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In vivo enhancement of sensory perception recovery in a tissue-engineered skin enriched with laminin

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Abstract

The use of autologous reconstructed skin appears to be a promising treatment for the permanent coverage of deep and extensive burns. However, the capability of reconstructed skin transplanted on wounds to promote recovery of sensory perception is a major concern. Our aim was to assess the effect of laminin on cutaneous nerve regeneration. We prepared collagen-chitosan sponges enriched with 0, 1, 10 or 50 μg of laminin/sponge to produce tissue-engineered reconstructed skins by culture of human fibroblasts and keratinocytes, then grafted on the back of athymic mice for 120 days. Immunohistochemical studies demonstrated that there were 7 times more neurofilament 150 kD-positive nerve fibers migrating in the graft in the samples enriched with 10 μg laminin/sponge, compared to reconstructed skin without laminin, 120 days after graft. A significant improvement in the current perception threshold of the Aβ and Aδ nerve fibers was measured using a Neurometer® in all grafts enriched with laminin. In addition, the type C nerve fibers reached an identical current perception threshold than mouse skin, in all reconstructed skins enriched or not with laminin. We conclude that the use of a tissue-engineered autologous skin graft enriched with laminin has the potential to efficiently optimize cutaneous sensory nerve regeneration in vivo.

Keywords: Burn; Collagen scaffold; Nerve regeneration; Wound healing

1. Introduction

Extensive burns induce a partial or total destruction of cutaneous peripheral nerves. Patients complain about poor discriminative sensibility and/or hyperaesthesia and dysaesthesia after healing following a conventional split-thickness skin graft [1–4].

The absence of cutaneous sensory recovery is due to the incomplete or disorganized nerve regeneration within the grafted tissue. Sensory handicaps deriving from loss of innervation usually lead to functional losses and impaired quality of life. There are three different types of sensory nerve fibers in the skin, the non-myelinated C fibers mediating nociception, temperature, slow pain perceptions, the myelinated Aδ fibers, mediating mechanical stimuli, pressure, temperature and fast pain, and the large myelinated Aβ fibers mediating the sense of touch and pressure through a connection with specialized structures such as corpuscles [5].

We developed a collagen-chitosan sponge which was used as a scaffold to grow dermis and epidermis. Carboxyl groups of the types I and III bovine collagens were linked by ionic bonds to free amino groups of chitosan, a deacetylated chitin. After lyophilization, the collagen sponge presents an alveolar structure with pores ranging between 50 and 150 μm [6,7]. We have previously demonstrated that our model of reconstructed skin transplanted on nude mice facilitated the migration of sensory nerve fibers in the graft between 90 and 120 days after transplantation [8]. However, the nature of these nerve fibers, and their functionality has not been assessed. Indeed, a preferential migration of unmyelinated C fibers has been described in the literature in scar tissue of burnt patients [2]. In addition, our reconstructed skin model was not specifically optimized to enhance nerve regeneration.

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The incorporation of different kinds of neurotrophic factors or molecules increasing nerve growth could be a powerful strategy to enhance sensory recovery. The incorporation of proteins into our collagen sponge is greatly to be facilitated because of the presence of chitosan. This polycationic polysaccharide creates ionic bonds with molecules that are negatively charged. Laminin is a large cross-shaped glycoprotein that is a major component of the meshwork of basement membranes, such as those surrounding nerves [9–11]. Laminin substrates are known to promote neurite extension from central as well as peripheral neurons [9,12,13]. Its large shape makes it a good candidate to be incorporated in the sponge structure on a long-term basis, after 30 days in vitro maturation period and 120 days in vivo graft of our reconstructed skin.

Our goal was to demonstrate that the incorporation of this molecule into our biomaterial would enhance the nerve regeneration process when grafting tissue-engineered skin on mice.

2. Materials and methods

2.1. Cell cultures

Fibroblasts were isolated from human skin biopsies following breast reductive surgeries as previously described [14]. Fibroblasts were grown in Dulbecco–Vogt modification of Eagle's medium (DMEM) (Flow Lab., Mississauga, Canada) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 100 U/ml penicillin, 25 μg/ml gentamicin (Shering, Pointe-Claire, Canada) in 8% CO_2 at 37 °C. Keratinocytes were grown and used at the 2nd passage as previously described [14,15].

2.1.1. Preparation of the reconstructed skin

Collagen sponges were prepared as previously described. Briefly, type I, III bovine collagen and chitosan (95% deacetylated) (SADUC, Lyon, France) were dissolved in 0.1% acetic acid and mixed. Human laminin (a mixture of laminin 1, 2, 3, 6, 8 and 10, principally laminin 10, extracted from human placenta; Chemicon International, Inc., Temecula, CA) was then added to the mixture at concentrations of 0, 1, 10 and 50 μg of laminin/sponge[16]. After mixing, 2.5 ml/well (9.1 cm^2) of the final solution were poured in 6 well plates (Becton Dickinson, Toronto, Canada) and frozen at −80 °C for 1 h. The frozen plates were then lyophilized in a Genesis 12EL vacuum freeze-dryer (Virtis, Gardiner, NY). Human fibroblasts (5th passage) were trypsinized and a suspension of 4.32×10^5 cells/cm^2 was added in each well on the top of the sponge and cultured for 10 days in the same medium described for fibroblast monolayer cultures supplemented with 100 μg/ml ascorbic acid (Sigma, Mississauga, Canada). Then, a suspension of 2.16×10^5 cells/cm^2 of keratinocytes was seeded on reconstructed dermis. These samples were cultured for 7 additional days in complete DME-Ham in a ratio 3:1 (Flow Lab.) supplemented with 24.3 μg/ml adenine, 10 ng/ml human epidermal growth factor (Chiron Corp., Emeryville, CA), 0.4 μg/ml hydrocortisone (Calbiochem, La Jolla, CA), 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 2×10^{-9} M 3, 3′, 5′, triiodo-L-thyronin, 100 μg/ml ascorbic acid, 10^{-10} M cholera toxin (Schwarz/Mann, Cleveland, OH), antibiotics, and 10% newborn calf serum (Fetal Clone II, Hyclone, Logan, UT). The reconstructed skins were then lifted to the air–liquid interface and cultured for 14 additional days in DME-Ham supplemented with 10% fetal bovine serum (Gibco), 0.4 μg/ml hydrocortisone, 5 μg/ml bovine insulin, 100 μg/ml ascorbic acid, and antibiotics. Culture media were changed every 2 days.

2.1.2. Animals and surgical manipulations

Adult male athymic nude mice (56 days old) (Charles River Laboratories, Lasalle, Canada) were used as surgical recipients. The mice were injected with ceftazidine (140 mg/mouse; Glaxo, Toronto, Canada) 24 and 48 h before surgery. Penicillin G and gentamicin (100 U/ml and 25 mg/ml, respectively; Sigma) were also added to their sterile drinking water. Animals were anaesthetized by inhalation of 3% isoflurane with 1.5 l/min oxygen. Isoflurane was lowered to 2% for surgery. A 2.5×2.5 cm^2 full-thickness skin area was excised to the muscle, on the back of the mouse. A Fusenig chamber was installed in the wound and stitched to the mouse skin. A 9 cm^2 reconstructed skin was put on the wound in the chamber. A cap was used to close the chamber for the first 7 days. The Fusenig chamber was removed after 30 days. Eight mice for each condition were sacrificed 120 days after graft for histological and immunohistochemical analysis of reconstructed skin biopsies.

2.1.3. Sensory recovery testing

Neurosensory recovery was evaluated by testing all three types of sensory nerve fibers, Aβ, Aδ and C type fibers, with a Neurometer® (Neurotron Inc. Baltimore, MD) 120 days after graft. The Neurometer® evaluation achieves neuroselectivity by using three different frequencies of an electrical sine wave stimulus (2000 Hz for Aβ, 250 Hz for Aδ and 5 Hz for C fibers) [17–20]. The apparatus was connected to each animal by two self-sticking electrodes. One was placed on the graft site whilst the other was placed on the proximal tail. Each animal was placed in a restraint system for easy manipulation and to prevent chewing of the electrodes. When the mice showed a tail flick the stimulus was immediately turned off and the intensity (in mAmp) was defined as the current stimulus threshold. This stimulation procedure was repeated until the same reaction stimulus intensity was obtained on three consecutive presentations.

All experimental procedures were approved by Laval University's Animal Care Committee with respect to the rules established from the Canadian Council on Animal Care.

2.1.4. Indirect immunofluorescence staining

Frozen sections (30 μm) were fixed in phosphate buffered saline (PBS) 3.7% (v/v) formol followed by methanol. Tissue sections were then blocked in PBS containing 1% (w/v) bovine serum albumin and incubated with antibodies. Rabbit anti-neurofilament 150 kD polyclonal antibody (Chemicon) was used. Immunoglobulins fixed on the antigen were recognized with goat anti-rabbit IgG conjugated to rhodamine (Chemicon). As controls, the primary antibody was omitted. Sections were viewed with a Nikon C1 laser scanning confocal microscope. A Nikon eclipse E600 fluorescence microscope (Nikon, Mississauga, Canada) was also used. Images were collected from the center of the reconstructed skin dermis. The serial images collected throughout one section with the confocal microscope were summed to give a two-dimensional representation of the full depth of the field. Images were processed with Adobe Photoshop CS2 (Adobe Systems Incorporated, San Jose, CA).

2.1.5. Data collection and statistical analysis

Neurites detected by immunofluorescent staining of the 150 kDa neurofilament were counted by a single observer on the total area of the transversal section of the different tissue-engineered skin biopsies. The total area of each transversal section was measured with MetaView imaging system 4.5 (Universal Imaging Corporation, Downington, PA). The number of neurites in each section was then divided by the area of the section to give a neurite density expressed as the number of neurites per squared millimetre of the biopsy. For each condition, a total of eight biopsies were assessed. Values are expressed as the means ± SD. Differences were tested by the bilateral Student's *t*-test. Probability level was regarded significant at $p < 0.05$.

3. Results

3.1. Effect of laminin on axonal migration

Positive staining of the skin graft with the neuronal marker, neurofilament 150 kDa, was observed by confocal