The Expression of Proinflammatory Genes in Epidermal Keratinocytes Is Regulated by Hydration Status

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Mucosal wounds heal more rapidly, exhibit less inflammation, and are associated with minimal scarring when compared with equivalent cutaneous wounds. We previously demonstrated that cutaneous epithelium exhibits an exaggerated response to injury compared with mucosal epithelium. We hypothesized that treatment of injured skin with a semiocclusive dressing preserves the hydration of the skin and results in a wound healing phenotype that more closely resembles that of mucosa. Here we explored whether changes in hydration status alter epidermal gene expression patterns in rabbit partial-thickness incisional wounds. Using microarray studies on injured epidermis, we showed that global gene expression patterns in highly occluded versus non-occluded wounds are distinct. Many genes including *IL-1β*, *IL-8*, *TNF-α* (tumor necrosis factor-α), and *COX-2* (cyclooxygenase 2) are upregulated in non-occluded wounds compared with highly occluded wounds. In addition, decreased levels of hydration resulted in an increased expression of proinflammatory genes in human *ex vivo* skin culture (HESC) and stratified keratinocytes. Hierarchical analysis of genes using RNA interference showed that both TNF-α and IL-1β regulate the expression of IL-8 through independent pathways in response to reduced hydration. Furthermore, both gene knockdown and pharmacological inhibition studies showed that COX-2 mediates the TNF-α/IL-8 pathway by increasing the production of prostaglandin E_2 (PGE_2). IL-8 in turn controls the production of matrix metalloproteinase-9 in keratinocytes. Our data show that hydration status directly affects the expression of inflammatory signaling in the epidermis. The identification of genes involved in the epithelial hydration pathway provides an opportunity to develop strategies to reduce scarring and optimize wound healing.

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INTRODUCTION

The skin has a critical role in maintaining water homeostasis. Dysfunction of the skin barrier not only causes delayed wound healing and hypertrophic scarring (Saulis *et al.*, 2002; Kloeters *et al.*, 2007; Mustoe, 2008; O'Shaughnessy *et al.*, 2009; Gallant-Behm and Mustoe, 2010; Jia *et al.*, 2011) but also contributes to the development of various skin diseases (Ghadially *et al.*, 1996; Sator *et al.*, 2003; Leung *et al.*, 2004; Segre, 2006). The mechanisms of barrier function in skin have been well studied for more than two decades. The correlation between dysfunction of the skin barrier and the initiation of atopic dermatitis was first stated in the 1990s (Elias *et al.*, 1999; Taieb, 1999). Wood *et al.* (1996, 1997) found that perturbation of the skin barrier leads to a marked increase of critical cytokines related to inflammation including IL-1α, IL-β, tumor necrosis factor-α (TNF-α), and granulocyte–macrophage colony-stimulating factor. These skin barrier injuries and related diseases and inflammatory processes underscore the importance of the skin epithelium in regulating the response of underlying connective tissue to skin damage. However, the comprehensive analysis of pathways indicating the behavior of skin in response to barrier disruption during wound healing is not yet fully characterized.

From superficial to deep, the human skin is composed of the stratum corneum, stratum granulosum, stratum spinosum, and stratum basale. The stratum corneum functions as a shield to protect the skin from oxidants, microbial infection, physical damage, and chemical erosion (Elias, 2008). In addition, its high lipid content allows it to act as the skin's primary barrier to moisture loss. The importance of the stratum corneum in maintaining water homeostasis has been widely described in

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Abbreviations: HESC, human ex vivo skin culture; HOW, high-occlusion wound; MMP, matrix metalloproteinase; NOW, non-occlusion wounds; PED3, postepithelialization day 3; PED5, postepithelialization day 5; PGE_2, prostaglandin E_2; SKC, stratified human keratinocyte culture model; TEWL, transepidermal water loss; TNF-α, tumor necrosis factor-α

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the literature (Harding, 2004; Rawlings and Harding, 2004; Elias, 2005; Feingold, 2007; Verdier-Sevrain and Bonte, 2007). Reduced hydration rates were found in skin with disrupted stratum corneum compared with skin covered with intact stratum corneum (Tagami, 2008). Also, the application of multiple layers of occlusive dressings has been shown to reduce drastically transepidermal water loss (TEWL) in a rabbit ear model of incisional grid wounds (Gallant-Behm and Mustoe, 2010). Disruption of the stratum corneum barrier function has been found in a variety of skin diseases, such as psoriasis (Ghadially *et al.*, 1996; Harding, 2004; Elias, 2005), atopic dermatitis (Taieb, 1999; Elias *et al.*, 2008; Cork *et al.*, 2009; Elias and Schmuth, 2009), and hereditary ichthyosis (Okulicz and Schwartz, 2003; Elias, 2005; Schmuth *et al.*, 2007). The stratum corneum maintains a stable gradient of water and solutes throughout the layers of the epidermis (Warner *et al.*, 1995, 1988a, b; Mauro *et al.*, 1998). Damage to the stratum corneum has been shown to result in a disturbance of this balance, with a subsequent increase in TEWL and alteration of gene expression in epidermal keratinocytes (Barel and Clarys, 1995; Wong *et al.*, 2006). A clinical study on scar formation found elevated levels of TEWL in hypertrophic scars and keloids and suggested that an increased water loss following skin injury is likely to promote the inflammatory reaction in the dermis (Suetake *et al.*, 1996). However, the mechanisms underlying the skin's response to TEWL and the signaling pathways involved are not known.

It has long been observed that mucosal wounds heal with minimal scarring, undergo faster healing, and exhibit less inflammation than equivalent cutaneous wounds (Szpaderska *et al.*, 2003; Mak *et al.*, 2009). Compared with skin wounds, mucosal wounds demonstrate a more highly regulated angiogenic response (Szpaderska *et al.*, 2005), have a differential expression of key profibrotic growth factors (Eslami *et al.*, 2009), and result in less scarring (Wong *et al.*, 2009). Unlike the stratum corneum cutaneous epithelium, the mucosal stratum corneum has little function in the maintenance of hydration because of its difference in structure (Squier and Brogden, 2011). However, the hydration status of the mucosal epithelium is maintained by its moist environment. Recent results from our group have shown that the difference in scar formation between mucosal and cutaneous wounds is attributable to differences in their ability to maintain hydration (Gallant-Behm *et al.*, 2011). In addition, microarray analyses have identified many genes that are differentially expressed in the epithelium of skin compared with mucosa following injury (Chen *et al.*, 2010; Gallant-Behm *et al.*, 2011). Thus, we hypothesize that alteration of gene expression is largely controlled by the hydration levels of wounds, and that treatment of injured skin with occlusion results in a phenotype of epithelial response that more closely resembles mucosa. The aim of this study was to identify the genes that have a critical role in the signaling response to changes in epidermal hydration status following injury. In addition, we addressed whether occlusion can modulate the expression of these genes. Using the rabbit ear wound model, we performed DNA microarray analyses on wounded epidermis to analyze comprehensively the transcriptome that results from changes in hydration status. Furthermore, we identified specific pathways that are involved in reduced hydration during wound healing. Our results suggest a previously unreported mechanism of reduced hydration in wound repair and will provide opportunities to develop strategies for wound care and scar treatment in clinics.

RESULTS

HOWs showed improved healing compared with NOWs in rabbit ears

We previously showed that cutaneous epithelium demonstrates an exaggerated response to injury compared with mucosal epithelium (Gallant-Behm *et al.*, 2011). We addressed whether different hydration conditions affect the healing process of cutaneous wounds. Once partial-thickness incisional wounds of rabbit ears were fully re-epithelialized (2 days after wounding; Figure 1a), wounds on one ear were covered with a water barrier consisting of multiple layers of a semiocclusive polyurethane dressing (highly occluded), mimicking a mucosal environment, whereas wounds on the contralateral ear were exposed to air (non-occluded). The appearance of wounds during healing in the two treatment conditions, high-occlusion wound (HOW) versus non-occlusion wound (NOW), was distinct. At postepithelialization day 3 (PED3), the incisional grid wounds in NOWs were immature (Figure 1b, right). Histological analysis by hematoxylin and eosin staining showed thickened epithelium in NOWs (Figure 1d). In contrast, HOWs demonstrated significantly improved repair by appearance (Figure 1b, left) and a thinner epithelial layer by histology (Figure 1e). Similarly, at PED5, NOWs had incompletely healed wounds (Figure 1c, right) and a thickened epithelium (Figure 1f), whereas HOWs showed complete healing (Figure 1c, left) and a thinner epithelium (Figure 1g).

Microarray analysis identified significant differences in gene expression between HOWs and NOWs following injury

Global gene expression was analyzed using RNA extracted from the epithelium of HOWs and NOWs at PED3 and PED5 to evaluate molecular differences in the context of wound healing. Cluster analysis based on the gene expression profiles in all samples showed apparent differences between HOWs and NOWs (Figure 2a). According to the gene expression heat map, the primary factor that affected expression patterns was the treatment of wounds (HOW vs. NOW). Within each treatment group, gene expression at PED3 and PED5 had apparent different overall gene expression patterns. At PED3, there were significant changes in gene expression level in 849 probes between HOWs and NOWs (adjusted $P<0.01$). Among these probes, 180 probes showed at least a 2-fold difference between HOWs and NOWs (138 upregulated, 42 downregulated; Figure 2b). At PED5, the number of differentially expressed gene probes was decreased to 314 (adjusted $P<0.01$), and 118 of them were up- or downregulated at least 2-fold in NOWs compared with HOWs. Interestingly, only 1 of the 118 probes detected markedly downregulated genes in NOWs, whereas all other 117 probes detected significantly