The Antimicrobial Protein Psoriasin (S100A7) Is Upregulated in Atopic Dermatitis and after Experimental Skin Barrier Disruption

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The innate defense of the skin against microbial threats is influenced by antimicrobial proteins (AMP). *Staphylococcus aureus* often colonizes the skin of patients with atopic dermatitis (AD). This was explained by diminished expression of AMP including cathelicidin/LL-37, human β-defensins-2 and -3, and dermcidin. The S100-protein psoriasin is an additional keratinocyte-derived AMP that preferentially kills *E. coli*. As *E. coli* infections are not observed in atopic skin we investigated the functional role of psoriasin in AD patients. Immunohistochemistry demonstrated enhanced epidermal psoriasin expression in AD. An up to 1500-fold increase in secreted psoriasin was detected by ELISA *in vivo* on the surface of AD skin compared to healthy control skin. Surprisingly, tumor necrosis factor-α-enhanced psoriasin release in primary keratinocytes was inhibited by the Th2-cytokines IL-4 and -13, whereas IL-17 and -22 induced psoriasin. Epidermal barrier disruption significantly enhanced psoriasin expression as demonstrated by tape stripping in healthy volunteers. The upregulation of psoriasin in AD maybe induced by the disrupted skin barrier offering a possible explanation why these patients do not suffer from skin infections with *E. coli*. This indicates that the antimicrobial response in AD is not generally impaired, but greatly differs according to the type of AMP produced by the skin.

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INTRODUCTION

In general, the epidermis is constantly exposed to a variety of microbial challenges. The permeability barrier, localized in the stratum corneum, is a mechanical protective hurdle against bacterial infection. The first column in antimicrobial defense is the physical barrier in the stratum corneum, which is supported by the (normal) microflora, antimicrobial lipids, and antimicrobial proteins (AMP; Bibel *et al.*, 1989; Elias, 2007; Jensen *et al.*, 2007; Aberg *et al.*, 2008). Impairment of the physical barrier by injury is a prerequisite for experimental skin infection by the application of a bacterial suspension (Singh *et al.*, 1971). In atopic dermatitis (AD) and psoriasis vulgaris, skin diseases that are associated with a disturbed permeability barrier function, the epidermis is colonized with a higher rate and potentially pathogenic microorganisms (Aly *et al.*, 1976, 1977; Leung, 2003; Baker, 2006). In a mouse model with a chronically impaired skin barrier induced by an essential fatty acid-deficient diet, microbial colonization with *Staphylococcus aureus* was enhanced (Bibel *et al.*, 1989).

Despite significant changes in bacterial colonization and an impaired permeability barrier, psoriatic epidermis is usually free of the symptoms of infection (Henseler and Christophers, 1995), whereas in AD, clinical signs of impetiginization are predominantly found in severe cases and after extensive scratching (Lubbe, 2003). Nevertheless, both diseases can be aggravated by bacterial ''superantigens'' that bypass the normal control of T-cell activation (Skov and Baadsgaard, 2000) and colonization with superantigen- and toxin-producing *S. aureus* can aggravate the disease.

The unexpected low incidence of infections in psoriatic skin suggests additional cutaneous defense mechanisms besides the physical barrier. One of these defense mechanisms comprises the production of AMP, which are found abundant in lesional psoriatic scales (Harder and Schröder, 2005a, b) and are generated by various epithelial cells (reviewed in (Ganz and Lehrer, 1998; Schröder and Harder, 2006)). Several types of AMP including the human β-defensins (hBD) 1–3 (Fulton *et al.*, 1997; Harder *et al.*, 1997, 2001, 2004), the cathelicidin LL-37 (Frohm *et al.*, 1997), dermcidin (Schittek *et al.*, 2001), RNase-7 (Harder and Schröder, 2002), and psoriasin (Gläser *et al.*, 2005) were

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Abbreviations: AD, atopic dermatitis; AMP, antimicrobial protein(s); hBD, human β-defensins; TNF-α, tumor necrosis factor-α

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identified in human skin. Keratinocyte-derived AMPs protect healthy skin from surrounding microorganisms and may regulate the composition of the normal flora (Dinulos *et al.*, 2003; Chung and Dale, 2004). Some of the AMPs (hBD-1, RNase-7, lysozyme) are constitutively expressed by keratinocytes (Harder *et al.*, 2007). A few—such as psoriasin—are focally present at different levels in healthy skin (Gläser *et al.*, 2005) and inducible upon skin inflammation (Madsen *et al.*, 1991) or wounding (Lee and Eckert, 2007), whereas others (hBD-2, -3, LL-37) are found only in inflamed but not in healthy skin (Abiko *et al.*, 2002; Sorensen *et al.*, 2003, 2005; Li *et al.*, 2004; Harder and Schröder, 2005a, b). AMP are inducible by proinflammatory cytokines including IL-1β, tumor necrosis factor-α (TNF-α) (Harder *et al.*, 2000, 2004), IL-17 and -22 (Liang *et al.*, 2006; Wolk *et al.*, 2006), after bacterial contact (Harder *et al.*, 2000; Kisich *et al.*, 2007), during epidermal differentiation (Harder *et al.*, 2004) or wound healing (Sorensen *et al.*, 2003). In addition, the expression of LL-37 in keratinocytes appears to be induced by $1,25(OH)_2$ vitamin D3 (Wang *et al.*, 2004; Weber *et al.*, 2005; Schauber *et al.*, 2007).

Recently, we identified the S100-protein psoriasin as an additional AMP in healthy human epidermis (Gläser *et al.*, 2005). At lower doses, psoriasin preferentially kills *E. coli*. At higher concentrations, however, it exhibits also bactericidal activity against *Pseudomonas aeruginosa* and *S. aureus*. Psoriasin, originally identified from psoriatic keratinocytes (Madsen *et al.*, 1991), is constitutively expressed at rather high levels in skin and upregulated by proinflammatory cytokines or bacterial compounds (Gläser *et al.*, 2005). Psoriasin was also shown to function as a potent T-cell- and neutrophil-chemotactic agent (Jinquan *et al.*, 1996; Zheng *et al.*, 2008).

Patients with AD often suffer from skin infections caused by bacteria and viruses (Leung, 2003; Wollenberg *et al.*, 2003). Among bacterial superinfections *S. aureus* is the predominant pathogen. Approximately 90% of AD patients were shown to be colonized with *S. aureus* compared to only 5–30% in a control population (Leung, 2003). Previously unknown observations suggest that an impaired expression of AMP may contribute to the increased susceptibility of AD skin to *S. aureus*. It has been demonstrated that the AMP hBD-2 and LL-37 are expressed to a lesser extent in the epidermis of AD patients, when compared to psoriatic epidermis (Ong *et al.*, 2002). Another study reported a disturbed upregulation of hBD-3 in AD skin (Nomura *et al.*, 2003a, b). This may be of particular importance because hBD-3 is known to be an important AMP produced by keratinocytes to control the growth of *S. aureus* (Kisich *et al.*, 2007). In addition, the sweat gland-derived AMP dermcidin was found to be diminished in AD patients (Rieg *et al.*, 2005). Together, these data support the hypothesis that a deficiency of AMP could contribute to the increased susceptibility of AD skin to infection with *S. aureus*.

Interestingly, AD patients do not suffer from skin infections caused by *E. coli*, although this is the most common gut bacterium (Baker, 2006). On the basis of the recently discovered potent *E. coli*-cidal activity (Gläser *et al.*, 2005; Li *et al.*, 2005), we hypothesized that psoriasin expression might be increased in AD patients, thereby contributing to the resistance of atopic epidermis to *E. coli* infections despite the impaired epidermal barrier. Here, we show that psoriasin indeed is enhanced in AD and thus differently regulated than the other AMP. We also provide evidence that the increased expression of psoriasin may be driven by the disrupted epidermal barrier, representing a kind of autoprotective mechanism.

RESULTS

Psoriasin immunoreactivity and secretion is enhanced in atopic dermatitis skin *in vivo*

The expression of psoriasin in the epidermis was first examined at the protein level by immunostaining using a specific antibody. For that purpose, biopsies were taken from four AD patients. Lesional skin of all four patients revealed increased psoriasin expression compared to nonlesional skin and control biopsies of healthy volunteers at the same anatomical localization (Figure 1a). The intensity of psoriasin immunostaining in the lesional skin of AD patients was comparable to that found in lesional psoriatic skin (Figure 1b).

To investigate whether the increased psoriasin expression in the skin of patients with AD leads to enhanced secretion of psoriasin, washing fluids of standardized areas of nonlesional and lesional skin were obtained and analyzed for psoriasin concentrations using a specific psoriasin ELISA. A markedly enhanced secretion with up to 1500-fold increase of soluble psoriasin was observed in the washing fluids derived from AD lesional skin when compared to nonlesional skin and normal controls (Figure 2). Notably nonlesional skin exhibited significantly enhanced levels of secreted psoriasin when compared to healthy control skin. The difference between nonlesional and lesional atopic skin was also shown to be significant ($P<0.0005$).

TNF-α-induced psoriasin secretion is inhibited by Th2 cytokines in primary keratinocytes

The Th2 cytokines IL-4 and -13 were found to be increased in AD skin and have been attributed to be responsible for the downregulation of hBD-2 and -3 and the cathelicidin LL-37 (Ong *et al.*, 2002; Nomura *et al.*, 2003a, b). Thus, we asked in turn whether both cytokines are involved in the regulation of psoriasin as well. To address this issue, cultured primary keratinocytes were stimulated with the Th2 cytokines IL-4 and -13 alone and in combination with the proinflammatory cytokine TNF-α that was shown to be upregulated in the epidermis of patients with AD (Sumimoto *et al.*, 1992; Ackermann and Harvima, 1998; de Vries *et al.*, 1998; Junghans *et al.*, 1998; Antunez *et al.*, 2006). Secreted psoriasin was determined in the supernatants by ELISA (Figure 3a). Stimulation with TNF-α significantly induced the release of psoriasin, whereas neither IL-4 nor IL-13 had a significant impact on the basal psoriasin expression. In contrast, both IL-4 and -13 markedly inhibited the induction of psoriasin by TNF-α. The combination of IL-4 and -13 did not yield an additive or synergistic inhibitory effect.

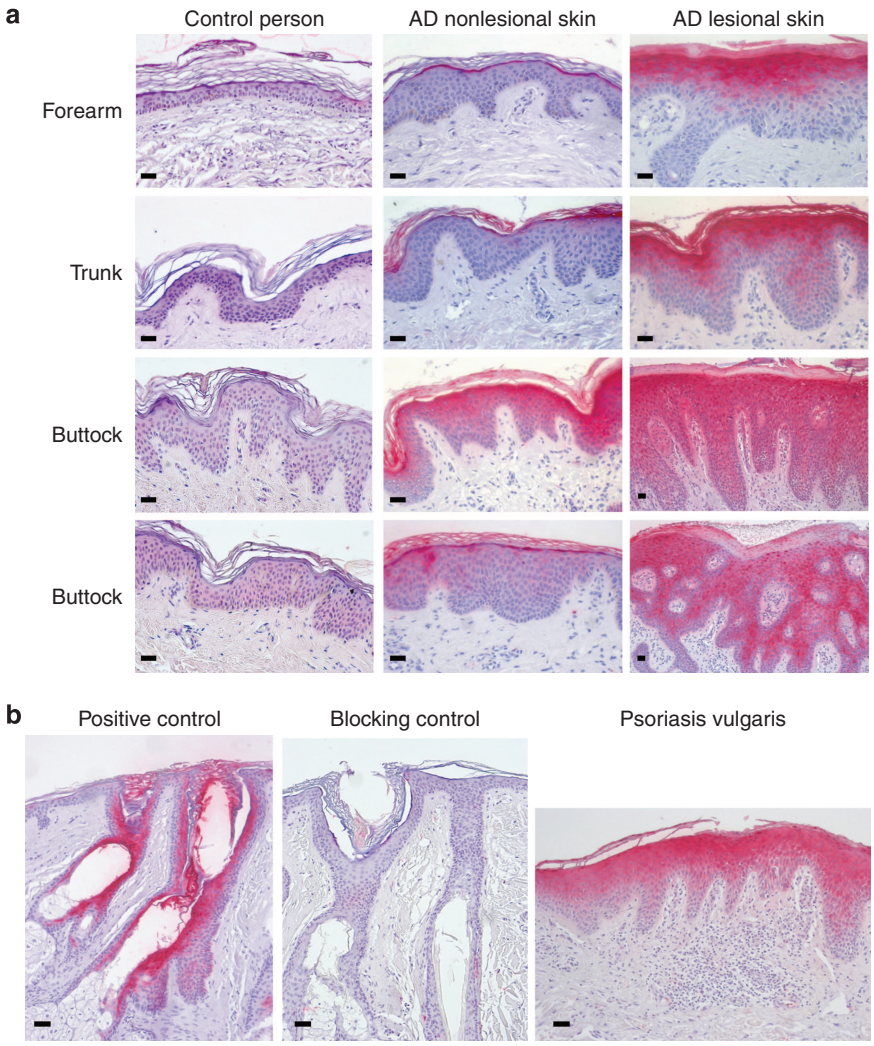

Figure 1. Psoriasin immunoreactivity is enhanced in lesional atopic dermatitis skin. (**a**) To localize the expression of psoriasin in human skin biopsies of AD patients and normal controls immunohistochemical staining was performed. Lesional skin of AD patients revealed increased psoriasin expression compared to nonlesional skin and healthy controls at correlating sites. Bars indicate 50 μm. (**b**) Specificity testing was performed by preincubation of the antibody with various concentrations of natural skin-derived psoriasin. Blocking the antibody with higher concentrations than 0.06 μg psoriasin resulted in complete inhibition of immunoreactivity in a tissue sample derived from the face of a healthy person. Immunostaining of psoriasis vulgaris served as a further positive control. Bars indicate 50 μm.

IL-17 and -22 induce psoriasin secretion in primary keratinocytes

As recently a possible pathogenic role of Th17 cells was reported for AD (Koga *et al.*, 2008), primary keratinocytes were additionally stimulated with IL-17 and/or -22. An enhanced secretion of psoriasin was observed 16 hour after stimulation with both cytokines and the combination of IL-17 and -22 resulted in an additive effect (Figure 3b). Stimulation of keratinocytes with IL-17 and -22 in the presence of Th2 cytokines IL-4 and -13 did not alter upregulated psoriasin secretion (data not shown).

Psoriasin secretion is enhanced after experimental disruption of the skin barrier

As it is known that patients with AD exhibit skin barrier disruption with enhanced levels of transepidermal water loss (Loden *et al.*, 1992; Jensen *et al.*, 2004), we hypothesized that an artificial disruption of the epidermal barrier could lead to an increase in psoriasin secretion. Indeed, a rapid increase of soluble psoriasin was observed directly after tape stripping. Increased soluble psoriasin was measured at nearly constant levels 30 and 120 minutes after skin injury performed in healthy volunteers ($n=12$, Figure 4a). Most interestingly, secretion of psoriasin remained elevated even 7 days after skin barrier disruption.

To investigate the influence of experimental barrier repair on psoriasin expression, additional experiments were performed under occlusion ($n=8$). Washing fluids collected at two time points showed enhanced expression 1 and 24 hour after tape stripping, independently from occlusion (Figure 4b). Median psoriasin concentrations rose from 2.63 ng/ml (0.86–7.61) after 1 hour to 10.95 ng/ml (5.26–67.90) without occlusion ($P<0.01$) and 5.88 ng/ml (1.87–36.85) under occlusion. At 24 hour after tape stripping median psoriasin

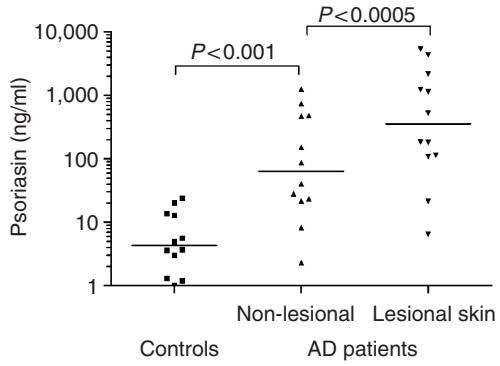

Figure 2. High concentrations of psoriasin are secreted in atopic dermatitis (AD) skin. A defined area (0.55 cm^2) of the skin of AD patients and matching normal controls was rinsed with sodium-phosphate buffer (pH 7.4). Soluble psoriasin concentrations were determined using a psoriasin-specific ELISA with two mAbs generated against natural psoriasin. Each sample was tested in triplicates. Bars indicate the median concentration. Groups were compared using the Wilcoxon matched pairs test ($n=12$).

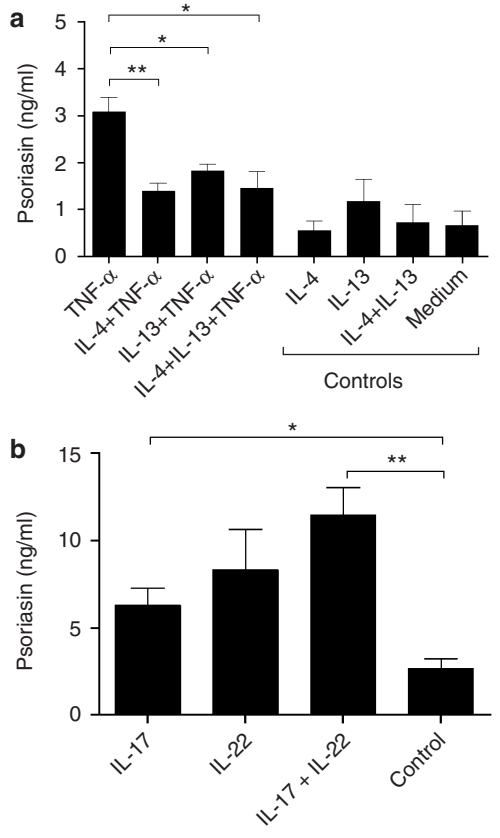

Figure 3. TNF-α induced psoriasin secretion is inhibited by Th2 cytokines and Th17 cytokines induce psoriasin secretion in primary keratinocytes. (**a**, **b**) Psoriasin concentration was measured by ELISA in the supernatants of cultured primary keratinocytes after incubation with different cytokines (TNF-α, IL-4, and -13: 50 ng/ml, IL-17, and -22: 20 ng/ml) for 16 hour. One representative out of three independent experiments is shown. Each stimulation was performed in triplicates. Bars indicate the SEM. Significance levels were determined in comparison to ''TNF-α-values'' (**a**) or the medium control (**b**) using the unpaired Student's *t*-test. *$P<0.05$; **$P<0.01$.

concentrations were at 7.52 ng/ml (1.54–27.13) without ($P<0.01$) and at 6.33 ng/ml (1.72–14.68) under occlusion. Differences at 1 hour between both procedures were statistically significant ($P<0.05$). Spearman's rank correlation analyses did not reveal any correlation between transepidermal water loss values and psoriasin concentrations in the individual groups (data not shown).

Accordingly, significantly enhanced psoriasin expression was also detected by immunohistochemistry in tissue samples obtained after tape stripping. Biopsies taken 24 hour after tape stripping ($n=2$) revealed impressive upregulation of psoriasin expression in the upper epidermal layers compared to the untreated control (Figure 4c) derived from the corresponding localization. Immunoreactivity appeared less intensive after occlusion (Figure 4c), decreased at later time points (day 4), and disappeared at day 7 ($n=3$, data not shown).

DISCUSSION

Human skin is permanently exposed to a variety of potentially harmful microorganisms but usually remains free of infection. The physical barrier is of central importance for antibacterial defense. However, disruptions of the barrier by small injuries like scratches are common and do not necessarily result in skin infection. The importance of keratinocyte-derived AMP as part of a ''chemical barrier'' in healthy and diseased skin is today well established (Nizet *et al.*, 2001; Elias, 2005, 2007). The identification of various AMP (hBD-2 (Harder *et al.*, 1997) and -3 (Harder *et al.*, 2001), psoriasin (Madsen *et al.*, 1991; Gläser *et al.*, 2005), and calprotectin (Clohessy and Golden, 1995)) from lesional psoriatic skin might explain the phenomenon that these patients suffer from significantly fewer skin infections than expected (Henseler and Christophers, 1995).

In contrast, hBD-2, -3, and LL-37 were shown to be expressed at decreased levels in acute and chronic lesions from patients suffering from AD (Ong *et al.*, 2002; Nomura *et al.*, 2003a, b) when compared with psoriasis vulgaris. Elevated amounts of Th2 cytokines present in atopic skin were discussed to cause the inhibition of the expression of AMP in the inflamed skin, as IL-4 and -13 *in vitro* suppressed the cytokine-mediated induction of hBD-2 and -3 (Ong *et al.*, 2002; Nomura *et al.*, 2003a, b). It was recently shown that the relative deficiency in hBD-3 expression may be an acquired defect as neutralization of the Th2 cytokine milieu in skin explants from AD patients restored the hBD-3 expression (Howell *et al.*, 2006). The same group reported that patients with ''extrinsic AD'', characterized by elevated serum immunoglobulin E and allergic sensitization, as well as patients with ''intrinsic AD'' showed both reduced expression of hBD-2, -3, and LL-37 (Howell *et al.*, 2005, 2006). Additionally, the sweat of subjects with AD was recently found to be deficient in the sweat gland-derived AMP dermcidin when compared with healthy subjects (Rieg *et al.*, 2005). These data suggest that an impaired induction of AMP may contribute to the increased susceptibility of skin infections in patients with AD.

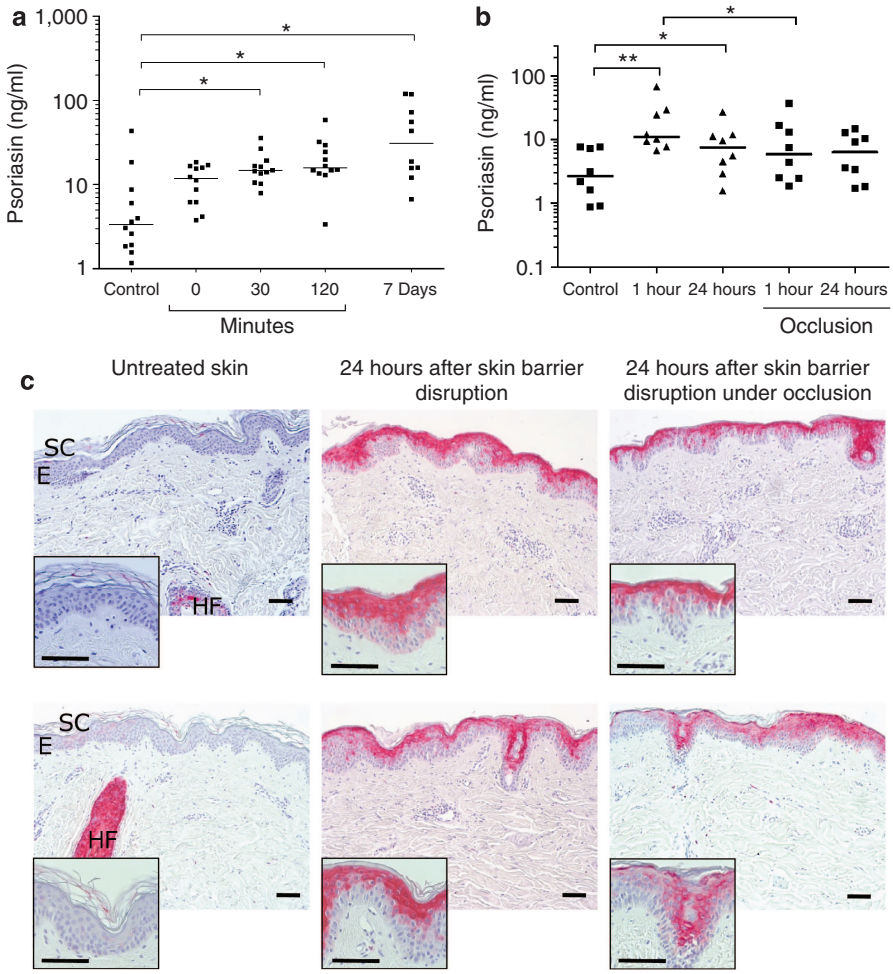

Figure 4. Psoriasin secretion is enhanced after experimental disruption of the skin barrier in healthy persons. (**a**) A defined area (0.55 cm^2) of the forearm skin of healthy volunteers ($n = 12$) was rinsed with sodium-phosphate buffer (pH 7.4) before (''control'') and after tape stripping at indicated time points. Psoriasin concentration in 1,000 µl washing fluid was determined using a psoriasin-specific ELISA. Each sample was tested in triplicates. Bars indicate the median concentration. All time points were compared to the control values using the Wilcoxon matched pairs test. *$P < 0.05$. (**b**) A defined area (0.55 cm^2) of both forearms of healthy volunteers ($n = 8$) was rinsed with sodium-phosphate buffer (pH 7.4) before (''control'') and after tape stripping at indicated time points and the test area of one arm was held under occlusion for 24 hour. Psoriasin concentration in the washing fluids was determined as indicated in (**a**). Both time points under investigation were compared to the control values using the Wilcoxon matched pairs test. *$P < 0.05$, **$P < 0.01$. (**c**) Immunohistochemical analysis of psoriasin expression in skin biopsies taken 24 hour after experimental skin barrier disruption by tape stripping without and under occlusion in comparison to the untreated control at the same localization (forearm). The upper and lower panels represent two different individuals. Bars indicate 50 µm. SC: Stratum corneum, E: epidermis, HF: hair follicle.

On the basis of these observations one may conclude that the antimicrobial response in atopic patients is generally impaired. However, in this context the fact that AD patients do not suffer from skin infections caused by the common gut bacterium *E. coli* is quite challenging. A first hint of an induced psoriasin expression in AD was reported by DNA microarray analysis (Sugiura *et al.*, 2005). Our finding that psoriasin protein is abundant on AD skin together with the known high *E. coli*-killing activity suggests that psoriasin may contribute to the resistance of atopic epidermis against colonization and infection with *E. coli*. The observation that psoriasin secretion was shown to be significantly upregulated in lesional and nonlesional AD skin may be explained by the generally impaired barrier and enhanced microbial colonization rate in these patients. The factors that are responsible for the high induction of psoriasin in AD skin are not known. Acute lesions of AD are characterized by the overexpression of Th2 cytokines, especially IL-4 and -13 (Jeong *et al.*, 2003; Homey *et al.*, 2006). But these cytokines do not appear to be responsible for the induction of psoriasin in AD as they did not alter the basal expression of psoriasin in human keratinocytes *in vitro*. However, TNF-α-induced upregulation of psoriasin in cultured keratinocytes was inhibited by cocultivation of keratinocytes with IL-4 and/or -13. In this context it has to be mentioned that the role of TNF-α in AD is controversially discussed and therapeutic inhibition of TNF-α was shown to be hardly efficient (Nomura *et al.*, 2003a, b; Buka *et al.*, 2005; Guttman-Yassky *et al.*, 2007; Machura *et al.*, 2007). On the first glance, these *in vitro* data may contradict our *in vivo* findings. However, the concept that AD is a purely Th2-driven disease is not any longer valid as it has been shown that in the chronic phase Th1 cytokines

appear to be more relevant (Grewe *et al.*, 1994; Leung and Bieber, 2003). Previously we could demonstrate that IFN-γ and IL-1β are able to induce psoriasin secretion besides TNF-α (Gläser *et al.*, 2005). Thus, other factors than TNF-α and/or Th2 cytokines may contribute to the extensive psoriasin induction observed in the *in vivo* situation of AD patients.

Th17 cells were identified as a previously unknown subset of $CD4^+$ T cells, characterized by the production of IL-17 and coexpression of IL-22 (Harrington *et al.*, 2005; Liang *et al.*, 2006). Recently, Th17 cells were reported to be increased in the peripheral blood of AD patients and infiltrated the papillary dermis of acute atopic skin lesions (Koga *et al.*, 2008). We demonstrate here that stimulation of primary keratinocytes with IL-17 and/or -22 resulted in an increased psoriasin secretion, a phenomenon that has been described before on the mRNA level (Liang *et al.*, 2006; Wolk *et al.*, 2006). In our *in vitro* studies, IL-17 and -22 were the most efficient factors to stimulate psoriasin secretion. However, one has to be aware that the definite functional role of Th17 cells and their cytokines in AD remains to be elucidated.

Another trigger for the upregulation of psoriasin may be the stimulation by bacteria and their products. Psoriasin was shown to be induced both *in vitro* and *in vivo* by bacterial culture supernatants (Gläser *et al.*, 2005) and flagellin has been identified as one of the responsible pathogen-associated molecular pattern molecule (Abtin *et al.*, 2008). Atopic skin is colonized by a high density of microorganisms, for example, for *S. aureus* 10^7 organisms per cm^2 (Leung, 2003). Thus, it remains to be determined whether *S. aureus* or other skin colonizing bacteria are able to induce psoriasin and why psoriasin—despite the high concentrations observed in AD patients *in vivo*—is not able to control their growth. The pathogenetic relevance of the chemotactic activity reported for psoriasin (Jinquan *et al.*, 1996) is still unclear as well.

In addition to our knowledge it is previously unreported that the disturbed epidermal barrier is a major inducer of psoriasin expression *in vivo*. AD is characterized by a chronically impaired skin barrier function (Jensen *et al.*, 2004; Rim *et al.*, 2005). One reason for this alteration may be the reduced expression of filaggrin (Palmer *et al.*, 2006). It was recently shown in mice that mRNA and protein expression of murine β-defensin-3 and cathelin-related antimicrobial peptide, representing the murine homologues of hBD-2 and LL-37, was increased 1–8 hour after acute permeability barrier disruption and normalized after 24 hour (Aberg *et al.*, 2008). The tape stripping experiments performed in this study are in accordance with these observations, indicating that skin barrier disruption may be one of the driving forces of the enhanced psoriasin expression and secretion in AD skin.

The pronounced and rapid induction of psoriasin may be an attempt to prevent skin infection in cases of a disrupted skin barrier. The increase in psoriasin secretion occurred early within the first 2 hour and maintained elevated up to 7 days. We speculate that preformed psoriasin located in the stratum corneum is released after experimental skin barrier disruption in normal controls as well as in diseases with endogenous skin barrier defects such as psoriasis and AD. Disruption of the epidermal barrier does not only lead to a fast release of preformed psoriasin from the stratum corneum, which is detectable in the washing fluids, but also to a prolonged protein release on the skin surface over several days after injury. Complete repair of the permeability barrier in humans after tape stripping takes about 1 week, implying that the increased secretion of psoriasin is present during the entire time frame of the disrupted skin barrier. Interestingly, experimental barrier repair by occlusion reduced the increase in psoriasin expression, supporting a direct link between skin barrier function and psoriasin expression.

This link may also explain the constant overproduction of psoriasin after experimental wounding. Lee and Eckert (2007) observed increased expression of psoriasin in wound exudates and granulation tissue obtained 72 hour after setting an abrasion wound in one human volunteer. These findings support the results of our skin barrier disruption experiments that included a higher number of individuals and also measured the *in vivo* secretion of psoriasin shortly after skin injury over a period of up to 7 days. In conclusion, skin wounding and barrier disruption may be the key factor leading to increased psoriasin secretion in AD, whereas the impact of other factors such as the cytokine milieu or pathogens remains to be determined.

MATERIALS AND METHODS

Patients and normal controls

In total, 12 Patients (2–41 years, women/men: 8/4) affected by AD and without specific pretreatment within the last 2 weeks were included in the study (Table 1). The severity of AD was assessed

Table 1. Characteristics of normal controls and patients enrolled in the study

				Psoriasin concentration (ng/ml)			
No.	Sex	Age	Localization	Controls	AD:NL	AD:L	SCORAD
1	M	20	Forearm	1.2	152	185	n.d.
2	F	2	Wrist	20.3	470	1,140	40
3	F	38	Forearm	1.3	87	1,234	23
4	F	3	Arm flexure	23.7	739	4,392	33
5	F	18	Wrist	1.0	23.4	182	18
6	M	37	Trunk	4.9	21.7	109	53
7	F	22	Buttock	3.6	1,252	5,409	69.5
8	F	23	Forearm	12.8	28.1	527	n.d.
9	M	21	Buttock	3.0	476	2,175	33.5
10	F	33	Forearm	3.7	8.2	21.5	49.5
11	F	36	Forearm	5.6	2.3	6.5	23
12	M	41	Lower leg	13.7	40.4	114	n.d.

AD, atopic dermatitis patients; F, female; NL, nonlesional skin (same localization as L); L, lesional skin; M, male; SCORAD, scoring of atopic dermatitis; n.d., not done.
Healthy volunteers (controls) and AD patients were matched by gender and age and washings fluids and biopsies were derived in each case from the same localization. Therefore sex, age, and localization were only listed once for both collectives.

using the ''SCORing Atopic Dermatitis'' according to the European Task Force on AD (Kunz *et al.*, 1997). Healthy persons matched by age and gender were chosen as controls. The study was approved by the ethical committee of the University Kiel (AZ A 104/06) in adherence to the Helsinki Guidelines and all patients and healthy normal controls gave written informed consent.

Collection of skin-derived washing fluids

To determine the active secretion of psoriasin, standardized areas (0.55 cm^2) of untreated lesional and nonlesional skin of AD patients were rinsed with 900 µl of 10 mM sodium-phosphate buffer (pH 7.4) and a corresponding localization was rinsed in the normal controls. Samples were mixed with 100 µl sodium-phosphate buffer (10 mM) containing 10% (w/v) bovine serum albumin (Sigma, Deisenhofen, Germany) and supernatants were stored after centrifugation of samples at −80 °C until further processing.

ELISA

To measure the psoriasin concentrations in skin-derived washing fluids and in primary keratinocyte culture supernatants, a psoriasin-specific sandwich ELISA was performed as described previously (Gläser *et al.*, 2005). A standard curve was created with serial dilutions of skin-derived psoriasin and all samples were processed in triplicates. The detection limit was 0.3 ng/ml psoriasin.

Immunohistochemistry

Punch biopsies were taken in local anesthesia from lesional and nonlesional skin in AD patients ($n=4$) and normal controls ($n=5$). Biopsies derived from the same anatomical localization (mostly contralateral site in AD patients and comparable site in the controls). Fixation of the tissue samples was performed in 4% paraformaldehyde. To localize the expression of psoriasin in human skin biopsies of AD patients and controls immunohistochemical staining was performed. Paraffin sections (5 µm) of the tissue samples were deparaffinized and rehydrated before heat-induced antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0). The slides were blocked with normal rabbit serum (1:75, Dako Cytomation, Glostrup, Denmark) before staining. Immunohistochemical staining was performed at room temperature for 1 hour using a monoclonal antibody (HL15-4, kindly provided by Hans Lange, Institute of Experimental Surgery, University Hospital of Schleswig-Holstein, Campus Kiel) directed against natural skin-derived psoriasin as described previously (Gläser *et al.*, 2005). A biotinylated secondary rabbit anti-mouse IgG (1:100, Dako Cytomation) antibody was used, followed by incubation with Vector Universal ABC Alkaline Phosphatase Substrate Kit (Vector, Burlingame, CA, USA) developed with Vector NovaRED Substrate (Vector) and counterstained with hematoxylin. Negative control straining was performed by omitting the primary antibody. Psoriatic lesions served as a positive control as they exhibit extensive positive staining within the differentiated epithelial layers from the stratum spinosum up to the stratum corneum. Specificity was determined by western blotting and by blocking experiments with preincubation of the antibody and different concentrations of the antigen as described previously (Gläser *et al.*, 2005).

Keratinocyte culture and stimulation with cytokines

Primary human keratinocytes were prepared from tissue obtained after plastic surgery procedures as described (Wiedow *et al.*, 1998) and were grown in EpiLife-Medium (Sigma, Taufkirchen, Germany). For stimulation, cells were grown in six-well-culture plates (9.6 cm^2 per well, Falcon) and were used after the second passage at a confluence of 70–80%. Cells were stimulated with IL-4 or -13 (50 ng/ml, PeproTech, Rocky Hill, NJ), IL-17 (20 ng/ml, PeproTech), IL-22 (20 ng/ml, R&D Systems, Wiesbaden, Germany), or TNF-α (50 ng/ml, PeproTech) for 16 hour and the culture supernatants were harvested and frozen at −80 °C until further processing by ELISA.

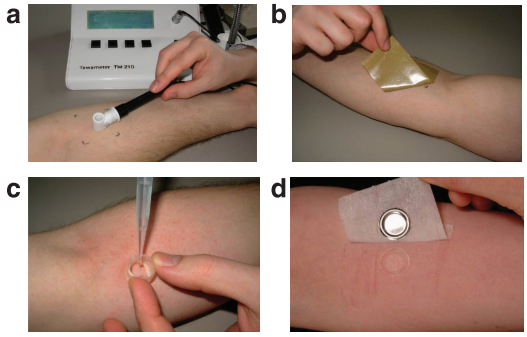

Figure 5. Experimental setting of the *in vivo* experiments to determine the active secretion of antimicrobial psoriasin. (**a**) Determination of the transepidermal water loss (TEWL) of a standardized area on the forearm before and during skin barrier disruption. (**b**) Serial tape-stripping to reach a TEWL of 40g/m^2 per hour. (**c**) Collection of washing fluids derived from the untreated forearm and the pretreated area at different time points. (**d**) Additional occlusion experiments were performed using Finn Chambers on Scanpor.

Experimental disruption of the skin barrier

Tape stripping (Tesa Pack original, ultra strong, Tesa Ag, Hamburg, Germany) of a standardized area of the forearm skin was performed in healthy volunteers ($n=12$, 24–48 years, women/men: 7/5) until a transepidermal water loss of 40 g/m^2 per hour monitored by the Tewameter TM210 (Courage and Khazaka, Cologne, Germany) was reached. In a second experiment both forearms were treated as described above ($n=8$, 25–50 years, women/men: 6/2) and occlusion was performed on one arm for up to 24 hour after experimental skin barrier disruption. Large Finn Chambers on Scanpor were used for effective occlusion (Epitest Ltd. Oy, Tuusula, Finnland). *In vivo* washing fluids (10 mM sodium-phosphate buffer, pH 7.4), derived from the untreated forearm and the pretreated area were collected at different time points, and the amount of psoriasin was determined by ELISA. Figure 5 demonstrates the experimental setting. In addition, punch biopsies were taken from the untreated forearms and 1, 4, or 7 days after skin barrier disruption for immunohistological analysis of psoriasin expression ($n=5$).

Statistics

Calculations were performed using Wilcoxon's signed rank test (secretion of AMP *in vivo*), the unpaired Student's *t*-test (expression of psoriasin *in vitro*) and Spearman's rank correlation analysis (correlation of transepidermal water loss and psoriasin secretion).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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