Immunohistological changes in skin wounds during the early periods of healing in a rat model

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ABSTRACT: The complexity of the wound healing process, which is still poorly understood, prompted us to perform an immunohistochemical investigation using rat skin as an *in vivo* model. Fifteen Sprague-Dawley rats were included in the experiment. Two round full thickness wounds, 4 mm in diameter, were made on the backs of all rats. Haematoxylin and eosin basic staining as well as antibodies against wide spectrum keratin, keratin 10, keratin 14, α-smooth muscle actin, vimentin, fibronectin, collagens Type 1 and 3, and the transcription factor Sox-2 were applied to paraffin and frozen sections of skin wound specimens two, six and fourteen days after surgery, respectively. New hair follicles with Sox-2-positive cells were present after fourteen days; keratin/vimentin positivity was restricted to specimens of day two. Collagen-3 expression prevailed over collagen-1 expression at all evaluated time intervals, except in the uninjured part of the dermis. In conclusion, rat skin wound healing is a dynamic process which can serve as a model for studying phenomena such as cell-cell interactions and transitions *in vivo*.

Keywords: tissue repair and regeneration; cell differentiation; transition; proliferation; wound healing

The integrity of the skin is necessary for creating a barrier between the outer and inner environment. In the case of surgery and/or trauma the skin is damaged and the healing process needs to be started to restore the integrity of the organism as soon as possible. Wound healing includes a series of processes which include cell migration (Woodley et al. 1993) proliferation, differentiation (Masur et al. 1996; Morasso and Tomic-Canic 2005; Novotny et al. 2011), and extracellular matrix formation (Clark 1990) including cell-cell and cell-matrix interactions (Adzick and Lorenz 1994; Werner et al. 2007; Dvorankova et al. 2011). Several molecules have been described which have significant modulatory roles in cell communication and thus have modulatory roles in tissue repair and regeneration processes (He and Baum 2006; Barrientos et al. 2008; Zaja-Milatovic and Richmond 2008; Hu et al. 2009; Yaman et al. 2010).

During epidermis regeneration and also during many pathological conditions such as carcinoma and/or psoriasis development, keratinocytes become activated. During this process these cells are characterised by the production of specific keratin proteins which reflect the level of their differentiation (Freedberg et al. 2001). Among these specific

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proteins keratin 10 has been considered as early marker of keratinocyte differentiation (Reichelt et al. 2001; Carter et al. 2003), and is expressed in the suprabasal epidermal layer, whereas keratin 14 has been considered as a marker of the basal epidermal layer (Reichelt et al. 2001; Peryassu et al. 2005).

Degradation, formation, and reorganization of the extracellular matrix (ECM) are processes which are indispensable for successful healing. Fibroblasts as a component of granulation tissue secrete both proteases which are able to digest the surrounding ECM and new proteins of the ECM including fibronectin and collagen that are changed into insoluble matrix (Kumar et al. 2003). Therefore, in the present experiment fibronectin and collagen Type 1 and 3 expression was monitored during the three basic phases of healing, i.e., inflammation – on Day 2, proliferation – on Day 6, and maturation – on Day 14 (Gal et al. 2008).

The economic and social impact of the non-healing of wounds in veterinary as well as human clinical practice calls for a better understanding of the biological mechanisms underlying the repair processes of organisms (Sen et al. 2009). In this regard, we performed the present investigation to better understand the basis of biological processes taking place during the first few days after wounding. Thus, this investigation was designed to study open skin wound healing on a histological level in a rat model.

MATERIAL AND METHODS

Animal model

This experiment was approved by the Ethical Committee of the Faculty of Medicine of Safarik University and by the State Veterinary and Food Administration of the Slovak Republic.

One-year-old male Sprague-Dawley rats (n = 15) were chosen for the experiment. In all rats, surgery was performed under general anaesthesia induced by administration of ketamine (40 mg/kg; Narkamon a.u.v., Spofa, Prague, Czech Republic), xylazine (15 mg/kg; Rometar a.u.v., Spofa, Prague, Czech Republic) and tramadol 5 mg/kg (Tramadol-K, Krka, Novo Mesto, Slovenia). Under aseptic conditions two round full thickness skin wounds, 4 mm in diameter, were performed on the back of all rats. Five rats were killed by ether inhalation, two, six, and fourteen days post-wounding, respectively.

Basic histology

One skin wound was removed from all rats killed at each evaluated time point and routinely processed for light microscopy (fixation in 4% buffered formaldehyde, dehydration using increasing concentrations of ethanol, paraffin embedding, sectioning, and staining). Deparaffinised sections,

Table 1. Reagents used for imunohistochemistry of frozen sections prepared from skin wounds

Primary antibody	Abbrevation	Host	Produced by	Secondary antibody	Produced by	Channel
Collagen I	Coll-1	mouse monoclonal	Sigma, St. Louis, USA	goat anti-mouse	Sigma-Aldrich, Prague, Czech Republic	TRITC-red
Collagen III	Coll-3	mouse monoclonal	Sigma, St. Louis, USA	goat anti-mouse	Sigma-Aldrich, Prague, Czech Republic	TRITC-red
Fibronectin	Fibronectin	rabbit polyclonal	Dakopatts, Glostrup, Denmark	swine anti-rabbit	Santa Cruz Biotechnology, Santa Cruz, USA	FITC-green
Keratin 10	K10	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	Sigma-Aldrich, Prague, Czech Republic	TRITC-red
Keratin 14	K14	mouse monoclonal	Sigma, St. Louis, USA	goat anti-mouse	Sigma-Aldrich, Prague, Czech Republic	TRITC-red
α-Smooth muscle actin	SMA	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	Both Sigma-Aldrich, Prague, Czech Republic	TRITC-red
Sox-2	Sox-2	rabbit polyclonal	Abcam, Cambridge Science, Cambridge, UK	swine anti-rabbit	Santa Cruz Biotechnology, Santa Cruz, USA	FITC-green
Vimentin	Vim	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	Both Sigma-Aldrich, Prague, Czech Republic	TRITC-red
Wide spectrum cytokeratin	K	rabbit polyclonal	Abcam, Cambridge Science, Cambridge, UK	swine anti-rabbit	Santa Cruz Biotechnology, Santa Cruz, USA	FITC-green

7 µm thick, were stained with haematoxylin-eosin (HE – basic staining).

Immunohistochemistry

The second wound was preserved using Tissue-Tek (Sakura, Zoeterwoude, the Netherlands) and frozen in liquid nitrogen. Frozen sections, 10 µm thick, were mounted on the surface of poly-L-lysine treated glasses (Sigma-Aldrich, Prague, Czech Republic). Later, they were fixed in 2% (w/v) paraformaldehyde in PBS (pH 7.2). Non-specific binding of secondary antibody was blocked by pre-incubation with normal swine serum (DAKO, Brno, Czech Republic) diluted in PBS (1 : 30) for 30 min.

The primary and secondary antibodies used in this study are described in Table 1. The commercially available antibodies were diluted as recommended by the manufacturer. Nuclei were counterstained by DAPI (Sigma-Aldrich, Prague, Czech Republic) and specimens were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). The analysis of specimens and data storage were performed using an Optiphot-2 fluorescence microscope equipped with specific filter-blocks for DAPI, FITC, and TRITC (Nikon, Tokyo, Japan). Images were captured and analysed using a Cool-1300Q CCD camera (Vosskühler, Osnabrück, Germany), and computer-assisted image analysis system LUCIA 5.1 (Laboratory Imaging, Prague, Czech Republic).

Histological assessment

The extent of re-epithelisation, presence of polymorphonuclear leukocytes (PMNL), fibroblasts, and newly formed vessels, were assessed in a blinded manner according to a semi-quantitative scoring system: – (absent), + (minimal), ++ (mild), +++ (moderate), and ++++ (marked) (Gal et al. 2008; Lacjakova et al. 2010). The extent of the immunohistochemical reaction of ECM proteins, such as collagen and fibronectin, was assessed by ranking the signal intensities according to the following scale: – (absent), + (mild), ++ (moderate), +++ (marked) (Gal et al. 2011).

RESULTS

Two days post-surgery

The epidermis was thickened at its cut edges; thus, the migration of keratinocytes over the wound was not always present (–/+). The dermis near the excision was rich in inflammatory cells (PMNL) (+++) and a demarcation line was formed and separated the necrosis from vital tissue (Figure 1). The number of fibroblasts slightly increased in the dermis near the wounded area (+). Simultaneous vimentin and keratin expression in cells separated from the epithelial leading edge was observed in this time period (Figure 2). Cells in the epithelial leading edge were positive for keratin 10 and 14 (not shown). The

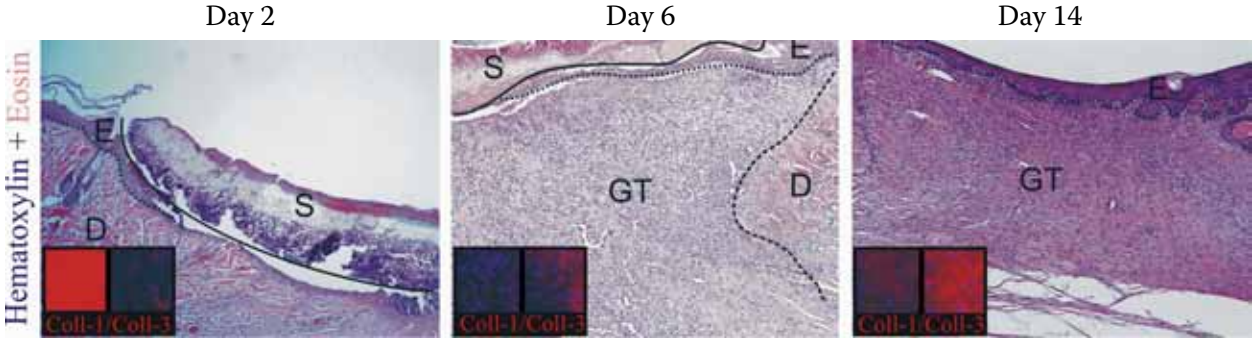

Figure 1. Basic staining of skin wounds 2, 6, and 14 days after surgery. **Day 2**: thickness of cut edge of the epidermis (E) and beginning of cell proliferation and migration beneath the demarcation line which separates vital tissue from necrosis/scab (S), insert – dermis (D) rich in collagen Type 1 (Coll-1) and no or minimal collagen Type 3 (Coll-3). **Day 6**: completed process of inflammation, prominent proliferation and migration of epithelial cells beneath the scab, well-formed granulation tissue (GT), insert – GT poor on Coll-3 and with no Coll-1. **Day 14**: completed process of re-epithelisation with formation of new hair follicles, granulation tissue with decreasing cellularity and with an increasing presence of collagen, insert – increasing expression of Coll-3, but small amounts of Coll-1; (dotted line sets epidermis apart from dermis and/or granulation tissue; the broken line distinguishes dermis from granulation tissue and the solid line separates scab/necrosis from vital tissue)

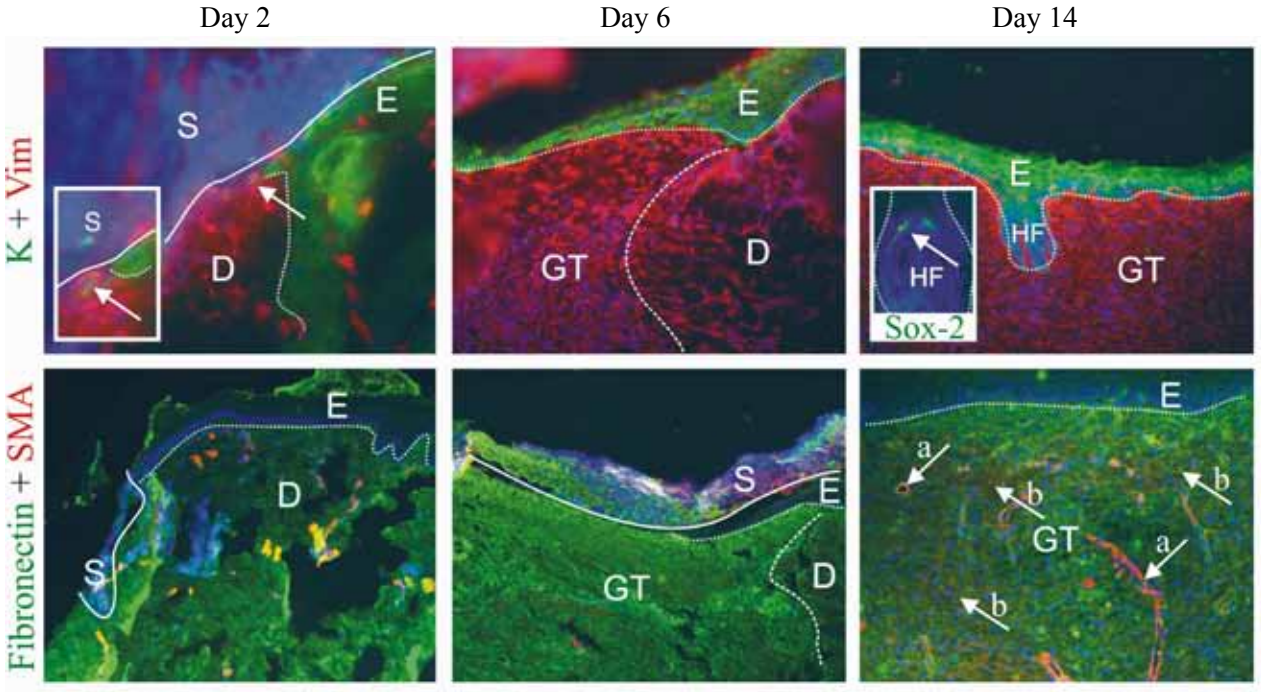

Figure 2. Immunohistochemistry of skin wounds 2, 6, and 14 days after surgery. **Day 2** (K + Vim): leading edge of the epidermis (E) located beneath the scab (S), insert – co-localisation of wide spectrum keratin (K) and vimentin (Vim). **Day 6** (K + Vim): migration of epidermal cells over the well-formed granulation tissue (GT) rich in vimentin+ cells, in contrast dermis (D) poor on vimentin+ cells. **Day 14** (K + Vim): completed process of re-epithelisation with formation of new hair follicles over the granulation tissue, insert – hair follicle with Sox-2+ cells. **Day 2** (Fibronectin + SMA): dermis poor on fibronectin, no α-smooth muscle actin positive (SMA+) structures. **Day 6** (Fibronectin + SMA): newly formed granulation tissue rich in fibronectin, absence of myofibroblasts in the granulation tissue. **Day 14** (Fibronectin + SMA): decreased expression of fibronectin, granulation tissue with moderate number of luminised vessels (arrow – a) and myofibroblasts (arrow – b); (dotted line sets epidermis apart from dermis and/or granulation tissue; the broken line distinguishes dermis from granulation tissue and the solid line separates scab/necrosis from vital tissue)

surrounding dermis contained collagen Type 1 (+++) and almost no collagen Type 3 (–/+) (Figure 1). At this evaluated time point granulation tissue was not yet formed; thus, its collagen content was not assessed.

Six days post-surgery

By six days after surgery the open wounds of animals were not yet completely bridged by a new layer of epithelial cells (++) (Figures 1 and 2) which were, however, positive for both keratin 10 and 14 not shown. The tissue was only lightly infiltrated with PMNL and monocytes/macrophages indicating that the inflammatory phase was almost finished (+). At the bottom of wounds newly created granulation tissue, rich in fibroblasts (++) and newly formed vessels (++), was observed. Granulation tissue contained vimentin-positive fibroblasts and a small amount of newly synthesized collagen Type 3 (+) and no collagen Type 1 (–). However, no myofibroblasts were observed at this time point.

Fourteen days post-surgery

Fourteen days post-surgery the regeneration of the epidermis was finished (Figures 1 and 2). In the samples the differentiation process of keratinocytes was confirmed by the normal process of keratinisation over the entire wound. Above the granulation tissue new hair follicles had started to develop. Cells in those follicles were keratin 14-positive not shown; some of them contained Sox-2-positive cells (Figure 2). Moreover, the presence of fibronectin in the granulation tissue decreased (+) and the content of collagens Type 1 (+) and 3 (++) increased. However, in contrast to intact dermis, the preva-

lence of collagen Type 3 over Type 1 was still observed (Figure 1). At this time point a small number of myofibroblasts (Figure 2) and luminized vessels (++) (Figure 1) was present in the granulation tissues of wounds.

DISCUSSION

During the first few days of healing the re-epithelisation of wounds is important for creating a barrier between the outer and inner environment to allow an undisturbed continuation of nascent repair processes. In addition to this function, epithelial cells, after a process called transition, might play also other roles in tissue repair. In this study we observed co-localization of vimentin and keratin, which is characteristic of the epithelial-mesenchymal transition, in cells separated from the epithelial leading edge two days post-wounding. This process has been extensively studied in developmental and cancer biology (Micalizzi and Ford 2009), since these separated cells might contribute to the formation of optimal cancer and/or tissue repair microenvironments. Hence, certain similarities, from the point of view of epithelial-mesenchymal interactions, between squamous cell carcinomas and skin wound healing do exist.

The maturation and remodelling phase of wound healing is characterised by a decrease in the cell population and an increase in collagen organization in granulation tissue which forms a scar (Kumar et al. 2003). Significant changes in this time period occur during epidermis regeneration as well. In our study we showed that at Day 14 hair follicles start to develop. Some cells in those follicles are Sox-2-positive. These data point to some parallels between mouse and rat skin; however, in a murine study Sox-2-positive cells were located at the bottom of so called "zigzag follicles" (Driskell et al. 2009), while we observed them apically. It is well known that Sox-2 is an evolutionarily conversed transcription factor and is expressed in embryonic stem cells and poorly differentiated cells (Collignon et al. 1996). This observation points to a high plasticity of epidermal cells following injury.

Furthermore, during the maturation phase myofibroblasts play an important role in wound contraction and scar formation (Li and Wang 2011). In spite of the good wound contraction in this animal species we found only a mild to moderate presence of myofibroblasts in the granulation tissues from sections of wounds which had already been contracting on Day 14.

In conclusion, in our study we have characterised selected proteins with regard to their roles in the course of skin wound healing in the rat model. Rat skin wound healing is a dynamic process which can serve as a model for studying several phenomena including cell-cell interactions and transitions *in vivo*. A correlation to the clinical situation would be possible if no inter-species variability would exist. When we compare our results to data obtained from pig skin (Klíma et al. 2009), quantitative aspects can indeed differ. Thus, the presented results cannot unambiguously be extrapolated to other animal species and humans. Thus, this study on the rat model should encourage further research into other species.

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