

II – 3.12 Analyses statistiques

Les résultats sont présentés ici en moyenne ± SEM et le taux de significativité est fixé à $p < 0,05$. Les résultats ont été comparés en utilisant une analyse de variance (ANOVA) à deux facteurs à l'aide du logiciel GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, USA). Les images de microscopie et les membranes des protéomes ont été analysées avec le logiciel ImageJ (NIH, USA).

II – 4 Résultats

II – 4.1 Analyse de l'effet de coculture 2D directe entre les fibroblastes et les cellules SH-SY5Y sur l'activation des fibroblastes

- Analyse immunocytochimique

L'effet des cellules SH-SY5Y sur les fibroblastes en coculture 2D directe a tout d'abord été étudié par immunofluorescence (Figure 22). La condition fibroblastes + TGF-β1 est un contrôle positif permettant de mettre en évidence l'activation des fibroblastes par la présence de filaments d'actine α-ML nombreux et épais. La présence de ces filaments d'actine α-ML n'est pas ou peu présente dans la condition fibroblastes seuls (contrôle négatif).

Lorsque les cellules SH-SY5Y sont cultivées en présence de ces fibroblastes, on remarque alors le développement de filaments d'actine α-ML au sein des fibroblastes. Ceci prouve donc que les cellules SH-SY5Y sont capables d'induire la différenciation des fibroblastes en myofibroblastes lors d'une coculture 2D directe entre ces 2 types cellulaires.

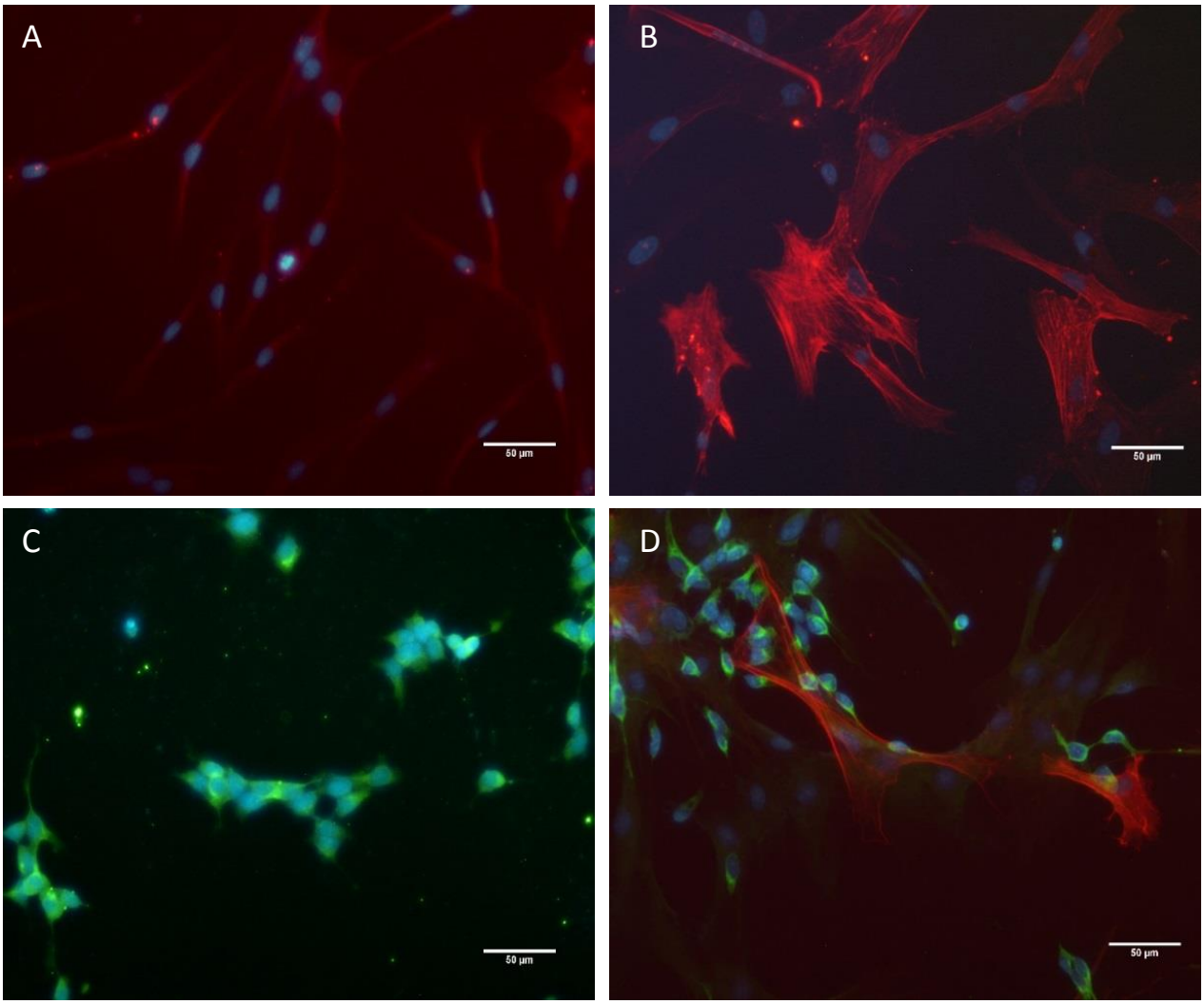


Figure 22 : Immunocytochimie dirigée contre l'actine α-ML (rouge) et NF-M (vert) après 72h de coculture 2D directe entre les fibroblastes dermiques et les cellules neuronales SH-SY5Y. L'ensemencement était de 14 600 cellules SH-SY5Y et 5500 fibroblastes par puits. A. fibroblastes seuls, B. fibroblastes + TGF-β1 à 5 ng/ml, C. cellules SH-SY5Y seules et D. fibroblastes + cellules SH-SY5Y.

- Analyse du sécrétome

Les fibroblastes sécrètent la matrice extracellulaire mais également de nombreuses molécules, en particulier des cytokines. Lorsque les fibroblastes sont activés en myofibroblastes, notamment lors du processus de cicatrisation, ils sont impliqués dans la fermeture de la plaie en rapprochant les berges de la plaie grâce à leur capacité de contraction. Les milieux conditionnés issus de la coculture 2D directe fibroblastes / cellules SH-SY5Y ont donc été analysés par « Proteome Profiler ». Parmi les 102 cytokines testées, ne seront présentées ici que celles présentant une différence d'expression entre la condition fibroblastes seuls et la condition fibroblastes + TGF-β1 (Figure 23).

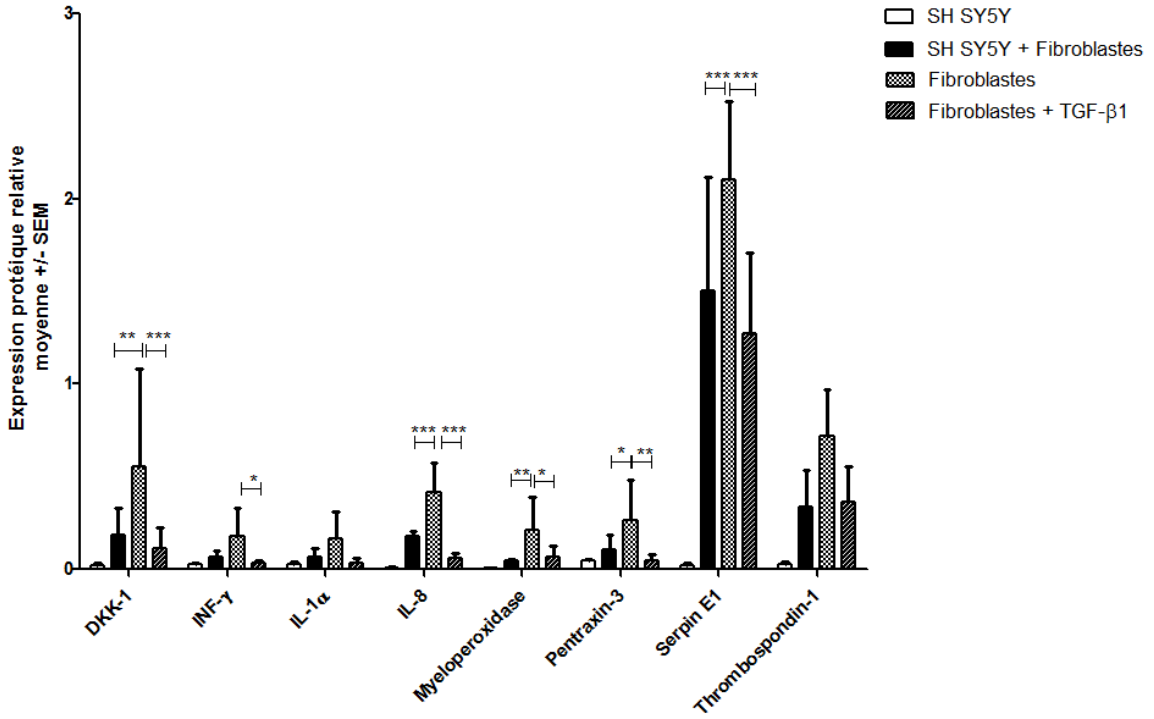


Figure 23 : Analyse comparative de la sécrétion des cytokines dans le milieu conditionné des cellules SH-SY5Y et des fibroblastes cultivés seuls ou en coculture 2D directe après 72h de coculture.

L'ensemencement était de 14 600 cellules SH-SY5Y et 5500 fibroblastes par puits.La condition fibroblastes + TGF-β1 à 5 ng/ml qui sert de contrôle positif est également représentée.

Après activation des fibroblastes (condition fibroblastes + TGF-β1), une diminution du profil sécrétoire est observée par rapport à la condition fibroblastes seuls. Cette réduction du profil sécrétoire est également observée lors de l'étude du sécrétome de la condition fibroblastes / cellules SH-SY5Y confirmant la capacité des cellules SH-SY5Y à induire un phénotype similaire à celui rencontré lorsque les fibroblastes sont engagés dans une différenciation myofibroblastique.

II – 4.2 Analyse de l'effet de coculture 2D indirecte entre les fibroblastes et les cellules SH-SY5Y sur l'activation des fibroblastes

Afin de savoir si le contact cellulaire est nécessaire entre les cellules SH-SY5Y et les fibroblastes pour induire la différenciation des fibroblastes en myofibroblastes, une étude sans contact a été réalisée à l'aide de « transwell » (Figure 24 et 25).

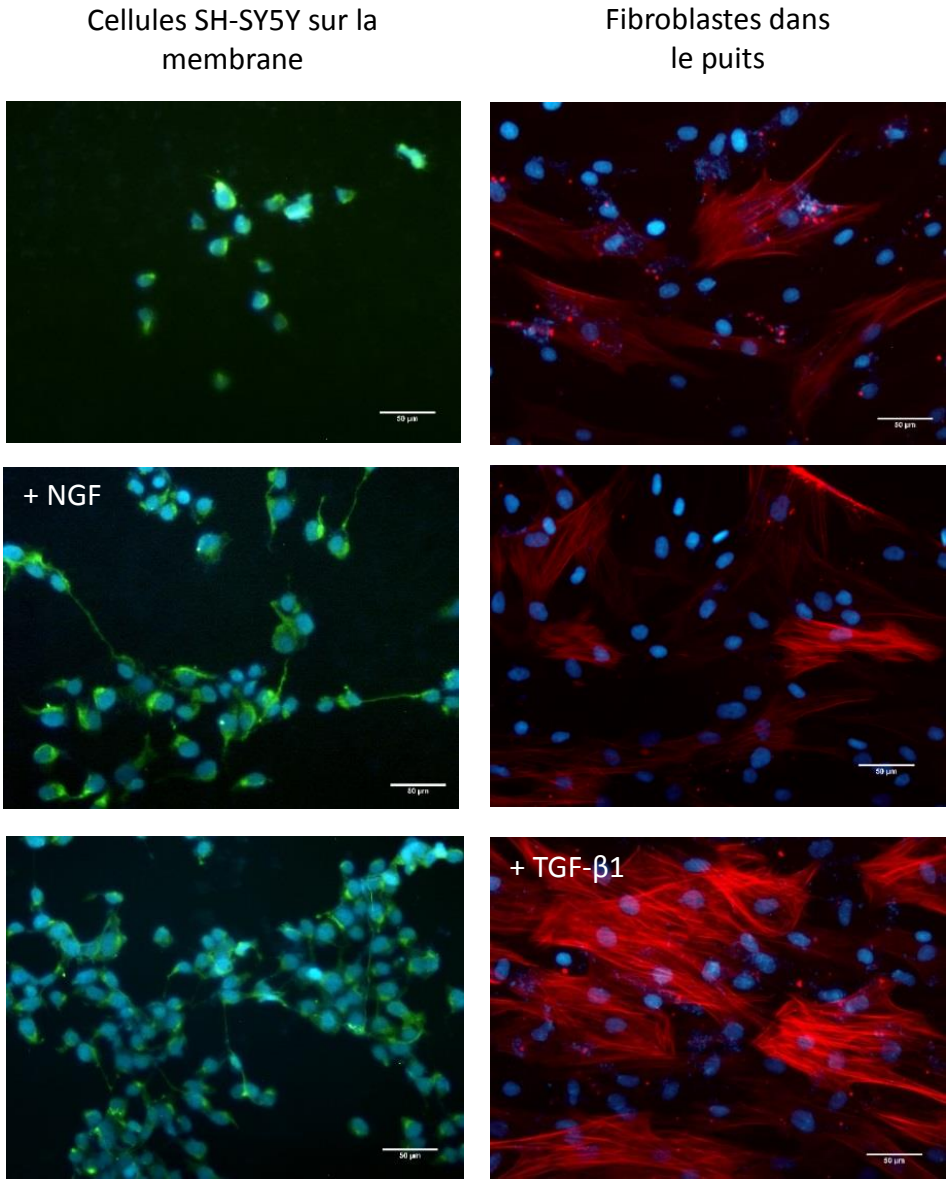


Figure 24 : Immunocytochimie dirigée contre l'actine α-ML (rouge) et NF-M (vert) sur les puits et les membranes des « transwells » après 72h de coculture indirecte entre les cellules SH-SY5Y et les fibroblastes. L'ensemencement était de 9400 cellules SH-SY5Y sur la membrane du « transwell » et 30 000 fibroblastes au fond du puits. Le NGF est utilisé à une concentration de 100 ng/ml et le TGF-β1 à 5 ng/ml.

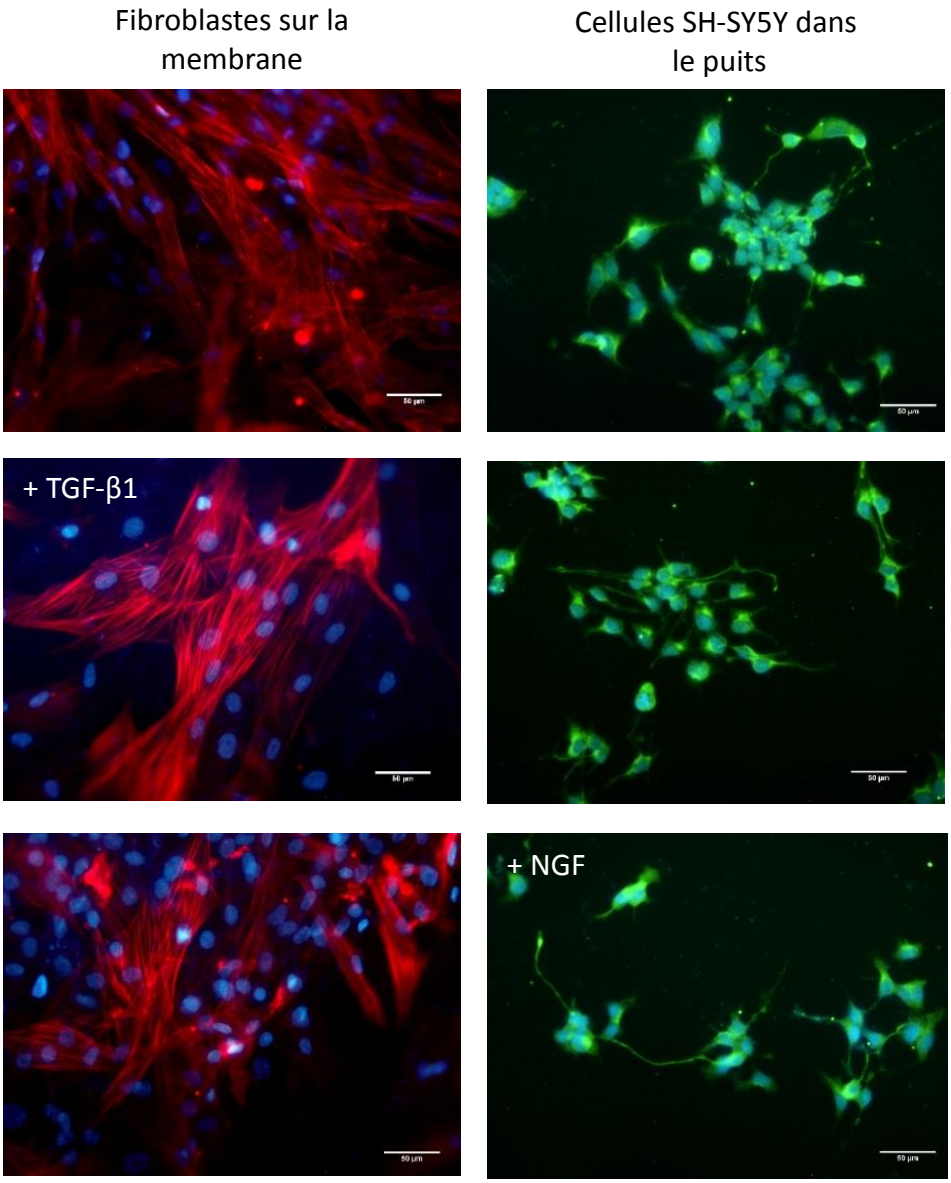


Figure 25 : Immunocytochimie dirigée contre l'actine α-ML (rouge) et NF-M (vert) sur les puits et les membranes des « transwells » après 72h de coculture indirecte entre les cellules SH-SY5Y et les fibroblastes. L'ensemencement était de 7000 fibroblastes sur la membrane du « transwell » et de 40 000 cellules SH-SY5Y au fond du puits. Le NGF est utilisé à une concentration de 100 ng/ml et le TGF-β1 à 5 ng/ml.

Ces différentes immunocytochimies montrent que lors de cocultures indirectes des cellules SH-SY5Y et des fibroblastes, les cellules SH-SY5Y sont capables d'induire la différenciation des fibroblastes en myofibroblastes. En effet, on remarque, dans chaque condition, la présence d'un marquage pour l'actine α-ML. Le contact cellulaire n'est donc pas nécessaire pour obtenir cette différenciation. Un ou des messagers chimiques seraient donc impliqués dans ce processus.

II – 4.3 Analyse de la structure de la matrice de collagène-chitosan

Pour développer un modèle de derme équivalent innervé, une matrice de collagène-chitosan a été mise au point. Après lyophilisation, la structure de cette matrice a été étudiée par microscopie électronique à balayage (Figure 26) et à transmission (Figure 27).

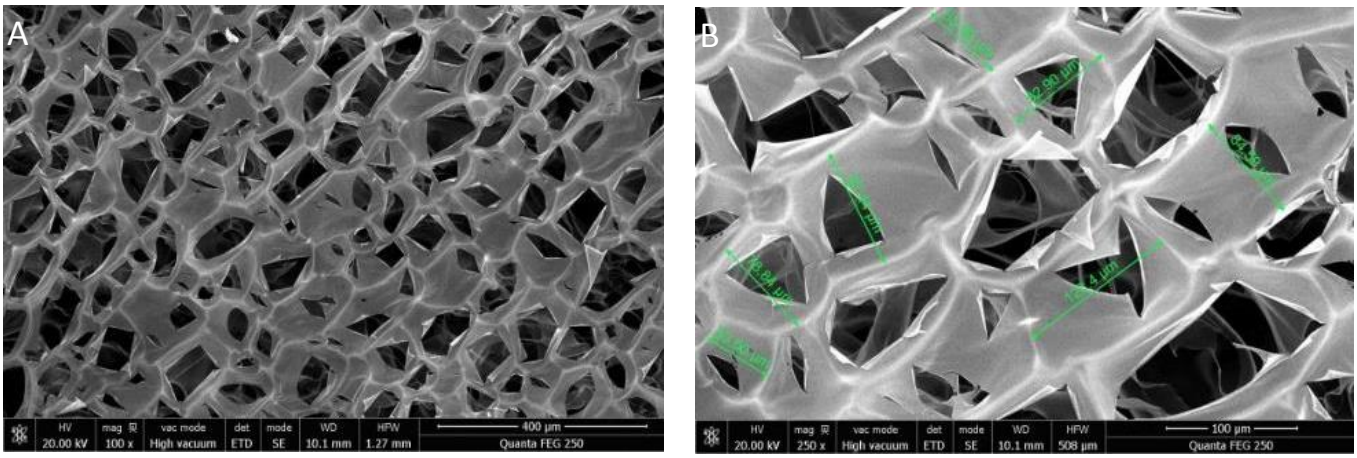


Figure 26 : Analyse de la structure de la matrice de collagène-chitosan par microscopie électronique à balayage après lyophilisation. A. faible grossissement (x100), B. fort grossissement (x250).

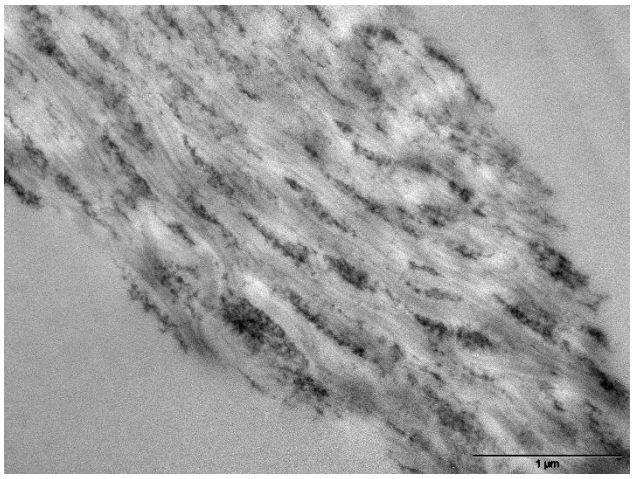


Figure 27 : Analyse de la structure de la matrice de collagène-chitosan par microscopie électronique à transmission après lyophilisation.

Comme le montre les images de la figure 26, la matrice de collagène-chitosan obtenue présente de nombreux pores de diamètres allant de 80 à 120 µm. La taille des pores est suffisamment importante pour permettre le passage de cellules (fibroblastes, SH-SY5Y) au travers de la matrice. L'image obtenue par microscopie électronique à transmission montre une partie de la paroi d'un pore.

II – 4.4 Analyse de l'effet de coculture 3D entre les fibroblastes et les cellules SH-SY5Y sur l'activation des fibroblastes

Une fois la structure de la matrice de collagène-chitosan validée, les fibroblastes et les cellules SH-SY5Y ont été cultivées en même temps au sein de cette matrice pour étudier si en 3D les cellules SH-SY5Y étaient capables d'activer les fibroblastes comme en 2D.

Pour cela, une analyse du sécrétome des différentes conditions a été réalisée en s'intéressant cette fois-ci aux protéases et aux inhibiteurs de protéases. Ces molécules étant très impliquées dans le processus de cicatrisation et notamment lors du remodelage de la MEC, il est important d'étudier l'effet de la coculture fibroblastes / cellules SH-SY5Y sur ces sécrétions. Parmi les 35 protéases et les 32 inhibiteurs de protéases testés, ne seront présentés ici que les « Matrix-Metalloproteinases » (MMPs) (Figure 28) et les « Tissue Inhibitor of Metalloproteinase » (TIMPs) (Figure 29).

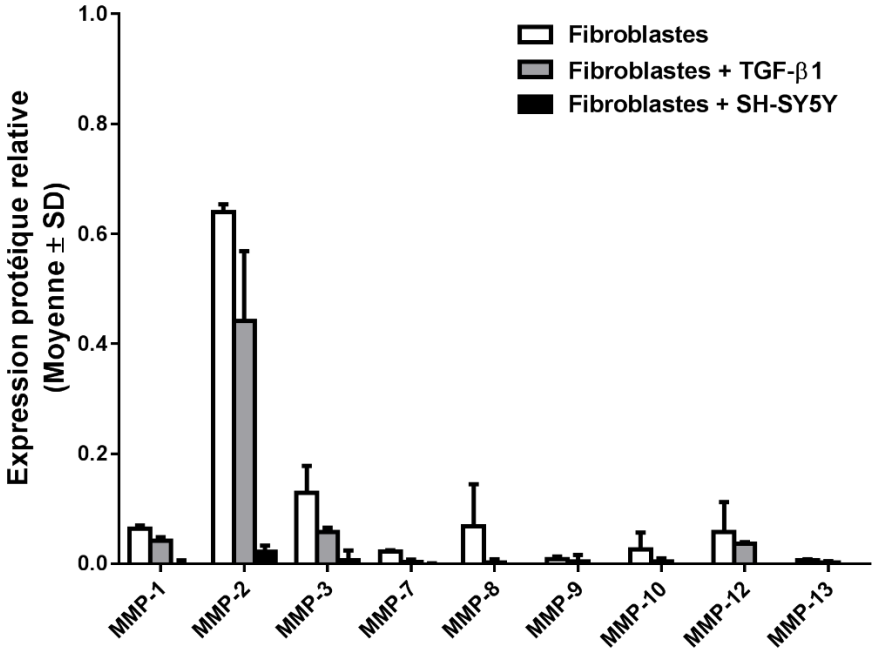


Figure 28 : Analyse comparative des MMPs sécrétées dans le milieu conditionné lors de la culture en 3D de fibroblastes seuls ou en présence de cellules SH-SY5Y après 72h de coculture. 600 000 fibroblastes sont ensemencés sur la matrice de collagène-chitosan. Après une semaine, 600 000 cellules SH-SY5Y sont alors ensemencées sur cette même matrice pour la condition fibroblastes + SH-SY5Y et la coculture est maintenu pendant 72h avant le recueil du milieu conditionné. La condition fibroblastes + TGF-β1 à 5 ng/ml sert de contrôle positif pour la différenciation des fibroblastes en myofibroblastes.

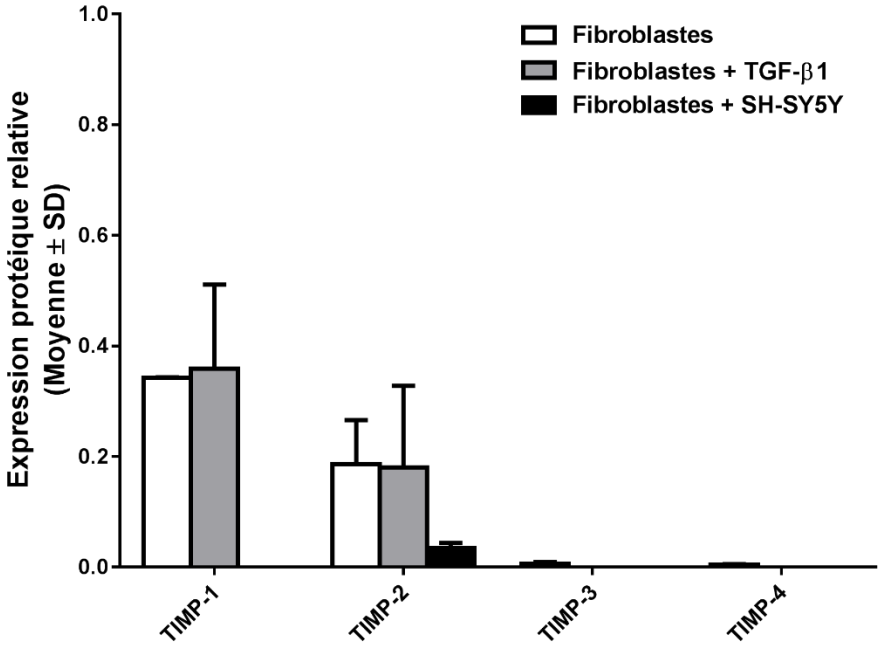


Figure 29 : Analyse comparative des TIMPs sécrétées dans le milieu conditionné lors de la culture en 3D de fibroblastes seuls ou en présence de cellules SH-SY5Y après 72h de coculture. 600 000 fibroblastes sont ensemencés sur la matrice de collagène-chitosan. Après une semaine, 600 000 cellules SH-SY5Y sont alors ensemencées sur cette même matrice pour la condition fibroblastes + SH-SY5Y et la coculture est maintenu pendant 72h avant le recueil du milieu conditionné. La condition fibroblastes + TGF-β1 à 5 ng/ml sert de contrôle positif pour la différenciation des fibroblastes en myofibroblastes.

Comme le montre ces figures, l'expression des MMPs est diminuée lors de la culture 3D de fibroblastes en présence de TGF-β1 par rapport à la culture de fibroblastes seuls. L'expression des TIMPs est quant à elle similaire que les fibroblastes soient différenciés en myofibroblastes ou non. Lors de la coculture 3D entre les fibroblastes et les cellules SH-SY5Y, l'expression des MMPs et des TIMPs sécrétées est fortement diminuée par rapport aux fibroblastes seuls.

II – 5 Discussion

L'objectif de ce travail *in vitro* était d'étudier les interactions possibles entre les cellules neuronales et les fibroblastes dermiques. Cela devrait permettre, à terme, le développement d'une peau reconstruite innervée pouvant être utilisée dans le traitement de la brûlure.

La première étape de ce travail a été de réaliser une coculture directe en 2D entre les fibroblastes dermiques humains et les cellules SH-SY5Y pour étudier si le contact entre ces deux types cellulaires avait un effet sur les fibroblastes, cellules indispensables lors du processus de cicatrisation. En immunocytochimie, on a pu observer qu'après 3 jours de coculture avec les cellules SH-SY5Y, les fibroblastes présentent des filaments d'actine similaire à ce qui est observé lors de la culture des fibroblastes avec le TGF-β1 (contrôle

positif). Les cellules SH-SY5Y sont donc capables d'induire la différenciation des fibroblastes en myofibroblastes. Fujiwara et al. ont également montré ce processus de différenciation des fibroblastes en myofibroblastes en présence de cellules PC12 (lignée cellulaire issue d'un phéochromocytome de rat, capable de former des neurites sous l'action du NGF) (Fujiwara et al., 2013).

Afin de poursuivre l'étude des interactions entre les cellules neuronales SH-SY5Y et les fibroblastes lors d'une coculture directe, nous avons étudié le profil de secrétions de différentes cytokines. Cette analyse confirme les résultats obtenus précédemment. En effet, le profil sécrétoire de la coculture est similaire à celui du contrôle positif fibroblastes + TGF-β1. Pour ces deux conditions, on observe une réduction de l'expression des cytokines étudiées par rapport à la condition fibroblastes seuls. Ces cytokines jouent un rôle important lors des processus inflammatoires et sont donc impliquées lors de la cicatrisation d'une plaie. Lors de la formation du tissu de granulation qui se déroule après la phase inflammatoire, il semble logique que l'expression de ces différentes cytokines impliquées dans l'inflammation soit réduite.

Pour mieux comprendre cette interaction cellulaire et savoir si le contact cellulaire était nécessaire pour obtenir une différenciation des fibroblastes en myofibroblastes lors de la coculture, nous avons réalisé cette coculture de manière indirecte. Le dialogue cellulaire n'était alors seulement possible que par des molécules solubles présentes dans le milieu de culture. Par immunomarquage de l'actine α-ML, nous avons pu montrer que sans contact cellulaire, les cellules neuronales SH-SY5Y étaient à nouveau capables d'induire la différenciation des fibroblastes en myofibroblastes. Certains facteurs solubles, non identifiés à ce jour, seraient donc impliqués dans ce processus.

Le fait que les cellules neuronales soient capables d'induire la différenciation des fibroblastes en myofibroblastes par contact direct et par la sécrétion de facteurs solubles est un point important. En effet, cela pourrait être une des actions de l'innervation lors du processus de cicatrisation.

Pour reproduire une condition plus physiologique, la coculture a été réalisée en 3D. Pour cela, une matrice de collagène-chitosan a été mise au point pour reproduire le derme. La structure de cette matrice est satisfaisante puisque, notamment, la taille des pores permet la migration de cellules à l'intérieur de cette matrice et la manipulation.

Lors de la coculture des cellules neuronales et des fibroblastes en 3D sur cette matrice, nous avons étudié le profil sécrétoire des MMPs et des TIMPs. Ces molécules sont notamment impliquées dans le remodelage du tissu lors de la cicatrisation. Nous avons pu observer que lors de l'activation des fibroblastes par le TGF-β1, la quantité de MMPs secrétées (notamment la MMP-2 et la MMP-3) est diminuée par rapport à la condition fibroblastes seuls. En effet, le TGF-β1 favorise le dépôt de MEC en augmentant la synthèse des protéines de la matrice et en diminuant l'expression des protéases capables de détruire cette matrice. Lors de la coculture des fibroblastes et des cellules SH-SY5Y, la quantité de ces MMPs secrétées est fortement réduite par rapport à la condition fibroblastes seuls. Cela est également le cas lorsque l'on étudie la sécrétion des TIMPs. Ces résultats sont en contradiction avec ceux obtenus par

Chéret et al. en 2014 (Chéret et al., 2014). Cette équipe a montré sur un modèle de peau lésée que l'apport de GDRs de souris au sein de cette plaie augmentait l'activité enzymatique de la MMP-2 par rapport à la condition sans GDRs. Ces résultats discordants peuvent être dus à des modèles d'étude définitivement très différents. De plus, au sein des GDRs, de nombreux types cellulaires sont présents, notamment des fibroblastes qui peuvent être impliqués dans l'expression des MMPs.

L'ensemble de ces résultats *in vitro* nous a permis de mieux comprendre l'implication de l'innervation lors de la cicatrisation. Un des mécanismes mis en évidence est la capacité des cellules neuronales à induire une différenciation des fibroblastes en myofibroblastes par contact direct ou de manière indirecte.

Chapitre 3 : Discussion Générale – Perspectives

L'objectif des études menées jusqu'à aujourd'hui sur les brûlures consistait à favoriser une fermeture rapide de la plaie pour éviter tout risque d'infection et pour ainsi améliorer la survie des patients. Différents substituts cutanées ont été développés dès lors permettant de combler la plaie et de restaurer la fonction barrière de la peau (Auxenfans et al., 2015; van Zuijlen et al., 2015).

Cependant, bien que la cicatrisation par ces méthodes ait permis de sauver de nombreux patients, des handicaps plus ou moins sévères persistent après la brûlure. En plus des séquelles esthétiques, les patients peuvent notamment souffrir de troubles de la sensibilité cutanée. Cela se manifeste par un prurit, une perte de la sensibilité au chaud ou au froid ou encore une perception douloureuse. Ces troubles sont provoqués par des anomalies de la réinnervation lors du processus de cicatrisation notamment lors de brûlures profondes.

Pour éviter le développement de ces troubles sensitifs cutanés, il est donc important de proposer une nouvelle stratégie thérapeutique à mettre en place après une brûlure thermique. L'objectif du projet de thèse (et du projet NERVAL dans son ensemble) était de s'intéresser au rôle de l'innervation lors de la cicatrisation après une brûlure. Ces premiers résultats devraient apporter les connaissances nécessaires pour la mise au point d'un substitut cutané permettant de retrouver une sensibilité normale après une lésion thermique.

Ces différents travaux de thèse ont permis d'étudier le rôle de l'innervation dans le processus de cicatrisation après une brûlure thermique mais également de mettre en lumière une action directe des cellules neuronales sur l'activité des (myo)fibroblastes, cellules essentielles lors du processus de cicatrisation.

La technique de brûlure utilisée pour notre expérimentation *in vivo* consiste en l'application d'une pièce de métal chauffée à une température donnée sur le dos de l'animal. Cet appareillage nous a permis de réaliser deux degrés différents de brûlure (second degré profond et troisième degré) en faisant seulement varier la température de la pièce de métal. Cette technique nous a également permis d'avoir des brûlures reproductibles entre chaque animal.

La brûlure était effectuée sur le dos de rats mâles. L'utilisation de rats permet d'avoir une surface cutanée totale suffisante pour que les brûlures ne représentent qu'un faible pourcentage de cette surface cutanée totale. Cependant, la structure de la peau des rats est différente de celle de l'homme. Cette limite de notre modèle peut être palier par l'utilisation de porcs. En effet, il est couramment admis que ces animaux possèdent une structure de la peau et un processus de cicatrisation cutanée similaires à la peau humaine. L'utilisation de ces animaux demande néanmoins une structure d'hébergement adaptée qui n'est pas facilement accessible à Limoges et qui est très couteuse.

Le traitement de la brûlure a été effectué de manière similaire à ce qui est classiquement fait chez l'homme. En effet, nous avons appliqué de la Flammazine ® juste après la réalisation de la brûlure et jusqu'à la fin de la cicatrisation. De plus, les plaies ont été détergées, étape nécessaire pour faciliter la cicatrisation. Avec ces traitements, les deux types de brûlures ont réussi à cicatriser en moyenne un mois après la brûlure sans nécessité de greffe. Ceci n'est cependant pas le cas pour des brûlures de même degrés chez l'homme. En effet, pour des

brûlures du deuxième degré profond ou du troisième degré chez l'homme, la greffe de peau ou de substituts cutanés est indispensable pour restaurer la fonction barrière de la peau et aider à la cicatrisation. Cette différence de cicatrisation est une autre limite de notre modèle.

Ce modèle nous aura cependant permis d'observer l'influence de l'innervation cutanée dans la cicatrisation après brûlure. En effet, nous avons montré par analyse histologique des plaies en cours de cicatrisation, que les animaux avec une innervation défectueuse présentaient un retard de cicatrisation par rapport aux animaux contrôles. Cependant, cela n'influençait pas la cinétique de fermeture de la plaie pour les deux types de brûlures. Ceci peut en partie s'expliquer par le fait que l'action de la RTX est réversible et que l'on observe un retour à une sensibilité normale, et donc à une innervation fonctionnelle, 35 jours après la brûlure. Une nouvelle injection de RTX avant ce retour à la normale aurait pu prolonger la non fonctionnalité de l'innervation et ainsi retarder la fermeture de la plaie chez ces animaux.

Ce rôle important de l'innervation dans la cicatrisation cutanée est actuellement très peu étudié dans le monde. Nous avons donc voulu comprendre par quels mécanismes l'innervation pouvait agir sur la cicatrisation. Pour cela, nous avons réalisé des cocultures de fibroblastes du derme et de cellules neuronales. Cela nous a permis d'étudier l'impact des cellules neuronales sur la différenciation des fibroblastes, processus indispensable lors de la cicatrisation.

Les résultats obtenus lors des cocultures 2D montrent que les cellules neuronales sont capables d'induire la différenciation des fibroblastes en myofibroblastes. Ceci a été montré par la mise en évidence de filaments d'actine α-ML dans les fibroblastes lors des cocultures mais également par l'étude du profil sécrétoire des cytokines. En effet, ce profil sécrétoire est similaire entre la condition contrôle positif (fibroblastes + TGF-β1) et la condition fibroblastes + cellules SH-SY5Y en coculture 2D directe. Cet effet des cellules SH-SY5Y sur la différenciation des fibroblastes est également retrouvé lors de la coculture 2D indirecte ce qui indique que des facteurs solubles sécrétés sont vraisemblablement responsables de ce résultat.

Pour se placer en conditions plus physiologiques, nous avons reproduit ces expériences en 3D. Pour cela, nous avons développé une matrice de collagène-chitosan qui représente un derme équivalent lors de nos études. Ce type de matrice a été mise au point par l'équipe de François Berthod (Laboratoire d'Organogénèse Experimentale, Université Laval, Québec, Canada) qui l'utilise pour développer différents modèles de peaux plus ou moins complexes (Parenteau-Bareil et al., 2011; Blais et al., 2013, 2014; Cadau et al., 2015). La différence entre notre modèle et le leur est que nous utilisons des cellules neuronales humaines (issue d'un neuroblastome) alors qu'ils utilisent des cellules neuronales issues de GDRs de souris.

L'analyse par microscopie électronique de la matrice de collagène-chitosan a permis de mettre en évidence la présence de pores qui, de par leurs diamètres, permettent aux cellules de migrer à l'intérieur et de s'y développer.

Pour étudier l'effet des cellules neuronales sur les fibroblastes lorsqu'ils sont cultivés en 3D et donc en conditions plus physiologiques, nous avons ensemencées les cellules sur la matrice de

collagène-chitosan. Cela nous a permis d'étudier le profil sécrétoire des protéases et des inhibiteurs de protéases lors de ces cultures. Nous nous sommes principalement intéressés à la sécrétion des MMPs et des TIMPs, molécules indispensables lors de l'étape de remodelage dans le processus de cicatrisation. Nous avons ainsi montré que lors d'une coculture 3D des cellules neuronales SH-SY5Y et des fibroblastes, on observe une réduction importante de la quantité des MMPs (notamment la MMP-2 et la MMP-3) et des TIMPs (notamment les TIMPs 1 et 2) sécrétés par rapport à la condition fibroblastes seuls. Cette réduction d'expression des MMPs est également observée lors de la culture des fibroblastes en présence de TGF-β1. Cela signifierait donc que, lorsque les fibroblastes sont différenciés en myofibroblastes, l'expression des MMPs est plus faible.

Ces résultats lors de la coculture 3D doivent être complétés notamment en réalisant un marquage de l'actine α-ML pour confirmer que les fibroblastes sont bien différenciés en myofibroblastes dans ces conditions. De plus, une analyse du profil sécrétoire des cytokines lors de ces cocultures 3D est en cours. Cela nous permettra de comparer ces futurs résultats avec les résultats obtenus lors de la coculture 2D.

L'ensemble des résultats obtenus lors de ce projet de thèse ont montré l'importance de l'innervation dans le processus de cicatrisation en induisant notamment la différenciation des fibroblastes en myofibroblastes, étape indispensable lors de la cicatrisation.

L'objectif final de mon projet de thèse et du projet NERVAL était de pouvoir proposer un nouveau concept de peau équivalente innervée qui pourrait être utilisé pour le traitement des brûlures et qui pourrait favoriser un retour à une sensibilité correcte chez les patients.

Pour répondre à cet objectif, nous avons développé cette peau équivalente innervée en utilisant la matrice de collagène-chitosan (Figure 30). Cette matrice, qui représente le derme, a été ensemencée de fibroblastes puis de kératinocytes pour reproduire la structure de la peau humaine. L'aspect innovant de notre modèle est l'apport de l'innervation au sein de cette peau équivalente. De plus, les cellules SH-SY5Y sont ensemencées sur un support original : un tapis de fibroïne de soie électrospinée qui permet de favoriser et d'orienter la croissance des neurites. Ces travaux utilisant la soie sont réalisés en collaboration avec l'équipe de Christophe Eglès (UMR CNRS 7338 "BioMécanique et BioIngénierie", Université de Technologie de Compiègne, France) Le montage final est inspiré des travaux de Blais et al. qui utilise des GDRs de souris (Blais et al., 2014).

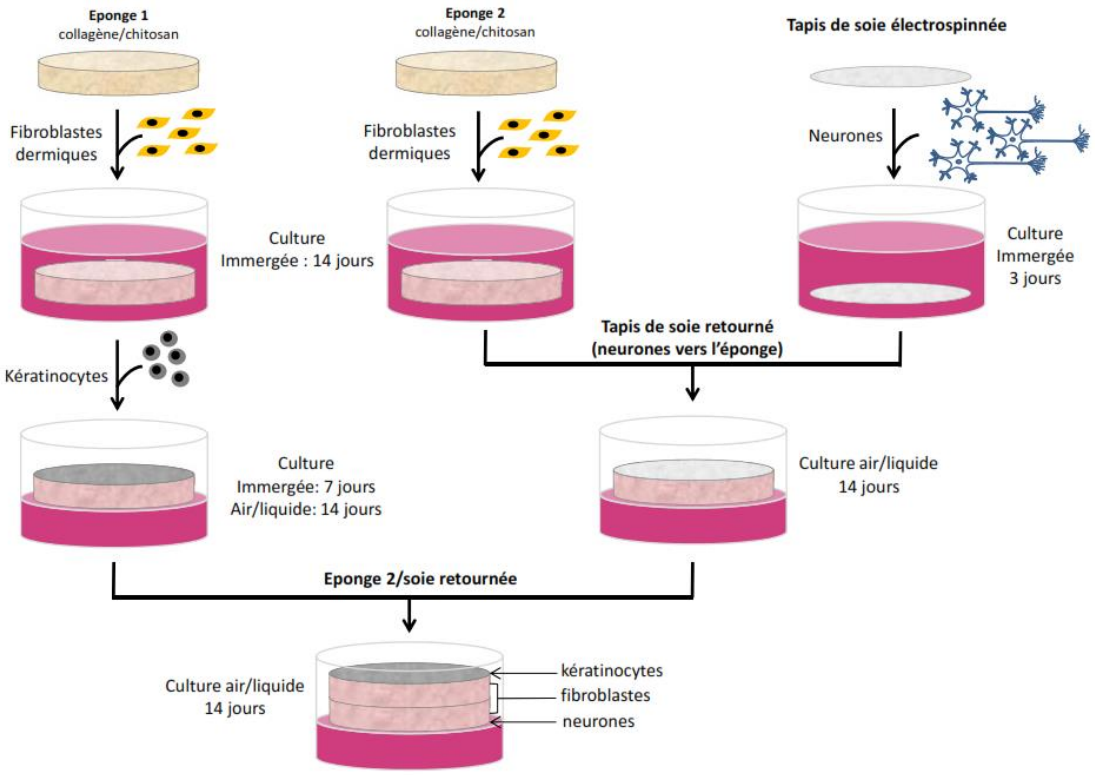


Figure 30 : Schéma récapitulatif pour la fabrication de notre modèle de peau équivalente innervée.

Après 6 semaines de culture, la peau équivalente est prête à être greffée. Pour étudier l'effet de cette peau équivalente innervée sur la cicatrisation et la réinnervation d'une plaie, cette peau équivalente a été greffé sur des souris « nude » après excision d'un morceau de peau. Après 15 jours de greffe, nous avons pu observer que macroscopiquement la cicatrisation était correcte (Figure 31).



Figure 31 : Analyse macroscopique de la cicatrisation à l'aide de notre modèle de peau équivalente innervée après 15 jours de greffe.

Lors de l'euthanasie des animaux, l'aspect microscopique de la cicatrisation et la réinnervation seront étudiés. Cela permettra de conclure sur l'apport des cellules neuronales dans ce modèle en comparant les résultats obtenus chez des animaux ayant reçu la peau équivalente comprenant ou non des cellules neuronales.

Pour poursuivre notre étude et nous intéresser à l'effet de notre peau équivalente innervée pour le traitement de la brûlure, des souris « nude », dont un morceau de peau a été excisé, ont été greffées avec un morceau de peau humaine. Après prise de la greffe, une brûlure plus ou moins profonde sera effectuée sur la peau humaine. Différents traitements seront alors testés : un tapis de soie seule ou la peau équivalente innervée. La soie utilisée lors de ces traitements pourra être neutre ou biofonctionnalisée. La biofonctionnalisation consiste à greffer des molécules bioactives à la fibroïne de soie. Les molécules greffées pourraient alors permettre d'améliorer la repousse axonale. Nous étudierons alors l'impact de ces traitements sur la cicatrisation et la réinnervation de la plaie. Ces résultats seront comparés à des animaux ayant subi la brûlure mais qui n'auront pas reçu de traitements spécifiques.

Nos résultats sont encourageants et nous souhaitons perfectionner notre nouveau concept de peau équivalente en y apportant la composante vasculaire. En effet, la mise en place d'une néoangiogenèse correcte est également un élément indispensable pour obtenir une cicatrisation satisfaisante (Park et al., 2015; Wang et al., 2016).

Lorsque sera mis au point le modèle de peau équivalente innervée et vascularisée et si les résultats sont toujours aussi prometteurs, une étude préclinique pourra être envisagée pour reproduire nos expériences à plus grande échelle et en maitrisant l'ensemble des paramètres (en utilisant les principes de « bonnes pratiques de fabrication »). Pour répondre à cet objectif, nous avons d'ores et déjà utilisé des cellules humaines dans l'ensemble de nos études. Les fibroblastes et les kératinocytes sont des cellules primaires issues de plasties mammaires et les cellules neuronales utilisées sont une lignée cellulaire issue d'un neuroblastome. Ce type cellulaire n'est malheureusement pas le meilleur modèle et ne pourra pas être utilisé pour notre peau équivalente innervée finale mais le recueil de cellules neuronales périphériques humaines est impossible. En effet, cela provoquerait des handicaps certains chez le patient. Une alternative serait l'utilisation de cellules souches cutanées humaines (« Skin Derived-Precursors » ou SKPs) qui peuvent être différenciées en cellules neuronales *in vitro*. Des premiers essais encourageants ont été réalisés. La revue « Role of innervation in scar formation and therapeutic potential of dermal stem cells » en annexe 2 introduit l'apport de ces SKPs lors du processus de cicatrisation pour aider à la restauration d'une innervation satisfaisante. Si les résultats de l'étude préclinique sont toujours positifs, l'application de nos nouveaux biomatériaux pourra être envisagée en clinique pour le traitement de différents types de blessures. Bien sûr, pendant le temps nécessaire à la construction de cette peau équivalente, la plaie du patient pourra être classiquement recouverte, comme cela est fait actuellement, pour éviter tout risque d'infection. Les brûlures seront concernées par ce nouveau traitement mais également d'autres plaies difficilement cicatrisables (plaies chroniques de types ulcères ou escarres). Si la plaie n'est pas trop étendue, l'objectif est de reconstruire une peau équivalente innervée et vascularisée à partir des cellules du patient pour éviter tout risque de rejet. A partir d'un morceau de peau saine, toutes les cellules composant

cette peau équivalente pourront être isolées (les cellules neuronales pourront être différenciées à partir des SKPs).

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Annexes

Annexe 1 : Myo(fibroblastes) et cicatrisation

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REVIEW

Fibroblasts and myofibroblasts in wound healing

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Abstract: (Myo)fibroblasts are key players for maintaining skin homeostasis and for orchestrating physiological tissue repair. (Myo)fibroblasts are embedded in a sophisticated extracellular matrix (ECM) that they secrete, and a complex and interactive dialogue exists between (myo)fibroblasts and their microenvironment. In addition to the secretion of the ECM, (myo)fibroblasts, by secreting matrix metalloproteinases and tissue inhibitors of metalloproteinases, are able to remodel this ECM. (Myo)fibroblasts and their microenvironment form an evolving network during tissue repair, with reciprocal actions leading to cell differentiation, proliferation, quiescence, or apoptosis, and actions on growth factor bioavailability by binding, sequestration, and activation. In addition, the (myo)fibroblast phenotype is regulated by mechanical stresses to which they are subjected and thus by mechanical signaling. In pathological situations (excessive scarring or fibrosis), or during aging, this dialogue between the (myo)fibroblasts and their microenvironment may be altered or disrupted, leading to repair defects or to injuries with damaged and/or cosmetic skin alterations such as wrinkle development. The intimate dialogue between the (myo)fibroblasts and their microenvironment therefore represents a fascinating domain that must be better understood in order not only to characterize new therapeutic targets and drugs able to prevent or treat pathological developments but also to interfere with skin alterations observed during normal aging or premature aging induced by a deleterious environment.

Keywords: myofibroblast, fibroblast, α-smooth muscle actin, mechanical signaling, fibrosis, scarring

Introduction to the myofibroblastic phenotype

Myofibroblasts were first described in healing skin wounds, where it was hypothesized that they were responsible for the phenomenon of wound contraction.[1] Since then, cells morphologically similar to myofibroblasts have been described in many tissues, predominantly in pathological states where their sustained presence is generally a marker of fibrosis and scarring.[2]

Early studies identified myofibroblasts on the basis of their ultrastructural morphology, with prominent microfilament bundles in their cytoplasm distinguishing them from "normal" quiescent tissue fibroblasts. Myofibroblasts also possessed fibronexus junctions between cells and the surrounding extracellular matrix (ECM), thus in some ways appearing to share some of the morphological characteristics of smooth muscle (SM) cells.[3]

Many tissues and pathologies have been described in which myofibroblasts have been identified, including hypertrophic and keloid scars in the skin, fibrotic liver as seen in liver cirrhosis and other liver pathologies, renal fibrosis, and idiopathic

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pulmonary fibrosis.[4] More recently, cells with phenotypic features of myofibroblasts have also been found in and around a number of epithelial tumors, where they have been termed cancer-associated fibroblasts or stromal myofibroblasts.[5–7] The role of myofibroblasts in driving fibrotic diseases, and the recent finding that cancer-associated myofibroblasts likely influence tumor growth and correlate with poor clinical prognosis, has increased our interest in their cellular origins, their regulation, and their role in repair and disease.[8]

After early studies that defined myofibroblasts on the basis of their ultrastructural morphology, later research using antibodies and immunohistochemistry resulted in myofibroblasts and their microenvironment being more clearly defined.[9] It is now accepted that myofibroblasts go through a precursor stage of expressing large stress fibers that are not seen in quiescent fibroblasts, prominent bundles of microfilaments that permit some contraction and pre-stressing and remodeling of the surrounding ECM.[10] Later, fully differentiated myofibroblasts show expression of the usually SM-specific cytoskeletal protein, α-SM actin, which is now often used to define the myofibroblast phenotype.[9,11]

The presence of a splice variant form of fibronectin (ED-A fibronectin) in the microenvironment adjacent to the myofibroblast is also a defining feature and appears to be required for their differentiation.[12] De novo expression of osteoblast (OB) cadherin has also been reported to be found on the surface of differentiated myofibroblasts, and is not seen on α-SM actin-negative fibroblasts.[13]

The other defining feature of myofibroblasts is that they fail to express the full repertoire of SM cell markers, allowing myofibroblasts to be distinguished from SM cells. Specifically, myofibroblasts in most cases are negative for SM cell markers such as SM myosin heavy chain,[14] n-caldesmon,[15] and smoothelin.[16] Desmin has also been used as a negative marker of myofibroblasts. Generally, SM cells express desmin and vimentin as well as SM myosin, while myofibroblasts express only vimentin. However, some situations have been reported in the literature where myofibroblasts in some pathologies have been found to be desmin positive.[17] Distinguishing myofibroblasts from pericytes is perhaps more problematic since pericytes can closely resemble myofibroblasts in being α-SM actin positive, vimentin and desmin positive, but SM myosin negative.[15,18] Indeed, pericytes may in some cases be a source of myofibroblasts in some conditions, including wound repair, where myofibroblasts may represent a pericyte that has lost some phenotypic features such as desmin expression.[19] Similarly, SM cells from the media of an injured blood vessel may lose late differentiation markers such as desmin, smoothelin, and SM myosin and acquire a myofibroblast phenotype.[20]

Lastly, myofibroblasts show both fibronexus junctions with other cells and specialized junctional complexes with the ECM; these large mature focal adhesions allow them to make strong attachments, contract and remodel the ECM, and provide a means of transducing mechanical force in the tissue.[21,22] The contractile nature of myofibroblasts has some similarities to SM cells, despite the differences in expression of cytoskeletal features. For example, the calcium signaling in myofibroblasts appears to be similar to that in SM cells, and the arrangement of cells into something resembling that of SM cells in tissues with single-unit SM is also similar, that is, with cells having junctional complexes and connections (including gap junctions) that allow the spread of contraction signals through the tissue. Contraction of myofibroblasts seems to be possible through Ca^{2+}-dependent mechanisms that are similar to those present in SM cells, with increased free Ca^{2+} regulating phosphorylation of myosin light chain. This may explain the rapid contractile responses of myofibroblasts in vitro to agonists such as angiotensin II or endothelin. Slower and more sustained contraction, which is perhaps more typical of what occurs during slow retractile contraction of connective tissue in granulation tissue, involves activity of the guanosine triphosphate (GTP)-ase RhoA and activation of its downstream target Rho-associated kinase (ROCK). This results in more continued phosphorylation of myosin light chain and thus sustained contraction.[23]

Role in wound healing

Immediately after wounding, the healing process commences, leading to (partial) restoration of injured tissue. Wound healing proceeds in three interrelated dynamic phases that temporally overlap (Figure 1). Based on morphological changes over the course of the healing process, these phases are defined as the inflammatory phase, the proliferative phase (the development of granulation tissue), and the regeneration phase, including maturation, scar formation, and re-epithelialization.[24] The inflammatory phase begins with damage of capillaries, triggering the formation of a blood clot consisting of fibrin and fibronectin. This provisional matrix fills in the lesion and allows a variety of recruited cells to migrate into the lesion. Platelets present in the blood clot release multiple chemokines, which participate in the recruitment of inflammatory cells, neutrophils, and macrophages, but also in chemotaxis and recruitment of fibroblasts and endothelial cells. The second stage of wound healing is the proliferative phase. Angiogenesis, which is

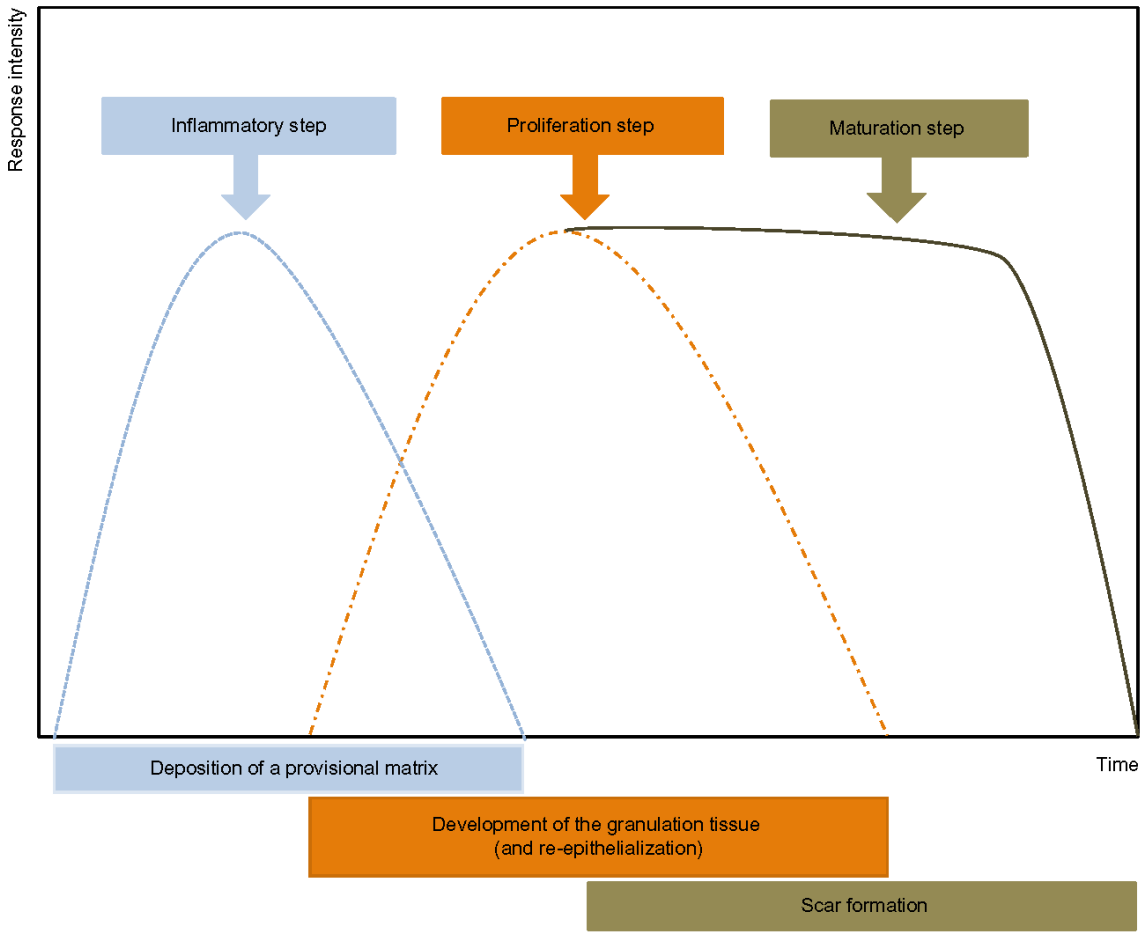


Figure 1 The various phases of the healing process.
Notes: After damage, inflammation leads to the formation of the granulation tissue, during which myofibroblasts appear. An important neoangiogenesis is also observed. On this granulation tissue, a new epidermis can then develop. Subsequently, remodeling of this granulation tissue occurs with apoptosis of the cells present in the granulation tissue (myofibroblasts and vascular cells) and reorganization of the extracellular matrix.

critical for the wound healing process, allows new capillaries to deliver nutrients to the wound, and contributes to the proliferation of fibroblasts. Initially the wound is hypoxic due to the loss of vascular perfusion, but with the development of a new capillary network, vascular perfusion is restored. Regulating wound angiogenesis in itself may represent a means for improving healing in some cases, particularly where delayed or defective angiogenesis is implicated in healing impairment.[25] In the granulation tissue, fibroblasts are activated and acquire α-SM actin expression and become myofibroblasts. These myofibroblastic cells synthesize and deposit the ECM components that eventually replace the provisional matrix (Figure 2). These cells exhibit contractile properties, due to the expression of α-SM actin in microfilament bundles or stress fibers, playing a major role in the contraction and maturation of the granulation tissue.[26]

Presently, it is accepted that the myofibroblastic modulation of fibroblastic cells begins with the appearance of the protomyofibroblast, whose stress fibers contain only β- and γ-cytoplasmic actins. Protomyofibroblasts generally evolve into differentiated myofibroblasts, the most common variant of this cell, with stress fibers containing α-SM actin (for review, see Tomasek et al[27]). Myofibroblasts can, depending on the experimental or clinical situation, express other SM-related contractile proteins, such as SM myosin heavy chains or desmin; however, the presence of α-SM actin represents the most reliable marker of the myofibroblastic phenotype.[27] The third phase of healing, scar formation, involves a progressive remodeling of the granulation tissue. During this remodeling process, proteolytic enzymes, essentially matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitor of metalloproteinases [TIMPs]) play a major

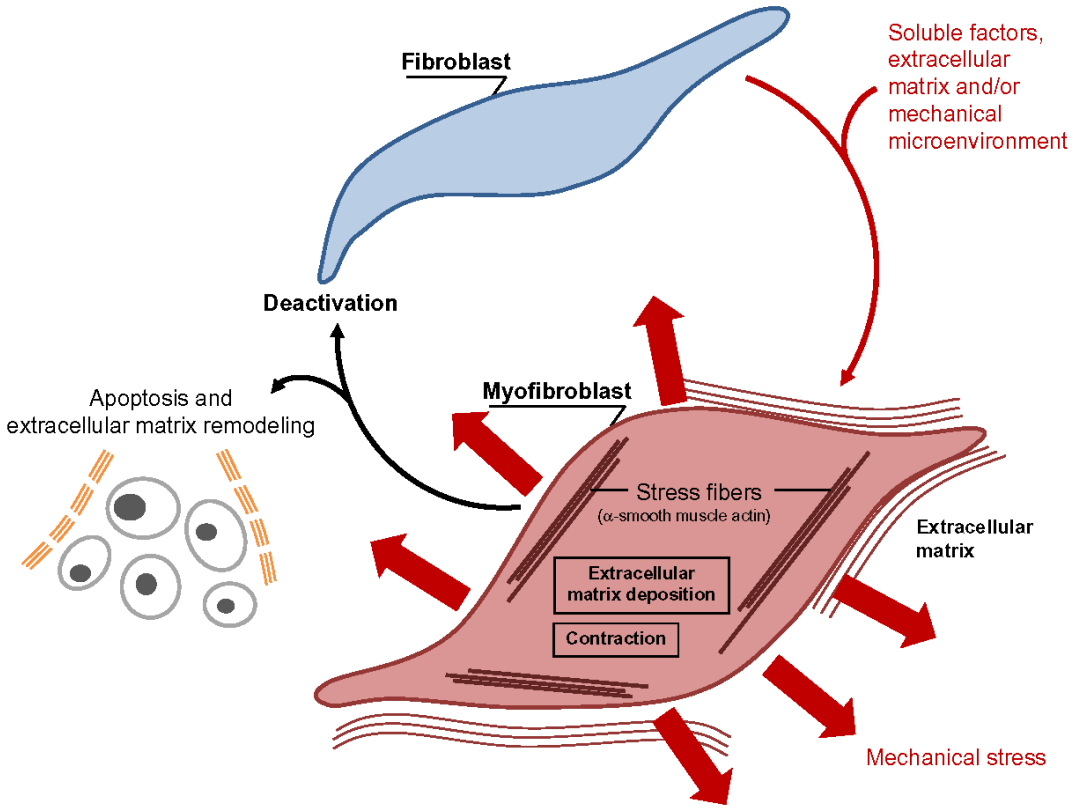


Figure 2 Schematic illustration showing the evolution of the (myo)fibroblast phenotype.
Notes: The myofibroblastic modulation of fibroblastic cells begins with the appearance of the proto myofibroblast, whose stress fibers contain only β- and γ-cytoplasmic actins and evolves, but not necessarily always, into the appearance of the differentiated myofibroblast, the most common variant of this cell, with stress fibers containing α-smooth muscle actin. Soluble factors, extracellular matrix components, and/or the mechanical microenvironment are involved in myofibroblastic differentiation. The myofibroblast can disappear by apoptosis; while deactivation leading to a quiescent phenotype has not been clearly demonstrated, at least in vivo.

role.[28] The synthesis of ECM is not totally stopped, but considerably reduced, and the synthesized components are modified as the matrix is remodeled. Progressively, collagen type III, the major component of the granulation tissue, is replaced by collagen type I, which is the main structural component of the dermis. Lastly, elastin, which contributes to skin elasticity and is absent in the granulation tissue, also reappears. In the resolution phase of healing, the cell number is dramatically reduced by apoptosis of both vascular cells and myofibroblasts.[29] To date, it is not known whether myofibroblasts can reacquire a quiescent phenotype, that is, return to a normal dermal fibroblast phenotype with no expression of α-SM actin (Figure 2).

Origin of wound myofibroblasts

It is generally accepted that the major source of myofibroblasts are local connective tissue fibroblasts that are recruited into the wound.[30] Dermal fibroblasts located at the edges of the wound can acquire a myofibroblastic phenotype and participate in tissue repair.[31] However, important heterogeneity in fibroblastic cell subpopulations has also been observed. These subpopulations reside in different locations within the skin and have specific activation and deactivation properties. At least three subpopulations have been identified in the dermis: superficial (or papillary) fibroblasts (papillary dermis is around 300–400 μm deep and is arranged as a ridge-like structure), reticular fibroblasts, which reside in the deep dermis (made of thick collagen and elastin fibers arranged parallel to the surface of the skin), and fibroblasts associated with hair follicles. These cell subpopulations can be isolated and exhibit, depending of the nature and age of the original skin, distinct phenotypic differences when cultured separately.[32,33]

Recently, the involvement in tissue repair of local mesenchymal stem cells has been increasingly raised. These progenitor cells have been described in the dermal sheath that surrounds the outside of the hair follicle facing the epithelial stem cells. They are involved in the regeneration

of the dermal papilla and can also became wound healing (myo)fibroblasts after a lesion or injury.[34,35]

Recent data have also implicated circulating cells, dubbed fibrocytes, in the tissue repair process. Fibrocytes enter injured skin together with inflammatory cells and may then acquire a myofibroblastic phenotype.[36] In post-burn scars, fibrocytes are recruited to the site of the lesion where they stimulate the local inflammatory response and produce ECM proteins, thus contributing to hypertrophic scar formation.[37]

Another type of circulating cell originating from bone marrow has also been suggested to play a role in tissue repair. Mesenchymal stem cells are bone marrow-derived non-hematopoietic precursor cells[38] that contribute to the maintenance and regeneration of connective tissues through engraftment.[39] Indeed, they have the capacity to seed into several organs and to differentiate into wound-healing myofibroblasts. This engraftment in injured organs is regulated by the severity of the damage.[40]

Finally, differentiated (or malignant) epithelial and endothelial cells can undergo a phenotypic conversion that gives rise to matrix-producing fibroblasts and myofibroblasts (through epithelial- and endothelial-to-mesenchymal transition processes).[41] This mechanism is increasingly recognized as an integral part of tissue fibrogenesis after injury, but seems to play a limited role during normal tissue repair.

Overall, mesenchymal stem cells, fibrocytes, bone marrow-derived cells, and cells derived from epithelial- and endothelial-to-mesenchymal transition processes may represent alternative sources of myofibroblasts when local fibroblasts are not sufficient to carry out tissue repair and remodeling.

Role of myofibroblasts in diseases (excessive scarring/fibrosis)

Myofibroblasts are implicated in many fibrotic and scarring diseases, where they carry out the important process after initial injury of providing mechanical support and integrity to the tissue. In normal physiological conditions, they are then lost via apoptosis, generally when the tissue integrity has been sufficiently restored to be mechanically coherent.[9,29] Thus, in normal physiological situations like skin wound healing, myofibroblasts disappear in a prominent wave of apoptosis, leaving a markedly less cellular scar. However, it is now assumed that, in many fibrotic and scarring conditions, as well as in the stromal response to tumors, myofibroblasts fail to undergo cell death, persist, and thus in turn lead to ongoing pathology and scarring (Figure 3). An example of reduced or inhibited apoptosis leading to scarring is in a model of hypertrophic scarring, where mechanical loading increases survival of myofibroblasts and was found to lead to greater scar formation.[42,43] A better understanding of the control and signaling that governs apoptosis (or autophagy) in myofibroblasts may lead to more targeted approaches to combatting fibrosis and scarring. It has been demonstrated that elevated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4-derived hydrogen peroxide, supported by concomitant decreases in nitric oxide signaling and reactive oxygen species scavengers, are central factors in the molecular pathogenesis of fibrosis.[44] Redox signaling could therefore represent an interesting target to restore the physiological fibroblast–myofibroblast ratio. Apoptosis in myofibroblasts is thought to be regulated by a reduction in the local growth factors that drive and sustain myofibroblast differentiation. In particular, local concentrations of transforming growth factor (TGF)-β1 and endothelin-1 play a role in stimulating myofibroblast survival via protein kinase B (AKT) activation.[45] However, changes in mechanical signaling such as unloading of mechanical force likely also plays a role (discussed below).

The importance of myofibroblasts in causing fibrosis in internal organs and the skin (hypertrophic scars), and the role that persistence of stromal myofibroblasts appear to play in tumor growth and spread, makes the (down)regulation of myofibroblasts and the potential regulation of myofibroblast disappearance through apoptosis of increasing interest (Figure 4) (for review, see Hinz and Gabbiani[46]).

Regulation of myofibroblasts by mechanical forces

Mechanical signals have been shown to play a role in myofibroblast differentiation as the ECM that surrounds the fibroblasts in damaged tissue changes its composition and its stiffness as tissue repair proceeds.[47] The early ECM present in damaged tissue, or provisional matrix, is rich in fibrin and has been estimated to be very compliant. Fibroblasts cultured in compliant ECM such as soft three-dimensional (3D) collagen gels, show little development of stress fibers. These fibroblasts then form only small adhesions with the ECM.[48,49] Fibroblasts grown in stiffer collagen matrices have been shown to form stress fibers and mature focal adhesions, though they are still negative for the myofibroblast marker α-SM actin. Lastly, the stiff matrix found either in 3D cultures using stiff (higher concentration) collagen matrix or in vivo in granulation tissue and fibrotic tissues is able to induce full myofibroblast differentiation in concert with

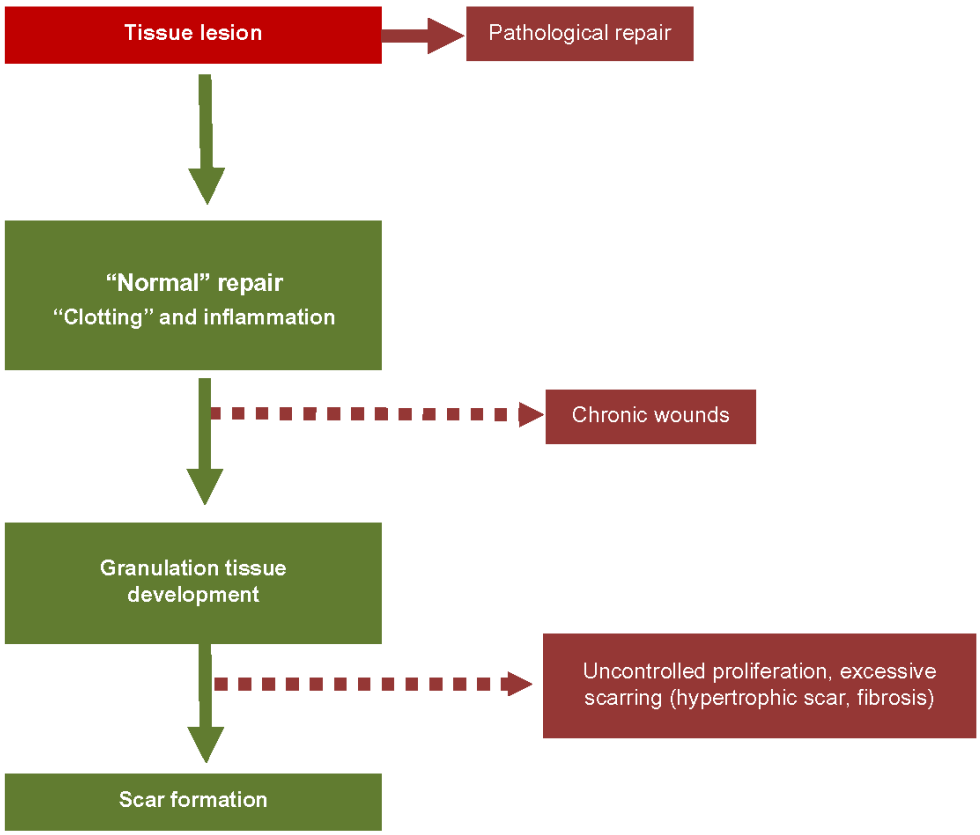


Figure 3 Pathological situations.
Notes: If the inflammation phase persists and the granulation tissue does not develop, a chronic wound may result. If the remodeling phase of the granulation tissue does not happen (neither apoptosis of the cells present in the granulation tissue, myofibroblasts, and vascular cells, nor reorganization of the extracellular matrix), myofibroblasts may persist, leading to pathological situations characterized by excessive scarring.

growth factor stimulation from TGF-β1.[22] The contractile nature of myofibroblasts itself leads to an increase in stiffness and mechanical stress of the ECM as healing progresses, leading to a positive-feedback loop where increased stress signals myofibroblast differentiation and also increases myofibroblast survival.[42] For this reason, mechanical feedback is considered to be important in driving pathological conditions such as contractures post-injury. The role of mechanical stress in stimulating myofibroblast activity has also been shown in experiments where dermal wounds in mice are mechanically stressed by stretching or splinting the wound, where increased myofibroblast activity results in increased scar formation, to some extent mimicking hypertrophic scarring that is seen in humans.[42]

In cancer biology, matrix stiffness can be used as a diagnostic indicator of the risk of malignancy and appears to correlate with increased invasiveness of tumors, for example in breast cancer where density of tissue on mammography correlates strongly with the risk of cancer formation. Recent studies have suggested this may be due to increased cell proliferation of epithelial and mesenchymal cells on stiffer matrices.

Conversely, releasing mechanical stress or reducing stiffness has been shown to induce both apoptosis and a reduction in α-SM actin expression and contractility in myofibroblasts.[50,51]

Mechanical signaling and stress modulate myofibroblast differentiation via a number of pathways and mechanisms. Stress may directly activate transcription of the α-SM actin gene, since application of force across integrin binding sites has been shown to up-regulate α-SM actin promoter activity.[52] As mentioned above, mechanical force alone is not generally sufficient to induce myofibroblast differentiation and other factors are needed to act in concert, specifically TGF-β1. Mechanical signaling and TGF-β1 stimulation also increase collagen gene expression by fibroblasts, emphasizing the role these factors play in stimulating a pro-fibrotic phenotype as is shown by activated myofibroblasts. TGF-β1

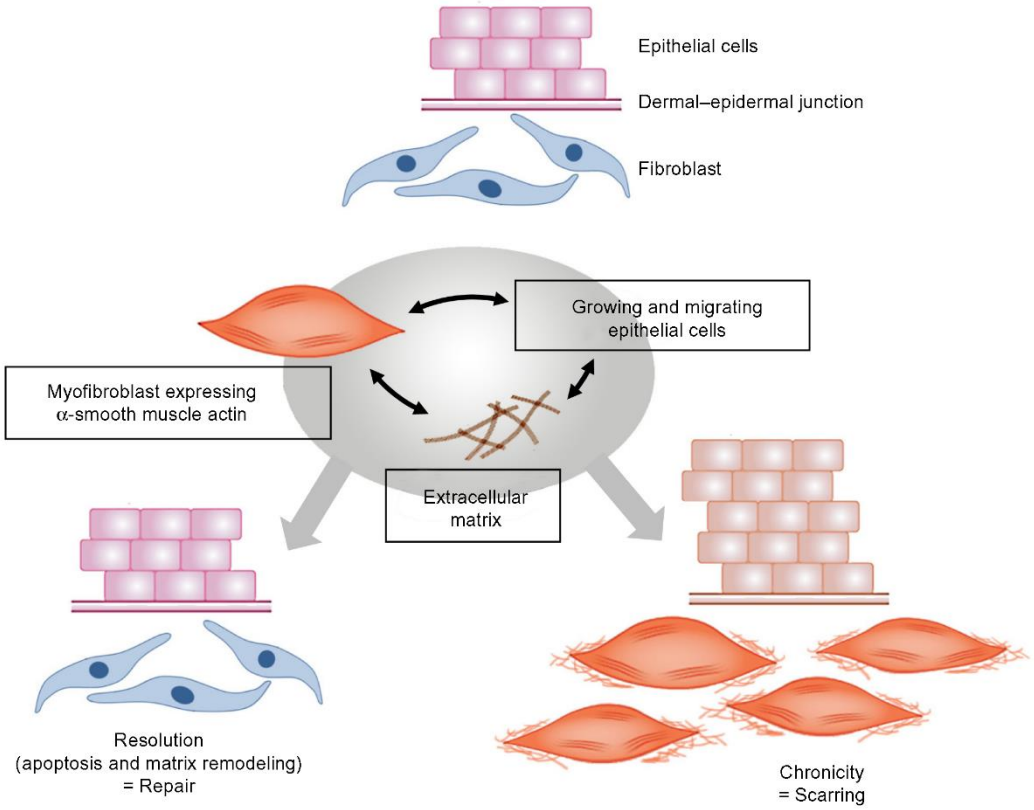


Figure 4 Processes leading to normal wound repair and pathological scarring.
Notes: In all of these situations, interactions between fibroblasts/myofibroblasts and the extracellular matrix, and also epithelial–mesenchymal cell dialogue, play a major role.

also favors deposition of ECM proteins over degradation by up-regulating TIMPs while decreasing expression of the MMPs themselves.[53]

Stimulation of myofibroblasts by TGF-β1 itself is affected by mechanical forces within the damaged or fibrotic tissue. TGF-β1 released from a variety of inflammatory cells and platelets in the microenvironment of damaged or fibrotic tissue is in a latent form. Indeed, myofibroblasts themselves release latent TGF-β1 complexed with latency-associated peptide (LAP). Together with a binding protein, TGF-β1 is bound to ECM proteins, providing a reservoir of latent TGF-β1 that can be activated as healing and scar formation progress.[54,55] Myofibroblasts express integrins that can bind to the LAP, and mechanical stress applied to the integrins, either by mechanical stress on the matrix and/or via myofibroblast contraction, can effectively activate TGF-β1 without cleaving the LAP and allow its binding to cell membrane receptors.[51] Thus, both increased mechanical stress and contraction can further increase myofibroblast contractile and matrix synthetic activity. This mode of activation provides another possible pathway for regulating myofibroblast activity by blocking integrin binding to latent TGF-β1, for example by blocking the integrin involved in latent TGF-β1 activation, αvβ5.[56] Inhibition of other integrin-binding sites has also been shown to inhibit myofibroblast development, including blocking of integrins α3β1,[57] α11β1,[58] αvβ5,[59] or β1.[60]

Hypoxia

Tissue oxygenation or hypoxia may play a role in both normal healing and pathological situations. In normal wound healing, the wound is transiently hypoxic as vascular perfusion is disrupted by the initial injury. Staining for the hypoxia-induced transcription factor, hypoxia inducible factor (HIF)-1α shows both areas of the early granulation tissue and the overlying migrating keratinocytes to be hypoxic. During normal healing, this hypoxia is resolved within a few days of injury and expression of HIF-1α declines. Hypoxia signaling can induce a number of growth factors that are beneficial to the healing process, prominent amongst them being vascular endothelial growth factor (VEGF) and, thus, acute hypoxia likely plays a beneficial role in healing. However, the same may not be true for chronic hypoxia and chronic hypoxia signaling.

Hypoxia has been reported to reduce myofibroblast activation and reduce collagen synthesis and α-SM actin expression.[61] In vivo studies using HIF-1-deficient mice showed that reducing HIF-1 availability during healing resulted in reduced collagen synthesis and delayed myofibroblast differentiation, suggesting that, overall, in vivo acute hypoxia during healing was normally compensated by induction of genes that allow tissue to adapt to transient hypoxia, such as VEGF.[62] In fact, fibroblasts that show reduced HIF-1α expression during hypoxia show inhibited migration and collagen synthesis in vitro. It is possible that, in some organs where there is pathological fibrosis and scarring, hypoxia and HIF signaling, possibly during more chronic hypoxic states, actually drives fibrosis, which has been suggested in the case of renal fibrosis. In some cells at least, there is cross talk between hypoxia signaling and TGF-β signaling that may exacerbate matrix synthesis and thus fibrosis.[63,64]

Therapies

Anti-fibrotic and anti-scarring therapies have proven to be a difficult and elusive area for research, with relatively few advances until quite recently. As the growth factor TGF-β is central to many of the mechanisms of pathological scarring and fibrosis, it has been the target of some therapeutic strategies. Some positive results have been reported with the drug pirfenidone, particularly in lung fibrosis, where the drug lowered TGF-β expression and both tissue and lavage fluid levels of the protein.[65] Interfering with activation of latent TGF-β is another potential target for anti-scarring therapies, and the role of integrin binding in TGF-β activation makes integrin-blocking antibodies a potential therapy for lowering TGF-β activation and thus downstream signaling.[66,67] Specific inhibitors of TGF-β signaling have also been suggested as possible treatments for scarring and fibrosis. TGF-β exerts its pro-fibrotic effects through transcription factor signaling Smad3, and selective inhibition of Smad3 phosphorylation and inhibition of Smad3 interaction with Smad4 has been shown to reduce fibroblast activation to the myofibroblast phenotype and also reduce ECM synthetic activity of the cells.[68] Other molecular targets include tyrosine kinases, and the drug imatinib mesylate has been reported to be anti-fibrotic through inhibiting downstream molecules that are required for the TGF-β response while having an additional anti-fibrotic role by also inhibiting platelet-derived growth

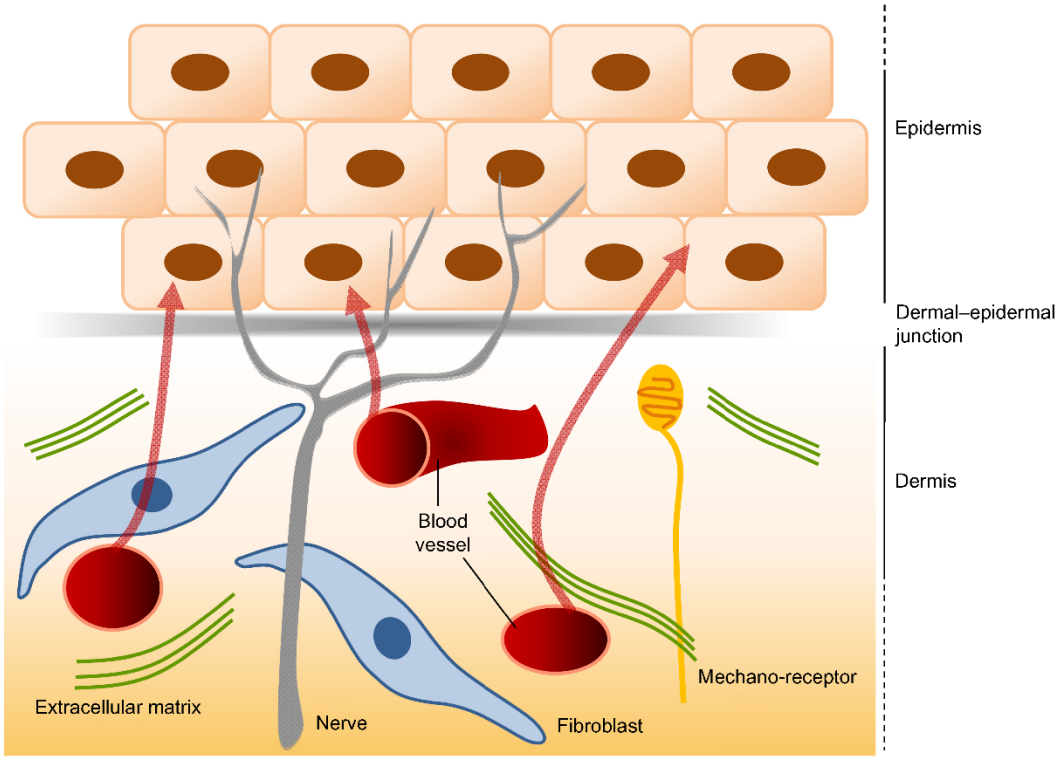


Figure 5 The interactions between the dermis and the epidermis.
Notes: The dermis and the epidermis contain nerves, but only the dermis is vascularized. The epidermis thus derives all its nutrients from dermal vessels (arrows). The dermal–epidermal junction plays a major role in the intense dialogue that exists between the keratinocytes of the epidermis and the cells of the dermis, notably the fibroblasts.

factor signaling.[69] Interestingly, HMG-coA reductase inhibitors such as statins have been shown to have anti-fibrotic effects, likely through inhibition of ROCK.[70] The widespread use of, and low rate of side effects associated with, these drugs may make them promising as anti-fibrotic therapies in the future.

Conclusion

Since their first description in the early 1970s, our knowledge about myofibroblast biology has increased greatly, and our interest in the biology of myofibroblasts has also increased, as this cell has been implicated in many pathological situations in addition to their role in normal wound repair. Despite major advances in our understanding of the origins of myofibroblasts and the factors that regulate their differentiation and activity, it remains a challenge and a major goal of researchers to find ways in which to regulate their activity to improve healing and scarring in the clinic. It is interesting that the skin is a highly sensitive organ. It is densely innervated by different sensory nerve fiber subtypes that react to tissue injury, temperature variation, and tactile stimuli (Figure 5). Cutaneous sensory nerve fibers are endings of dorsal root ganglia (or spinal ganglia) neurons that carry signals from sensory organs toward the appropriate integration center of the brain or of the spinal cord. Several clinical observations indicate that damage to the peripheral nervous system influences wound healing, sometimes resulting in chronic wounds within the affected area. Patients with cutaneous sensory defects due to spinal cord injury or diabetic neuropathy have an increased risk of developing ulcers that fail to heal. In addition, in aged patients, cutaneous repair processes are less efficient and this could be partly due to a deterioration of the peripheral nervous system at the skin level. Interestingly, factors that are required for sustaining peripheral nerves, the neurotrophin network, have also been shown to have direct effects on dermal fibroblasts in regulating ECM secretion, fibroblast differentiation, and tensile strength via effects on myofibroblasts.[71] Understanding the role of innervation during wound healing and myofibroblastic differentiation therefore represents an interesting domain. In addition, cutaneous innervation is certainly necessary to provide good hemostasis and to maintain the mechanical and cosmetic properties of the skin.[72] In conclusion, the recent advances made in understanding control of differentiation, proliferation, and survival of myofibroblasts will hopefully lead to new therapeutic approaches to limit scarring and improve healing in the not-too-distant future.[73]

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Disclosure

The authors report no conflicts of interest directly relevant to the content of this paper.

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Special Issue: Scar

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Role of Innervation in Scar Formation and Therapeutic Potential of Dermal Stem Cells

Abstract

During the resolution phase of normal healing, a considerable loss of various cell types, including myofibroblasts, occurs by apoptosis. Inappropriate delay of apoptosis, and thus increased survival of myofibroblasts, may be a factor which leads to pathological situations and excessive scarring. Many observations clearly suggest that innervation and neuromediators play a major role in wound healing. An abnormal level of neurogenic factors is implicated not only in the development of chronic wounds, but also in excessive scar formation. Understanding interactions between neuromediators and myofibroblasts and allowing normal reinnervation and adequate levels of neuromediators during the healing process are certainly important to avoid the appearance of pathological situations. Skin-derived precursors which can differentiate into neurons and Schwann cells could be involved during the healing process and could be also used to help restore normal innervation after skin damage and for the treatment of various neurodegenerative diseases. The aim of this review is to first discuss the mechanisms leading to normal or excessive scarring. Then, the roles of innervation and neuromediators during wound healing will be presented. Finally, the interest in using skin-derived precursors to help restore adequate innervation after skin damage will be introduced.

Keywords: myofibroblast, apoptosis, excessive scarring, innervation, neuromediator, skin-derived precursor

Introduction

Considering the important functions of the skin as a barrier against foreign pathogens and water loss, in the regulation of body temperature and sensory perception, normal wound healing can be considered as a critical survival event. In current medical practice, severe scarring disorders can affect the patient at a functional level, but also aesthetically and psychosocially. Understanding the mechanisms involved in normal and pathological healing processes is fundamental. Many different cell types play important roles after skin damage and a dialogue between these cells is essential. Among these cells, myofibroblasts due to their contractile properties and to their capacities to synthetize and remodel the extracellular matrix play a major role. Close by, the roles of nerve fibre endings and of neuromediators are not well understood. Finally, although several studies have discussed the fact that skin-derived precursors (SKPs) represent interesting cells for skin repair in cutaneous wounds, the regeneration of injured skin is still in the distant future. Several issues need to be resolved before considering the wide use of SKPs for regenerative medicine and in particular the field of skin repair. In this review, these different aspects will be discussed.

General mechanisms of scar formation

GRANULATION TISSUE REMODELLING

In the granulation tissue, fibroblasts are activated and acquire a myofibroblastic phenotype. These myofibroblastic cells synthesize and deposit the extracellular matrix components which replace the provisional matrix. This provisional matrix which appears after the blood vessel damage is mainly composed of fibrin and fibronectin, contributes to the initiation of the inflammatory response and allows the migration of fibroblasts into the wound bed. Myofibroblasts also exhibit contractile properties, due to the expression of α-smooth muscle actin in microfilament bundles or stress fibres, playing a major role in the contraction and in the maturation of the granulation tissue.[1] Presently, it is accepted that the myofibroblastic modulation of fibroblastic cells begins with the appearance of the protomyofibroblast, whose stress fibres only contain β- and γ-cytoplasmic actins. Protomyofibroblasts generally evolve into differentiated myofibroblasts, the most common variant of this cell, with stress fibres containing α-smooth muscle actin.[2] Myofibroblasts can, depending on the experimental or clinical situation, express other smooth muscle related contractile proteins, such as smooth muscle-myosin heavy chains or desmin; however the presence of α-smooth muscle actin represents the most reliable marker of the myofibroblastic phenotype.[3] The third phase of healing, scar formation, involves a progressive remodelling of the granulation tissue. During this remodelling process, proteolytic enzymes, essentially matrix metalloproteinases (MMPs), and their inhibitors (TIMPs for tissue inhibitor of metalloproteinases) play a major role.[4] The synthesis of extracellular matrix is not totally stopped, but considerably reduced, and the synthesized components are modified as the matrix is remodelled. Progressively, collagen type III, the major component of the granulation tissue, is replaced by collagen type I which is the main structural component of the dermis. Lastly, elastin which contributes to skin elasticity and which is absent in the granulation tissue, also reappears. In the resolution phase of healing, the cell number is dramatically reduced by apoptosis of both vascular cells and myofibroblasts.[5] The signal triggering this cell death is unknown but may be related to modifications in the concentrations of local trophic factors, including neuromediators, or in specific myofibroblast adhesion to

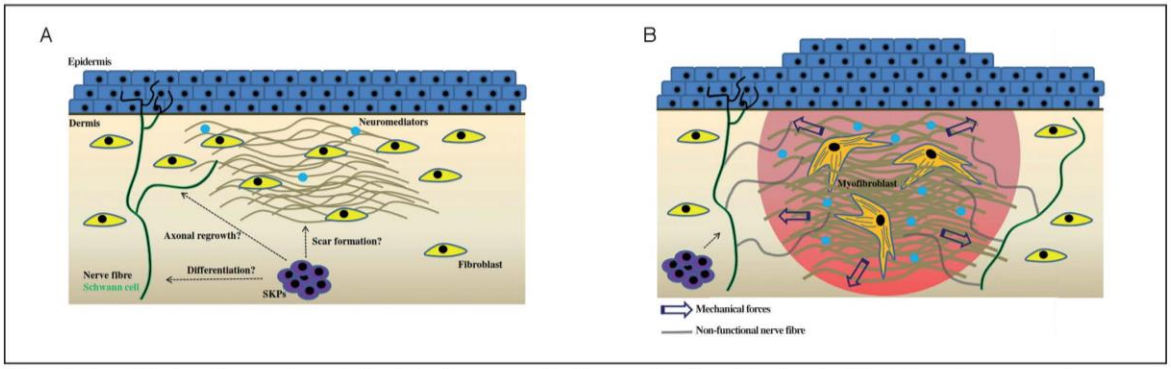


Figure 1. Potential roles of innervation and skin-derived precursors in skin wound healing. *A: During physiological scarring, progressive extracellular matrix remodeling is mediated by (myo)fibroblasts. It is now known that nerve fibers contribute to the healing process by releasing neuromediators and in parallel the process of wound reinnervation occurs. Local or transplanted skin-derived precursors (SKPs) could be involved at different stages such as the overall scar formation, axonal regrowth and could as well differentiate into neurons and/or Schwann cells. B: During hypertrophic scarring, the contractile activity of α-smooth muscle actin expressing myofibroblasts associated with abnormal production of extracellular matrix leads to a pathological scarring phenomenon. Hypertrophic scars are associated with thicker epidermis, local inflammation and the ongoing generation of mechanical forces due to the activity of myofibroblasts. The abnormal release of neuromediators by nerve endings could also promote this phenomenon while increase axonal regrowth of non-functional nerve fibres has also been described.*

the extracellular matrix. To date, it is not known if myofibroblasts can reacquire a quiescent phenotype and return to a normal dermal fibroblast phenotype with no expression of α-smooth muscle actin.

PATHOLOGICAL SCARRING

Myofibroblasts are implicated in many fibrotic and scarring diseases where they carry out the important process, after initial injury, of providing mechanical support and integrity to the tissue. In normal physiological conditions, they are then lost via apoptosis, generally when the tissue integrity has been sufficiently restored to be mechanically coherent.[6,7] Thus, in normal physiological situations like skin wound healing, myofibroblasts disappear in a prominent wave of apoptosis, leaving a markedly less cellular scar. However, it is now assumed that, in many fibrotic and scarring conditions, as well as in the stromal response to tumors, myofibroblasts fail to undergo cell death, persist and thus in turn lead to ongoing pathology and excessive scarring *(Figure 1)*. In clinical research, it has been shown that α-smooth muscle actin is detected in hypertrophic scars but not in keloids.[8] Indeed, it seems that α-smooth muscle actin is detected in these both types of excessive scarring.[9] Nevertheless, contractures develop in hypertrophic scars due to the important presence of α-smooth muscle actin expressing myofibroblasts but not in keloids. An example of reduced or inhibited apoptosis leading to excessive scarring is in a model where mechanical loading increases survival of myofibroblasts inducing excessive scarring formation.[10,11] A better understanding of the control and signalling that govern apoptosis (or autophagy) in myofibroblasts may lead to more targeted approaches for combatting fibrosis and excessive scarring. It has been demonstrated that elevated NADPH oxidase 4-derived hydrogen peroxide level supported by concomitant decreases in nitric oxide signalling and in reactive oxygen species scavengers, is a central factor in the molecular pathogenesis of fibrosis.[12] Redox signalling could therefore represent an interesting target to restore physiological fibroblast-myofibroblast ratio. Apoptosis in myofibroblasts is thought to be regulated by a reduction in the local growth factors that drive and sustain myofibroblast differentiation, in particular local concentrations of transforming growth factor-β1 and endothelin-1 which play a role in stimulating myofibroblast survival via AKT activation.[13] However, changes in mechanical signalling such as unloading of mechanical force, also play a role.[14]

The importance of myofibroblasts in causing fibrosis in internal organs and in the skin (hypertrophic scars), and the role that persistence of stromal myofibroblasts appear to play in tumour growth and spread, makes the (down)regulation of myofibroblasts and the potential regulation of myofibroblast disappearance through apoptosis of increasing interest.[15]

Roles of innervation and neuropeptides during healing

Globally, the role of innervation in organ repair is poorly known. Nerves, essentially sensory nerves, are present within the skin and influence a variety of physiological and pathophysiological cutaneous functions.

Cutaneous sensory nerve fibres are endings of dorsal root ganglia (or spinal ganglia) neurons that carry signals from sensory organs toward the appropriate integration centre of the brain via the spinal cord. The skin is a highly sensitive organ which is densely innervated both in the dermis and the epidermis with different types of nerve endings that could be associated with specific receptors[16], which discriminate between pain, thermal and tactile sensations. When deep skin damage occurs, cutaneous nerves and sensory receptors are while sensory neuron cell bodies persist in the dorsal root ganglia.

Mechanical stimuli are detected via mechanoreceptors associated with sensory corpuscles through Aß fibres or with Aδ free nerve endings, temperature via the thermoreceptors through Aδ and C fibres, and pain via the nociceptors through Aδ and C fibres.[17] Cutaneous sensory nerves are classified according to diameter and speed of impulse as Aß, Aδ, and C nerve fibres, from the biggest and fastest to the smallest and slowest, respectively. All kinds of fibres are accompanied by Schwann cells in the dermis but not in the epidermis. The larger nerve fibres, Aß and free nerve endings Aδ, are associated with Schwann cells, which secrete a basal lamina around them and also produce myelin sheaths. C free nerve endings are the termination of unmyelinated fibres and are →

associated in the dermis with Schwann cells which do not produce myelin. In addition to mechanical, thermal and pain stimuli, both C and Aδ free nerve endings respond to a range of stimuli such as physical (trauma, heat, osmotic changes, ultraviolet light) as well as chemical (toxic agents, allergens, proteases, microbes) agents.[18]

In unstimulated nerves, neuromediators are barely detectable within the skin tissues. Upon direct stimulation by physical or chemical means, or during pathological situations such as inflammation or trauma, a significant increase in neuromediator levels is observed. Thus, mediators derived from sensory or autonomic nerves may play an important regulatory role in the skin under many physiological and pathophysiological conditions, particularly during wound healing.[19]

Overall, little is known about the role of sensory fibres on myofibroblast differentiation and activity. Skin lesions and peripheral nerve damages cause resident and infiltrating immune cells, but also the sensory nerve terminals themselves, to release inflammatory mediators including interleukin-1α, tumour necrosis factor-α, bradykinin, substance P, calcitonin gene related peptide, nerve growth factor, and prostaglandins, contributing to the "inflammatory soup".[20]

Interestingly, altered substance P levels may contribute to impaired cutaneous healing responses associated with chronic wounds or hypertrophic scar formation.[21] Topical application of exogenous substance P enhances wound closure kinetics in diabetic rats suggesting that diabetic wounds have insufficient substance P levels to promote a neuroinflammatory response necessary for normal wound repair. In contrast, increased nerve fibre numbers and neuropeptide levels with reduced neutral endopeptidase (a membrane bound metallopeptidase that degrades substance P at the cell membrane) levels in hypertrophic scar samples suggest that excessive neuropeptide activity induces exuberant inflammation and extracellular matrix deposition with a persistent activation of myofibroblasts in hypertrophic scars. Chéret *et al.*[22] report that the adhesion of human dermal fibroblasts and their differentiation into myofibroblasts, are promoted after incubation with vasoactive intestinal peptide, calcitonin gene related peptide, and substance P. Recently, Fujiwara *et al.*[23] demonstrated that direct contacts of fibroblasts with neuronal processes are necessary for the differentiation into myofibroblasts and for the induction of collagen gel contraction, important processes able to promote wound healing; the molecular mechanism of fibroblast differentiation by direct contact with neuronal processes was not identified in this study. Interestingly, symptoms of itching and pain, and abnormal thermosensory thresholds, are present in excessive skin scarring suggesting that these pathological situations are closely associated with small nerve fibres. Moreover, the density of neuropeptide containing nerve fibres was greater in excessive scarring than in normal skin samples.

Interests of dermal stem cells for healing and/or regenerative medicine

ORIGIN AND ISOLATION OF DERMAL STEM CELLS

Adult stem cells can be isolated from the skin. These SKPs possess capacities of self-renewal and multipotency. They can differentiate into both neural and mesodermal progeny, including cell types that are never found in the skin, such as neurons. Neural crest stem cells have a similar broad potential and contribute to the development of the dermis and, in this regard, SKPs form a neural crest-related stem cell niche that arises in the skin during embryogenesis and persists in lower numbers into adulthood.[24] SKPs are present in different locations in the dermis, the largest and most studied source is located in the dermal papillae at the base of the hair follicle.[25,26] Other sources of dermal SKPs include the hair bulge, sebaceous gland, sweat gland as well as a perivascular niche recently described.[27]

After isolation, SKPs are maintained in culture as spheroids in a serum free medium supplemented with fibroblast growth factor-2 and epidermal growth factor. They express specific embryonic transcription factors such as nestin, vimentin and fibronectin.[28,29] The addition of serum to the culture medium induces cell adhesion to the culture plastic with cells migrating out of the adherent sphere colony. Then, neuronal differentiation can be promoted using cocktails of neurotrophins such as brain-derived neurotrophic factor, neurotrophin-3, nerve growth factor and AMPc while glial differentiation is mediated by the addition of forskolin and heregulin 1ß to the culture medium.[30-33] Thus, upon neuronal or glial differentiation, SKP-derived neurons or SKP-derived Schwann cells can be generated and used for *in vitro* studies and tested in *in vivo* wound healing models.

POTENTIAL INVOLVEMENT OF SKPS DURING THE HEALING PROCESS AND THERAPEUTIC STRATEGIES

Little is known regarding the role of SKPs in the wound healing process but several studies have shown that SKP-derived Schwann cells help promoting peripheral nerve regeneration.[34-36] It suggests that SKP-derived Schwann cells are fully functional in promoting axonal regrowth. More recently, Sato *et al.*[37] have showed that intradermal injections of SKPs around full-thickness excisional cutaneous wounds in diabetic mice mediate faster wound closure and re-epithelization, earlier angiogenesis and might promote wound reinnervation. Interestingly, another study has also shown that SKPs transplantation in denervated cutaneous wounds in nude mice promotes wound closure and local secretion of neuromediators such as substance P, calcitonin gene related peptide as well as nerve growth factor.[38] More studies have to be performed in order to determine if local or transplanted SKPs can either directly differentiate into Schwann cell and/or neurons following skin injury or if they somehow help mediating the migration of local Schwann cells and/or axonal regrowth of nerve fibres during scar formation *(Figure 1)*. Thus, the isolation of SKP-derived autologous precursors from adult human skin represents an easily accessible and a very promising source of neurons and Schwann cells to help restore normal innervation after skin damage and for the treatment of various neurodegenerative diseases.

Conclusion

Understanding the processes involved in excessive scarring formation, particularly the roles played by the mechanical environment, remains an important question to be solved. Certainly, peripheral innervation and released neuromediators are important factors contributing to skin healing and future studies could shed light on new mechanisms involved in normal and pathological scarring. Finally, because of their capacity of multipotency and their localization in the skin dermis, SKPs are definitively an attractive tool for tissue engineering and in the field of regenerative medicine. ■

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Titre : Innervation périphérique et réparation cutanée : rôle de l'innervation dans la cicatrisation après brûlure et sur l'activité cellulaire des fibroblastes dermiques

La peau est un organe sensoriel qui permet notamment à l'organisme de s'adapter à son environnement. Ainsi, de nombreuses fibres nerveuses sont présentes au sein de cette peau permettant de détecter différents stimuli tels que la pression, la douleur ou encore une variation de température. A la suite d'une brûlure profonde, ces terminaisons nerveuses sont détruites et la repousse de ces fibres lors du processus de cicatrisation est inadéquate conduisant à des handicaps souvent sérieux chez les patients. Dans un premier travail, la réalisation d'une brûlure chez des animaux présentant ou non une neuropathie périphérique induite par la résinifératoxine a permis d'étudier le rôle de l'innervation lors de la cicatrisation. Chez les animaux traités, une cicatrisation plus lente et un défaut de réinnervation par rapport aux animaux contrôles étaient observés. Pour approfondir le rôle de l'innervation dans la cicatrisation, des études *in vitro* ont ensuite été réalisées afin d'évaluer les interactions possibles entre les cellules neuronales et les fibroblastes dermiques. Même en l'absence de contacts directs, il a été montré que les cellules neuronales sont capables d'induire la différenciation des fibroblastes en myofibroblastes. Au vue de l'implication de l'innervation sur la différenciation des fibroblastes et sur la cicatrisation, il semble important de pouvoir proposer aux patients brûlés, pour leur traitement, un nouveau concept de substitut cutané favorisant une repousse axonale fonctionnelle au sein du tissu cicatriciel.

Mots clés : innervation cutanée, brûlure, cicatrisation, interactions cellulaires.

Title: Peripheral innervation and cutaneous repair: role of innervation in wound healing after burn and on cellular activity of dermal fibroblasts

The skin is a sensitive organ which notably allows the body to adapt to its environment. Many nerve fibers are present in the skin and are implicated in the detection of various stimuli such as pressure, pain or temperature variation. After a deep burn injury, these nerve fibers are destroyed and their regrowth during the wound healing process is imperfect which induces serious disabilities for patients. In a first study, a burn model was developed on animals presenting or not a peripheral neuropathy induced with resiniferatoxin and had allowed to study the role of sensory innervation on wound healing. On animals treated with resiniferatoxin, wound healing was delayed and nerve regeneration was imperfect compared to control animals. To further investigate the role of innervation in wound healing, *in vitro* studies were then performed to evaluate possible interactions between neuronal cells and dermal fibroblasts. Even in the absence of direct contacts, it has been shown that neuronal cells are able to induce the differentiation of fibroblasts into myofibroblasts. Considering the involvement of innervation on the differentiation of fibroblasts and on wound healing, it seems important to provide burned patients with a new concept of skin substitute promoting functional axonal regrowth in the scar tissue.

Key words: cutaneous innervation, burn, wound healing, cell interactions.