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Influence of sensory neuropeptides on human cutaneous wound healing process

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ABSTRACT

Background: Close interactions exist between primary sensory neurons of the peripheral nervous system (PNS) and skin cells. The PNS may be implicated in the modulation of different skin functions as wound healing.
Objective: Study the influence of sensory neurons in human cutaneous wound healing.
Methods: We incubated injured human skin explants either with rat primary sensory neurons from dorsal root ganglia (DRG) or different neuropeptides (vasoactive intestinal peptide or VIP, calcitonin gene-related peptide or CGRP, substance P or SP) at various concentrations. Then we evaluated their effects on the proliferative and extracellular matrix (ECM) remodeling phases, dermal fibroblasts adhesion and differentiation into myofibroblasts.
Results: Thus, DRG and all studied neuromediators increased fibroblasts and keratinocytes proliferation and act on the expression ratio between collagen type I and type III in favor of collagen I, particularly between the 3rd and 7th day of culture. Furthermore, the enzymatic activities of matrix metalloprotesases (MMP-2 and MMP-9) were increased in the first days of wound healing process. Finally, the adhesion of human dermal fibroblasts and their differentiation into myofibroblasts were promoted after incubation with neuromediators. Interestingly, the most potent concentrations for each tested molecules, were the lowest concentrations, corresponding to physiological concentrations.
Conclusion: Sensory neurons and their derived-neuropeptides are able to promote skin wound healing.

1. Introduction

The skin is densely innervated by primary afferent sensory nerve endings issued from the dorsal ganglia root (DRG). This cutaneous innervation plays a pivotal role in encoding afferent sensory function as temperature, touch, pain and also efferent function by releasing neuropeptides upon activation. Furthermore, these free nerve endings are in close contact with skin cells (fibroblasts, keratinocytes), or with dermal structures as blood vessels, sweat glands or hair follicules [1]. For all these reasons, cutaneous innervation is defined as one of the key actor of the maintenance of the skin homeostasis and there are increasing evidences that it participates in numerous physiological processes such as skin wound healing (SWH) [2]. Indeed, an impaired SWH is often observed in patients suffering from peripheral nerve lesions following trauma, spinal cord lesions or diabetic neuropathy [3,4]. However, the mechanisms involved in the interaction between sensory nerves and skin cells in such a process remain poorly understood.

The SWH process can be divided into 3 distinct stages: inflammation, proliferation (matrix component synthesis, re-epithelialization, neovascularization) and remodeling. The second phase is the most metabolic active phase during which fibroblasts migrate into the injured area, proliferate, synthetize extracellular matrix (ECM) components and differentiate into myofibroblasts, the specialized activated fibroblasts of the dermis [5]. These specialized cells deposit collagen to form a neo-ECM, in particular collagen types I and III. This neo-ECM is firstly composed of collagen type III progressively replaced by collagen type I, more resistant. Thus, collagen types I and III serve as structural and regulatory molecules playing a pivotal role during wound healing [6]. In addition to the secretion of collagen, fibroblasts secrete matrix metalloproteinases (MMPs) and their tissue inhibitors, involved in the remodeling of the new ECM. Among them, MMP-2 and MMP-9 are known to control the ECM turnover; MMP-9 is

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mainly expressed during the early phases of wound healing, and contributes to keratinocyte migration during epithelialization, whereas MMP-2 is mainly expressed in the final phases and is important for the collagen turnover [7,8].

This new granulation tissue allows keratinocytes to proliferate and migrate to reconstitute the epidermis. All of the cellular and biochemical events observed during SWH are regulated by mediators that orchestrate cell–cell and cell–matrix interactions.

Sensory neurons (SN) and skin cells communicate *via* a variety of molecules such as neuropeptides, neurohormones and neurotrophins, and their specific receptors are expressed by both neuronal and non-neuronal skin cells. These molecules may participate in SWH [9]. Thus, several neuropeptides have been shown to stimulate cellular proliferation and the synthesis of extracellular matrix components. The concentration of substance P (SP) and the density of SP-immunoreactive nerve fibers in dermis are known to increase during wound healing [10]. Calcitonin gene-related peptide (CGRP) and SP have been shown to stimulate fibroblasts, neurogenic inflammation as well as epidermal organization and keratinocyte renewal [2,11–13]. The role of the neuropeptide vasoactive intestinal peptide (VIP) in SWH has been suspected due to its ability to stimulate the proliferation and migration of keratinocytes [14,15]. On the other hand, the action of VIP, CGRP or SP on MMP enzymatic activity and collagen turnover remains poorly known (for review [9,16–20]). So, the mechanistic details that govern the activity of these molecules in SWH remain poorly understood.

Several *in vitro* and *in vivo* models to study SWH process have been reported in the literature [21]. However, although providing precious information concerning SWH, in these organotypic models from animal origin or reconstructed human skin, the peripheral nervous system is lacking. To assess the influence of SN-derived neuropeptides on the human SWH process, we used an organotypic model of injured human skin cocultured either with rat primary SN derived from DRG neurons or cultured only with neuropeptides. We investigated their effects on the proliferative and ECM remodeling phases, dermal fibroblast adhesion and differentiation into myofibroblast.

2. Materials and methods

2.1. Primary culture and coculture conditions

Human skin wound healing assay was obtained by performing an internal injury on the 6 mm Ø human skin explants (HSE) with a smaller punch of 3 mm Ø [22]. HSE were cocultured with primary sensory neurons from rat DRG (named as "DRG neurons" then in the text) in the same dish as previously described [2] (Fig. 1). In some conditions, NGF-β was added (25 ng/ml; Sigma–Aldrich, Saint Quentin Fallavier, France). In a simple culture, injured HSE were maintained in the same medium and supplemented with SP, CGRP or VIP (PolyPeptide Laboratories, Strasbourg, France) at 10^{-10} M, 10^{-8} M or 10^{-6} M for 3, 7 and 10 days of culture (Fig. 1). Every two day, the half of the culture was discarded and freshly culture medium containing neuropeptides was added to the culture.

Human dermal fibroblasts (HDFs) were extracted from the same skin that was acquired from donors for a skin culture. The HDF medium culture was comprised of DMEM containing 10% of fetal bovine serum (FBS, PAA Laboratories, Les Mureaux, France). All cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Immunohistochemistry

HSE were fixed in 4% paraformaldehyde (PAF 4%) and frozen in isopentane chilled with liquid nitrogen. HSE sections (HSES) were obtained with a cryostat (10 μm) and permeabilized with Triton X-100 and saponin (0.05% and 0.1%, respectively, in Dulbecco's Phosphate Buffered Saline (DPBS)). Non-specific binding sites were blocked with 2.5% fetal calf serum (PAA Laboratories, Les Mureaux, France) and 2.5% goat serum (Abcam, Paris, France) in 0.1% Tween-20 in DPBS for 15 min. Primary and secondary antibodies were incubated with 1% goat serum and 0.01% Tween-20 in DPBS for 2 h at room temperature. The primary antibodies used were (i) rabbit anti-Ki-67 (1:100), (ii) mouse anti-Cytokeratin-6 (1:100), (iii) rabbit anti-collagen III (1:250; Abcam, Paris, France), and (iv) mouse anti-collagen IA (1:1000; Santa Cruz Biotechnology, Heidelberg, Germany). Goat anti-rabbit immunoglobulin G chromeo 488 (1:1000; Abcam, Paris, France), goat anti-mouse FITC (1:1000, Abcam, Paris, France) and goat anti-mouse TRITC antibodies (1:500; Sigma–Aldrich, Saint Quentin Fallavier, France) were used as secondary antibodies. A negative control was obtained by omitting the primary antibody, and 4′,6-diamidino-2-phenylindole (DAPI, 100 μg/ml) was used to stain nuclei. Fluorescence analysis was performed using an Axiostar plus system (Carl ZEISS, Microlmaging Gmbh, Germany).

2.3. Collagen I/III fluorescence intensity measurement

Immunostained collagen fluorescence intensity was measured by selecting an area of 628 × 628 pixels at the lesion area using the Paint.net software (Rick Brewster and contributors, Microsoft developer division) and the collagen fluorescence intensity of this area was analyzed using the ImageJ software by measuring RGB (Red-Green-Blue), according to a protocol described previously [23,24]. To minimize staining variability, immunostaining of each experiment have been realized at the same time, the same control section was made for each batch and the same microscopy conditions (light source, light intensity and magnification) have been used for all the images.

2.4. Cell proliferation determination (Ki-67 and Cytokeratin-6)

At each culture time, 6 HSES by sample were realized and on each section, 6 pictures including the wound area and close areas around it, were realized, avoiding the edges of the HSE. Then, to determine the number of proliferative cells in the dermis, we counted all the cells stained by Ki-67 and realized a ratio with the total number of cells present in the dermis determined by the DAPI staining (Ki-67/DAPI). The same proceeding was applied to evaluate the number of proliferative cells in the epidermis with the cytokeratin-6 staining instead of Ki-67. The same microscopy conditions (light source, light intensity and magnification) have been used for all the images.

2.5. Gelatin zymography

Supernatant samples (25 μl) were collected at different coculture times, with 5 μl of non-reducing sample buffer and separated on a 10% polyacrylamide gel containing 0.1% gelatin as a protease substrate. After electrophoresis, gels were washed three times with 2,5% Triton X-100 to remove SDS. Substrate digestion was performed by incubating the gel for 16–20 h at 37 °C in developing buffer (50 mM Tris–HCl, pH 7,6, containing 200 mM NaCl and 5 mM $CaCl_2$). The gel was stained with 0.1% Coomassie Brilliant Blue for 1 h, and the location of gelatinolytic activities were detected as clear bands on a blue background after discoloration with distilled water containing 30% methanol and 10% acetic acid. These bands were identified as pro-enzymes (por-MMP-2 and por-MMP-9) and activated MMP (MMP-2 and MMP-9) forms based on their molecular weight. Gels were scanned and the quantification of the white bands was performed by densitometric