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Comparison of Wound Closure after Burn and Cold Injury in Human Skin Equivalents

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TO THE EDITOR

Full thickness burns, in contrast to cold injury wounds, heal with significant (hypertrophic) scarring and contraction (Martin, 1997; Camp *et al.*, 2003). Optimal scar formation is dependent on early factors regulating wound closure and inflammation.

Upon wounding, keratinocytes immediately adjacent to the wound edge proliferate and migrate into the wound gap in order to close the wound (Saarialho-Kere *et al.*, 1994, 1995). This process of re-epithelialization has been shown to involve interactions between keratinocytes and fibroblasts. The inflammatory response of wound healing occurs parallel to re-epithelialization. It involves the secretion of inflammatory mediators (chemokines) which regulate infiltration of neutrophils macrophages and lymphocytes into the wound bed (Engelhardt *et al.*, 1998). These inflammatory mediators also stimulate angiogenesis, mitogenesis, and dermal remodeling (Martin, 1997; Gillitzer and Goebeler, 2001). In this study, we established in how far human skin equivalents (HSEs) (human reconstructed epidermis on fibroblast-populated rat-collagen gels) (Bell *et al.*, 1981; Spiekstra *et al.*, 2005) can be used to reproduce histological observations associated with burn and cold injury *in vivo*. We determined novel similarities and differences involved in the closure of these two very different types of wound in the fully defined, *in vitro*, HSEs wound-healing model. As immune infiltrating cells and other skin residential cells (e.g., Langerhans Cells, macrophages, monocytes, mast cells, and endothelial cells) are not present in the HSEs, we are able to report events entirely due to keratinocyte–fibroblast interactions.

The unwounded region of HSEs consists of fully reconstructed epidermis containing a compact basal layer, spinous layer, granular layer, and stratum corneum on a fibroblast-populated collagen gel. Burn injury resulted in destruction of the epidermis and death of fibroblasts (seen by elongated fibroblasts become rounded and necrotic) (Figure 1a). After burning, wound contraction occurred (width of device used to inflict wound = 2.0 mm; width of wound after 24 hours = 1 mm $\pm$ 0.21, $n = 12$). These findings correlate to those obtained after burning *ex vivo* skin (data not shown) and also to *in vivo* observations after burning (Li *et al.*, 1980; Ehrlich and Hembry, 1984). In contrast to burns, extreme cold injury did not result in destruction of the epidermis of HSEs (Figure 1b). Cells showed characteristics of necrosis (not apoptosis) with rounded nuclei in both the frozen areas of the epidermis and dermis. Notably, a clear separation of the epidermis from the dermis at the basement membrane zone (blistering) was observed in HSEs only in the area of the cold injury. No wound contraction occurred after 24 hours. On the contrary the wound margins were 2.56 mm $\pm$ 0.77, $n = 12$ which is slightly wider than the width of the device used to inflict wounds (2 mm). These findings correlate to those after inflicting cold injury to *ex vivo* skin (data not shown) and also to *in vivo* observations after extreme cold injury (Shepherd and Dawber, 1984; Matook *et al.*, 1994).

After both burn and cold injury, regenerating epidermis formed underneath the dead epidermal tissue (Figure 1a and b). Re-epithelialization occurred from both sides of the wound margins until the fronts met and closed the wound. At 2 days after wounding, the intermediate zone showed a fully differentiated morphology including the presence of stratum corneum. Fibroblast migration lagged behind keratinocyte migration and was not observed until 7 days after injury. The rate of re-epithelialization was significantly slower after introduction of burns compared to cold injury. At 2 days after wounding, the burn wound front had

Abbreviations: HSEs, human skin equivalent

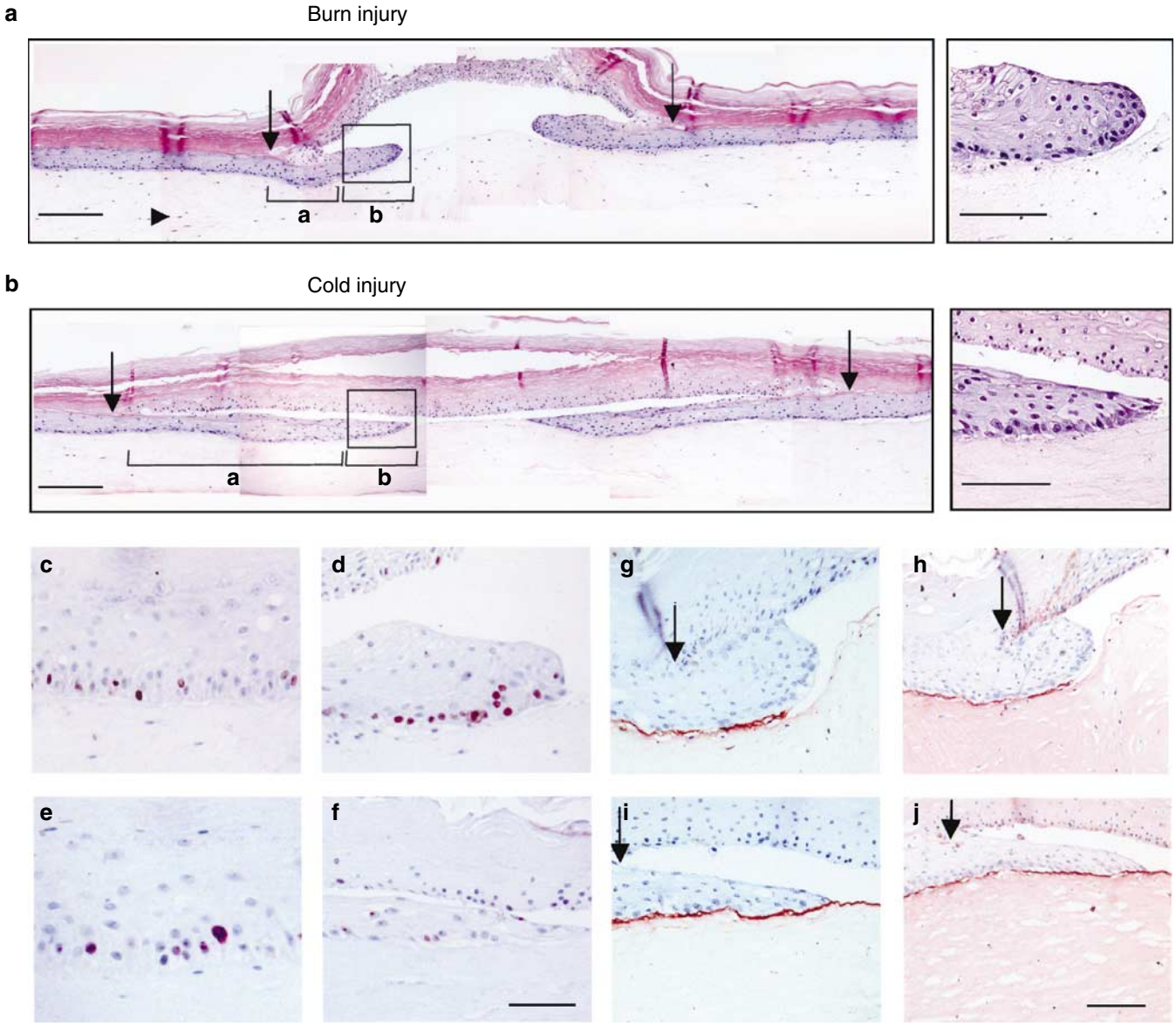

Figure 1. After burn and cold injury HSEs show significant differences regarding histology of migrating epidermal fronts, keratinocyte proliferation, and expression of basement membrane proteins. At 2 weeks after culturing HSEs, full-thickness wounds were introduced. For burn injury, a metal device attached to a Weller® soldering station (WSD 81Cooper Tools, Besigheim, Germany), heated continuously at 70°C, was applied for 10 seconds to the stratum corneum. For cold injury, a similar metal device, first cooled to −196°C in liquid nitrogen, was applied for 10 seconds to the stratum corneum. In both cases, the area of the device in contact with the HSEs was 2 cm long and 2.0 mm wide. All experiments were performed from three independent donors in 4-fold. (**a** and **b**) Hematoxylin and eosin (H&E) staining of HSEs sections. a = intermediate region; b = migrating front; arrow = wound margin; arrowhead = fibroblast; bar = 200 μm; inset bar = 100 μm. (**a**) Re-epithelialization after burn injury. Inset shows rounded, multilayered migrating front with the outermost region being clearly detached from the dermal matrix. (**b**) Re-epithelialization after cold injury. Inset shows entire front making close contact with dermal matrix. (**c**–**f**) Show keratinocyte proliferation (Ki67 immunostaining). (**c** and **e**) unwounded areas of HSEs; (**d**) migrating front after burning showing proliferating cells in the basal and suprabasal layers; and (**f**) migrating front after cold injury showing sporadic proliferating keratinocytes; (**g**–**j**): HSEs 24 hours after injury showing immuno-staining for basement membrane proteins collagen type IV and laminin 5. (**g** and **h**): Note absence of collagen IV and laminin 5 in unepithelialized regions after burning and presence in re-epithelialized regions. (**i** and **j**): note after cold injury collagen IV and laminin 5 expression remains. (**c**–**j**) Bar = 75 μm; arrow indicates wound margin.

migrated 450 ± 30 μm compared to the cold injury wound front which had migrated 950 ± 60 μm ($P < 0.01$). The morphology of the migrating fronts of the two types of wounds also showed different morphological characteristics. In burns, the migrating front was rounded and multi-layered (Figure 1a, inset). Many proliferating keratinocytes were present in the basal layer and also suprabasal layers indicating that the migrating front was hyper-proliferative (Figure 1d) compared to unwounded areas of the HSEs (Figure 1c). The outermost region was clearly detached from the collagen gel. In contrast, after cold injury only sporadic proliferating keratinocytes could be detected and the

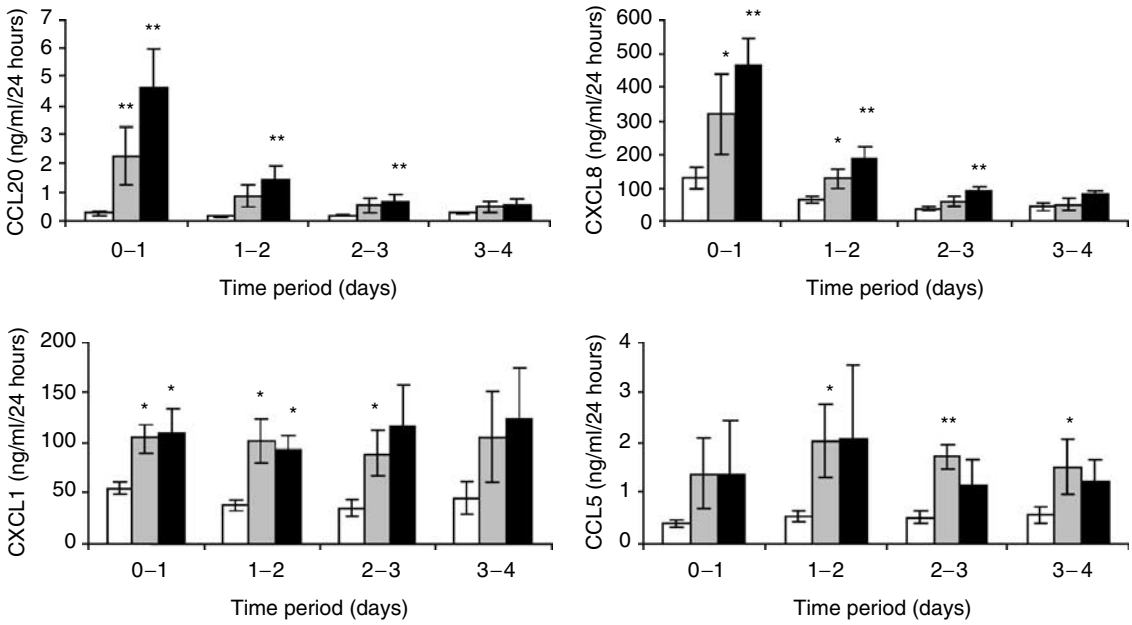

Figure 2. Chemokine profiles are similar during closure of burn and cold injury-inflicted wounds. Burn and cold injury was inflicted as described in Figure 1. Culture supernatants were collected and new medium (see Spiekstra *et al.*, 2005) was added each day for a period of 4 days. Chemokine secretion was measured by ELISA (antibody pairs were purchased and used as described by supplier: R&D systems, Minneapolis, MN). Each bar represents the mean ± SEM of 5 independent experiments each performed in duplicate; $*P<0{,}05$; $**P<0.01$; $***P<0.001$ wounded *vs* unwounded cultures; Mann-Whitney test. Open bar: unwounded HSEs; grey bar: burn; black bar: cold injury.

migrating front was pointed with a single keratinocyte at the outermost point (Figure 1b, e, and f). Also in contrast to burns, the outermost region was clearly attached to the collagen gel.

These observations led us to determine whether the expression of basement membrane proteins was affected upon wounding. After burning, basement membrane proteins (collagen type IV, collagen type VII, heparan sulfate proteoglycan, laminin) could no longer be detected by immuno-histochemical analysis indicating that these components of the basement membrane had been destroyed in both excised skin (data not shown) and HSEs (Figure 1g and h shows representative staining for collagen type IV and laminin 5, respectively). In HSEs, just behind the outermost migrating front and in the intermediary region, the basement membrane proteins could again be detected indicating that they have been resynthesized by keratinocyte–fibroblast interactions (El Ghalbzouri *et al.*, 2005). In contrast to burning, after cold injury – all four basement membrane proteins could still be detected (Figure 1i and j). Retainment of the basement membrane after freezing is in agreement to results published by Langdon *et al.* (1988). Our results indicate that after burning, in contrast to after cold injury, the basement membrane is damaged and has to be re-synthesized in order to permit re-epithelialization. *In vivo*, this may contribute to poor scar quality.

Next we determined whether differences also occurred after burn and cold injury with regards to the secretion of inflammatory mediators which have already been implicated to play a role in (acute surgical) wound healing and epidermal damage *in vivo* (Gillitzer and Goebeler, 2001; Schmuth *et al.*, 2002). CCL5/RANTES, CCL20/MIP3α, CXCL1/GRO-α, CXCL8/IL-8 secretion was therefore investigated during closure of burn and cold injury wounds (Figure 2). An increase in secretion of all four inflammatory mediators was observed already within 24 hours after wounding. CCL20/MIP3α and CXCL8/IL-8 secretion declined when the wound closed (after 3 days) whereas CXCL1/GRO-α and CCL5/RANTES secretion remained high after wound closure. In our model, the secretion of all four inflammatory mediators in response to cellular damage was similar for both types of wound. It should not be forgotten however that we investigate only keratinocyte and fibroblast responses to wounding. These cells are at the first line of assault/tissue damage and give signals to the immune system thus initiating the immune response. Differences in the immune response/chemokine secretion may occur in the presence of other skin residential cells (Langerhans cells, macrophages, monocytes, mast cells, and endothelial cells) and once infiltrating cells and platelets have entered the damaged region and responded to the extent of tissue damage (this is currently under investigation). *In vivo*, major tissue repair is required after burn injury which results in more contraction and scar formation in contrast to cold injury (Li *et al.*, 1980; Ehrlich and Hembry, 1984).

In conclusion, while important morphological differences were observed between the two types of wound, no differences were observed in the profiles of secreted chemokines. Future studies are underway to extensively

characterize the model with respect to dermal matrix damage/regeneration, re-epithelialization, and inflammatory mediators and also to incorporate other skin residential cells and infiltrating cells into the model. The *in vitro* wound-healing model described in this study can be used in the future to investigate early differences between healing after burn and cold injury and may provide a pre-clinical means to investigate novel therapeutics designed for optimal wound healing in a fully defined *in vitro* setting before progressing to animal or clinical studies.

The VU University Medical Center has approved the experiments described in this letter.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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