Induction of Inflammatory Cytokines by a Keratin Mutation and their Repression by a Small Molecule in a Mouse Model for EBS

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Epidermolysis bullosa simplex (EBS) is a skin disorder caused by mutations in keratin (K) 5 or K14 genes. It is widely regarded as a mechanobullous disease, resulting from a weakened cytoskeleton, causing extensive cytolysis. It was postulated by others that certain K14 mutations induce tumor necrosis factor-α (TNF-α) and increase apoptosis. Here, we report that in $K5^{-/-}$ mice and in a cell culture model of EBS, the mRNA and protein levels of TNF-α remain unaltered. Transcriptome analysis of $K5^{-/-}$ mice revealed, however, that the proinflammatory cytokines IL-6 and IL-1β were significantly upregulated at the mRNA level in $K5^{-/-}$ mouse skin. These results were confirmed by TaqMan real-time PCR and ELISA assays. We hypothesize that keratin mutations contribute to EBS in a mouse model by inducing local inflammation that mediates a stress response. Following clinical reports, we applied the small molecule doxycycline to $K5^{-/-}$ mice. We demonstrate that doxycycline extended the survival of neonatal $K5^{-/-}$ mice from less than 1 to up to 8 hours. Microarray and TaqMan real-time PCR showed a downregulation of matrix metalloproteinase 13 and IL-1β, indicating an effect of doxycycline on transcription. Our data offer a novel small molecule-based therapy approach for EBS.

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INTRODUCTION

Epidermolysis bullosa simplex (EBS) is a dominantly inherited skin disorder caused by mutations in keratin (K) 5 or K14 genes that is characterized by cytoplasmic protein aggregates and extensive cytolysis. It is widely regarded as a mechanobullous disease, resulting from an altered cytoskeleton unable to provide support against mechanical stress. Previously, we and others have generated K5 and K14 mutant mice serving as models for EBS (Lloyd *et al.*, 1995; Cao *et al.*, 2001; Peters *et al.*, 2001). Of these, $K5^{-/-}$ mice develop the most severe condition owing to the absence of keratin filaments in the basal epidermis, followed by a complete detachment of the epidermis from the underlying dermis. The resulting cytolysis causes death of neonates during the first hour after birth with full penetrance (Peters *et al.*, 2001).

Although it is well established that EBS is mainly caused by mechanical trauma of an epidermis that expresses mutant keratins, additional EBS pathomechanisms have recently emerged. Tumor necrosis factor-α (TNF-α) has been hypothesized to contribute to the severe Dowling–Meara form of EBS, caused by a point mutation at Arg 125 in K14 (Yoneda *et al.*, 2004). TNF-α is a proinflammatory cytokine that induces apoptosis in some cell types (Yeh *et al.*, 1998; Hsu and Twu, 2000). It exerts its effects by binding to TNF-α receptors TNF-R1 and TNF-R2. The cytotoxic effects of TNF-α are mediated by TNF-R1, which has a cytoplasmic death domain that interacts with an adaptor protein, TNF-α receptor-associated death domain, following ligand binding. TNF-α receptor-associated death domain subsequently interacts with additional adapter proteins receptor-interacting protein, TNF-R-associated factor 2, and Fas-associated death domain, which recruits and activates pro-caspase-8. Caspase-8 then oligomerizes and is activated by self-cleavage, and initiates a protease cascade that leads to apoptosis. Yoneda *et al.* (2004) have suggested that TNF-α mediates cytotoxicity in a keratinocyte model of EBS, based on HaCaT keratinocytes transiently transfected with a K14 mutant (K14Arg125Cys). Using this cell culture model of EBS, they reported that K14 aggregates led to the release of TNF-α and the subsequent activation of the TNF-α receptor by an autocrine/paracrine pathway, which resulted in cell death.

In view of these reports, we considered the hypothesis that additional mechanisms and not only mechanical weakness

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Abbreviations: EBS, epidermolysis bullosa simplex; MMP, matrix metalloproteinase; RT, reverse transcription; TNF-α, tumor necrosis factor-α; wt, wild-type

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contribute to EBS. Identification of such mechanisms would offer potential targets for novel therapy approaches. Given the significant problems accompanying gene therapy approaches of EBS, alternative efforts are indeed necessary (Kirfel *et al.*, 2002; Arin and Roop, 2004). One clinical study has recently suggested that the systemic application of tetracycline to patients suffering from the severe Dowling–Meara form of EBS leads to a significant reduction of the blistering and less fragile epidermis (Retief *et al.*, 1999). Tetracyclines can act as antibiotics by inhibiting bacterial protein synthesis, but recent work has shown that they are pluripotent drugs that affect many cellular functions: minocycline reduces inflammation and protects against focal cerebral ischemia (Yrjanheikki *et al.*, 1999); tetracyclines affect prion infectivity through a direct interaction with proteinase-resistant forms of the prion protein (Forloni *et al.*, 2002); doxycycline modulates smooth muscle cell growth, migration, and matrix remodeling after arterial injury (Bendeck *et al.*, 2002).

Here, we report on the systemic treatment of $K5^{-/-}$ mice serving as an animal model for EBS, by doxycycline. We demonstrate that the systemic application of physiological concentrations of doxycycline prolonged the life of neonatal $K5^{-/-}$ mice. Affymetrix analysis revealed that this was accompanied by changes at the transcriptional level. Proinflammatory cytokines IL-6 and IL-1β were significantly upregulated at the mRNA and protein level in $K5^{-/-}$ mice skin. In contrast to other studies, however, the mRNA and protein levels of TNF-α remained unchanged in mice and in a cell culture model. At the same time, genes involved in tissue remodeling were repressed. We hypothesize that mutations in genes that cause EBS and related disorders not only lead to cell fragility, but cause local inflammation, possibly by mediating a stress response.

RESULTS

Absence of TNF signaling in $K5^{-/-}$ mice and in a cell culture model of EBS

EBS is a blistering cutaneous disease featuring protein aggregates. Recently, TNF-α-mediated cytotoxity in a keratinocyte model of EBS was reported (Yoneda *et al.*, 2004). In that study, HaCaT keratinocytes transiently transfected with K14 (R125C) were found to release TNF-α with a subsequent activation of the TNF-α receptor by an autocrine/paracrine pathway, which resulted in cell death. A well-established EBS mouse model, $K5^{-/-}$ mice (Peters *et al.*, 2001) and a HaCaT keratinocyte line expressing mutant K14 (R125C) serving as EBS cell models (Werner *et al.*, 2004) were used to investigate whether TNF-α was induced in these models. First, we examined the potential role of TNF-α, which is expressed in the epidermis, in K5-deficient mouse skin (Kondo and Sauder, 1997). We quantified TNF-α protein expression by ELISA (Figure 1g) and found no significant difference of TNF-α expression between $K5^{-/-}$ (202 ± 9.8 pg/mg total protein) and wild-type (wt) control animals (192 ± 13 pg/mg total protein), $P = 0.49$ (Figure 1g). Therefore, neither the extensive cytolysis nor K14 aggregates present in $K5^{-/-}$ epidermis stimulated TNF-α secretion. Consistent with these data, immunofluorescence analysis of frozen skin sections using TNF-α antibodies showed no difference between $K5^{-/-}$ and wt back skin (Figure 1a and b). Second, we used mouse MGU74Av2 microarrays (Affymetrix, Santa Clara, CA) for large-scale gene expression profiling in mouse skin between $K5^{-/-}$ and wt controls (GSE760). A comprehensive analysis of genes relevant to the TNF-α pathway failed to reveal differentially expressed genes related to the TNF-α pathway when mRNA expression profile from $K5^{-/-}$ and wt mice was compared (Table 1).

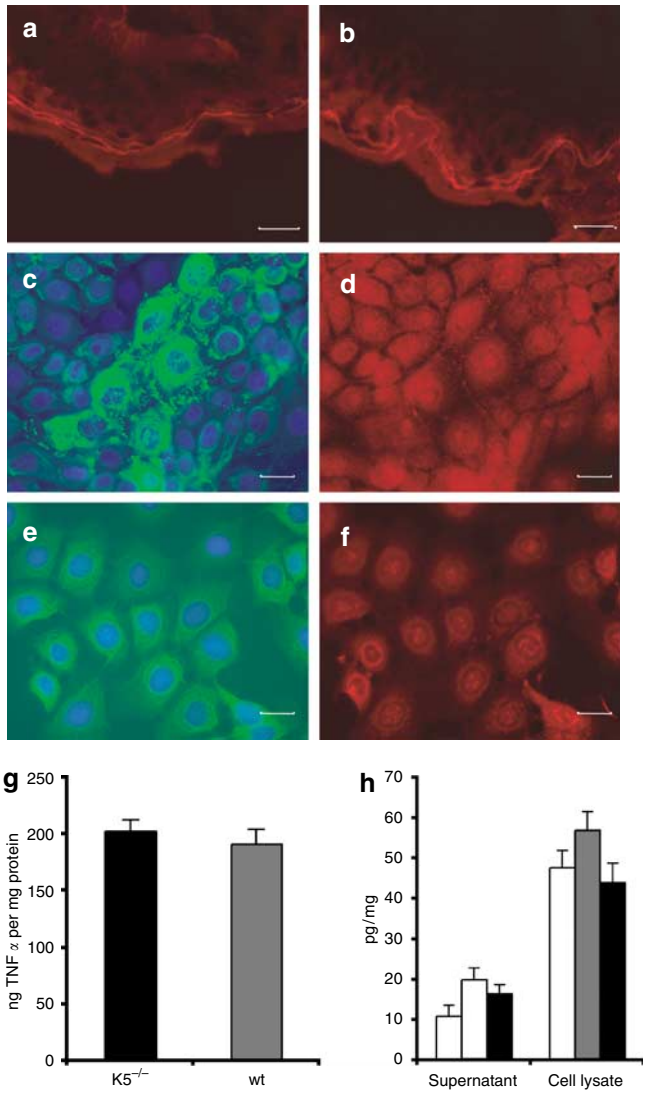

Figure 1. TNF-α expression in an EBS mouse and a cell culture model. (**a**) Immunofluorescence of TNF-α in $K5^{-/-}$ and (**b**) wt skin (bar = 10 μm); TNF-α immunofluorescence in cultured MCF7 cell lines, (**c**) K14 and (**d**) TNF-α staining in stably transfected mutant K14 ArgR125Cys MCF-7 cell line, (**e**) K14 and (**f**) TNF- α staining in stably transfected wt K14wt MCF-7 cell line. Bar = 10 μm. (**g**) Quantification of TNF-α protein expression in $K5^{-/-}$ *versus* wt epidermis. Amount of TNF-α protein in $K5^{-/-}$ epidermis (black bar, 202 ± 9.8 pg/mg total protein, $n = 6$) and wt (white bar, 192 ± 13 pg/mg total protein, $n = 6$). Student's *t*-test showed no TNF-α difference between $K5^{-/-}$ and wt epidermis, $P = 0.49$. (**h**) Quantification of TNF-α protein expression. Measurements were taken in K14 ArgR125Cys *versus* wt K14wt MCF-7-transfected cell lines using ELISA ($n = 3$).

Given that these data were in contrast to a previous study using HaCaT keratinocytes transiently transfected with the most frequent EBS mutant K14 Arg125Cys, we reasoned that the failure to induce TNF signaling in $K5^{-/-}$ mice might be due to the presence of wt K14 (Yoneda *et al.*, 2004). To extend our *in vivo* analysis to a cell culture model, we switched to HaCaT and to MCF-7 cells, which were both stably transfected with the mutant K14 Arg125Cys created previously (Werner *et al.*, 2004). In both cell types, we found no changes in TNF-α by immunofluorescence analysis (Figure 1c–f). Next, TNF-α levels secreted into the medium and inside cells were measured with a solid-phase ELISA. Repeated measurements revealed that TNF-α was not increased in the media supernatants of K14 mutant (16.6 pg/ml) compared to those from control transfectants (20.1 pg/ml; Figure 1h). The expression level of TNF-α protein in mutant-transfected cells (44 pg/ml) was lower as in mock (47.7 pg/ml)- or K14 wt-transfected cells (57 pg/ml; Figure 1h). Collectively, our data have demonstrated that neither the absence of K5 nor the presence of mutant K14 or of keratin aggregates stimulate TNF-α secretion in both EBS models. To our knowledge, TNF-α levels have not been examined in EBS patients.

Increased IL-6 and IL-1 mRNA levels in $K5^{-/-}$ skin

The absence of TNF signaling does not exclude a role of other proinflammatory cytokines. Therefore, we performed a microarray analysis from neonatal $K5^{-/-}$ and wt skin. This revealed that 85 genes were up- and 31 were downregulated upon loss of K5. Of these, the mRNA for IL-6 was increased 3.46-fold in $K5^{-/-}$ skin *versus* wt skin, the one encoding chemokine (C–X–C motif) ligand 1 was upregulated 4.05-fold (Table 2). This was confirmed by TaqMan real-time quantitative reverse transcription (RT)-PCR (Figure 2a). IL-6 was significantly upregulated in the skin of $K5^{-/-}$ mice (8.47-fold increase, $P<0.05$). The transcript of chemokine (C–X–C motif) was little in wt, but statistics show no significant difference comparing with $K5^{-/-}$ skin (Figure 2b, $P=0.26$). IL-1β was also increased in the skin of $K5^{-/-}$ mice (5.72-fold increase, $P<0.05$; Figure 2a). Furthermore, we analyzed IL-6 and IL-1β at the protein level in $K5^{-/-}$ and wt epidermis. In the former, the content of IL-1β was increased significantly (8.80 ± 0.18 pg/mg total protein, $P<0.05$) compared with wt controls (7.25 ± 0.15 pg/mg total protein). Owing to the very low levels of IL-6, however, statistic analysis showed that there was no significant difference of IL-6 expression between $K5^{-/-}$ (0.086 ± 0.036 pg/mg total protein) and wt control mice (0.057 ± 0.028 192 13 pg/mg total protein, $P=0.52$, Figure 2c).

Extended survival of $K5^{-/-}$ mice following small molecule treatment

K5, together with K14 and K15, forms the keratin cytoskeleton of basal epidermis. Previously, we reported that $K5^{+/-}$ mice die in the first hour after birth ($n=200$) with full penetrance because the inability to form a keratin cytoskeleton in their basal epidermis causes extensive fragility. In a first attempt to improve this condition, pregnant $K5^{+/-}$ females were systemically treated with doxycycline, starting

Table 1. Expression of genes related to the TNF-α pathway analyzed by MGU74Av2 microarray

Genes	Knockout_*vs* wt_skin; signal fold	
X87128: *Mus musculus* p75 TNF receptor DNA	1.231144413	NC
U21050: Tnf receptor-associated factor 3	0.870550563	NC
X92346: Tnf receptor-associated factor 4	1.071773463	NC
D84196: mouse strain NOD (TNFA) gene	1.231144413	NC
D78141: Tnf receptor-associated factor 5/cds=(322,1998)	1.319507911	NC
AF010600: *Mus musculus* TNF-response element-binding protein mRNA	0.933032992	NC
U37522: *Mus musculus* TNF-related apoptosis-inducing ligand (TRAIL) mRNA	1.319507911	NC
L35302: Tnf receptor-associated factor 1	1.071773463	NC
U90926: *Mus musculus* putative TNF-resistance related protein mRNA	1.319507911	NC
AJ007749: *Mus musculus* mRNA for caspase-8	1	NC
U54803: caspase-3, apoptosis-related cysteine protease	0.707106781	NC
Y13090: *Mus musculus* mRNA for caspase-12	1.414213562	NC
U67321: caspase-7	1.148698355	NC
D28492: caspase-2	1	NC
AB005663: *Mus musculus* mRNA for JNK1	1.071773463	NC
AB005664: *Mus musculus* mRNA for JNK2, complete cds	1.071773463	NC
AB029482: *Mus musculus* mRNA for JNK-binding protein JNKBP12	1.148698355	NC
AB005662: *Mus musculus* mRNA for JNK/SAPK-associated protein-1	1.319507911	NC
U18310: SAPK/Erk/kinase 1	1.148698355	NC

NC, no change; TNF-α, tumor necrosis factor-α; wt, wild-type.

Table 2. Selected upregulated genes in K5$^{-/-}$ compared to wt skin

Genes	Fold
Chemokine (C–X–C motif) ligand 2	7.62
Chemokine (C–X–C motif) ligand 1	4.79
Hypoxanthine guanine phosphoribosyl transferase	3.66
IL-6	3.46
Chemokine (C–X–C motif) ligand 1	2.81
Keratin complex 2, basic gene 6a	2.01

at E13.5. This was based on our previous finding that the formation of keratin aggregates typical of EBS started at E13.5 in embryonic stage (Lu *et al.*, 2005). Treatment with the drug prolonged the survival time of 34 K5$^{-/-}$ pups up to 8 hours (Table 3).

To analyze the mechanisms responsible for the prolonged survival at the molecular level, we used Affymetrix Genechips mouse MGU74Av2 for large-scale gene expression profiling in epidermis and in skin after treatment with doxycycline and compared it to vehicle-treated controls (GSE760). In K5$^{-/-}$ epidermis, 159 genes were differentially expressed after doxycycline treatment. Fifty-nine genes were upregulated after doxycycline treatment and 100 genes were suppressed. Here, we focus on genes relevant to inflammation and tissue repair (Table 4). Most importantly, in K5$^{-/-}$ epidermis, doxycycline downregulated matrix metalloproteinase 13 (MMP-13, also called collagenase 3) mRNA expression (0.4-fold decrease), and upregulated various procollagens (procollagen type III α1, 39.95-fold increase; procollagen type I α1, 18.38-fold increase; procollagen type VI α3, 15.03-fold increase). Moreover, doxycycline repressed the expression of genes encoding IL-1β (0.28-fold decrease), IFN-activated gene 202B (0.24-fold decrease), chemokine ligand 1 (0.21-fold decrease), serine protease 12 (0.11-fold decrease). On the other hand, it activated the serine proteinase inhibitor gene (6.41-fold increase).

Next, TaqMan real-time relative quantitative RT-PCR was performed with glyceraldehyde-3-phosphate dehydrogenase as the reference gene to validate the microarray results (Figure 3a and b). In line with the microarray data, mRNA expression of MMP-13 and IL-1β were significantly downregulated in the epidermis of doxycycline-treated K5$^{-/-}$ mice (0.263, 0.339, 0.295, $P<0.05$; Figure 3a). On the other hand, real-time RT-PCR result did not confirm the downregulation of IFN-activated gene 202B and the upregulation of procollagen. The statistical data showed no significant changes (Figure 3b) because of significant deviation between samples. This result was consistent with previous data on the doxycycline treatment following myocardial infarction. In this setting, left ventricular remodeling occurred in the post-infarction period by inhibition of collagenase activity without changing collagen (Villarreal *et al.*, 2003). However, there was no significant difference when we checked the content of IL-1β in K5$^{-/-}$epidermis after doxycycline *versus* no treatment by ELISA (data was not shown). Using Western blotting, we found a slightly decreased amount of active MMP-13 in the epidermis after doxycycline treatment (Figure 4). We hypothesize that these changes represent the initiation of ''matrix remodeling'' and contributes to a reduced bullae formation and prolongs the life of K5$^{-/-}$ mice. These results indicate that doxycycline can partially

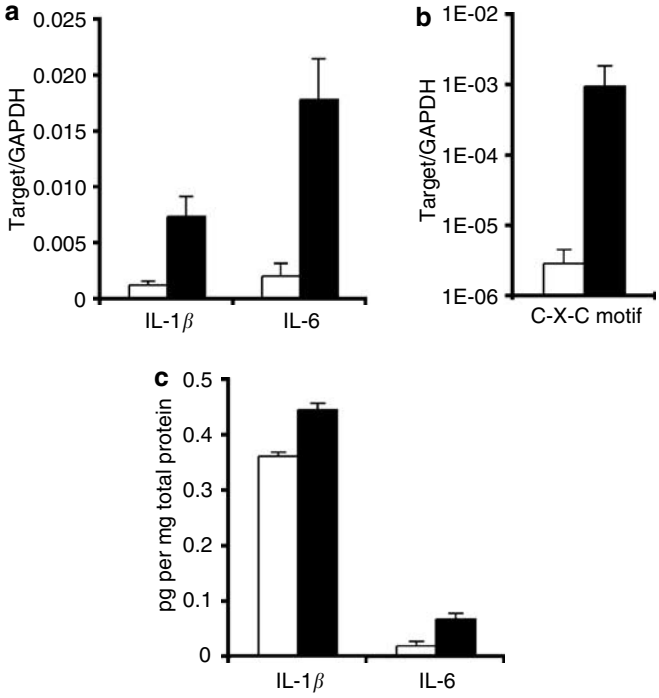

Figure 2. IL-6 and IL-1β induction in K5$^{-/-}$ mice. (**a**) Changes in TaqMan real-time relative quantitative RT-PCR with glyceraldehyde-3-phosphate dehydrogenase as reference gene. Both IL-1β and IL-6 were increased in the skin of K5$^{-/-}$ mice (5.72- and 8.47-fold increase, $P<0.05$), white: controls ($n=6$), black: K5 $^{-/-}$ ($n=6$). (**b**) C–X–C motif real-time relative quantitative RT-PCR with glyceraldehyde-3-phosphate dehydrogenase expression, $P=0.26$. (**c**) The content of IL-1β in K5$^{-/-}$ epidermis (black bar, 447.3$\pm$11 pg/mg total protein, $n=6$) and wt (white bar, 362$\pm$7.4 pg/mg total protein $n=6$). $P<0.05$. The content of IL-6 in K5$^{-/-}$ epidermis (black bar, 69.0$\pm$10.9 pg/mg total protein, $n=6$) and wt (white bar, 20.1$\pm$7.6 pg/mg total protein, $n=6$) $P<0.05$.

Table 3. Surviving time of doxycycline-treated and -untreated K5 knockout neonatal mice

Survival time (hours)	Untreated	Treated
<1	40	0
3	0	3
5	0	10
7	0	13
8	0	8
Total	40	34

Table 4. Differentially expressed genes in the microarray profiling in K5$^{-/-}$ epidermis following doxycycline treatment

	Fold
Genes downregulated	
Bcl2-like 10	0.40
MMP-13	0.37
IFN-activated gene 202B	0.37
Phosphatidylinositol-4-phosphate 5-kinase, type 1α	0.36
Regulator of G-protein signaling 7	0.34
IL-1β	0.28
IFN-activated gene 202B	0.24
Insulin receptor	0.23
Distal-less homeobox 6	0.22
Chemokine (C–C motif) ligand 1	0.21
Paired box gene 6	0.20
S100 calcium binding protein A (calgranulin A)	0.13
Protease, serine 12 neurontrypsin (motopsin)	0.11
Genes upregulated	
Procollagen type III α1	39.95
Insulin-like growth factor binding protein 1	32.9
Procollagen type I α1	18.38
Procollagen type VI α3	15.03
Fibrinogen gammapolypeptide	11.79
Leptin receptor	11.31
Serine (or cysteine) proteinase inhibitor, clade F, member 1	6.41
Procollagen type I α2	5.74
Twist gene homolog 1 (*Drosophila*)	4.50
Procollagen type V α2	3.29
Delta-like 1 homolog (*Drosophila*)	2.93
Procollagen type I α1	2.60

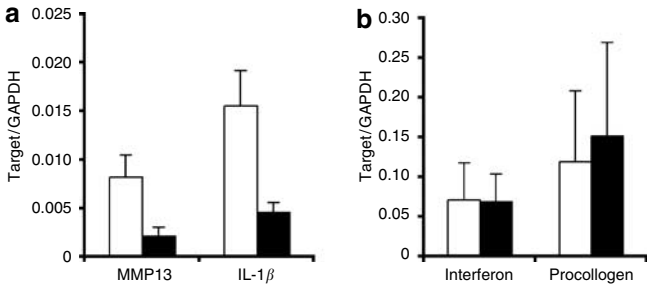

Figure 3. Validation of gene expression changes in K5$^{-/-}$ after doxycycline treatment using TaqMan real-time relative quantitative RT-PCR. (**a**) MMP-13 and IL-1β were decreased in the epidermis of doxycycline-treated K5$^{-/-}$ mice (0.263, 0.295, $P<0.05$). (**b**) No mRNA expression changes of IFN and procollagen. White bar, doxycycline untreated ($n=6$); black bar, doxycycline treated ($n=6$).

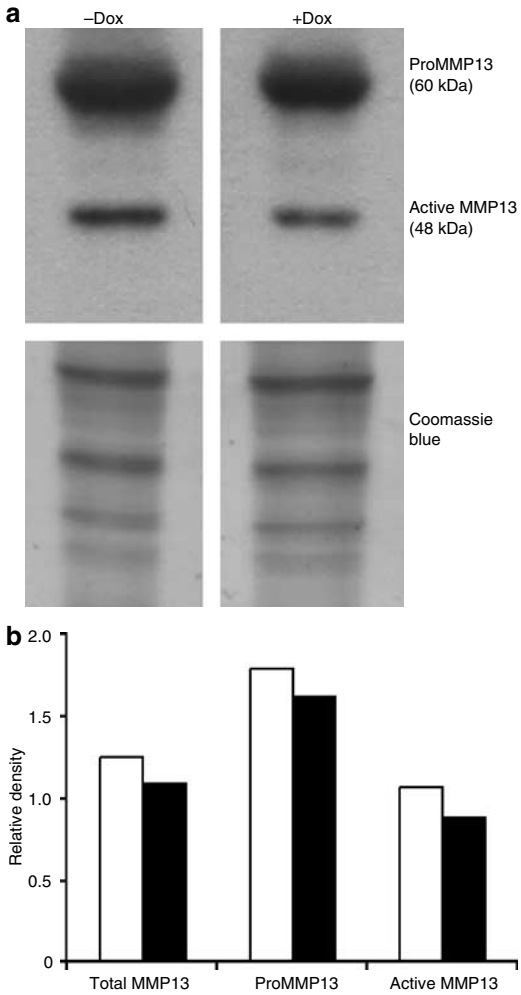

Figure 4. Analysis of MMP-13 in protein extracts of skin of K5$^{-/-}$ mice. (**a**) Equal amounts (30 μg) of protein extracts, isolated from epidermis (epi) of K5$^{-/-}$ mice treated/untreated with doxycycline were assayed by immunoblot analysis for the presence of latent (60 kDa) and active (48 kDa) and MMP-13. Coomassie Blue staining (lower panel) of a gel run in parallel was used as loading control. (**b**) Protein levels normalized to Coomassie Blue staining, normalization of this representative experiment is shown.

rescue K5$^{-/-}$ mice and support the previous clinical study (Retief *et al.*, 1999; Weiner *et al.*, 2004).

DISCUSSION

TNF-α is not involved in blister formation in EBS

TNF-α is a multifunctional cytokine and belongs to a family of ligands that can bind to a family of receptors. The pleiotropic actions of TNF range from proliferative responses such as cell growth and differentiation to inflammatory effects and the mediation of immune responses, to destructive cellular outcomes such as apoptotic and necrotic cell death mechanisms (MacEwan, 2002). Although some skin disease like psoriasis – a disease of keratinocyte proliferation and inflammation, which follow infiltration by activated T-helper cells and mononuclear cell and the release of TNF-α, EBS is

clearly a well-established mechanobullous disease resulting from mutations in epidermal keratins with subsequent skin fragility and keratinocyte cytolysis, particularly following mild mechanical stress (Fuchs and Cleveland, 1998). Recently, however, the secretion of TNF-α was reported from keratinocytes transiently transfected with K14 Arg125Cys. The subsequent stimulation of TNF-R1 and caspase-dependent apoptosis in an autocrine/paracrine pathway were suggested to play an important role in the mechanism linking keratin aggregates to cell death (Yoneda *et al.*, 2004). Using the same mutant K14 ArgR125Cys stably transfected into the same keratinocytes, we were unable to identify any changes in TNF-α release by several, highly sensitive methods. In support, the analysis of $K5^{-/-}$ mice by microarray analysis and quantitative analysis of TNF-α at the protein level have not shown any difference compared with wt mouse samples. Therefore, our *in vitro* and *in vivo* data strongly argue that TNF-α is not involved in EBS. Possibly, the setting of the previous experiment – transiently transfected cells – has contributed to the apparent difference.

We note, that on the other hand, IL-6 and IL-1β are significantly upregulated in $K5^{-/-}$ skin. IL-1 is an important mediator of inflammation and tissue damage in multiple organs, both in experimental animal models of disease and in human diseases. The IL-1 family consists of two agonists, IL-1α and IL-1β, two receptors, biologically active IL-1RI and inert IL-1RII, and a specific receptor antagonist, IL-1Ra. Epidermal keratinocytes have been shown to produce IL-1α and IL-1β and their receptors. IL-6 is similar to IL-1 in that it stimulates T- and B-cell proliferation and production of acute-phase reactants (Kishimoto, 1989), and it has been found to stimulate keratinoncyte growth *in vitro* (Gallucci *et al.*, 2004). Both IL-1β and IL-6 mediate local inflammation. This is consistent with local inflammation that can accompany rare and severe cases of EBS (Fine and Johnson, 1988). We posit that cytokine secretion in EBS can result from cytolysis, a process known to involve mechanical and/or oxidative stress in keratinocytes. Furthermore, EBS keratinocytes are in an activated state, as indicated by the expression of K6 (Fuchs and Cleveland, 1998).

Doxycycline can repress inflammation and remodeling tissues in an EBS mouse model

In the past 20 years, remarkable advances have been made in our understanding of major subtypes of EBS. These include the more complete characterization of the breadth, severity, and relative frequency of cutaneous and extracutaneous manifestations within each EBS, and elucidation of the molecular basis of most EBS subtypes (Fuchs *et al.*, 1994; Fine and Eady, 1999). Although eventually some of this knowledge may result in the successful development of beneficial gene therapies, at present there are no treatments available that appear to result in long-term reduction in skin blistering or improvement of mechanical skin fragility in EBS. This was particularly disappointing, because even those patients with the most localized forms of EBS experience tremendous alterations in lifestyle and quality of life. Malkinson and co-workers described their experience with oral tetracycline treatment of a father and son affected with the Dowling–Meara subtype of EBS (Retief *et al.*, 1999). Tetracycline was initially administered to a patient to treat acne, and significant reduction in both the number of blisters and skin fragility were noted. A dose-dependent response was observed, with an effective dose of 1,500 mg/day. An identical response was observed in the affected parent. Most importantly, a long-standing benefit was observed, with recurrence of disease activity as the dosage of tetracycline was reduced. This study did not address the mechanism of tetracycline affecting skin blister formation in a keratin disorder such as EBS.

In this study, we applied doxycycline to $K5^{-/-}$ mice, an animal model of EBS, to get a first insight into the mechanism of tetracycline action in diseases including EBS. Doxycyline was chosen due to several reasons. First, it is used widely in tet-on or tet-off systems in mice, where the correct dose and additional parameters have been optimized (Gossen and Bujard, 1992; Boy *et al.*, 2006). Second, doxycycline can be administered orally and is rapidly and almost completely absorbed from the gastrointestinal tract and the interference by food with absorption of doxycycline is not significant. The most important finding in our study is that doxycycline extended the survival time of $K5^{-/-}$ mice from 1 to 8 hours. This was accompanied by changes in the transcriptional activity of ~160 genes. This change puts a cautionary note on the widespread use of tetracycline derivatives in conditional transgenesis in mice.

By microarray and Taqman realtime PCR analysis, the mRNA-encoding MMP-13 decreased after doxycycline treatment. This demonstrated that doxycycline might downregulate MMP-13 activity that controls matrix degradation and thereby reduce bullae formation. This is also consistent with observation from patients in which tetracycline suppressed the formation of new bullae (Retief *et al.*, 1999; Weiner *et al.*, 2004). Doxycyclines and other derivatives of tetracycline are potent MMP inhibitors (Sapadin and Fleischmajer, 2006). Studies with chemically modified tetracyclines have shown that the antibiotic and anti-MMP activities lie in different regions of the molecule; with the antibiotic activity residing at the dimethylamino group at the carbon-4 position of the A ring, whereas the anti-MMP activity resides in the carbon-11 carbonyl oxygen and carbon-12 hydroxyl groups (Ryan *et al.*, 1996). On the basis of this anti-MMP activity, they have been used to reduce tissue degradation in aortic aneurysms (Petrinec *et al.*, 1996; Boyle *et al.*, 1998; Curci *et al.*, 1998, 2000; Kaito *et al.*, 2003), periodontal disease (Ryan *et al.*, 1996), as well as arthritis (Greenwald *et al.*, 1987, 1998; Brandt, 1995; Steinmeyer *et al.*, 1998; Shlopov *et al.*, 1999), and are used to prevent tumor cell invasion, metastasis (Seftor *et al.*, 1998), tumor angiogenesis (Tamargo *et al.*, 1991), arterial remodeling (Bendeck *et al.*, 2002), and ocular surface repair (Smith and Cook, 2004).

Using transcriptional profiling, our study has revealed that the positive effect of doxycycline on blister formation is not only exerted by dowregulation of MMP-13, but also by a significant downregulation of IL-1β, decreasing

inflammation. Of note, the Dowling–Meara subtype of EBS is characterized by the presence of inflammatory blister (Fine and Eady, 1999). Moreover, EBS with circinate erythema, accompanied by local inflammation has been described (Gu *et al.*, 2003). It will be informative to investigate inflammatory cytokines in such patients. The effect of tetracycline derivatives are in line with an inhibited induction of IL-1 β-converting enzyme to reduce inflammation and to protect against focal cerebral ischemia (Yrjanheikki *et al.*, 1999). Moreover, doxycycline was reported to inhibit IL-1 in the corneal epithelium and to be most effective in the treatment of inflammatory ocular conditions (Solomon *et al.*, 2000; Smith and Cook, 2004). Although our results were not confirmed in protein level, this might be due to the oral application of doxycycline to pregnant $K5^{+/-}$ females starting from E13.5. Possibly, the level of the drug decreases quickly in newborn $K5^{-/-}$ embryos that might prevent further improvement. We did not succeed in administering drugs to neonatal $K5^{-/-}$ pups. In the clinical report, a long-term improvement was observed, with recurrence of disease activity as the doses of tetracycline was reduced (Retief *et al.*, 1999).

Recently, a number of reports have revealed that well-known antibiotics play multiple roles beyond their antibacterial activities: tetracyclines reduce prion infectivity through a direct interaction with the proteinase-resistant form of the prion protein (Forloni *et al.*, 2002), and penicillin and ceftriaxone have potential as neurotherapeutics for treating neurodegenerative disease by acting at the trancriptional level (Rothstein *et al.*, 2005). Given the large number of drugs already approved by the FDA, a systematic screen of these compounds for additional targets may not only unravel their mode of action but also provide novel therapy options in diseases difficult to treat.

MATERIALS AND METHODS

Animal treatment

$K5^{+/-}$ and $K5^{-/-}$ mice (BALB/c) were originally generated in our lab (Peters *et al.* 2001). All mice were kept under the rule of the animal license 50.203.2-BN 24, 20/04. Experiments were approved by the Regierungspraesidium Koeln (Germany). To get doxycycline-treated or -untreated neonatal pups, doxycycline (50 μg/ml doxycycline with 5% sucrose) was administered by oral route to pregnant $K5^{+/-}$ females (mated to $K5^{+/-}$ males) starting from E13.5. Five percent sucrose solution was administered to the control group.

Total RNA isolation

Skin was removed from neonatal mice and immediately frozen in liquid nitrogen. RNA was extracted with TRIzol reagent (Life Technologies-BRL, Karlsruhe, Germany) according to the protocol of the supplier. Briefly, 50–100 mg of tissue samples were homogenized in 1 ml TRIzol reagent with a power homogenizer and then extracted with 0.2 ml chloroform. The aqueous phase was precipitated by 0.5 ml isopropyl alcohol. The RNA pellets were washed with 75% ethanol and dissolved in RNase-free water. RNA concentration was determined by UV spectrophotometer, and then RNA quality was checked on a 0.8% agarose gel. Total RNA samples were stored at -80°C.

Microarray analysis

Mouse MGU74Av2 microarrays (Affymetrix, Santa Clara, CA) were used for large-scale gene expression profiling in epidermis or skin after doxycycline treatment *versus* controls. The following six groups of tissues were used in the study. Three groups were $K5^{-/-}$ epidermis, $K5^{-/-}$ skin, and wt skin treated with doxycycline, another three groups were control. Six animals were included in each group. All the total RNA samples were isolated from tissues taken immediately after birth, and were pooled for later microarray experiments. Array hybridization was carried out at the core facility of the University of Münster. Scanned images were analyzed to identify the differentially expressed genes with MA5.0 software (Affymetrix).

Real-time RT-PCR

Quantification of five selected transcripts was performed by relative quantitative real-time RT-PCR (ABI PRISM 7700 SDS; PE Biosystems, Foster City, CA) (Table 5) (Chen *et al.*, 2001). We carried out relative quantification of starting mRNA copy numbers according to the threshold cycle (Ct) method (Fink *et al.*, 1998). Glyceraldehyde-3-phosphate dehydrogenase was used as the endogenous reference gene as it does not exhibit significant expression changes between EBS and control mice, or doxycycline-treated and -untreated mice. The reaction was performed in a MicroAmp Optical 96-Well Reaction Plate. Eurogentec Reverse Transcriptase qPCR Master Mix (cat. no. RT-QPRT-032X, Eurogentec, Scraing, Belgium) was used for a one-tube-one-step RT-PCR in a total reaction volume of 12.5 μl. Relative quantification started from each reaction containing 10 ng of total RNA. Reaction conditions were optimized for primers, probes, and $MgCl_2$ concentration as outlined. Cycling conditions were 48°C for 30 minutes for RT step, followed by 95°C for 10 minutes, and a two-step PCR with 60 cycles of 95°C for 15 seconds and 59°C for 1 minute.

Cell culture

Four cell lines were used throughout this study: human HaCaT keratinocytes stably transfected with wt K14 or with K14(Arg125Cys), human MCF7-wt K14-, and MCF7-K14Arg125Cys-transfected (Werner *et al.*, 2004). Cells were grown on polystyrene flasks or dishes at 37°C in a 95% O_2/5% CO_2 atmosphere. Cells were maintained in DMEM with 10% fetal calf serum, penicillin/streptomycin (2 mM), and L-glutamine (100 U/ml).

ELISA

The content of TNF-α, IL-6, and IL-1β in mouse skin or epidermis, as well as TNF-α in cell culture medium and lysate were measured with a solid-phase ELISA kits (Biosource, Camarillo, CA), according to the manufacturer's instruction. Briefly, mice were killed immediately after birth. Skin and epidermis of $K5^{-/-}$ mice was obtained by using tweezers. For preparation of wt epidermis and whole-cell homogenates, we used the method described by Arnott et al. (2004). The total protein concentration was determined using the BCA protein assay. Identical amounts of total proteins were applied for ELISA detection. The absorbance of each well was measured at 450 nm with a BioRad spectrophotometer.

Immunofluorescence analysis

Preparation of tissue samples and immunofluorescence analysis were carried out as before (Peters *et al.*, 2001). The following primary antibodies were used: AF 138 against K5 (1:600, Hiss

Table 5. Sequences and concentration ratios of primers and hydrolysis probes of reference genes with labeling dyes (F for FAM as reporter dye, T for TAMRA as quencher dye), which were used for real-time RT-PCR

Gene	Forward (f)/reverse (r) primer/hydrolysis probe	Primer probe concentration (nM) (f/r/p)	$MgCl_2$ concentration (nM)	GenBank accession number
GAPDH	(f), 5′-TGCCAAGTATGATGACATCAAGAAG-3′ (r), 5′-TAGCCCAGGATGCCCTTTAGT-3′ F5′-TGGTGAAGCAGGCGGCCGAG-3′T	300/300/100	5	BC083065
Chemokine (C–C motif) ligand 1	(f), 5′-TCCAGTGTTACAGAAAGATGGGC-3′ (r), 5′-TTCTCTACCTTTGTTCAGCCTGAAT-3′ F5′-TCCTGTCCTGATCCCCCAGCTGTG –3′T	50/50/100	5	M23501
Procollagen, type III, á1	(f), 5′-CATGTTTGGTTTGGAGAATCTATGA-3′ (r), 5′-CACATCAACGACATCTTCAGGAA-3′ F5′-TGGTGGTTTTCAGTTCAGCTATGGCCC -3′T	300/900/100	7	AK079113
Collagenase 3	(f), 5′-GCAGTTCCAAAGGCTACAACTTGT-3′ (r), 5′-GGAGTGATCCAGACCTAGGGAGT-3′ F5′-TTGTTGCTGCCCATGAGCTTGGC–3′T	300/300/100	5	X66473
IL-1β	(f), 5′-CAAAGGAAAGAATCTATACCTGTCCTGT-3′ (r), 5′-CTTGGGATCCACACTCTCCAG-3′ F5′-TGAAAGACGGCACACCCACCCTG -3′T	300/900/100	5	M15131
IL-6	(f), 5′-ACACATGTTCTCTGGGAAATCGT-3′ (r), 5′-AAGTGCATCATCGTTGTTCATACA-3′ F5′ TGAGAAAAGAGTTGTGCAATGGCAATTCTGA 3′T	300/300/100	5	X54542
IFN-activated gene 202B	(f), 5′-AAAGCTACTAAAAGAATCTTTTGAAGGG-3′ (r), 5′-TAAATAGTTTTGTTGCTTTCAATGCC-3′ F5′-TGGTTACCACAAAGGTCCCAAACAAGTGG-3′T	300/300/100	5	M31418

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Diagnostics, Freiburg, Germany), α-CK14.2 against K14 (1:700, kindly provided by L. Langbein, German Cancer Research Center, Heidelberg, Germany), M18 against TNF-α (1:10, Santa Cruz Biotechnology, Santa Cruz, CA), Image analysis and processing were performed using the AxionVision 4.3 (Carl Zeiss, Goettingen, Germany) and Adobe Photoshop 6.0 software. Secondary antibodies used for immunofluorescence studies in recommended dilutions were Alexa 488A- or Alexa 594-conjugated IgG (Molecular Probes, Leiden, The Netherlands). Immunofluorescence analysis of cells was described by our lab (Werner *et al*., 2004).

Immunoblot analysis

For preparation of mouse skin extracts, frozen mouse tissue was minced and extracted in 2% SDS/phosphate-buffered saline solution, sonicated, and incubated overnight at 4°C. The homogenate was cleared by centrifugation at 14,000 r.p.m. at 4°C for 15 minutes and processed for immunoblot analysis. Protein concentration was assessed using the BCA® Protein assay according to the manufacturer's instructions (Pierce, Rockford, IL). Proteins were separated on SDS-polyacrylamide gels (10% acrylamide) with 10 μg of the samples. Gels were run in duplicate, one gel was stained with Coomassie Blue (Serva, Heidelberg, Germany), the other was transferred to nitrocellulose membranes and transfer efficiency controlled by Ponceau Red staining (Sigma-Aldrich, St Louis, MO). Membranes were blocked with 5% milk in TBST for 1 hour at room temperature. The MMP-13 antibody K73/6, kindly provided by Dr R M Hembry (School of Biological Sciences, University of East Anglia, Norwich, UK), was used at a dilution of 1:500 in TBST. After several washings with TBST, bound primary antibody was revealed using a peroxidase-conjugated secondary antibody and specific immunocomplexes were detected using an ECL western blotting detection kit (Amersham, Freiburg, Germany). Quantification of signal intensities was performed using ImageQuant software supplied to ''Personal Densitometer'' from Molecular Dynamics (Krefeld, Germany). Signal intensities of specific bands were normalized to the intensity of the total proteins detected by Coomassie Blue staining.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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