Sensory Neurons Accelerate Skin Reepithelialization via Substance P in an Innervated Tissue-Engineered Wound Healing Model

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Keratinocytes are responsible for reepithelialization and restoration of the epidermal barrier during wound healing. The influence of sensory neurons on this mechanism is not fully understood. We tested whether sensory neurons influence wound closure via the secretion of the neuropeptide substance P (SP) with a new tissue-engineered wound healing model made of an upper-perforated epidermal compartment reconstructed with human keratinocytes expressing green fluorescent protein, stacked over a dermal compartment, innervated or not with sensory neurons. We showed that sensory neurons secreted SP in the construct and induced a two times faster wound closure *in vitro*. This effect was partially reproduced by addition of SP in the model without neurons, and completely blocked by a treatment with a specific antagonist of the SP receptor neurokinin-1 expressed by keratinocytes. However, this antagonist did not compromise wound closure compared with the control. Similar results were obtained when the model with or without neurons was transplanted on CD1 mice, while wound closure occurred faster. We conclude that sensory neurons play an important, but not essential, role in wound healing, even in absence of the immune system. This model is promising to study the influence of the nervous system on reepithelialization in normal and pathological conditions.

Introduction

THE ABSENCE OF CUTANEOUS sensory innervation affects negatively all stages of wound healing.¹ The mediator of the positive influence of nerves on reepithelialization is unclear, but skin injuries are known to induce a neuronal release of neuropeptides such as substance P (SP) in the skin.² SP induces neurogenic inflammation by regulating blood flow and it modulates local immune function upon binding to its neurokinin-1 (NK1) cell receptor.³ It was shown to increase the density of leukocytes and macrophages 3 days after a topical application on a full-thickness wound in mice.⁴ Thus, nerve fibers may enhance reepithelialization through the stimulation of inflammation, which is known to play a key role in wound healing.^{5,6} But they may also play a direct effect on keratinocytes since these cells express the NK1 receptor. Upon binding to SP, NK1 activates autocrine production of SP in keratinocytes and cell proliferation in mouse skin organ culture.^{7,8} Still, it is unclear whether direct paracrine neuronal to keratinocyte interactions contribute to reepithelialization independently of inflammatory cells *in vivo*.⁹ However, Hsieh and Lin demonstrated that in absence of skin injury, denervation

alone in rat hind paw skin is sufficient to reduce keratinocyte proliferation and induce an epidermal thinning within 4 days.¹⁰

It is difficult to discriminate between the roles played by neuropeptides on inflammation versus a direct effect on keratinocytes to investigate their effect on wound healing in animal models. In addition, murine skin wound closure is mostly achieved by contraction of the wound margins, while human skin heals through reepithelialization. Our aim was to define precisely, using human keratinocytes, whether sensory neurons can directly promote reepithelialization independently of the immune system and whether such modulation was mediated by the release of SP.

Tissue-engineered reconstructed human skin offers the opportunity to study reepithelialization in absence of immune cells and we have previously established a protocol to innervate these constructs.^{11–15} In the present study, we developed a new innervated wound healing model. The model was innervated or not with sensory neurons and the keratinocytes were transduced to express the green fluorescent protein (GFP) to follow up accurately in real time the wound closure and answer long-standing questions concerning reepithelialization.

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Materials and Methods

Cell isolation and amplification

Fibroblasts and keratinocytes were isolated and cultured from human skin biopsies after surgeries as previously described.¹⁶ The study was approved by the CHA research ethical committee DR-002-951 protocol. Briefly, human fibroblasts between the third and the sixth passages were grown in DMEc: DMEM (Invitrogen) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and antibiotics [100 U/mL penicillin G (Sigma-Aldrich) and 25 µg/mL gentamicin (Shering)]. Keratinocytes were cultured on a feeder layer of lethally irradiated 3T3 mouse fibroblasts in DHc: a 3:1 ratio of DMEM/Ham's (Invitrogen) supplemented with 5% HyClone FetalClone II (Thermo Fisher Scientific), 5 µg/mL insulin (Sigma-Aldrich Canada), 0.4 µg/mL hydrocortisone (Cedarlane), 10^{-10} M cholera toxin (ICN Biochemicals), 10 ng/mL human epidermal growth factor (EGF; Austral Biologicals), and antibiotics. Human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HDMECs) were obtained as previously described¹⁷ and were grown on collagen-coated surface in EGM-2: EGM-2 MV bullet kit (Lonza). Sensory neurons were extracted from the dorsal root ganglia of mouse embryos (E13) and cultured as previously described.¹¹

Keratinocyte proliferation and migration assays

To assess whether SP influences the proliferation of epithelial cells, keratinocytes in exponential proliferation phase were cultured in DHc without EGF in presence of 0, 10^{-6} , 10^{-7} , and 10^{-8} M of recombinant SP (Bachem). A proliferation assay with a colorimetric method was used according to the manufacturer's protocol to quantify differences in cell number after 24, 48, 72, and 96 h of culture (cell counting kit-8; Dojindo Molecular Technologies, Inc.).

To monitor the effect of SP on keratinocyte migration, a scratch wound assay was performed.^{18,19} Keratinocytes were cultured for 7 days in 24-well plates, and a manual scratch was performed in the confluent cell layer with a pipette tip. Recombinant SP at 0, 10^{-6} , 10^{-7} , and 10^{-8} M was added for 24 h in DMEM-Ham medium without any other supplement. Cell migration was analyzed each 2 h using time-lapse imaging with a Zeiss axio observer microscope (Zeiss Canada) and quantified with Image J 1.46 software.

Production of GFP lentiviral expression vector and transduction of keratinocytes

To produce lentivirus, 293FT cells were transfected using Lipofectamine 2000 (Invitrogen) as per manufacturer's instruction with 12 µg of an equimolar mixture of P1p1, P1p2, pVSVg packaging plasmids (gracious gift from Dr. Sylvain Guérin), and plenti6.3/V5/Gw/EmGFP expression vector (Invitrogen). Three days post-transduction, lentivirus-containing supernatant was collected and cleared through a 0.45-µm filter, titrated using human fibroblasts. The multiplicity of infection (MOI) was determined by flow cytometry with log amplification using a fluorescence-activated cell sorter (FACScan; BD Biosciences) for detection of GFP-expressing cells 1 week after transduction. Human keratinocytes were cultured 3 days in DHc and then treated with a 2 mL solution

per 75 cm² of DHc containing 10 µg/mL Polybrene (Sigma-Aldrich Canada) and three MOI of lentivirus. After 6 h, 18 mL of DHc was added.

Preparation of innervated wound healing model and wound healing model

Thin collagen sponges were developed from a modified version of our previously published method.²⁰ Briefly, types I and III bovine collagen (Symatase) and chitosan (Kemestrie, Inc.) were dissolved but without chondroitin 4–6 sulfates in 0.1% acetic acid. Then, 1.3 mL/well (9.6 cm²) of the final solution was poured in six-well plates (BD Biosciences), frozen at -80°C , and lyophilized in a vacuum lyophilizer (Dura-Stop™ Freeze-Dryer; FTS Systems, Inc.).

The epidermal construct (sponge A) was seeded with 2.1×10^5 fibroblasts/cm² and cultured in DMEc with 100 µg/mL ascorbic acid for 14 days. Then, keratinocytes were seeded (2.1×10^5 cells/cm²) over it and cultured by immersing in DHc with 100 µg/mL ascorbic acid for 7 days and then 14 days at the air–liquid interface in the same medium but without EGF.

The dermal construct (sponge B) was seeded with a 1:1 ratio suspension of fibroblasts and HUVECs or HDMECs (passage 5) (2.1×10^5 cell type/cm²). The constructs were cultured in a 1:1 ratio of EGM-2 and DMEc plus 75 µg/mL ascorbic acid (Sigma) for 1 week, and in a 3:1 ratio of DMEM-Ham's F-12 medium supplemented with 10% FBS, 0.4 µg/mL hydrocortisone, 5 µg/mL bovine insulin, 100 µg/mL ascorbic acid, and antibiotics (A/Lc) for 1 additional week. On day 14, mouse sensory neurons were seeded at 2.1×10^5 cells/cm² on the dermal construct, which was cultured at the air–liquid interface for 14 days with A/Lc, 100 µg/mL ascorbic acid, and 10 ng/mL NGF [nerve growth factor 2.5S (natural murine; Invitrogen)] (Fig. 1).

The epidermal construct was perforated from side to side with a punch biopsy (Acuderm, Inc.) of 8 mm in diameter. Then, the dermal construct was flipped upside down so the side B that was seeded with neurons was positioned in the bottom. Finally, the perforated epidermal construct was stacked over the dermal construct and both tissues were held together using ligaclips (Ethicon Endo-Surgery). Constructs were subsequently cultured at the air–liquid interface in DHc without EGF plus 100 µg/mL ascorbic acid.

SP at 10^{-7} M (Bachem) and 2 µM Spantide II (Enzo life Science) were diluted in the culture medium at every medium change after wounding.

Animals and surgical manipulations

Adult male CD1 mice (44 days old; Charles River Laboratories) were used as surgical recipients as previously described.¹¹ Briefly, the mice were injected with ceftazidime (140 mg per mouse; Glaxo) 24 and 48 h before surgery. Animals were anesthetized via inhalation of 3% isoflurane with 1.5 L/min oxygen (lowered to 2% for surgery). A 2.5-cm² full-thickness skin area was excised to the muscle, on the back of the mouse. The innervated wound healing model (iWHM) and WHM (with a 6-mm wound diameter) made of GFP-expressing keratinocytes were transplanted in a Fuesnig chamber to protect them after grafting. To assess the reepithelialization process, the mice were anesthetized prior to imaging via inhalation of 2% isoflurane with 1.5 L/min

FIG. 1. Schematic representation of the preparation of the innervated wound healing model (iWHM). The iWHM was made of two compartments: the upper one corresponding to the wounded epidermal part, and the lower one, to the innervated (or not) wound bed. In (A), the sponge biomaterial was seeded with fibroblasts and cultured for 14 days immersed in culture medium, and then seeded with keratinocytes cultured for 7 days immersed, and 14 additional days at the air–liquid interface to promote epidermal differentiation. In (B), the sponge biomaterial was cultured with fibroblasts and endothelial cells for 14 days, and then seeded with mouse sensory neurons cultured 14 additional days at the air–liquid interface. In (C), the epidermal construct (sponge A) was perforated from side to side (after a total of 35 days of culture) just before it was stacked up over the intact dermal construct (sponge B) that was previously flipped upside down to have the neurons underneath the sponge. The migrating keratinocytes from sponge A will grow over the dermal construct to close the wound. Color images available online at www.liebertpub.com/tea

oxygen. All the manipulations of the animals were performed according to the rules established by the Canadian Council on Animal Care.

SP at 10^{-7} M and 2 μ M Spantide II were diluted in 30% Pluronic® F-127 (Anaspec) and deposited as one topical application of 50 μ L on wounds after grafting.

Indirect immunofluorescence

Indirect immunofluorescence assays were performed on paraformaldehyde (4%)–fixed culture cells or frozen tissue sections embedded in O.C.T. compound (Sukura, Finetek U.S.A., Inc.). A further step of permeabilization was performed with PBS containing 0.5% (v/v) Triton X-100 (Bio-Rad). The primary antibodies used were sheep polyclonal anti-human PECAM-1 (R&D Systems); mouse monoclonal IgG anti-human K14 (Sigma), mouse monoclonal IgG1 anti-K10 (Cederlane), rabbit polyclonal anti-mouse SP (Immunostar), rabbit polyclonal anti-human NK1 (Millipore), Chicken polyclonal IgY anti-NFM (Millipore), and rabbit polyclonal anti-TRPV1 (Novus Biological). The secondary antibodies used were as follows: donkey anti-sheep IgG Alexa 488®, goat anti-mouse IgG biotin (Jackson), Alexa405-conjugated Streptavidin (Invitrogen), and rabbit anti-chicken IgY rhodamine (Chemicon). Cell nuclei were counterstained with either Hoechst reagent 33258 (Sigma Aldrich) or DRAQ5 (Biostatus Limited). As a control, the primary an-

tibody was omitted. Immunostaining was performed on tissues in which keratinocytes did not express GFP.

ELISA analysis

Supernatants were harvested, clarified by centrifugation, and frozen at -80°C . For treatment with transient receptor potential vanilloid 1 (TRPV1) agonist, 0.01 ng/mL capsaicin (Cayman Chemical) dissolved in 1 mM DMSO or 1 mM DMSO for controls were added in the medium for 30 min. The quantification of SP was performed as described in the manufacturer's protocol (Substance P Parameter Assay Kit; R&D Systems).

Quantification of reepithelialization

The reepithelialization process was monitored through quantification of GFP fluorescence. Macroscopic pictures of the wounds were gathered using IVIS® 200 Imaging System (CaliperLS, Xenogen). Pictures were analyzed using Living image 4.2 (CaliperLS) and NIH imageJ 1.46. The percentage of reepithelialization was calculated from the ratio and difference between the area of the reepithelialized surface compared with the initial wound area for each treatment and measurements. Values are expressed as the means $\pm$ SDs. Differences were tested by Student's *t*-test with a bilateral distribution and equal variances. The probability level was regarded as significant at $p < 0.05$.

Results

Histological characterization of the iWHM

To study reepithelialization in presence of sensory neurons but in absence of immune cells, we prepared the iWHM by stacking a perforated epidermal constructs on an endothelialized dermal construct that was innervated or not with mouse sensory neurons (Fig. 1). The histology showed that the keratinocytes formed a thick and well-differentiated epidermis (Fig. 2A, B). The fibroblasts colonized both of the sponges and they also synthesized extracellular matrix (in pale blue). Keratinocytes migrated from the wound margins over the dermal construct (Fig. 2A, C, D), forming an epidermal tongue partly differentiated up to the stratum corneum (Fig. 2A, asterisk).

Characterization of nerve fibers, neuropeptides, capillary-like tubes, and epidermis outgrowth in the WHM and the iWHM

To verify that our new iWHM model displays all the components to study reepithelialization in presence of sensory neurons, we characterized it using confocal microscopy. The network of nerve fibers, stained with the 150-kDa neurofilament (NFM), was observed in the entire dermal construct in the iWHM in close proximity to the migrating epidermis (Fig. 3C, D). A staining for PECAM-1 (CD31), a cell-adhesion molecule expressed only by endothelial cells, was also performed in the iWHM and the WHM to show the network of capillaries (Fig. 3A–D) and the epidermis was stained for the intermediate filament cytokeratin K14 (Fig. 3A–D). Keratinocytes behind the leading front edge of the migration tongue were more differentiated, expressing the differentiation marker K10 (Fig. 3E).

We validated the expression by nerves of SP, and by keratinocytes of the NK1 receptor in the iWHM by immunohistochemistry. The NK1 cell receptor for SP was expressed in the epidermis including the epidermal migration tongue (Fig. 3E) and by fibroblasts both in the iWHM and

when cultured in monolayer (Fig. 3E, F). SP was expressed in the neuronal cell body and nerve fibers of the sensory neurons in the iWHM (Fig. 3G–I).

To confirm the functionality of sensory neurons in the iWHM, we validated their potential to release SP after stimulation of the TRPV1 with the agonist capsaicin. The activation of TRPV1 is known to induce nerve fibers to release neuropeptides through degranulation of the large dense-core vesicles.²¹ The presence of SP was detected by ELISA analysis of the culture supernatant from dermal constructs with and without sensory neurons after 7 days of innervation (Fig. 3M). In absence of neurons, a low amount of SP secretion was observed, which could be expressed by fibroblasts since they were shown to produce SP.²² However, there was five times more SP released in presence of neurons (Fig. 3M). Sensory neurons in the iWHM expressed TRPV1 (Fig. 3J–L). When the TRPV1 agonist capsaicin was added for 30 min in the medium, a four times increase of the amount of SP secretion was detected in presence of neurons, while the concentration of SP remained unchanged without neurons (Fig. 3M).

Influence of sensory neurons, SP, and the NK1 antagonist Spantide II on wound closure in vitro

To accurately assess the reepithelialization process, we transduced human keratinocytes with GFP and analyzed wound closure using the IVIS Lumina imaging system. The transduction efficiency of the keratinocytes was 35% (data not shown), and was sufficient to perform an accurate follow-up of the wound closure (Fig. 4).

The percentage of wound closure was analyzed by assessing the percentage of wounded area over time (Fig. 4B). Three days after wounding, the percentage of wound closure was 2.2 times higher in presence of sensory neurons (iWHM vs. WHM). Based on the percentage of wound closure, the speed of reepithelialization from day 0 to 3 was three times higher with neurons, and complete wound closure was reached after 4 days in the iWHM, versus up to 9 days in the WHM.

FIG. 2. Histological aspect of the model. The histology of the reconstructed skin graft was assessed by observation of 4- μ m-thick tissue cross-sections stained with Masson's Trichrome. The reepithelialization process from side to side of the wound can be observed (A). The boxes in (A) are enlarged from (B) to (D). They show an intact portion of two stacked sponges (B), a reepithelialized area with the keratinocytes from the epidermal upper compartment migrating over the lower dermal compartment (C) and the front of the epidermal tongue of migration over the dermal construct (D). The asterisk in (A) shows a portion of the epidermal tongue differentiated up to the stratum corneum. Scale bars in A: 500 μ m; B–D: 100 μ m. Color images available online at www.liebertpub.com/tea

FIG. 3. Neurite and capillary-like tube outgrowth in the iWHM and WHM. Twenty-micron-thick sections of the WHM (A, B) and iWHM (C, D) were observed using a confocal microscope after staining of keratinocytes with Keratin 14 antibodies (in blue, A–D), endothelial cells organized into capillaries with PECAM-1 antibodies (in green, A–D), and nerve fibers with Neurofilament M antibodies (in red, C, D). Panels (A) and (C) correspond to areas of the epidermal tongue of migration and (B) and (D) to the wound margin. The white-dotted lines show the sponge of the upper compartment that was perforated, while the white arrows designate the initial wound margin (B, D). Keratinocytes in the tongue of migration express SP receptor NK1 (E; in green) and the differentiation marker Keratin 10 (E; in red). Fibroblasts also express the NK1 receptor (F; in green). Nerve fibers (G and I; NFM in green) in the iWHM express substance P (H and I; in red). Most nerve fibers in the model (J and L; NFM in green) express TRPV1 (K and L; in red). WHM and iWHM were stimulated or not with a TRPV1 agonist to induce the release of SP, quantified by ELISA in the supernatant (M). The graph is a representation of the mean results $\pm$ SDs obtained for each condition (* $p \leq 0.05$; ** $p \leq 0.002$; $n = 3$). Scale bars A–D: 100 μ m; E: 50 μ m; F: 10 μ m; G–I: 20 μ m; J–L: 20 μ m. Color images available online at www.liebertpub.com/tea

To confirm that the increased speed of reepithelialization induced by innervation was due to release by nerves of SP, it was added at 10^{-7} M in the culture medium every 2 days during the reepithelialization process in absence of innervation. Three days after wounding, the percentage of wound closure was 1.5 times higher with SP in the WHM. To determine whether the en-

hanced reepithelialization induced by neurons in the iWHM was due to SP, we added Spantide II, an antagonist for the NK1 receptor of SP.²³ In presence of Spantide II, neurons failed to enhance wound closure, and there was no significant difference between the WHM and the iWHM, but Spantide II did not significantly inhibit reepithelialization in the WHM (Fig. 4).

FIG. 4. Influence of sensory neurons, SP, and the NK1 antagonist Spantide II on the reepithelialization process *in vitro*. Wound closure was measured after imaging of green fluorescent protein (GFP) expressed by keratinocytes in the WHM and the iWHM after 3, 6, and 9 days, following addition of SP or Spantide II in the culture medium. Representative pictures of the keratinocytes-GFP radiant efficiency in the wound area show the progress of the reepithelialization process over time within the different conditions (A). The wound areas appear as dark gray. The GFP radiant efficiency is depicted as dark red for highest values of GFP illumination (larger number of cell layers) and yellow for lower values (lower number of cell layers). The percentage of wound closure for the different conditions over time has been quantified (B). The graph is a representation of the mean results $\pm$ SDs obtained for each condition (* $p \leq 0.05$; ** $p \leq 0.0002$; $n = 4$). Color images available online at www.liebertpub.com/tea

When keratinocytes cultured in monolayer on plastic were treated with increasing concentrations of 10^{-8} , 10^{-7} , and 10^{-6} M of SP, no effect on cell proliferation was observed (data not shown), but a 1.6-fold increase in the cell migration speed was detected at 10^{-7} M compared with the control (Fig. 5).

Influence of sensory neurons, SP, and the NK1 antagonist Spantide II on reepithelialization *in vivo*

To assess whether the presence of sensory nerve fibers influences reepithelialization *in vivo*, iWHM or WHM was grafted on the back of immunocompetent CD1 mice as previously described.¹¹ The transplantation of iWHM and WHM on mice enabled the analysis of the human keratinocyte reepithelialization in presence of the immune system, but with or without innervation, since we previously showed that it takes more than 30 days for our reconstructed skin to be reinnervated from the wound bed in mice.^{12,15,24} The presence of nerve fibers significantly improved the percentage of reepithelialization by 1.5 times after 34 h (Fig. 6A, B). All the wounds were closed at day 2. Spantide II completely abrogated the enhancing effect on wound closure mediated by neurons of the iWHM, but did not alter reepithelialization in the WHM. In contrast, the addition of SP in the WHM increased the percentage of reepithelialization by 1.4 times (Fig. 6B).

Discussion

Sensory nerves play a major role in the wound healing process. However, their influence has mostly been described for the recruitment of immune cells through the induction of neurogenic inflammation. Only few studies were performed on the direct modulation of nerves on reepithelialization, and were limited by the challenge of removing both nerve fibers and immune cells from the wound area. In addition, existing data rely mostly on mouse and rat models that heal their wounds primarily by contraction, as opposed to human skin that heals mostly through reepithelialization (keratinocyte migration).

FIG. 5. Effect of SP on keratinocyte migration. Keratinocyte migration was measured using the scratch wound assay. Cell migration was analyzed by time-lapse microscopy after stimulation with 10^{-8} , 10^{-7} , and 10^{-6} M of SP. The graph is a representation of the mean results $\pm$ SDs obtained for each condition (* $p \leq 0.02$; $n = 5$).

FIG. 6. Influence of sensory neurons, SP, and NK1 antagonist Spantide II on the reepithelialization process *in vivo*. Wound closure was measured after imaging of GFP expressed by keratinocytes in the WHM and the iWHM transplanted on CD1 mice after 1 and 2 days, following addition of SP or Spantide II on the wound. Representative pictures of the keratinocytes-GFP radiant efficiency in the wound area show the progress in the reepithelialization process over time within the different conditions (A). The wound areas appear in gray color. The GFP radiant efficiency is depicted as dark red for highest values of illumination and yellow for lower values. The percentage of wound closure for the different conditions over time has been quantified (B). The graph is a representation of the mean results $\pm$ SDs obtained for each condition (* $p \leq 0.00001$, $n = 8$; ** $p \leq 0.0001$, $n = 4$). Color images available online at www.liebertpub.com/tea

Whereas wound healing is known to be a very complex process involving multiple cell types and cytokines, the purpose of this work was to focus on the investigation of the direct effect of sensory nerves on the human keratinocyte reepithelialization process *in vitro* and *in vivo* independently of inflammation and granulation tissue remodeling, which are already known to be major players in wound healing. To investigate epidermal wound closure in carefully monitored conditions, we developed a tissue-engineered human reconstructed skin model made of two compartments. The lower compartment features the dermis that combines a capillary network with or without a nerve network. The capillary network will be essential to promote a rapid connection of the graft to the host blood circulation after transplantation on mice, and subsequently, recruitment of inflammatory cells.²⁵ Thus, to develop similar models for both *in vitro* and *in vivo* experiments, a capillary-like network was added in all samples. The upper compartment features a thin dermis over which an epidermis is reconstructed. The rationale of preparing two compartments is to enable wounding using a punch biopsy only of the upper part that contains the epidermis, but not of the lower part that will serve as a wound bed on which keratinocytes will migrate. The expression of GFP in the epidermis allowed for a noninvasive and very accurate imaging of the wound closure in real time. Indeed, since the epidermal migrating tongue remains transparent when the tissue is observed macroscopically, the visualized wound corresponds to the margins on the epidermal tongue that has been fully differentiated up to the stratum corneum (see asterisk in Fig. 2), and thus macroscopic observation alone markedly underestimates the speed of wound closure. Imaging of keratinocytes with GFP expression allows for a very accurate measurement of the epidermal tongue migration.

Using our unique iWHM, we showed that sensory neurons induced *in vitro* the complete reepithelialization of the innervated wound 2.2 times faster compared with the WHM (after 4 days instead of 9). This effect was confirmed *in vivo* after graft of the iWHM on CD1 mice. The main advantage of transplanting the iWHM and the WHM on immunocompetent mice is to assess the effect of inflammation in presence or absence of innervation while using human keratinocytes to analyze reepithelialization. Indeed, we previously showed that nerve regeneration from the mouse wound bed necessitates at least 1 month to occur, precluding any innervation of the WHM in the first 48 h after graft.^{12,15,24} Reepithelialization was shown to be 1.4 times higher in the iWHM compared with the WHM after 34 h, while all wounds were closed after 2 days instead of 9 *in vitro*. Thus, our results show that sensory neurons directly influence reepithelialization, regardless the presence of immune cells.

The second step was to investigate how the sensory innervation promotes reepithelialization. Since the main paracrine secretion from sensory neurons corresponded to neuropeptides released from large dense-core vesicles, and because SP was already known to promote keratinocyte proliferation,⁸ this molecule was the most likely candidate. We showed that neurons in the iWHM contain SP and its NK1 receptor was expressed by keratinocytes, and that SP was released in the medium. When SP was added in the culture media (*in vitro*) or on the wound (*in vivo*) in the WHM, it promoted a faster wound closure. When a specific

antagonist of the SP receptor NK1 was added to the iWHM, the increase of the wound closure induced by neurons was completely abolished. However, this inhibitor did not ultimately prevent complete reepithelialization in the WHM. It was reported in organ culture of rabbit cornea that reepithelialization is accelerated by exogenous application of SP with EGF²⁶ and wound healing is slowed by SP depletion in aged rats.²⁷ This effect could be mediated by a direct effect of SP on keratinocytes through an increased proliferation and/or migration of keratinocytes. We showed that SP did not enhance keratinocyte proliferation, but increased their migration speed in monolayer culture.

Conclusion

We demonstrated for the first time direct evidence that nerve-derived SP can specifically increase the reepithelialization process of a wounded human skin independently of inflammation. These results suggest that administration of SP, or inhibitors of the neutral endopeptidase, which is known to degrade SP in the skin,²⁸ could be a promising therapeutic option to enhance wound healing, especially in denervated wounds such as in diabetic patients. The iWHM should also be a useful model to study *in vitro* the healing of skin wounds in pathological conditions, such as diabetes or aging.

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Disclosure Statement

The authors declare that no competing financial interests exist.

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