The Role of Chronic Inflammation in Cutaneous Fibrosis: Fibroblast Growth Factor Receptor Deficiency in Keratinocytes as an Example

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Fibrosis is associated with a variety of skin diseases and causes severe aesthetic and functional impairments. Functional studies in rodents, together with clinical observations, strongly suggest a crucial role of chronic injury and inflammation in the pathogenesis of fibrotic diseases. The phenotype of mice lacking fibroblast growth factor (FGF) receptors 1 and 2 in keratinocytes supports this concept. In these mice, a defect in keratinocytes alone initiated an inflammatory response, which in turn caused keratinocyte hyperproliferation and dermal fibrosis. As the mechanism underlying this phenotype, we identified a loss of FGF-induced expression of claudins and occludin, which caused abnormalities in tight junctions with concomitant deficits in epidermal barrier function. This resulted in severe transepidermal water loss and skin dryness. In turn, activation of keratinocytes and epidermal γδ T cells occurred, which produced IL-1 family member 8 and S100A8 and S100A9. These cytokines attracted immune cells and activated fibroblasts, resulting in a double paracrine loop through production of keratinocyte mitogens by dermal cells. In addition, a profibrotic response was induced in fibroblasts. Our results highlight the importance of an intact epidermal barrier for the prevention of inflammation and fibrosis and the role of chronic inflammation in the pathogenesis of fibrotic diseases.

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THE CONNECTION BETWEEN INFLAMMATION AND FIBROSIS IN THE SKIN

Fibrosis, the replacement of parenchymal tissue by (nonfunctional) connective tissue, can affect multiple tissues and organs, and constitutes a severe and frequently life-threatening health problem. Important examples are fibrosis of the lung, the kidney, and the liver. Fibrosis can also occur in the skin, which is most obvious in scleroderma, hypertrophic scars, and keloids (Shaw *et al.*, 2010). Furthermore, various other skin diseases are frequently associated with fibrotic processes, including acne and acne rosacea. In the latter, it manifests predominantly in the skin of the nose, resulting in the development of rhinophyma (Payne *et al.*, 2002).

The pathomechanisms underlying the development of fibrosis have only partially been elucidated. Fibrosis is considered as the result of abnormal repair in response to chronic tissue damage, which may be caused by different insults such as viruses, bacteria, radiation, mechanical injury, allergic responses, and autoimmune processes (Wynn, 2008). As a consequence, a chronic inflammatory response develops, which results in upregulation of various proinflammatory cytokines and chemokines. Many of them induce the expression of growth factors, which directly stimulate proliferation of fibroblasts, their differentiation into myofibroblasts, and production of extracellular matrix by these cells. These growth factors include platelet-derived growth factor, transforming growth factors β1 and β2, as well as activin. They are overexpressed in a variety of fibrotic diseases, including hypertrophic scars and keloids (Werner and Alzheimer, 2006; Krieg *et al.*, 2007; Trojanowska, 2008).

Several recent studies highlight the important roles of proinflammatory cytokines and chemokines in the pathogenesis of cutaneous fibrosis (Wynn, 2008). Thus, IL-33 and thymic stromal lymphopoietin caused inflammation and cutaneous fibrosis when intradermally injected into mouse skin (Jessup *et al.*, 2008; Rankin *et al.*, 2010). This was also seen in transgenic mice inducibly expressing IL-13 in keratinocytes (Zheng *et al.*, 2009).

The role of inflammation in the development of fibrosis is further documented in wound healing. Thus, scarless healing in the mammalian fetus is associated with a strikingly reduced inflammatory response compared with the situation in the adult organism (Cowin *et al.*, 1998; Stramer *et al.*,

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Abbreviations: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor

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2007; Gurtner *et al.*, 2008). Furthermore, mice lacking macrophages and functional neutrophils due to deficiency in the purin-rich box 1 transcription factor showed strongly reduced scarring postnatally (Martin *et al.*, 2003). Therefore, inhibition of the inflammatory response in injured tissues is considered as a promising strategy to limit the development of fibrosis (Shaw *et al.*, 2010).

THE ROLE OF EPIDERMAL DEFECTS IN CUTANEOUS FIBROSIS

Recent studies with genetically modified mice revealed a crucial role of an intact epidermis and hair follicles in the prevention of dermal scarring. For example, mice lacking the β1-integrin subunit in keratinocytes developed a progressive inflammatory response, followed by severe dermal scarring (Brakebusch *et al.*, 2000). Loss of Notch1 in keratinocytes caused inflammatory skin disease resembling mild atopic dermatitis (Demehri *et al.*, 2009). Interestingly, these mice also developed a hyperplastic and fibrotic dermis. This was accompanied by the development of spontaneous skin tumors (Demehri *et al.*, 2009), a finding that is consistent with the important role of inflammation and fibrosis in the pathogenesis of cancer (Schafer and Werner, 2008). The wound-like microenvironment that was established in the Notch1-deficient mice resulted from a defect in epidermal barrier function, which caused upregulation of cytokines, including thymic stromal lymphopoietin (Demehri *et al.*, 2009). The latter, when overexpressed in keratinocytes of transgenic mice, was sufficient to provoke an atopic dermatitis-like skin disease and dermal fibrosis (Yoo *et al.*, 2005).

In the following, we report on a recent study from our laboratory that highlights the tight connection between barrier function impairment, inflammation, and dermal fibrosis. These abnormalities occurred in mice lacking fibroblast growth factor (FGF) receptors (FGFR) 1 and 2 in keratinocytes. Our results indicate that a defect in keratinocytes can initiate a strong dermal response, consistent with the important role of stromal–epithelial interactions in the skin (Werner *et al.*, 2007).

FGFS AND THEIR FUNCTIONS IN THE SKIN

The FGF family includes 22 polypeptides that control proliferation, migration, and survival of different cell types through activation of four transmembrane tyrosine kinase receptors (FGFR1–4; Ornitz and Itoh, 2001; Beenken and Mohammadi, 2009). These functions of FGFs are also important in development, repair, and disease of the skin (Steiling and Werner, 2003). In particular, FGF7, FGF10, and FGF22, which activate specific splice variants of FGFR1 and FGFR2 on keratinocytes (the FGFR1–IIIb and FGFR2–IIIb splice variants), are strongly expressed in normal and, particularly in, wounded skin (Werner *et al.*, 1992, 1993; Komi-Kuramochi *et al.*, 2005). This is functionally important, as transgenic mice expressing a dominant-negative FGFR2–IIIb mutant in keratinocytes showed epidermal atrophy, hair follicle abnormalities, impaired wound re-epithelialization, and progressive dermal fibrosis (Werner *et al.*, 1994). As the dominant-negative FGFR mutant blocks the action of all FGFRs in response to common FGFs, the type of FGFR that is responsible for these abnormalities remained to be identified. To address this question, we generated mice lacking FGFR1 and/or FGFR2 in keratinocytes by breeding of mice with floxed *fgfr1* and *fgfr2* alleles with transgenic mice expressing Cre recombinase under the control of a keratin 5 promoter.

THE CONSEQUENCES OF THE LOSS OF FGFR1 AND FGFR2 IN KERATINOCYTES

Whereas loss of FGFR1 had no obvious consequences, loss of FGFR2 caused mild skin abnormalities, including loss of sebaceous glands and hair follicle abnormalities (Grose *et al.*, 2007; Yang *et al.*, 2010). A much more severe phenotype was seen in the double knockout mice (designated K5-R1/R2 mice). These animals were much smaller than control littermates and they progressively lost their hairs. This resulted in complete baldness by the age of 2–4 months (Yang *et al.*, 2010; Figure 1a).

Histological analysis revealed that hair morphogenesis is not impaired, which is consistent with the finding that the loss of FGFR1 and FGFR2 was only completed after birth. However, the double knockout mice failed to regenerate their hair follicles and remained in the telogen stage instead of initiating a new hair cycle. This finding is consistent with the upregulation of FGF7 and FGF10 in the dermal papilla during the transition from early to late telogen. This was suggested to be important for the stimulation of hair germ cell proliferation and subsequent hair cycle activation (Greco *et al.*, 2009). The failure of K5-R1/R2 mice to regenerate hair follicles supports this predicted FGF function. Following the first hair cycle, the remaining hairs were completely lost and no follicle remnants could be detected in the dermis at the age of 3–4 months (Figure 1b; Yang *et al.*, 2010). Long-term labeling with BrdU revealed that this hair follicle loss

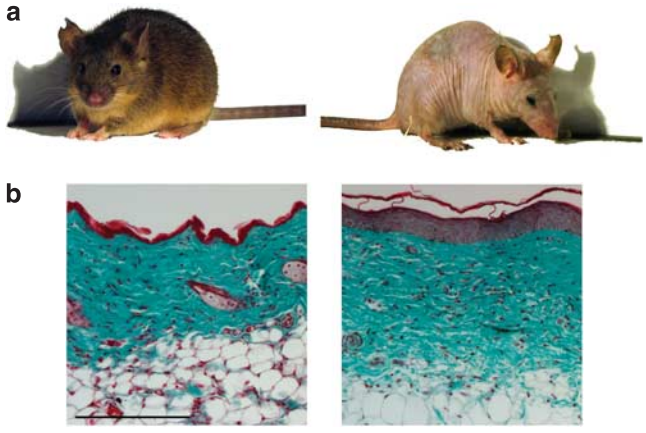

Figure 1. Macroscopic and histological abnormalities in fibroblast growth factor receptor mutant mice. (**a**) Pictures were taken from control (left panel) and K5-R1/R2 mice (right panel) at the age of 5 months. (**b**) Paraffin sections from the back skin of control and K5-R1/R2 (5 months old) were stained using the Masson trichrome procedure. Bar = 200 μm.

correlated with a complete loss of hair follicle stem cells (unpublished data).

A second abnormality that we observed in the FGFR1/R2-deficient mice was the progressive epidermal hyperthickening (acanthosis; Figure 1b). This was not due to impaired keratinocyte differentiation or reduced apoptosis, but resulted from keratinocyte hyperproliferation in the basal layer of the epidermis. The observed increase in keratinocyte proliferation upon aging suggests that the hyperproliferation is not a cell-autonomous phenotype but mediated via the stroma. Consistent with this hypothesis, cultured primary keratinocytes from FGFR1/R2-deficient mice showed a similar proliferation rate as cells from control mice (Yang *et al.*, 2010). This finding suggested that loss of FGFR1 and FGFR2 in keratinocytes results in activation of stromal cells, which in turn express keratinocyte mitogens. Expression studies using dermal RNA from these mice indeed revealed a strong upregulation of hepatocyte growth factor, transforming growth factor-α, GM-CSF, and others, which are likely to be responsible for the increased keratinocyte proliferation.

To determine whether inflammatory cells are directly or indirectly responsible for the upregulation of the above-mentioned growth factors, we stained skin sections from control and K5-R1/R2 mice with antibodies against different immune cells. A particularly striking finding was the strong increase in the number in epidermal γδ T cells (also called dendritic epidermal T cells), which was already seen in very young animals. Dendritic epidermal T cells are able to initiate a ''stress-surveillance'' response and are believed to quickly limit the dissemination of infected or malignant cells to sustain tissue integrity (Hayday, 2009). Therefore, the increase in these cells suggests the presence of stressed/abnormal keratinocytes in K5-R1/R2 mice.

In addition, there was an increase in the number of mast cells and dermal αβ and γδ T cells in adult K5-R1/R2 mice. Finally, B-cell activation occurred in these mice as reflected by the deposition of IgG1, IgG2a, and IgE in the dermis, and the presence of high levels of IgE in the serum. The progressive inflammation was further documented by the upregulation of various proinflammatory cytokines in the skin of adult K5-R1/R2 mice (Yang *et al.*, 2010).

Concomitant with the appearance of an inflammatory infiltrate, a progressive dermal fibrosis developed. This is reflected by the severe dermal thickening as determined by Masson trichrome staining (Figure 1b), and by the increase in connective tissue density over time (data not shown). These findings highlight the important role of inflammation in the development of dermal fibrosis.

We next determined whether various cytokines are differentially expressed in the epidermis of control and K5-R1/R2 mice. Mice at P18 were used for this purpose, as the loss of FGFR expression was almost complete at this stage but the epidermis was not yet hyperthickened. We found a strongly increased expression of the genes encoding S100A8, S100A9, and IL-1 family member 8 (IL-1F8) in the epidermis of K5-R1/R2 mice. Real-time reverse transcription-PCR analysis of RNA from cells that had been isolated by FACS of epidermal cell suspensions revealed that IL-1F8 is predominantly expressed by γδ T cells, whereas S100A8 and S100A9 are mainly expressed by keratinocytes. Therefore, both cell types contribute to the proinflammatory phenotype (Figure 2). It has previously been shown that S100A8 and S100A9 are important chemoattractants for inflammatory cells (Gebhardt *et al.*, 2006), and their upregulation in K5-R1/R2 mice is likely to contribute to the inflammatory process in these mice. An important role of IL-1F8 in the phenotype of K5-R1/R2 mice is suggested by our finding that this cytokine stimulates the expression of IL-6 and of various growth factors in fibroblasts, including the growth factors that are upregulated in the dermis of K5-R1/R2 mice (see above; Yang *et al.*, 2010). Taken together, our results revealed that loss of FGFR1 and FGFR2 in keratinocytes activates these cells, as well as γδ T cells, and they in turn produce S100A8, S100A9, and IL-1F8. These cytokines attract immune cells and activate fibroblasts, which then produce additional proinflammatory cytokines and growth factors that stimulate keratinocyte proliferation. In addition, inflammatory cell-derived factors induce a profibrotic phenotype in dermal fibroblasts, resulting in progressive dermal fibrosis (Figure 2).

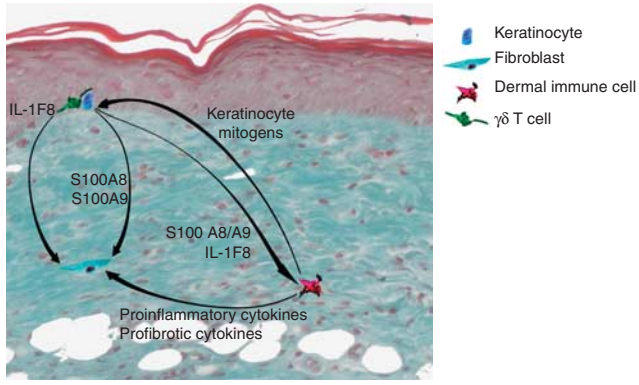

Figure 2. Model describing the pathomechanisms in the skin of fibroblast growth factor receptor (FGFR)1/R2-deficient mice. Loss of FGFR1 and FGFR2 in keratinocytes resulted in reduced expression of claudins and occludin. The resulting defect in the epidermal barrier, together with the loss of sebaceous glands, caused skin dryness. This induced a stress–response in keratinocytes, resulting in activation of γδ T cells and enhanced expression of cytokines in the epidermis. The latter stimulate keratinocyte proliferation directly (IL-1 family member 8, IL-1F8) and/or indirectly (IL-1F8 and S100A8/A9) via a double paracrine loop that involves several keratinocyte mitogens that are produced by various cell types in the dermis. In addition, invasion of irritants, allergens, and pathogens may further activate immune cells and accelerate the inflammatory process. Concomitantly, this enhances expression of proinflammatory cytokines and growth factors and stimulates production of extracellular matrix proteins in fibroblasts, resulting in dermal fibrosis.

CUTANEOUS INFLAMMATION AS A RESULT OF IMPAIRED EPIDERMAL BARRIER FUNCTION

What are the mechanisms underlying the onset of an inflammatory response in K5-R1/R2 mice? A direct FGF-mediated suppression of proinflammatory cytokine expression seems unlikely, as S100A8/A9 and IL-1F8 were expressed at similar levels in cultured keratinocytes from control and K5-R1/R2 mice. A second possibility is

inflammation as a result of hair follicle degeneration. Although this cannot be fully excluded, it seems also unlikely as the phenotype progressed upon loss of all follicles. By contrast, our findings strongly suggest that the cutaneous inflammation is the consequence of impairments in the epidermal barrier. Although expression of various components of the cornified envelope was not reduced in K5-R1/R2 mice, mRNA and protein levels of several tight junction components, including claudins 1, 3, 8, and occludin, were much lower in the epidermis and in cultured keratinocytes of K5-R1/R2 mice compared with control littermates. This resulted in the formation of defective tight junctions as indicated by ultrastructural analysis of the epidermis and by the reduced transepithelial electrical resistance of a confluent keratinocyte monolayer formed by cells from K5-R1/R2 mice compared with control mice (Yang *et al.*, 2010). It has previously been shown that alterations in the composition of tight junctions affect the junctional permeability and cause a defect in barrier function (Inai *et al.*, 1999; Furuse *et al.*, 2002; Tunggal *et al.*, 2005). The latter was confirmed in our study, as K5-R1/R2 mice showed a strong transepidermal water loss, which progressed with age. This is consistent with the high consumption of drinking water by K5-R1/R2 mice, as well as with the dry and fragile appearance of their skin. Together with the loss of sebaceous glands, which normally produce the moisturizing sebum, this causes severe skin dryness. The latter stimulates proliferation of keratinocytes and causes dermal mast cell hypertrophy, their degranulation, and subsequent inflammation (Denda *et al.*, 1998). In addition, dryness was shown to stress and damage keratinocytes, which in turn results in activation and proliferation of γδ T cells (Jameson and Havran, 2007). The role of an impaired barrier and subsequent dryness in the skin phenotype of K5-R1/R2 mice is supported by our results that topical treatment of the skin of these animals with moisturizing cream reduced the number of mast cells and γδ T cells (Yang *et al.*, 2010). In addition to the water loss, it may well be that irritants/allergens and microorganisms can invade into the fragile skin, in particular at sites of minor injury, resulting in immune cell activation and enhancement of the inflammatory response. As a consequence, inflammatory cell-derived cytokines activate fibroblasts, which in turn acquire a profibrotic phenotype and deposit large amounts of extracellular matrix, resulting in dermal fibrosis.

Taken together, our findings reflect the importance of an intact epidermal barrier for the prevention of fibrotic processes and highlight the important role of inflammation for the development of dermal fibrosis.

CONFLICT OF INTEREST

The authors state no conflict of interest.
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