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In vitro study of the impact of mechanical tension on the dermal fibroblast phenotype in the context of skin wound healing

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ABSTRACT

Skin wound healing is finely regulated by both matrix synthesis and degradation which are governed by dermal fibroblast activity. Actually, fibroblasts synthesize numerous extracellular matrix proteins (*i.e.*, collagens), remodeling enzymes and their inhibitors. Moreover, they differentiate into myofibroblasts and are able to develop endogenous forces at the wound site. Such forces are crucial during skin wound healing and have been widely investigated. However, few studies have focused on the effect of exogenous mechanical tension on the dermal fibroblast phenotype, which is the objective of the present paper. To this end, an exogenous, defined, cyclic and uniaxial mechanical strain was applied to fibroblasts cultured as scratch-wounded monolayers. Results showed that fibroblasts' response was characterized by both an increase in procollagen type-I and TIMP-1 synthesis, and a decrease in MMP-1 synthesis. The monitoring of scratch-wounded monolayers did not show any decrease in kinetics of the filling up when mechanical tension was applied. Additional results obtained with proliferating fibroblasts and confluent monolayer indicated that mechanical tension-induced response of fibroblasts depends on their culture conditions. In conclusion, mechanical tension leads to the differentiation of dermal fibroblasts and may increase their wound-healing capacities. So, the exogenous uniaxial and cyclic mechanical tension reported in the present study may be considered in order to improve skin wound healing.

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1. Introduction

After human skin injury, wound healing occurs as a restorative process, divided into three overlapping phases: hemostasis/inflammation, granulation tissue formation/re-epithelialization and tissue remodeling (Haertel et al., 2014; Wong et al., 2013). Human dermal fibroblasts (HDF) play a key role in this phenomenon by proliferating into the wound space, synthesizing extracellular matrix (ECM) components, developing mechanical forces and remodeling the scar (Singer and Clark, 1999). The accumulation and the remodeling of ECM by fibroblasts during the entire wound healing process could be schematized as a balance between matrix accumulation and degradation. For this, HDF synthesize ECM components, which initially are fibronectin and hyaluronic acid. Then, proteoglycans, type III and type I collagen are deposited and become the major components of wound ECM (Clark et al., 2007). Fibroblasts synthesize also proteolytic enzymes, matrix metalloproteinases (MMPs), which are a family of zinc endopeptidases capable of degrading all ECM components. Different identified MMPs are secreted by fibroblasts during cutaneous wound healing: MMP-1,-2,-3 and -19. One of the most important is the MMP-1 (collagenase) which can degrade the major structural ECM proteins, namely type I collagen (Toriseva and Kähäri, 2009). MMP activity is regulated by tissue inhibitors of metalloproteinases (TIMPs) of which four isoforms have been identified. During wound healing, dermal fibroblasts express TIMP-1, -2 and -3. Into the wound, HDF differentiate into myofibroblasts, which express alpha smooth muscle actin (α-SM actin) (Grinnell, 1994). They develop mechanical forces which create

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a mechanical feedback loop between cells and their environment (Tomasek et al., 2002). Myofibroblasts contract and compact the neo-formed ECM, contributing to bring wound edges together in physiological conditions. But sometimes, the presence at the wound site of local mechanical forces, *i.e* tension forces, can contribute to an abnormal wound healing process and to the development of excessive scars such as keloids (Ogawa et al., 2011). The effects of mechanical forces on cells have already been investigated in various types of cells, such as endothelial and smooth muscle cells or chondrocytes that are constantly subjected to fluid shear stress or pressure forces (Wilson et al., 2014; Chiquet, 1999). The effects of endogenous mechanical tension have been studied on HDF by culturing them into anchored collagen lattices (Ehrlich, 2003). In these models, tension forces are developed in response to the compaction of collagen fibers by HDF (Grinnell, 2003; Rhee and Grinnell, 2007). This results in the differentiation of fibroblasts into myofibroblasts and in the induction of mechano-responsive genes, including gene coding for ECM proteins and cytoskeleton (Kessler et al., 2001). This mechanical tension increases also fibroblast proliferation and decrease apoptosis (Grinnell, 2003). Transmission of mechanical signals across the plasma membrane to the intracellular compartment occur through integrins that act like "strain-gauges" (Harburger and Calderwood, 2009; Humphries et al., 2006). Such signals lead to the regulation of several signaling pathways by initiating the transduction of mechanical forces into chemical signals, resulting in modifications in gene expression (Wang et al., 2014; Hinz et al., 2012; Eckes et al., 2006; Katsumi et al., 2004). Only few studies have focused on the effects of exogenous mechanical tension on HDF, only with short-term exposure (Huang et al., 2013; Nishimura et al., 2007; Kessler et al., 2001; Parson et al., 1999). However, to our knowledge, no study has investigated the effects of such mechanical tension on scratch-wounded monolayers. Consequently, the purpose of this study was to investigate the influence of a uniaxial and cyclic mechanical tension (controlled for magnitude and frequency) on HDF cultured as scratch-wounded monolayers. Knowing the importance of collagen I, MMP-1 and TIMP-1 synthesis and myofibroblast differentiation in skin wound healing, synthesis modulation of these factors under the action of exogenous mechanical tension was explored. The "healing" of monolayers, procollagen type-I, MMP-1 and TIMP-1 synthesis were studied throughout culture time. In order to further explore the HDF response, the mechanical tension was applied to confluent monolayers and proliferating fibroblasts for which α-SM actin expression was assessed.

2. Methods

2.1. Materials

Phosphate Buffer Solution (PBS), Dulbecco's Modified Eagle's Medium (DMEM), Fetal Calf Serum (FCS) and Penicillin–Streptomycin were purchased from Dutscher (Brumath, France); Formaldehyde, NaOH, triton X100, BSA (Bovine Serum Albumin), FITC-labeled mouse anti-human α-SM actin antibody (F3777), isotype-matched control antibody (F8521) and a protease inhibitor cocktail (consisiting in: Aprotinin, Bestatin, E-64, Leupeptin, Pepstatin A) were purchased from Sigma-Aldrich (P1860, France). Type I collagen-coated 6-well Uniflex™ plates from Dunn Labortechnik GmbH (Asbach, Germany) were used. Procollagen Type I C-peptide (PIP) EIA kit (MK101) was purchased from Ozyme (Saint-Quentin en Yvellines, France). RayBio® Human MMP-1 Elisa kit (ELH-MMP1-001) and RayBio® Human TIMP-1 Elisa kit (ELH-TIMP1-001) were purchased from Clinisciences (Montrouge, France).

2.2. Isolation and culture of human dermal fibroblasts

Biopsies of healthy human skin were obtained from abdominal skin collected during plastic surgery after written informed consent of the patient. HDF were obtained by cell outgrowth from these explants and cultured in DMEM supplemented with 10% FCS, penicillin ($100\ U\ ml^{-1}$) and streptomycin ($0.1\ mg\ ml^{-1}$) at 37 °C with 5% CO_2. For each experiment, triplicates were done using three different lines of dermal fibroblasts, isolated from three donors. For all experiments described above, HDF were plated onto type I collagen-coated 6-well Uniflex™ plates at a rate of 2.5×10^5 cells per well in DMEM supplemented with Penicillin (100 U/mL) and Streptomycin (0.1 mg/mL) and 10% FCS. Prior to the application of mechanical tension, medium was replaced by DMEM containing Penicillin (100 U/mL) and Streptomycin (0.1 mg/mL) and a lower level of FCS (1%). Cells were used between the 4th and 8th passage in all experiments. Experiments were done in triplicate, and, at least, three independent experiments were performed.

2.3. Monitoring of scratch-wounded monolayers and kinetics of colonization

HDF were cultured until confluence onto type I collagen-coated 6-well Uniflex™ plates. Scratch-wounded monolayers were done by creating a "wound gap" with a sterile plastic tip in each well. This gap, crossing diametrically the well, was parallel to the direction of applied mechanical tension. The filling up of the gap was monitored both in tension conditions and in control conditions by numerical photography at 0; 6; 12; 24; 48; 72 and 96 h using an Olympus IX50 microscope equipped with an Olympus DP50 camera. The size of the denuded area was determined by computer using the Carl Zeiss Axiovision Rel. 4.6 software. The colonized area was expressed as a percentage of the initial area of the wound.

2.4. Strain system and applied mechanical tension

An FX-4000™ Flexcell® Tension System (Flexcell® International, Hillsborough, USA) was used in order to apply uniaxial and cyclic strain to HDF (Matheson et al., 2006). This uniaxial mechanical tension was applied to the flexible bottom of 6-well Uniflex™ culture plates, where cells were cultured. To this purpose, and according to manufacturer instruction, the bottom of each culture plate was exposed to a regulated vacuum controlled by a computer, and was therefore stretched over an Arctangle® Loading Station™ when negative pressure was applied (Fig. 1). The amount of deformation due to mechanical strain was 10% and the frequency was 1 Hz. The waveform of the mechanical signal was square in order to provide the fastest possible rise to 10% elongation followed by the fastest possible fall to 0%. Controls were unloaded during each experiment.

2.5. α-SM actin expression

Proliferating HDF, cultured onto type I collagen-coated 6-well Uniflex™ plates, were submitted (or not submitted in the case of controls) to mechanical tension and harvested by trypsinization at 0; 6; 12; 24; 48; 72 and 96 h of culture. Cells were then fixed in 10% formol, permeabilized with Triton X-100 (0.4% in PBS) and washed in PBS-EDTA (2 mM). For staining, cells were incubated with the FITC-labeled mouse anti-human α-SM actin antibody or an isotype-matched control antibody (1:250, 4 °C, overnight), prepared in PBS, BSA (1%), Triton X-100 (0.1%), washed twice and suspended in PBS-EDTA (2 mM). Relative expression of α-SM actin was determined by the measurement of Mean Fluorescence Intensity (MFI) of anti-α-SM actin antibody compared with MFI of isotype control antibody. MFI was assessed by flow cytometry analysis (FC500 Beckman Colter) and data were expressed as a percentage of positive cells.

2.6. Procollagen type I, MMP-1 and TIMP- 1 assays

To assess the synthesis of Procollagen type I, MMP-1 (pro and active forms) and TIMP-1 by HDF under mechanical tension (or w/o for controls), three culture conditions were compared: proliferating HDF, HDF confluent monolayer and wounded HDF monolayer. At 6, 12, 24, 48, 72 and 96 h of culture, supernatants were collected and a protease inhibitor cocktail was added (10%). Cells were then incubated with NaOH 0.1 N in order to extract total protein for assay normalization. Total protein concentration was assessed by Bradford's assay. Procollagen type I,

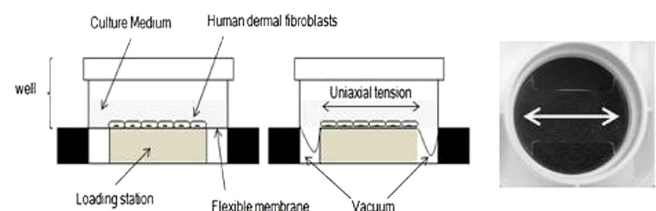

Fig. 1. (A and B) Principle of the application of a uniaxial mechanical tension to the flexible bottom of a Uniflex 6-well plate by the Flexcell® system (Dunn Labor Technic). (C) Top view of a single Uniflex well where the uniaxial strain field is represented by the white arrow (scale bar = 150 μm).

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