Constitutive Expression and Regulated Release of the Transmembrane Chemokine CXCL16 in Human and Murine Skin

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The CXC-chemokine ligand 16 (CXCL16) is expressed as a transmembrane adhesion molecule and can be released as a chemoattractant. Both functions are carried out by binding of CXCL16 to its receptor, CXC-chemokine receptor 6 (CXCR6). We here provide early evidence that CXCL16 is expressed *in situ* by epidermal keratinocytes of normal skin on messenger RNA and protein level and released into the wound exudate upon injury. Cultured human and murine keratinocyte cell lines (HaCaT and PAM212, respectively) as well as primary keratinocyte cultures constitutively express transmembrane CXCL16 on the cell surface. Soluble CXCL16 is released by its limited proteolytic cleavage involving the disintegrin-like metalloproteinase (ADAM)10 but not the closely related ADAM17, as shown by specific inhibitors and small-interfering RNA knockdown experiments. This shedding of CXCL16 is reduced by serum starvation but enhanced by cell stimulation with ionomycin or by UVB irradiation. Soluble CXCL16 from keratinocytes was shown to bind and activate CXCR6, and marked expression of this receptor was found on a subpopulation of T cells in the dermis. Thus, CXCL16 is constitutively expressed on the surface of human epidermal keratinocytes, released upon cell activation or photodamage and may then target CXCR6-expressing T cells in the dermis.

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INTRODUCTION

Chemokines play an important role as paracrine and autocrine mediators in healthy and diseased skin where they have been implicated in homeostatic and inflammatory processes, and in the pathology of skin tumors. They function as mediators of constitutive cell trafficking, promote inflammatory cell recruitment, and in some cases may control cell proliferation and wound healing (Caux *et al.*, 2000; Gillitzer and Goebeler, 2001; Payne and Cornelius, 2002; Homey and Bunemann, 2004). Most chemokines are soluble polypeptides of about 10 kDa either expressed and secreted constitutively or released upon cell activation (Rossi and Zlotnik, 2000; Rot and von Andrian, 2004). However, there are two exceptional chemokines that are expressed as type I transmembrane molecules, the CX3C-chemokine ligand 1/Fractalkine, and the CXC-chemokine ligand 16 (CXCL16) (Bazan *et al.*, 1997; Matloubian *et al.*, 2000). These molecules consist of a short cytoplasmatic tail, a single membrane spanning α- helix, an extracellular, highly glycosylated, mucine-like stalk and a globular, 10 kDa chemokine domain at the N-terminus.

Different biological activities have been attributed to CXCL16. As a surface-expressed molecule CXCL16 functions as a scavenger receptor for oxidized low-density lipoprotein (Shimaoka *et al.*, 2000). In addition, surface-expressed CXCL16 is capable of binding to its receptor CXC-chemokine receptor 6 (CXCR6) expressed on leukocytes and thereby establishes firm cell to cell contact (Shimaoka *et al.*, 2004). However, CXCL16 is not exclusively expressed on the cell surface. It is also found as soluble molecule that is constitutively generated by proteolytic cleavage of its transmembrane variant, a process called shedding. In fibroblasts and endothelial cells the disintegrin-like metalloproteinase (ADAM)10 has been implicated in constitutive shedding of CXCL16 (Abel *et al.*, 2004; Gough *et al.*, 2004). CXCL16 cleavage can be further enhanced by activation with phorbol-12-myristate-13-acetate, which is independent of ADAM10 but rather involves the related metalloproteinase

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Abbreviations: ADAM, a disintegrin and metalloproteinase; CXCL16, CXC-chemokine ligand 16; CXCR6, CXC-chemokine receptor 6; HEK, human embryonic kidney; mRNA, messenger RNA; POD, peroxidase; PBS, phosphate-buffered saline; PBS-T, Tween in PBS; RT, room temperature; siRNA, small-interfering RNA

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ADAM17 (Abel *et al.*, 2004; Ludwig *et al.*, 2005b). Shedding results in the release of soluble CXCL16 which then functions as a chemoattractant for CXCR6-expressing cells such as T-cell subtypes and bone marrow plasma cells (Matloubian *et al.*, 2000; Nakayama *et al.*, 2003; Geissmann *et al.*, 2005).

CXCL16 expression has first been reported for antigen-presenting cells (Matloubian *et al.*, 2000; Shimaoka *et al.*, 2000; Wilbanks *et al.*, 2001) and was subsequently detected in cytokine-stimulated endothelial cells, smooth muscle cells (Abel *et al.*, 2004), and in transformed glial cells (Ludwig *et al.*, 2005b). The chemokine and its receptor CXCR6 have been found in inflamed vasculature (Minami *et al.*, 2001), liver (Geissmann *et al.*, 2005), intestine (Hase *et al.*, 2006), lung (Morgan *et al.*, 2005), rheumatoid joints (van der Voort *et al.*, 2005), and brain (Ludwig *et al.*, 2005b), where they have been implicated in disease processes such as acute and chronic inflammation and tumor development. Nothing is known, however, about the expression and function of both molecules in the skin.

Here we provide first *in vivo* and *in vitro* evidence for the constitutive expression of CXCL16 in epidermal keratinocytes and its regulation by ADAM. We further demonstrate the chemokine's activity on its receptor CXCR6, which we could localize to the dermis of healthy skin.

RESULTS

CXCL16 is expressed in human and murine skin

Expression of CXCL16 was investigated in paraform-fixed paraffin sections of healthy human skin using a purified polyclonal rabbit antibody directed against the chemokine domain of human CXCL16. This antibody had been successfully used in several other studies to demonstrate the expression of CXCL16 *in situ*. By chromogenic labeling the antibody showed strong binding to keratinocytes throughout the nucleated epidermal layers but not to cells of the dermal layer or to the *stratum corneum*. (Figure 1a). This binding was also seen using a polyclonal goat antibody to human CXCL16 (not shown) but neither with goat nor rabbit isotype control antibodies (Figure 1c). As described previously for endothelial cells in the brain (Ludwig *et al.*, 2005b) CXCL16 was also found on small blood vessels within the skin (Figure 1d). In addition, a few distinct cells within the dermis were also CXCL16 positive. But compared to the over all dermal staining, the signal for CXCL16 in the epidermal layer was more intense and therefore keratinocytes appear to be the major source of this chemokine in healthy skin.

We next asked whether the metalloproteinase ADAM10 that has been implicated in cleavage of CXCL16 in fibroblasts and endothelial cells is also expressed in human skin. We found strong expression of the protease in the epidermal layer (Figure 1b). Therefore we propose that CXCL16 and ADAM10 interact in human skin, which may lead to the cleavage and release of soluble CXCL16.

We then investigated freshly prepared skin of healthy mice for the presence of CXCL16 messenger RNA (mRNA). *In situ* hybridization demonstrated a signal for CXCL16 mRNA in the epidermal layer of murine skin (Figure 2a) and within the dermis a specific signal was associated with blood vessels (data not shown). To investigate the release of soluble

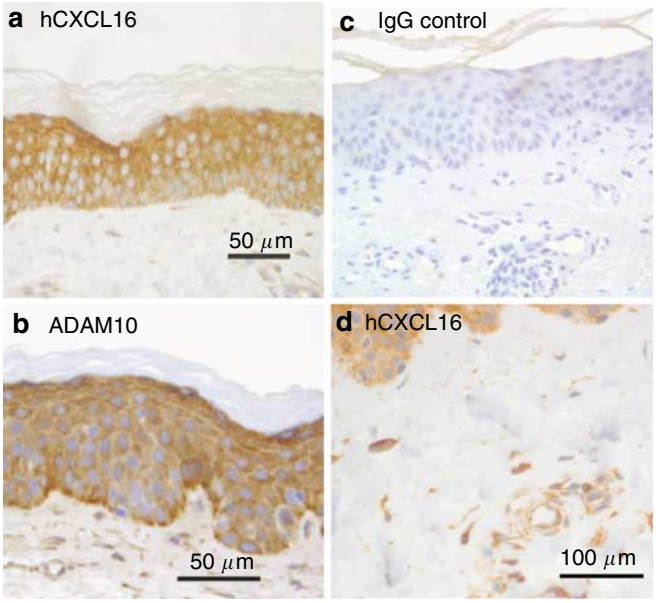

Figure 1. Expression of human CXCL16 in normal human skin. For chromogenic staining of CXCL16 and ADAM10 in healthy human skin, acetone-fixed sections were incubated with (**a**, **d**) rabbit anti-hCXCL16 (**b**) rabbit anti-ADAM10 or an irrelevant (**c**) rabbit IgG control antibody followed by incubation with POD-coupled goat anti-rabbit IgG antibody, enzymatic staining and counterstaining with hemalumn. In (**a**–**c**) epidermal skin and in (**d**) dermal skin containing a blood vessel (bottom right) is shown. Bar = 50 μm.

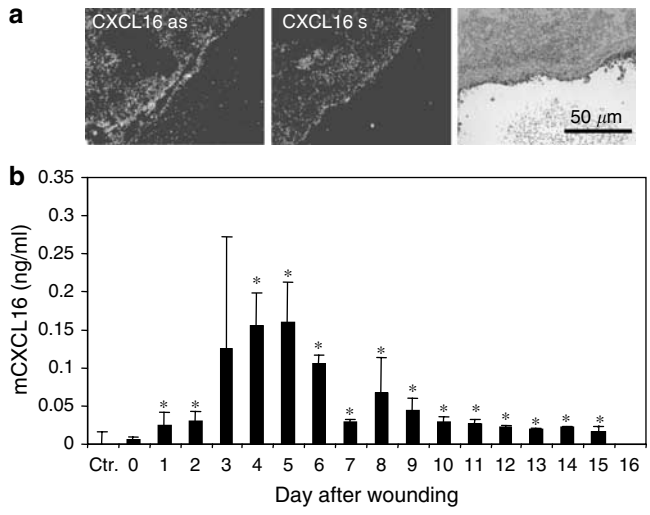

Figure 2. *In situ* expression and *in vivo*-release of murine CXCL16. (**a**) Paraformaldehyde-fixed fresh frozen sections of murine skin were incubated with radioactively labeled antisense cRNA probes (as) for mCXCL16 and sense-controls (s), respectively. Hybridized cRNA probes were subsequently visualized by autoradiography. Bar = 20 μm. (**b**) Wound fluid from injured mice was collected every 24 hours over a period of 15 days, cleared by centrifugation and subsequently analyzed for the presence of CXCL16 by an ELISA specific for mCXCL16. Data are given as mean and SD ($n = 5$). Statistically significant release of mCXCL16 ($P < 0.05$) is indicated by asterisks.

CXCL16 in the murine skin we next analyzed wound fluids of injured mice for the presence of the chemokine. Quantification of mCXCL16 by ELISA revealed that CXCL16 was present in the cell-free wound fluid already within the first day after injury. The release of CXCL16 was maximal after 3–5 days, afterwards decreasing gradually to almost background levels (Figure 2b).

CXCL16 is constitutively expressed and released by human and murine keratinocyte cell lines

In vitro experiments were performed to further investigate the expression of CXCL16 by keratinocytes. Cultured human HaCaT cells and murine PAM212 cells, both representing well-established keratinocyte cell lines, were analyzed for surface expression of CXCL16 by flow cytometry. Using antibodies directed against human or murine CXCL16, respectively, we were able to demonstrate surface expression of human and murine CXCL16 on human HaCaT and on murine PAM212 cells, respectively (Figure 3a and c). We then investigated whether soluble CXCL16 would be released by the cultured cell lines. Using different ELISAs for human and murine CXCL16, respectively, human HaCaT as well as murine PAM212 cells were found to release CXCL16 in a time-dependent manner. Moreover, in the presence of a broad-spectrum metalloproteinase inhibitor (marimastat) the CXCL16 release of both cell lines was considerably reduced suggesting that a metalloproteinase activity is involved in this process (Figure 3b and d).

To confirm CXCL16 expression by keratinocytes, we prepared primary keratinocytes from human foreskin and murine skin. Consistent with our previous observation the primary cell cultures released soluble CXCL16 in a time-dependent manner yielding concentrations of up to 1.2 ng/ml after 4 hours of incubation as detected by an ELISA for murine and human CXCL16, respectively (data not shown, compare Figure 4c).

Release of CXCL16 from keratinocytes involves ADAM10

We next asked which metalloproteinase is responsible for the release of CXCL16 from keratinocytes. In previous studies we and others have demonstrated that ADAM10 and ADAM17 contribute to the release of transmembrane chemokines from murine fibroblasts, endothelial cells, and CXCL16-transfected ECV304 cells. As shown by Western blot analysis ADAM10 and ADAM17 are expressed in HaCaT cells and converted into the mature, proteolytically active form (Figure 4a). To

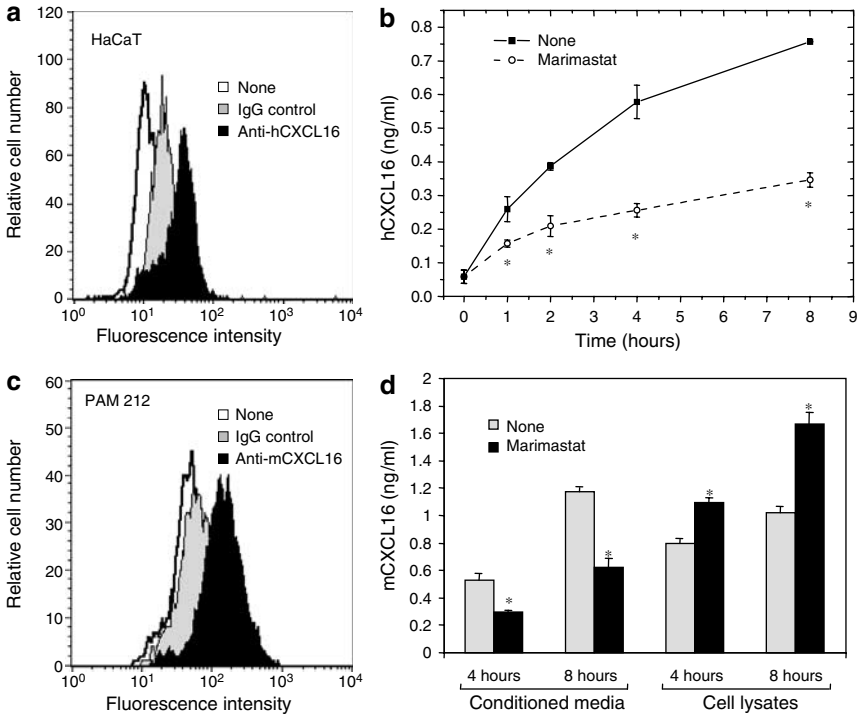

Figure 3. Surface expression and release of CXCL16 by cultured keratinocytes. (**a**) HaCaT cells were harvested and analyzed for CXCL16 surface expression by flow cytometry using a purified rabbit anti-hCXCL16 antibody that was detected by a phycoerythrin-conjugated secondary antibody. The fluorescence signal of the cells stained for hCXCL16 is shown in comparison to that of unstained cells or cells receiving an isotype control antibody. (**b**) HaCaT cells were incubated in serum-free medium for different periods of time in the presence or absence of marimastat (5 μM). Subsequently, conditioned media were harvested and analyzed for released CXCL16 by a specific ELISA for hCXCL16. (**c**) PAM212 cells were incubated with a rat anti-mCXCL16 antibody, an isotype control antibody, or left unstained. After incubation with a FITC-conjugated secondary antibody cells were analyzed by flow cytometry. (**d**) PAM212 cells were incubated in serum-free medium for 4 hours in the presence or absence of marimastat (5 μM). Subsequently, released CXCL16 in the conditioned media was determined by a specific ELISA for mCXCL16. Data are representative for three experiments and shown as mean and SD ($n=3$). Asterisks indicate statistically significant differences ($P<0.05$) between inhibitor-treated cells and untreated cells.

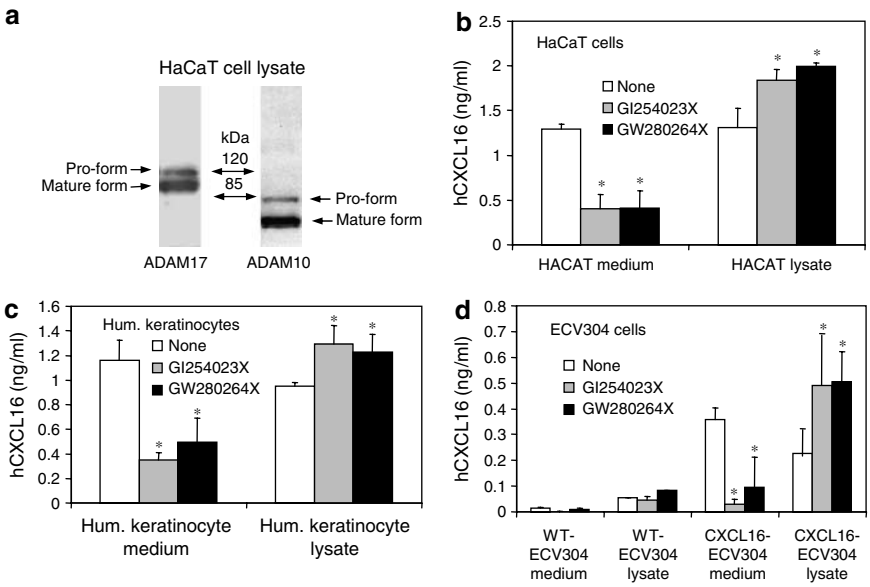

Figure 4. Effect of ADAM10/ADAM17-inhibitors on CXCL16-release from cultured keratinocytes. (**a**) Cell lysate of cultured HaCaT cells was investigated for the presence of immature and processed forms of the metallproteinaseses ADAM10 and ADAM17. For Western blotting purified rabbit antibodies against the C-terminus of ADAM10 and ADAM17, respectively were used. (**b–d**) (**b**) HaCaT cells (**c**) human primary keratinocytes, (**d**) WT-ECV304 and CXCL16-ECV304 cells and were incubated for 4 hours in the presence or absence of 5 μM GI254023X or GW280264X. Subsequently, conditioned media were harvested and cell lysates were prepared. Released and cell-associated CXCL16 in media and lysates, respectively, was then quantified by ELISA. Statistically significant differences ($P<0.05$) between inhibitor-treated cells and untreated cells are indicated by asterisks.

investigate the contribution of ADAM10 and ADAM17 to shedding of CXCL16 by keratinocytes we first employed two previously described specific inhibitors, one of which referred to as GI254023X, preferentially blocks ADAM10, but not ADAM17, whereas the other, designated as GW280264X, blocks both proteinases (Hundhausen *et al.*, 2003; Ludwig *et al.*, 2005a). In cellular cleavage assays both compounds dose dependently reduced the release of CXCL16 in HaCaT cells, CXCL16-transfected ECV304 cells, and in primary human keratinocytes (Figure 4b–d). Moreover, the reduced release was associated with an accumulation of cellular CXCL16 in the three different cell types. Same results were obtained in primary murine keratinocytes (data not shown). These findings indicate that ADAM10 rather than ADAM17 is involved in the constitutive cleavage of CXCL16 in keratinocytes.

To obtain further evidence for the role of ADAM10 in CXCL16 cleavage we decided to downregulate the endogenous expression of either ADAM10 or ADAM17 in primary human keratinocytes by small-interfering RNA (siRNA) technique. As demonstrated by RT-PCR for human ADAM10 or ADAM17 respectively, siRNA transfection led to specific and concentration-dependent downregulation of either metalloproteinase (Figure 5a and b). This effect was most pronounced in the time period between 48 and 72 hours after transfection and no side effect of ADAM10 siRNA on ADAM17 expression and *vice versa* was observed (data not shown). Next we performed release assays for endogenous CXCL16 with siRNA-treated cells. In line with the inhibitor studies, the downregulation of endogenous ADAM10 protein expression after siRNA treatment (Figure 5c) turned out to reduce the release of CXCL16 (Figure 5d) whereas the targeting of ADAM17 had no effect (Figure 5e).

Release of soluble CXCL16 is upregulated upon cell activation

As cultured HaCaT cells release considerable amounts of CXCL16 under normal cell culture conditions we tested whether the growth medium by itself might upregulate CXCL16 release. When the cells were incubated in medium without serum for 4 or 8 hours, CXCL16 release was more than 3-fold reduced compared to cells that received 2.5% serum. This effect was not due to reduced cell proliferation, as the total expression of CXCL16 in the cell lysates was not significantly altered (data not shown). We then investigated a number of different stimuli for their potential to modulate the release of CXCL16. Cell stimulation with the proinflammatory cytokines tumor necrosis factor-α, IFNγ, or IL4 (each 10 ng/ml, for 24 hours) did not affect protein expression of CXCL16 in the cell lysates or in the corresponding conditioned media (data not shown). This was in contrast to cultured endothelial cells or monocytic cells (THP-1 cells) which do not express CXCL16 constitutively but can be stimulated with IFNγ to express the chemokine on the protein level (Abel *et al.*, 2004).

In the next experiment we addressed the short-term regulation of CXCL16 shedding by treating serum-depleted keratinocytes with 4-β-phorbol 12-myristate 13-acetate, which is widely used to rapidly upregulate shedding events that are predominantly mediated by ADAM17 (Garton *et al.*, 2001; Sahin *et al.*, 2004). However, 4-β-phorbol 12-myristate 13-acetate treatment induced no significant increase in the release of CXCL16, nor did it modulate cell-associated CXCL16 (data not shown), suggesting that ADAM17 is not

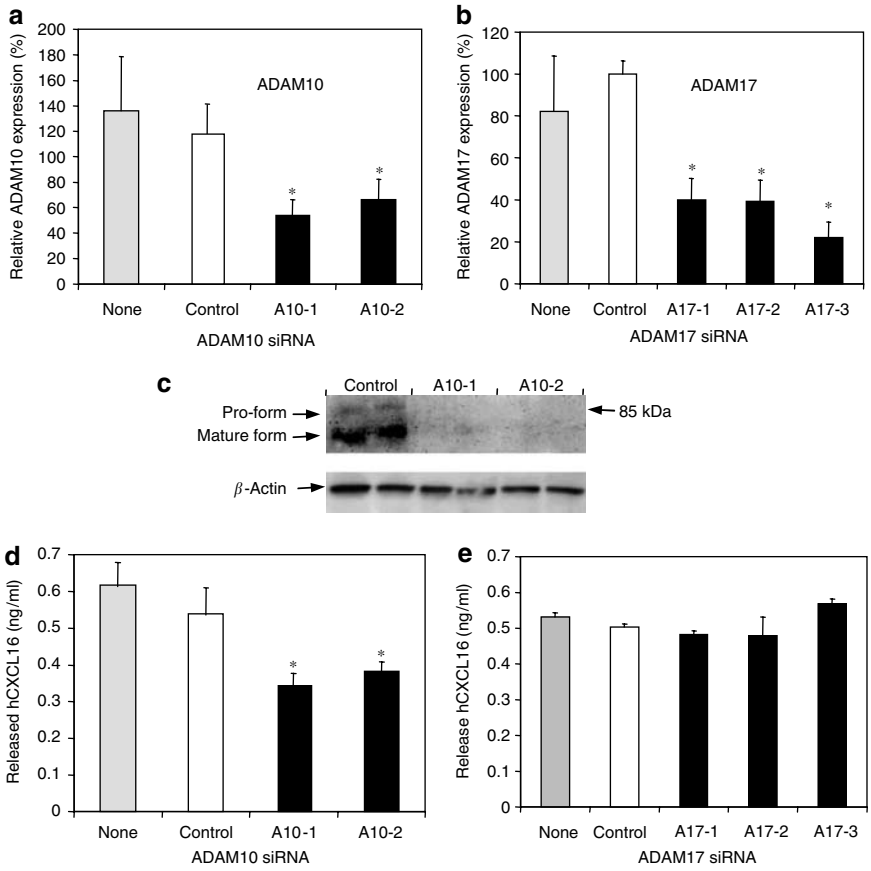

Figure 5. Effect of ADAM10/ADAM17 downregulation by siRNA on CXCL16-release from kreatinocytes. Cultured keratinocytes prepared from human foreskin were transfected with different heteroduplexed siRNA oligonucleotide constructs for downregulation of ADAM10 (A10-1, 2, 3) or ADAM17 (A17-1, 2, 3), an irrelevant siRNA control or without siRNA. At 48 hours after transfection mRNA and protein was extracted. (**a** and **b**) The mRNA expression level of ADAM10 and ADAM17 was determined by real-time RT-PCR and expressed in relation to that of glyceraldehyde-3-phosphate dehydrogenase. (**c**) Lysates of siRNA-transfected cells were analyzed for expression of the pro- and mature form of ADAM10 and controlled for β-actin content by Western blotting. (**d** and **e**) For CXCL16 release experiments siRNA-treated cells were washed and incubated for 4 hours in fresh medium. (**c** and **e**) Subsequently, cells were harvested for analysis of ADAM10 and ADAM17 mRNA expression and media were collected for quantification of released CXCL16 by ELISA. Data are representative for three experiments and shown as mean and SD ($n=3$). Asterisks indicate statistically significant differences ($P<0.05$) between cells receiving specific siRNA and control cells treated with irrelevant siRNA.

the relevant CXCL16 sheddase. We then investigated ionomycin as another potent inducer of shedding events which are predominantly mediated by ADAM10 (Nagano *et al.*, 2004; Reiss *et al.*, 2005). Ionomycin induced a rapid time-dependent increase in CXCL16 release, which was associated with a reduced content of the transmembrane molecule in the cellular lysates as measured by ELISA (Figure 6a and b). This ionomycin-induced shedding was blocked by the preferential ADAM10 inhibitor GI254023X suggesting that ADAM10 is responsible for the ionomycin-induced shedding of CXCL16 (Figure 6c).

In search of more physiological stimuli of shedding we induced photodamage in keratinocytes by UVB irradiation. Keratinocytes were serum starved for 16 hours and treated with different dosages of UVB. Subsequently, cells were further cultured in serum-free medium and after different periods of time, released and cell-associated CXCL16 was determined by ELISA. UVB radiation at a dosage of 10 mJ/cm^2 lead to a more than 3-fold increase of soluble CXCL16 within 4 hours of incubation (Figure 7a). In the presence of metalloproteinase inhibitors the release was blocked resulting in an accumulation of the molecule in the cell lysates (Figure 7b and c). This effect was seen with both, the preferential ADAM10- and the combined ADAM10/ADAM17-inhibitor (GI254023X and GW280264X, respectively), suggesting that ADAM10 inhibition is sufficient to suppress constitutive and UVB-induced release of CXCL16.

The receptor CXCR6 is activated by keratinocyte-derived CXCL16 and is expressed by distinct cells of the dermis

The biological activity of keratinocyte-derived CXCL16 was determined by the use of CXCR6-transfected human embryonic kidney (HEK)293 cells. As keratinocyte supernatants may contain a number of stimuli potentially activating HEK293 cells, the cellular response to CXCL16 was directly measured at the receptor level in terms of CXCR6-downregulation from the cell surface. HaCaT-conditioned media were collected and concentrated. The CXCL16 concentration was deter-

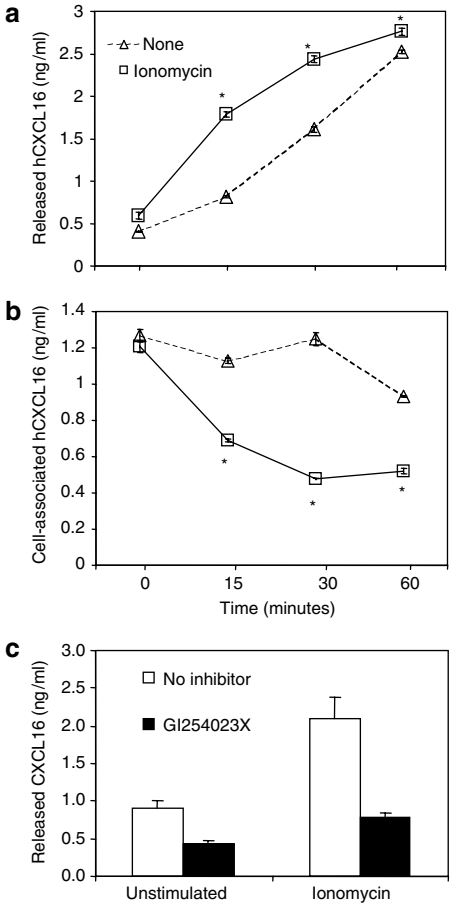

Figure 6. Effect of ionomycin on CXCL16-release from keratinocytes. (**a** and **b**) Serum-deprived HaCaT cells were treated with 1 μM ionomycin for different periods of time. Subsequently, the amount of (**a**) CXCL16 released into the media and that remaining in (**b**) the cell lysates was determined by hCXCL16-ELISA. (**c**) HaCaT cells were either left untreated or pretreated with the preferential ADAM10 inhibitor GI254023X for 5 minutes. Subsequently, cells were stimulated with 1 μM ionomycin or left unstimulated for 30 minutes. Released and cell-associated CXCL16 was then determined by ELISA. Data are representative for three experiments and shown as mean and SD ($n=3$). Asterisks indicate statistically significant differences ($P<0.05$) between inhibitor-treated and untreated cells.

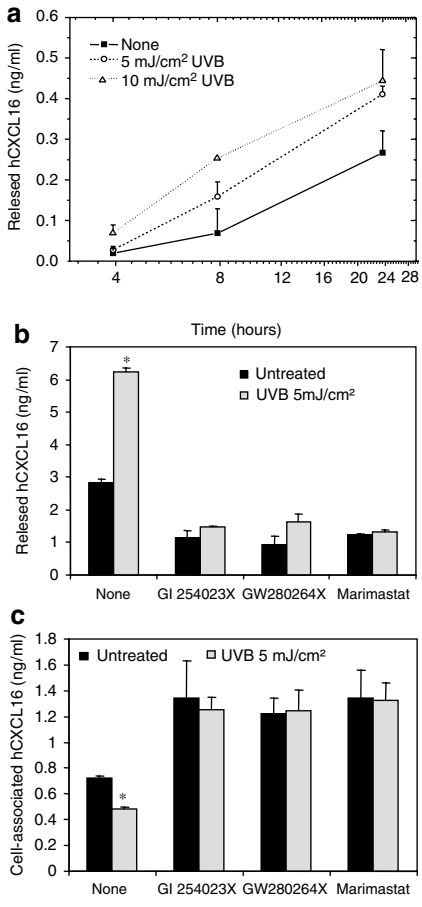

Figure 7. Effect of UVB on CXCL16-release from keratinocytes. (**a**) Serum-deprived HaCaT cells were treated with different dosages of UVB (5 and 10 mJ/cm^2) and subsequently incubated in serum-free medium for the indicated periods of time. (**b** and **c**) HaCaT cells were treated with UVB (5 mJ/cm^2) and cultured for 24 hours in serum-free medium in the absence or presence of the inhibitors GI254023X and GW280264X. (**b**) Conditioned media and (**c**) cell lysates were then analyzed for the presence of CXCL16 by ELISA. Data are representative for three experiments and shown as mean and SD ($n=3$). Statistically significant differences ($P<0.05$) between UVB-treated and untreated cells are indicated by asterisks.

mined by ELISA and the conditioned medium was then added to CXCR6-transfected HEK293 cells yielding a final concentration of 18 ng/ml CXCL16. After 30 minutes of incubation the expression level of CXCR6 at the cell surface was investigated by flow cytometry. To verify that CXCR6 is internalized upon ligand-engagement, cells were incubated with recombinant human CXCL16 chemokine domain in parallel. As expected, recombinant CXCL16 and the HaCaT-conditioned medium profoundly reduced the surface expression of CXCR6 (Figure 8a). This effect was time- and concentration-dependent (not shown) and could be completely prevented when the keratinocytes were stimulated with either recombinant CXCL16 or HaCaT-conditioned medium at 4°C (Figure 8b), demonstrating the temperature dependence of the internalization process. A purified rabbit antibody against CXCL16 (5 μg/ml), but not the respective IgG control antibody was capable of blocking the receptor internalization induced by either recombinant CXCL16 or HaCaT-conditioned medium demonstrating that keratinocyte derived CXCL16 was responsible for the internalization of its receptor. (Figure 8c and d). Thus, CXCL16 produced by keratinocytes was capable of binding and regulating its receptor.

Finally, we questioned whether healthy human skin contains CXCR6-expressing cells that could be target cells for keratinocyte-derived CXCL16. We used a mAb against human CXCR6 for immunofluorescence detection. A marked expression of CXCR6 was found in a small number of distinct cells in the papillary dermis (Figure 9a). Notably, CXCR6-expressing cells were absent in the epidermis of healthy skin and were therefore distinct from CXCL16-expressing epidermal keratinocytes (Figure 9b). This was consistent with our finding that cultured keratinocytes release CXCL16 but do not express CXCR6 at protein or mRNA level (data not shown).

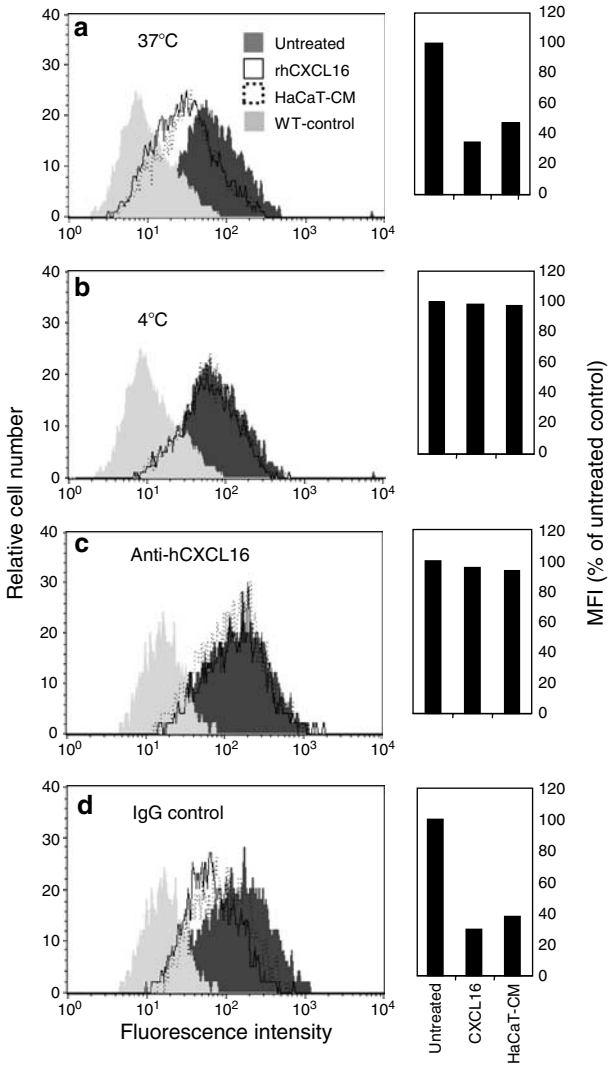

Figure 8. Activity of keratinocyte-derived CXCL16 on CXCR6-transfected HEK293 cells. CXCR6-expressing HEK293 cells were treated with recombinant CXCL16 or concentrated HaCaT-conditioned (HaCaT-CM) medium for 30 minutes at either (**a**) 37°C or (**b**) 4°C. In a separate experiment recombinant CXCL16 or concentrated HaCaT-conditioned medium were preincubated with a neutralizing antibody to (**c**) CXCL16 or an (**d**) isotype control and then added to the CXCR6-expressing HEK293 cells for 30 minutes at 37°C. Subsequently, cells were assayed for CXCR6 surface expression using a phycoerythrin-labeled mAb to CXCR6. As a control, WT-HEK293 cells expressing no CXCR6 were stained in parallel. The mean intensity of the fluorescence signal from CXCL16 or HaCaT-CM-treated cells was calculated as percentage of that from untreated cells and is shown as insets. Data are representative for three experiments.

Double staining for CD3 and CD 68 surface markers revealed that most CXCR6-positive cells belonged to the CD3-positive T-cell lineage (Figure 9c). However, not all CD3-positive cells were stained for CXCR6 suggesting that only a subpopulation of T cells expresses CXCR6.

DISCUSSION

We here provide *in vivo* and *in vitro* evidence for the expression and shedding of the transmembrane chemokine

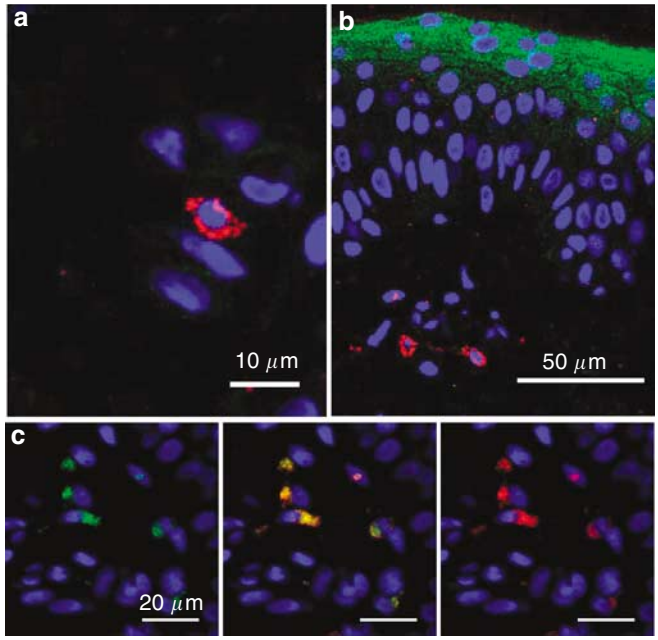

Figure 9. Immunfluorescence staining of human and murine skin for CXCR6. Paraformaldehyde-fixed sections of healthy human paraffin-embedded skin were incubated with (**a**) rabbit anti-hCXCR6 antibody or coincubated with (**b**) rabbit anti-hCXCL16 and monoclonal mouse anti-hCXCR6 antibodies. Bound antibodies were then detected with Cy2-coupled goat anti-rabbit IgG or Cy3-coupled goat anti-mouse IgG, respectively. (**c**) Sections were incubated with rabbit anti-hCD3 and mouse anti-hCXCR6 antibodies followed by incubation with Cy2-coupled goat anti-rabbit IgG and Cy3-coupled goat anti-mouse IgG and subsequently analyzed for immunfluorescence indicating expression of CD3 (left), CXCR6 (right) or both (middle). Bar = 20 μm.

CXCL16 in the skin. We show that CXCL16 is constitutively expressed by epidermal keratinocytes of healthy human skin. We found that CXCL16 is constantly released from the cell surface of cultured keratinocytes by the activity of ADAM10. This shedding is diminished upon depletion of growth factors, but enhanced by ionomycin and UVB irradiation. We further demonstrate that CXCL16 released from keratinocytes is functionally active in terms of binding to CXCR6 and inducing receptor internalization. CXCR6 is expressed in healthy skin and could be located on distinct cells of the papillary dermis. CXCL16 and CXCR6 are therefore expressed in different compartments of healthy skin.

Keratinocytes have been identified as important producers of chemokines in the skin (Caux *et al.*, 2000; Gillitzer and Goebeler, 2001; Homey and Bunemann, 2004). Notably, most chemokines are expressed only upon stimulation of keratinocytes. Bacterial products or proinflammatory cytokines are major inducers of the so-called inflammatory chemokines. Among these are T-helper-1 cell type chemokines inducible by IFNγ and tumor necrosis factor-α, and T-helper-2 type chemokines inducible by IL4. In a number of cell types including smooth muscle cells and endothelial cells IFNγ has been found to stimulate expression of CXCL9/10/11, CX3C-chemokine ligand 1 and also CXCL16 (Abel *et al.*, 2004; Wuttge *et al.*, 2004). In keratinocytes, IFNγ

induces CX3C-chemokine ligand 1, CXCL10, and CXCL9 (Kaplan *et al.*, 1987; Tensen *et al.*, 1999; Sugaya *et al.*, 2003), but surprisingly, as we describe here IFNγ does not induce CXCL16. Instead, CXCL16 is constitutively expressed by keratinocytes *in situ* and *in vitro* and the expression is not further enhanced by cytokine stimulation. These findings reveal that a pool of CXCL16 is constantly present in healthy skin suggesting a function different from that of most other chemokines that are absent in healthy skin with exception of the homeostatic chemokine CXCL4 (Fedyk *et al.*, 2001).

The metalloproteinase ADAM10 has been implicated in the processing of CXCL16 in endothelial cells, fibroblasts, and glioma cells (Abel *et al.*, 2004; Gough *et al.*, 2004; Ludwig *et al.*, 2005b). As we demonstrate here by the use of previously described specific inhibitors (Ludwig *et al.*, 2005a), as well as siRNA knockdown experiments, this metalloproteinase also is the major candidate for shedding of CXCL16 in keratinocytes. Interestingly, 4-β-phorbol 12-myristate 13-acetate, which is known to induce several ADAM17-mediated shedding processes, including that of CX3C-chemokine ligand 1 (Garton *et al.*, 2001), does not enhance the release of CXCL16. However, shedding is enhanced by ionomycin, which has been used previously to upregulate ADAM10-mediated cleavage processes (Nagano *et al.*, 2004; Reiss *et al.*, 2005). Moreover, UVB irradiation, within physiological doses also enhances the release of CXCL16, a process that can be blocked by ADAM10 inhibition. Taken together, these findings suggest that ADAM10 is not only constitutively active but can be further activated in response to cellular stress imposed by ionomycin or UV radiation to release more CXCL16 from keratinocytes.

Various biological activities have been attributed to CXCL16. The transmembrane chemokine has been demonstrated to function as a scavenger receptor on macrophages mediating uptake of oxidized low-density lipoprotein *in vitro* (Shimaoka *et al.*, 2000). Additionally, it promotes the binding and phagocytosis of bacteria (Shimaoka *et al.*, 2003) by macrophages. Thus, there is evidence that pathogenic molecules may be recognized by CXCL16. Some chemokines, including truncated forms of CTAPIII/CXCL7, exhibit bactericidal activity (Krijgsveld *et al.*, 2000). It therefore remains to be elucidated whether CXCL16 might exert a protective role in the skin as a component of the innate defense system. On the other hand, CXCL16 is well known to bind and activate the chemokine receptor CXCR6 (Matloubian *et al.*, 2000; Wilbanks *et al.*, 2001). In healthy human and murine skin we found CXCR6-immunoreactive cells in the papillary dermis but not in the epidermal layer. Dermal skin usually contains fibroblasts, endothelial cells, nerve endings, mast cells, dendritic cells, and T lymphocytes even in the absence of an immune response. Among these different cell types, T-cell subsets and natural killer T-cells have been described to express CXCR6 in various tissues including peripheral blood (Sharron *et al.*, 2000), Peyers' patches (Hase *et al.*, 2006), rheumatoid joints (van der Voort *et al.*, 2005), and the liver (Geissmann *et al.*, 2005). We here could demonstrate the expression of CXCR6 on CD3-positive cells, but importantly, within healthy skin not all CD3-positive cells were stained for CXCR6. In a recent study, CXCR6 could also be detected on isolated T-helper type 1 cells from human healthy skin using flow cytometry (Clark *et al.*, 2006). Therefore, resident CXCR6 positive cells in the dermal skin are very likely to represent a T lymphocyte subpopulation and further investigation is required to define this T-cell phenotype. Notably, the ligand CXCL16 is exclusively located in the epidermis of healthy skin and therefore unlikely to activate CXCR6 within the dermis. However, we here provide evidence that soluble CXCL16 is released into the wound fluid of injured skin in mice. We also show that increased amounts of CXCL16 are released from keratinocytes in response to photodamage. It can be envisaged that disruption of skin integrity induced by mechanical injury or sunburn leads to infiltration of released CXCL16 into the dermis where it will get in contact with CXCR6-positive cells within the dermis and then cause the activation of these cells. The CXCR6-positive cells could either migrate to the site of injury or they could be stimulated to carry out further mediatory functions that allow to coordinate an appropriate response for skin repair. We therefore propose an instrumental role of proteolytic shedding by ADAM10 for the function of the CXCL16/CXCR6 axis in skin.

MATERIALS AND METHODS

Cytokines, antibodies, and inhibitors

Recombinant human CXCL16 extracellular domain and chemokine domain, recombinant human IFN-γ, recombinant human tumor necrosis factor-α, goat anti-human CXCL16 (hCXCL16) antibody, rat anti-murine CXCL16 (mCXCL16) mAb (clone 142417), phycoerythrin-conjugated and unconjugated mouse anti-human CXCR6 (hCXCR6) mAb (clone 56811), and rat anti-murine CXCR6 (mCXCR6) mAb (clone 221002) were obtained from R&D Systems (Wiesbaden, Germany). Unconjugated and biotinylated rabbit anti-hCXCL16 antibody and goat anti-mCXCL16 antibody were from PeproTech (London, UK). The rabbit antiserum B 42.1 against the C-terminus of murine/human ADAM10 was kindly provided by Dieter Hartmann (Leuven and Flanders Interuniversity, Belgium) and characterized previously (Hartmann *et al.*, 2002). The purified rabbit antibody against the C-terminus of human ADAM17 was from Chemicon (Chandlers Ford, UK). The rabbbit anti-human CD3 antibody was obtained from Dako (Hamburg, Germany). The metalloproteinase inhibitors GW280264X ((2R,3S)-3-(Formyl-hydroxyamino)-2-(2-methyl-1-propyl) hexanoic acid [(1S)-5-benzyloxycarbamoylamino-1-(1,3-thiazol-2-ylcarbamoyl)-1-pentyl] amide) and GI254023X ((2R,3S)-3-(Formyl-hydroxyamino)-2-(3-phenyl-1-propyl) butanoic acid [(1S)-2,2-dimethyl-1-methylcarbamoyl-1-propyl] amide) were synthesized as described in US Patents US 6 172 064, US 6 191 150, and US 6 329 400. Marimastat was from GlaxoSmithKline (Stevenage, UK) (Chapman *et al.*, 2000). The compounds were assayed for inhibition of recombinant human ADAM17 and ADAM10 ectodomains as described (Hundhausen *et al.*, 2003; Ludwig *et al.*, 2005a).

Immunohistochemistry

For hCXCL16 chromogenic staining, paraform-fixed sections of normal, healthy human skin were incubated with 1% BSA in

Tris-buffered saline to block unspecific binding and incubated with 0.3% H_2O_2 to block endogenous peroxidase (POD) activity. Sections were incubated with rabbit anti-hCXCL16 (250 ng/ml PeproTech Inc., Rocky Hill, NJ), rabbit anti-ADAM10 (5 μg/ml, Chemicon, Temecula, CA) or normal rabbit IgG in Tris-buffered saline at 4°C overnight. Bound antibody was detected with a POD-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany) and chromogenic reagents (BioGenex, San Ramon, CA). Nuclei were counterstained with hemalumn (Merk, Darmstadt, Germany).

For hCXCL16 indirect immunofluorescence staining, paraffin-embedded human skin fixed in 8% formalin was used. Unspecific antibody binding was blocked with 0.1% BSA (PAA Laboratories, Linz, Austria) and 0.2% glycine in Tris-buffered saline. For blocking of unspecific binding of antibodies to Fc-receptors, 2 ng/ml sheep IgG Fc-fragments (Dianova) were added for 1 hour at room temperature (RT). Human CXCL16 was detected with a purified rabbit anti-hCXCL16 antibody (250 ng/ml, PeproTech). For detection of human and murine CXCR6 a mouse mAb to hCXCR6 (2.5 μg/ml, R&D Systems) or a rat mAb to mCXCR6 (1 μg/ml, R&D Systems) was used, respectively. Sections were incubated overnight at 4°C. For fluorescent staining of T-cell sections were incubated with a rabbit anti-human CD3 antibody (Dako) for 1 hour at RT. As secondary reagents Cy2-coupled goat anti-rabbit-IgG, Cy3-coupled goat anti-mouse-IgG and FITC-coupled goat anti-rat-IgG antibodies (Dianova) were applied according to the manufacturers recommendations. Nuclei were counterstained with bisbenzimide (Hoechst 33258) and sections were analyzed by confocal laser scanning microscopy (Zeiss, LSM 510 UV, Jena, Germany).

In situ hybridization

Radioactive-labeled RNA probes were generated by *in vitro* transcription from a cDNA fragment corresponding to nt 632–965 of mCXCL16 (accession no. NM_023158) in a pGEM-T easy plasmid (Promega, Mannheim, Germany) using (^{35}S)-uridine triphosphate and (^{35}S)-cytidine triphosphate as labeled nucleotides. Probes were diluted in hybridization buffer (50% formamide, 10% dextran sulfate, 0.05% tRNA, 0.6 M NaCl, 10 mM Tris·HCl pH 7.4, 1 × Denhardt's solution, 100 μg/ml sonicated salmon sperm DNA, 1 mM EDTA, and 10 mM dithiothreitol) to a final concentration of 5×10^4 c.p.m./μl. Feshly prepared murine skin was embedded in Tissue-Tek medium (Sakura Finetek, Torrance, CA) and frozen on dry ice. Sections (25 μm) were cut on a cryostat (Leica, Bentheim, Germany), thaw-mounted on silane-treated slides, fixed in a 4% phosphate-buffered paraformaldehyde solution (pH 7.4) for 1 hour at RT, rinsed with phosphate-buffered saline (PBS) and treated with 0.4% phosphate-buffered Triton × 100 solution for 10 minutes. After washing with PBS and water, tissue sections were incubated in 0.1 M triethanolamine (pH 8) containing 0.25% (v/v) acetic anhydride for 10 minutes, rinsed several times with PBS, washed with increasing ethanol concentrations, and air dried. After application of the labeled cRNA probes, sections were incubated in a humid chamber at 58°C for 16 hours. After hybridization, sections were rinsed with 2 × standard saline citrate (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), and treated with RNAse A (20 μg/ml), and RNAse T_1 (1 U/ml) at 37°C for 30 minutes. Successive washes followed at RT in 1 ×, 0.5 ×, and 0.2 × standard saline citrate for 20 minutes each and in 0.2 × standard saline citrate at 65°C for 1 hour. The tissue was then dehydrated and sections were dipped in NTB2 (Kodak, Integra Biosciences, Germany) nuclear emulsion and stored at 4°C. After exposure for 14 days, autoradiograms were developed. If required, sections were counterstained with cresyl violet and then photographed under darkfield or brightfield illuminations. Sense probes that were used to confirm the specificity of the hybridization did not show any signal.

In vivo wounding

Five hairless mice (SKH-1, Charles River, Wilmington, MA) were anaesthetized and wounded by excision of 200 mm^2 skin on the right flank. The animals were bandaged with Comfeel (Coloplast, Hamburg, Germany). To collect the exudates, wounds were washed every day with 200 μl PBS and then re-bandaged. The washing fluid was then analyzed for presence of CXCL16 by ELISA as described below.

Tissue samples, preparation, and culture of keratinocytes

Primary human keratinocytes were prepared from foreskin as described before (Harder *et al.*, 2004). The medical ethical committee of the Christian-Albrechts-University Kiel (D410/04) approved all experiments with primary human keratinocytes. The study was conducted according to the Declaration of Helsinki Principles. Experiments were performed with written informed consent of tissue donors. Cells were maintained in EpiLife medium (Cascade Biologics, Portland, OR) containing EpiLife defined growth supplement, 0.06 mM $CaCl_2$, 100 U/ml Penicillin, 100 μg/ml Streptomycin (both from Gibco, Grand Island, NY). Cells were grown in 12-well plates and received fresh medium every second day.

Primary murine keratinocytes were prepared from 2 d old mice (Balb/C, Charles River, Wilmington, MA). The skin was digested with 0.48 U/ml of dispase II (Roche Diagnostics, Mannheim, Germany) in serum-free keratinocyte growth medium (Gibco) for 2.5 hours at RT. The epidermis was mechanically separated from the dermis and digested with Trypsin-EDTA solution (Invitrogen, Carlsbad, CA) for 10 minutes. The cells were passaged through a cellstrainer of 100 μm mesh size (VWR International, Darmstadt, Germany) and digestion was stopped with soybean-trypsin inhibitor (Sigma, Munich, Germany, 1 mg/ml). After centrifugation at 1,200 *g* for 5 minutes the cells were seeded on bovine collagen IV (Becton Dickinson, Franklin Lakes, NJ) coated to 12-well plates (Becton Dickinson) and grown in serum-free KSFM medium containing 100 mM $CaCl_2$, 5 μg/ml recombinant human epidermal growth factor, and 50 μg/ml 0.1 ng/ml bovine pituitary extract.

HEK293 cells, stably expressing hCXCR6 or hCXCL16 were generated by transfection with hCXCR6- or hCXCL16-cDNA inserted into the expression vector pcDNA3.1 and subsequent selection with geneticin (500 μg/ml, PAA Laboratories). Separate clones grown at limiting dilution were harvested and analyzed for surface expression of hCXCR6 and hCXCL16 by flow cytometry (see below).

The spontaneously transformed human keratinocyte cell line HaCaT (From Dr Fusenig, DKFZ Heidelberg, Germany) and the murine keratinocyte cell line PAM212 were cultured in DMEM and Rosewell Park Memorial Institute 1640 medium, respectively, both supplemented with 10% fetal bovine serum (PAA Laboratories).

Analysis of CXCL16 and CXCR6 surface expression by flow cytometry

For flow cytometric analysis adherent cells were harvested by scraping into ice-cold PBS containing 0.1% BSA, and washed once.

To investigate human CXCL16 surface expression, cells were resuspended in ice-cold PBS containing 0.1% BSA and 0.01%NaN_3 at 3×10^5 cells/ml and incubated with purified rabbit anti-hCXCL16 or rabbit IgG control (both diluted at 2 μg/ml in PBS with 0.1%BSA and 0.01%NaN_3) for 1 hour on ice. To analyze mCXCL16 surface expression a monoclonal rat anti-mCXCL16 and a respective isotype control (both at 3 μg/ml) were used. After washing, cells were incubated with secondary fluorescein-conjugated goat $F(ab')_2$-antibody fragments against rabbit IgG (Dako) or phycoerythrin-conjugated goat $F(ab')_2$-fragments against rat IgG (Dianova), respectively, for 1 hour on ice. To examine hCXCR6 surface expression, an anti-hCXCR6 mAb directly coupled to phycoerythrin and a respective isotype control antibody (both at 3 μg/ml) were used. Cells were washed twice and suspended in ice-cold PBS containing 2% paraformaldehyde (PFA, Roth, Karlsruhe, Germany). The fluorescence signal of the labeled cells was then analyzed by flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany) and calculated as median fluorescence intensity of the cell population. The specificity of the detection method was controlled by the use of non-transfected HEK293 cells that showed no fluorescence staining in contrast to that of hCXCR6- or hCXCL16-transfected HEK293 cells.

CXCL16 release assays

Keratinocytes were grown until they reached 70–90% confluency, washed with PBS, and 0.75 ml fetal calf serum-free medium with or without metalloproteinase inhibitors was added. After 5 minutes, the cells were stimulated with 4-β-phorbol 12-myristate 13-acetate, ionomycin (both from Sigma) or left unstimulated for various periods of time. The conditioned media were harvested, cleared by centrifugation and a protease inhibitor cocktail (Complete, Roche Diagnostics) was added according to the instructions of the manufacturer. The cells were washed with ice-cold PBS and then lysed in PBS containing proteinase inhibitors and 1% Triton X-100. The lysates were then cleared by centrifugation at 10,000 *g* for 10 minutes. The amount of CXCL16 in the conditioned media and cell lysates was analyzed by ELISA (see below).

UV irradiation

HaCaT cells were seeded in tissue culture dishes at a density of 2×10^5 cells/ml and grown to 70–90% confluency. Before UV irradiation cells were serum-starved for 16 hours. Subsequently, cells were washed with prewarmed PBS and exposed to UV light in the presence of PBS. For UVB irradiation, six FS20 fluorescent lamps were used (emission peak 313 nm, Philips, Eindhoven, Netherlands). Immediately after UV treatment, PBS was replaced by serum-free DMEM medium and the cells were further cultured for up to 24 hours. Control cells were subjected to the identical procedure without UV exposure.

CXCL16 specific ELISA

The ELISA for human CXCL16 was carried out as described before (Abel *et al.*, 2004). In brief, goat anti-human CXCL16 (2 μg/ml) was coated to a microtiter plate (Microlon, Greiner, Frickenhausen, Germany), followed by blocking with 0.05% Tween in PBS (PBS-T) containing 2% BSA for 2 hours. The samples and a standard, prepared as serial 1:2 dilutions of 6.25 ng/ml recombinant human CXCL16 in PBS-T with 1% BSA, were added for 2 hours, followed by 2 hours incubation with 200 ng/ml biotinylated rabbit anti human CXCL16 in PBS-T containing 1% BSA. After 1 hour incubation with 100 mU/ml streptavidin-POD conjugate (Roche Diagnostics) in PBS-T with 1% BSA, the bound enzymatic activity was quantified using chromogenic POD substrate (BM blue, Roche Diagnostics). The specificity of the ELISA was demonstrated by its positive reaction with lysates and conditioned media of CXCL16-transfected COS7 cells or ECV-304 cells as described previously (Abel *et al.*, 2004). The ELISA specific for murine CXCL16 was purchased from R&D Systems and performed following the manufacturer's instructions. The ELISA reacted with lysates and supernatants of COS7 cells transfected with murine CXCL16 but not with that of cells transfected with human CXCL16 or empty vector (Abel *et al.*, 2004).

siRNA

For downregulation of endogenous ADAM10/ADAM17 expression in human keratinocytes the following duplexed RNA oligonucleotides (Stealth RNAi) were used: ADAM10-construct 1, 5′-UAC ACC AGU CAU CUG GUA UUU CCU C-3′; ADAM10 construct 2, 5′-AAC AGU AGU CAU CAU GAU UCU GCU C-3′ ADAM10-construct 3, 5′-AGA AUU AAC ACU CUC AGC AAC ACC A-3′; ADAM17-construct 1, 5′-AUG AGU UGU AAC CAG GUC AGC UUC C-3′; ADAM17-construct 2, 5′-AUA CAU GAC AUA UUU CCC UCC CUG G 3′; ADAM17-construct 3, 5′-UAC UGU ACA GGG CUU UCC UUU CCU C-3′. As negative control unspecific stealth RNA duplexes (Invitrogen) with low GC content were used. For transfection human primary keratinocytes were grown to 60% confluence in 12-well plates. The siRNA constructs were preincubated with Lipofectamin 2000 (1 μl/well, Invitrogen) for 20 minutes at RT and then applied to the cells at final concentrations of 10, 20, and 30 pM in 500 μl EpiLife medium (Cascade Biologics) without antibiotics. After 6 hours of incubation the medium was replaced and the cells were further incubated for 48 or 72 hours. Subsequently, efficiency of mRNA knockdown was measured by RT-PCR. Conditioned media were harvested and analyzed for the presence of released CXCL16 by ELISA.

RNA isolation, RT-PCR, and quantitative real-time RT-PCR

The cells were lysed in 1 ml TRIzol (Invitrogen) followed by chloroform (Sigma-Aldrich, Munich, Germany) and isopropanol (Sigma-Aldrich) precipitation as described by the manufacturer. RNA quantity and quality were determined by photometry and gel electrophoresis. RNA (2 μg) was digested with DNAse I (Fermentas, St Leon-Rot, Germany) followed by reverse transcription with Superscript II (Invitrogen). Quantitative real-time RT-PCR was performed in a flourescence temperature cycler (LightCycler; Roche Diagnostics) as described elsewhere (Harder *et al.*, 2004). The following primers were used: ADAM10, 5′-TCC AC AGC CCA TTC AGC AA-3′ and 5′-GCG TCT CAT GTG TCC CAT TTG-3′; ADAM17 5′-GAA GTG CCA GGA GGC GAT TA-3′ and 5′-CGG GCA CTC ACT GCT ATT ACC 3′; glyceraldehyde-3-phosphate dehydrogenase, GA1 5′-CCA GCC GAG CCA CAT CGC TC-3′ GA2 5′-ATG AGC CCC AGC CTT CTC CAT-3′ Reactions were performed in 10 μl volumes containing 5 μl SYBR Premix Ex Taq (MoBiTec; Göttingen, Germany), 0.2 μM of each each primer and serial dilutions of cDNA. Following denaturation at 95°C for 30 s, a touch down PCR was run with 45 cycles of 10 s denaturation at 95°C, followed by 20 s annealing from 66°C down to 55°C and 15 s amplification at 72°C.

To confirm specificity of the amplified DNA a melting curve was determined at the end of each run by cooling down to 65°C for 15 s and heating slowly at 0.2°C/s to 95°C with continuous measurement of the fluorescence. Standard curves were determined for each primer pair with serial dilutions of cDNA. All samples were quantified in relation to the expression of glyceraldehyde-3-phosphate dehydrogenase. Results were expressed as ratio of target gene and glyceraldehyde-3-phosphate dehydrogenase expression.

Western blot analysis of ADAM10 and ADAM17

Western blot analysis of endogenous ADAM10 and ADAM17 was performed as described previously (Hundhausen *et al.*, 2003). In brief, the cells were washed with PBS and either directly lysed in reducing SDS-sample buffer or used for simultaneous extraction of proteins and mRNA (Macherey and Nagel, Düren, Germany) following the manufacturer's recommendations. Samples were then subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions using 10% Tris-glycine gels. Proteins were transferred onto polyvinylidene difluoride membranes (Hybond-P, Amersham, Freiburg, Germany) that were probed with dilutions of rabbit antiserum against ADAM10 (1:10,000) or rabbit antiserum against ADAM17 (1:1,000). Bound Ig was detected with POD-coupled goat anti-rabbit Ig (Perbio, Bonn, Germany, diluted 1:10,000 in PBS-T) and subsequent addition of enhanced chemiluminescence substrate (ECLplus, Amersham). Signals were recorded using a luminescent image analyzer (Fujifilm Image reader, LAS1000, Tokyo, Japan). Equal loading and transfer of proteins to the membrane was controlled by subsequent detection of β-actin using a specific mAb, mAbcam 8226 (Abcam, Cambridge, UK) followed by POD-coupled goat anti-mouse Ig (Perbio, diluted 1:10,000 in PBS-T).

Statistical analysis

Two populations of data were statistically analyzed using the unpaired two tailed *t*-test and considered significantly different at *P*-values smaller than 0.05.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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