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Methods to study differences in cell mobility during skin wound healing *in vitro*

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ABSTRACT

Wound healing events which occur in humans are difficult to study in animals due to differences in skin physiology. Furthermore there are increasing restrictions in Europe for using animals for testing the therapeutic properties of new compounds. Therefore, in line with the 3Rs (reduction, refinement and replacement of test animals), a number of human *in vitro* models of different levels of complexity have been developed to investigate cell mobility during wound healing. Keratinocyte, melanocyte, fibroblast and endothelial cell mobility are described, since these are the residential cells which are responsible for restoring the main structural features of the skin. A monolayer scratch assay is used to study random fibroblast and endothelial cell migration in response to EGF and bFGF respectively and a chemotactic assay is used to study directional fibroblast migration towards CCL5. In order to study endothelial sprouting in response to bFGF or VEGF, which involves continuous degradation and resynthesis of a 3D matrix, a fibrin gel is used. Human physiologically relevant tissue-engineered skin models are used to investigate expansion of the stratified, differentiated epidermis (keratinocytes and melanocytes) over a fibroblast populated dermis and also to study migration and distribution of fibroblasts into the dermis. Together these skin models provide a platform for testing the mode of action of novel compounds for enhanced and scar free wound healing.

1. Introduction

The skin is the largest organ of our body and its primary function is to provide a protective barrier against (i) transcutaneous water loss, possibly leading to dehydration and (ii) harmful environmental UV, chemicals (allergens, irritants) and pathogens. Upon wounding, it is most important that the skin barrier function is restored as quickly as possible. This is achieved by a complex wound healing process which involves four overlapping phases (hemostasis, inflammation, proliferation and tissue remodeling) finally resulting in the formation of a scar (Gurtner et al., 2008; Martin, 1997; Martin and Leibovich, 2005; van den Broek et al., 2014b). Each phase involves the migration of different cell types into the wound area. Hemostasis occurs directly after injury resulting in vasoconstriction and activation of platelets. The platelets release many soluble wound healing factors that activate the coagulation pathway leading to the deposition of insoluble fibrin known as the fibrin clot or scab. This fibrin clot functions as a temporary cover over the wound but also serves as a network enabling cells to migrate into the wound bed. The fibrin clot forms a reservoir for cytokines and growth factors (*e.g.* TNF-α, CCL5, bFGF, VEGF, EGF). These are released from platelets as well as secreted by cells within the damaged tissue and infiltrating immune cells, in order to further promote wound healing (Barrientos et al., 2008; Kroeze et al., 2009; Lawrence and Diegelmann, 1994). The inflammatory phase involves the infiltration of monocytes, macrophages (M1) and other immune cell types necessary to combat infection and remove damaged tissue. During the proliferation phase, extensive proliferation and migration of keratinocytes, melanocytes, fibroblasts and endothelial cells takes place in response to *e.g.* CCL5, bFGF, VEGF and EGF (Adelmann-Grill et al., 1990; Behm et al., 2012; Kroeze et al., 2009). Enhanced keratinocyte and melanocyte proliferation ensures the supply of enough

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epidermal cells to migrate directly underneath the fibrin clot in order to regenerate an intact differentiated and pigmented epidermis thus restoring the outermost skin barrier. Melanocyte proliferation and migration is closely regulated by keratinocytes (Gibbs et al., 2000). Fibroblast proliferation and migration into the newly forming granulation tissue has been described to lag behind keratinocyte migration and is therefore most probably dependent on soluble mediators secreted by the epidermal keratinocytes (Braiman-Wiksman et al., 2007; Breetveld et al., 2006). Epidermal expressed integrins are upregulated during wound healing and are crucial for keratinocyte migration as they facilitate the step-by-step migration of keratinocytes over the basement membrane (Koivisto et al., 2014). Endothelial cells sprout from existing vessels into the fibrin and fibrinogen rich granulation tissue which forms underneath the clot, thus ensuring adequate vascularization to the healing wound. Throughout wound healing tissue synthesis and remodeling occurs (Greaves et al., 2013). Fibroblasts synthesize extracellular matrix and some will also differentiate into myofibroblasts, which causes wound contraction (Broughton et al., 2006). This initial wound contraction is important as it decreases the area of the open wound. When the skin is superficially injured, wound healing mostly results in a thin, hardly visible (normotrophic) scar. However, wound healing of deep and/or large skin wounds often results in abnormal scars due to excessive wound contraction and extracellular matrix formation, for example hypertrophic scars forming after 3rd degree burn wounds (Bayat et al., 2003). Oral wound healing is notably faster than skin wound healing and occurs with negligible scar formation (Engeland et al., 2006; Larjava et al., 2011). Therefore, understanding the cellular events regulating wound healing is of vital importance if we are to develop novel therapeutic strategies aimed towards enhanced and scar free skin wound healing.

Wound healing events which occur in humans are difficult to study in animal models due to differences in skin physiology. For these reasons, animals do not form abnormal scars representative of human scars such as keloids and hypertrophic scars (van den Broek et al., 2014b). Therefore we have developed a series of human *in vitro* models of different levels of complexity to investigate human skin integrity, wound closure and scar formation (Boink et al., 2015; Gibbs et al., 2006; Kroeze et al., 2009, 2012b, 2012a; Oudhoff et al., 2009; Reijnders et al., 2015; Spiekstra et al., 2007; van den Broek et al., 2012, 2014a, 2014b). In this study we focus on cell mobility during wound closure. In particular, we focus on keratinocyte, melanocyte, fibroblast and endothelial cell mobility, since these are the residential cells which are responsible for restoring the main structural features of the skin.

2. Materials and methods

2.1. Cell culture

Dermal fibroblasts: fibroblasts were isolated from human adult skin and cultured as previously described (Kroeze et al., 2009). Human adult skin was derived from healthy individuals undergoing abdominal dermolipectomy. All tissue was used in compliance with the Dutch code for proper secondary use of human tissue in agreement with the declaration of Helsinki. Dermal fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) (Lonza, Verviers, Belgium), 1% UltroSerG (UG) (Biosepra SA, Cergy Saint-Christophe, France) and 1% penicillin/streptomycin (P/S) (Invitrogen, Paisley, UK). Passage 2–4 cells were used in experiments consisting of a $>99\%$ pure population of CD31−/CD90+/vimentin+ fibroblasts as confirmed by flow cytometry.

Endothelial cells (EC): stromal cells (fibroblasts and endothelial cells) isolated from dermis were seeded onto 1% gelatin (Sigma-Aldrich, St. Louis, USA) precoated flasks in fibroblast medium in order to promote endothelial cell growth. Cells were grown for approximately 3–5 days until the flasks were 70–80% confluent and then trypsinized. Separation of endothelial cells from the dermal stromal cell population was performed by a MidiMACS separator using microbeads against CD31 (Miltenyi Biotec, Leiden, The Netherlands) following the manufacturers protocol. Endothelial cells were further cultured on gelatin-coated flasks in HMVEC medium, consisting of M199 (Lonza, Verviers, Belgium), 10% New Born Calf Serum (Invitrogen, Paisley, UK), 10% Human Serum (Sanquin, the Netherlands), 1% P/S, 2 mM L-Glutamin (Invitrogen, Paisley, UK), 5 U/ml Heparin (Pharmacy VUmc, The Netherlands) and 0.04 mg/ml endothelial cell growth factor (prepared from bovine brain). This procedure was repeated until a $>99\%$ pure population of CD31+/CD90− endothelial cells was obtained, as confirmed by flow cytometry. Cells were used for experiments between passage 5 and 9.

2.2. Fibroblast and endothelial cell migration scratch assay

The migratory potential of fibroblasts was assessed with a wound healing scratch assay (Gabbiani et al., 1984; Kroeze et al., 2009). Fibroblasts were cultured until confluency in 48-well culture plates (Corning, New York, US) as described above. Hereafter, fibroblasts were cultured for 4 days in DMEM, 1% P/S and 0.1% BSA in order to obtain growth arrest. A scratch was made in the confluent monolayer with a plastic disposable pipette tip (1000 μl). Cultures were washed twice with PBS to remove all detached cells. Hereafter fibroblasts were cultured in the presence or absence of 10 ng/ml rhEGF (Sigma-Aldrich, St-Louis, USA) in DMEM, 1% P/S and 0.1% BSA for 4 days. The wound area was photographed at $t=0$ and $t=4$ days with a phase contrast microscope coupled to a digital camera (Coolpix 5400, Nikon Corporation, Japan). The pictures were analyzed using an image processing algorithm wherein the wound area is determined (Topman et al., 2012). This algorithm distinguishes cell-free areas from cell-populated areas based on differences in local texture homogeneity. The migrated area was determined by subtracting the wound area at time point $t=4$ days from $t=0$ days.

Endothelial cells were seeded onto gelatin-coated 12-well culture plates at a density of 12×10^3 cells/cm^2 in HMVEC medium and cultured until confluency. Hereafter, cells were cultured for 8 h in M199 medium, 10% Human Serum, 10% New Born Calf Serum, 1% P/S, 2 mM L-glutamin (HMEC medium). A scratch was made in the confluent monolayer with a plastic disposable pipette tip (1000 μl), after which the cell cultures were washed three times with M199 medium to remove all detached cells. Hereafter cells were cultured in the presence or absence of 10 ng/ml bFGF (Preprotech, London, UK) in HMEC medium for 16 h. Migration was analyzed as described above for fibroblasts.

2.3. Fibroblast chemotaxis assay

Fibroblast chemotactic migration was assessed with the Boyden well chamber technique using 24-transwell plates (8 μm pore size; Costar Corning Incorporated, Corning, New York, US) (Boyden, 1962; Kroeze et al., 2009). 0.5×10^5 fibroblasts (pre-incubated in DMEM, 1% P/S and 0.1% BSA for 4 days) were seeded in 200 μl DMEM, 1% P/S and 0.1% BSA into the upper transwell compartment and allowed to attach overnight. Hereafter, the culture medium in the lower compartment only was supplemented with CCL5 (250 ng/ml) or in both the upper and lower compartment (both 250 ng/ml). After 24 h the assay was stopped and the non-migrated cells were removed from the upper transwell surface with a cotton swab so that only migrated cells attached to the lower surface could be counted. The transwells were washed in PBS, fixed using 4% formaldehyde (Klinipath, Duiven, The Netherlands), washed in PBS and stained with 10 μM propidium iodide (Invitrogen, Paisley, UK). The number of fibroblasts that had migrated from the upper to the lower surface of the transwell in 24 h were counted. The result is expressed relative to control cultures without CCL5.

2.4. Endothelial cell in vitro tube formation assay

In vitro EC angiogenesis was determined essentially as described previously (Koolwijk et al., 1996). Briefly, 3-dimensional fibrin matrices were prepared by addition of 0.5 U/ml thrombin (MSD, The Netherlands) to a 3 mg/ml fibrinogen solution (Enzyme Research Laboratories, Leiden, The Netherlands) in M199 medium. The fibrinogen–thrombin mixture (100 μl) was pipetted into a 96-well plate. After 2 h of polymerization, the thrombin was inactivated by incubating the matrices with HMEC medium. Hereafter, EC (2×10^4 cells) were seeded confluently onto the surface of each matrix. After 16 h of attachment the EC were stimulated with HMEC medium supplemented with 2 ng/ml TNF-α in presence or absence of VEGF (25 ng/ml) (Invitrogen, Carlsbad, USA) or bFGF (10 ng/ml). After 48–72 h the gels were formaldehyde-fixed, paraffin-embedded. The sprouts formed by EC into the fibrin matrices were photographed and analyzed using a Nikon Eclipse 80i microscope and NIS elements AR software 3.2. The amount of sprouting is expressed as surface area of the sprouts as a percentage of the total surface of the picture.

2.5. Skin equivalent

Skin equivalents were constructed as described previously (Gibbs et al., 2006; Spiekstra et al., 2007). In brief, acellular human dermis (AS210) (A-Skin, Amsterdam, The Netherlands) was used as the connective tissue matrix. Two 3 mm