Enhanced Expression and Secretion of Antimicrobial Peptides in Atopic Dermatitis and after Superficial Skin Injury

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Human skin can defend itself against potentially invading microorganisms by production of antimicrobial peptides (AMPs). The expression of AMPs in atopic dermatitis (AD) is still emerging. To gain more insight into the role of AMPs in AD, we systematically analyzed the expression of ribonuclease 7 (RNase 7), psoriasin, and human β-defensins (hBD)-2 and -3 in AD compared with psoriatic and healthy control skin as well as after experimental barrier disruption. Immunostaining revealed enhanced expression of all AMPs in the lesional skin of untreated AD and psoriasis when compared with non-lesional skin and controls. Accordingly, induced *in vivo* secretion of RNase 7, psoriasin, and hBD-2 was detected using ELISA on lesional skin in AD and in even higher concentrations in psoriasis. The secretion of AMPs did not correlate with severity of AD and *Staphylococcus aureus* colonization. Skin barrier disruption caused enhanced immunoreactivity of hBD-2 and hBD-3 after 24 hours. Strong secretion of RNase 7 was already detected after 1 hour, whereas hBD-2 secretion was significantly enhanced after 24 hours only under occlusion. Thus, a disturbed skin barrier may trigger AMP induction in AD and psoriasis. The functional role of AMP in AD, especially with regard to the control of *S. aureus* colonization, needs further analysis.

Journal of Investigative Dermatology (2010) **130,** 1355–1364; doi:10.1038/jid.2009.432; published online 28 January 2010

INTRODUCTION

In addition to serving as a physical barrier, human skin harbors a chemical defense system based on the production of antimicrobial lipids and proteins. Antimicrobial peptides (AMPs) and proteins represent a diverse group of small, mainly cationic endogenous proteins that show antimicrobial activity against bacteria, fungi, and viruses. Extensive research during the past decade has shown that human skin and other epithelial tissues as well as various leukocytes are able to produce a wide variety of AMPs (Harder and Schröder, 2005b).

There is increasing evidence that several AMPs have an important role in cutaneous defense, in particular the human β-defensins (hBD)-2 and hBD-3 (Harder *et al.*, 1997, 2001; Schröder and Harder, 1999; Pazgier *et al.*, 2006), the S100 protein psoriasin (S100A7c) (Gläser *et al.*, 2005), the ribonuclease 7 (RNase 7; Harder and Schröder, 2002), the cathelicidin LL-37 (Gudmundsson *et al.*, 1996; Dorschner *et al.*, 2001; Nizet *et al.*, 2001; Harder and Schröder, 2002), and the sweat gland-derived dermcidin (Schittek *et al.*, 2001). Many of these AMPs are upregulated in keratinocytes upon contact with microorganisms or microbial products (Harder and Schröder, 2005a). This indicates that keratinocytes do not merely function as a physical epidermal barrier, but rather are active participants in innate defense by recognition of microorganisms and subsequent initiation of innate immune responses, such as the rapid induction of AMPs. AMPs are also upregulated under inflammatory conditions and are induced by inflammatory cytokines such as IL-1, IL-17, IL-22, and tumor necrosis factor-α (Harder *et al.*, 2007). Epidermal differentiation and wound healing are other factors that induce the expression of AMPs (Sorensen *et al.*, 2003, 2006; Harder *et al.*, 2004). Various AMPs (e.g., hBD-2 and hBD-3 as well as LL-37) show immunomodulatory functions such as recruitment and activation of antigen-presenting cells (Oppenheim and Yang, 2005).

The role of AMPs in cutaneous biology may be reflected by the increasing number of publications showing an association of AMPs with skin diseases. Several AMPs have been isolated from psoriatic scales. Psoriatic skin is characterized by a high expression of various AMPs that may be locally

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Abbreviations: AD, atopic dermatitis; AMP, antimicrobial peptide; CFU, colony-forming units; hBD, human β-defensin; RNase 7, ribonuclease 7; SCORAD, SCORing of Atopic Dermatitis

Received 18 September 2009; revised 11 November 2009; accepted 16 November 2009; published online 28 January 2010

induced by microorganisms and endogenous proinflammatory cytokines (Harder and Schröder, 2005b). The abundance of AMPs in psoriatic skin offers an explanation as to why patients with psoriasis suffer from fewer cutaneous bacterial and viral infections than expected (Henseler and Christophers, 1995). In contrast to psoriasis, it has been reported that the skin of patients suffering from atopic dermatitis (AD) is characterized by an impaired induction of AMPs such as hBD-2, hBD-3, LL-37, and dermcidin (Ong *et al.*, 2002; Nomura *et al.*, 2003; de Jongh *et al.*, 2005). Potential reasons may include the lack of major AMP inducers in AD skin, such as the cytokines IL-1, IL-17, and IL-22, as well as the suppression of AMP induction by elevated levels of T-helper 2 cytokines, such as IL-4, IL-10, and IL-13. It has been hypothesized that a reduced induction of AMPs in AD may contribute to the increased susceptibility of AD skin to *Staphylococcus aureus* infection (Boguniewicz and Leung, 2006). Most of the studies analyzing AMPs in AD, however, compared AMP expression levels primarily with psoriatic but not with healthy skin (Ong *et al.*, 2002; de Jongh *et al.*, 2005; Guttman-Yassky *et al.*, 2008). Recently, we observed that the expression and secretion of psoriasin was strongly induced in AD when compared with healthy skin, indicating that the expression of AMPs is not generally impaired in AD (Gläser *et al.*, 2009a).

To gain more insight into the role of AMPs in AD, we systematically analyzed both the expression and the *in vivo* secretion of two constitutively produced skin-derived AMPs, RNase 7 and psoriasin, and of two inducible AMPs, hBD-2 and hBD-3, in healthy, psoriatic, and chronic as well as acute atopic skin. In this study, we show that all four AMPs are induced in both non-lesional and lesional skin of psoriatic and atopic patients when compared with the skin of healthy individuals. As already reported for psoriasin, superficial skin barrier disruption by tape stripping in healthy skin also induced the expression of hBD-2, hBD-3, and RNase 7, suggesting that the enhanced expression of AMPs in psoriatic and AD skin may be driven by disrupted epidermal barrier.

RESULTS

Enhanced AMP immunoreactivity in lesional and non-lesional AD and psoriatic skin when compared with healthy controls

We used immunostaining to perform a comparative analysis of the expression of RNase 7, psoriasin, hBD-2, and hBD-3 in the same biopsies derived from normal as well as untreated psoriatic and AD skin. Immunoreactivity of all analyzed AMPs was increased in non-lesional AD (except hBD-2) (Figure 1a) and psoriatic skin (Figure 1b) when compared with skin derived from the same localizations of healthy individuals. A further increment of immunoreactivity of all AMPs was observed in the lesional skin of AD (Figure 1a) and psoriasis (Figure 1b). Quantitative verification of AD immunostainings by systematically scoring immunoreactivity of all epidermal layers confirmed an upregulation of AMP expression (Supplementary Table S1 online). These data indicate that both AD and psoriasis are characterized by an enhanced protein expression of the AMPs RNase 7, psoriasin, hBD-2, and hBD-3, with the highest expression levels in psoriasis.

Enhanced secretion of AMPs in lesional and non-lesional skin of patients with AD and psoriasis when compared with healthy controls

To analyze whether increased AMP expression, detected using immunostaining, is associated with an enhanced secretion of AMPs on the skin surface, we analyzed the *in vivo* secretion levels of AMPs by determining the AMP concentrations in skin-derived washing fluids. For this purpose, washing fluids of standardized skin areas from lesional and non-lesional skin of patients with untreated AD (total $n=38$; chronic AD $n=24$ and acute AD $n=14$) and psoriasis vulgaris ($n=8$) as well as matched controls ($n=34$) were analyzed for AMP levels using ELISA.

Significantly higher RNase 7 levels were detected in the lesional skin of chronic and acute AD as well as psoriasis patients when compared with non-lesional skin and healthy controls (Figure 2a). There was no significant difference when comparing the amounts of RNase 7 in non-lesional skin of AD or psoriasis patients with healthy controls (Figure 2a).

The psoriasin concentration of lesional and non-lesional skin of chronic and acute AD patients was significantly increased with respect to healthy controls (Figure 2b). These data confirm our recent study (Gläser *et al.*, 2009a), but include now a higher sample size and a characterization of the respective bacterial load of the skin areas (see below). A similar pattern was observed for psoriatic skin. The levels of psoriasin were significantly increased in the non-lesional and lesional psoriatic skin when compared with healthy controls (Figure 2b).

The amounts of hBD-2 derived from the surface of lesional skin of AD patients were significantly higher than the amounts detected on the surface of healthy controls (Figure 2c). The same pattern was observed in psoriasis with much higher mean concentrations of secreted hBD-2. No significance was found when comparing non-lesional skin of AD patients and healthy controls, despite a clear trend toward higher levels in non-lesional AD skin (Figure 2c). Levels of hBD-2 were higher in lesional skin of acute AD when compared with chronic AD. Lesional skin of acute AD was characterized by significant higher amounts of hBD-2 when compared with non-lesional skin of acute AD and controls, a finding not observed for chronic AD.

hBD-3 concentrations above the detection limit ($1.25\,ng\,ml^{-1}$) could only be detected in a couple of lesional (total $n=6$; chronic $n=1$ and acute $n=5$) and non-lesional (total $n=3$; chronic $n=2$ and acute $n=1$) AD as well as lesional ($n=3$) and non-lesional ($n=2$) psoriasis samples and were therefore not included in the statistical analysis.

In general, the secreted amounts of AMPs in psoriasis were higher than the levels detected in AD (Figure 2). The secreted amounts of AMPs in AD were highest for psoriasin followed by RNase 7 and hBD-2.

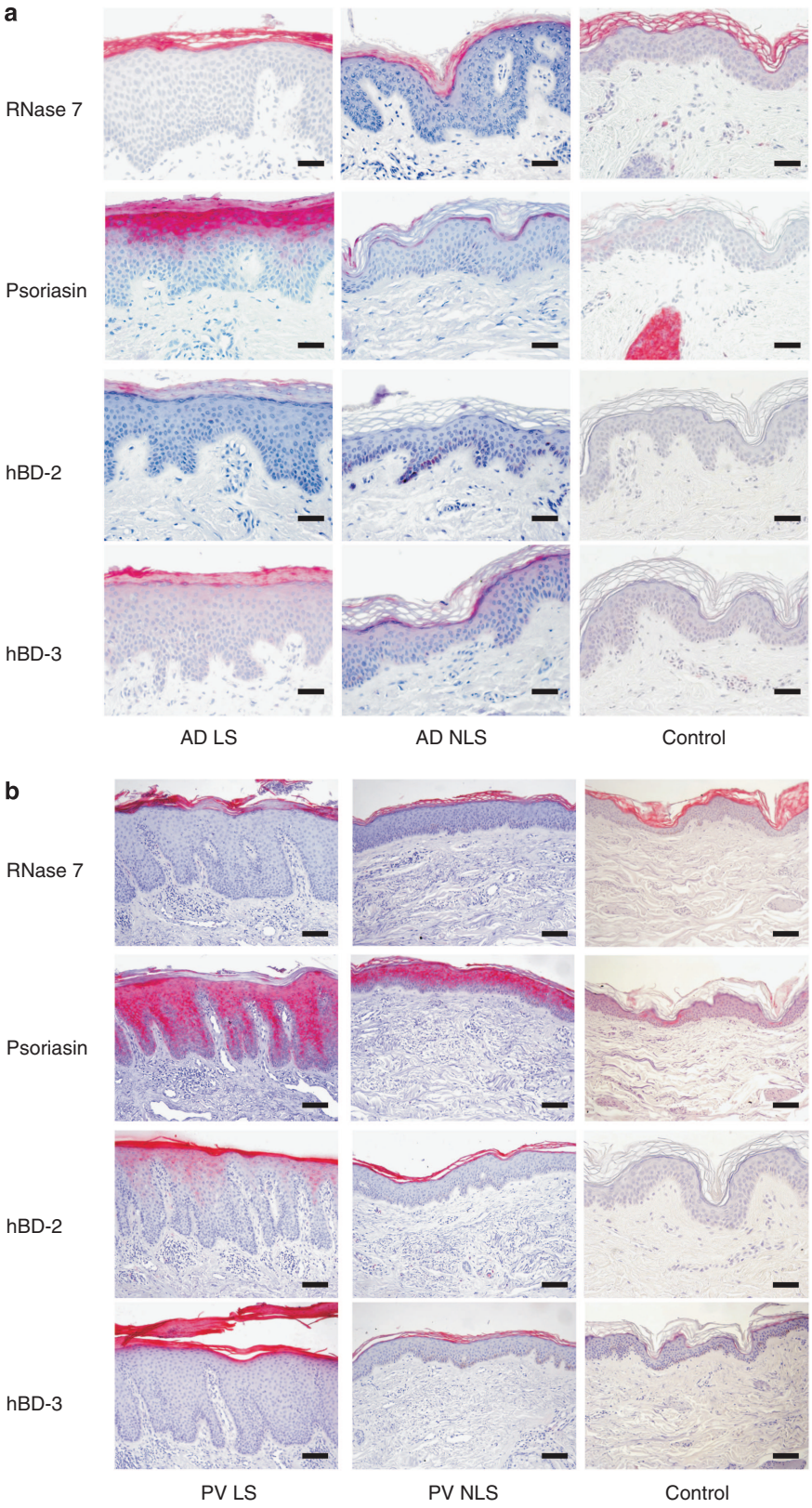

Figure 1. Induction of antimicrobial peptide (AMP) expression in untreated atopic dermatitis and psoriasis skin samples. (**a**) Immunohistochemical analysis of RNase 7, psoriasin, human β-defensin (hBD)-2, and hBD-3 of serial slides of the same skin biopsies derived from forearm lesional (AD LS) as well as non-lesional skin from AD patients (AD NLS) and healthy controls. (**b**) Immunohistochemical analysis of RNase 7, psoriasin, hBD-2, and hBD-3 of serial slides of the same skin biopsies derived from forearm lesional (PV LS) and non-lesional (PV NLS) skin from psoriasis vulgaris (PV) patients and healthy controls. Samples from one representative person (AD = 4, PV = 4, controls = 8) are shown. Scale bar = 100 μM.

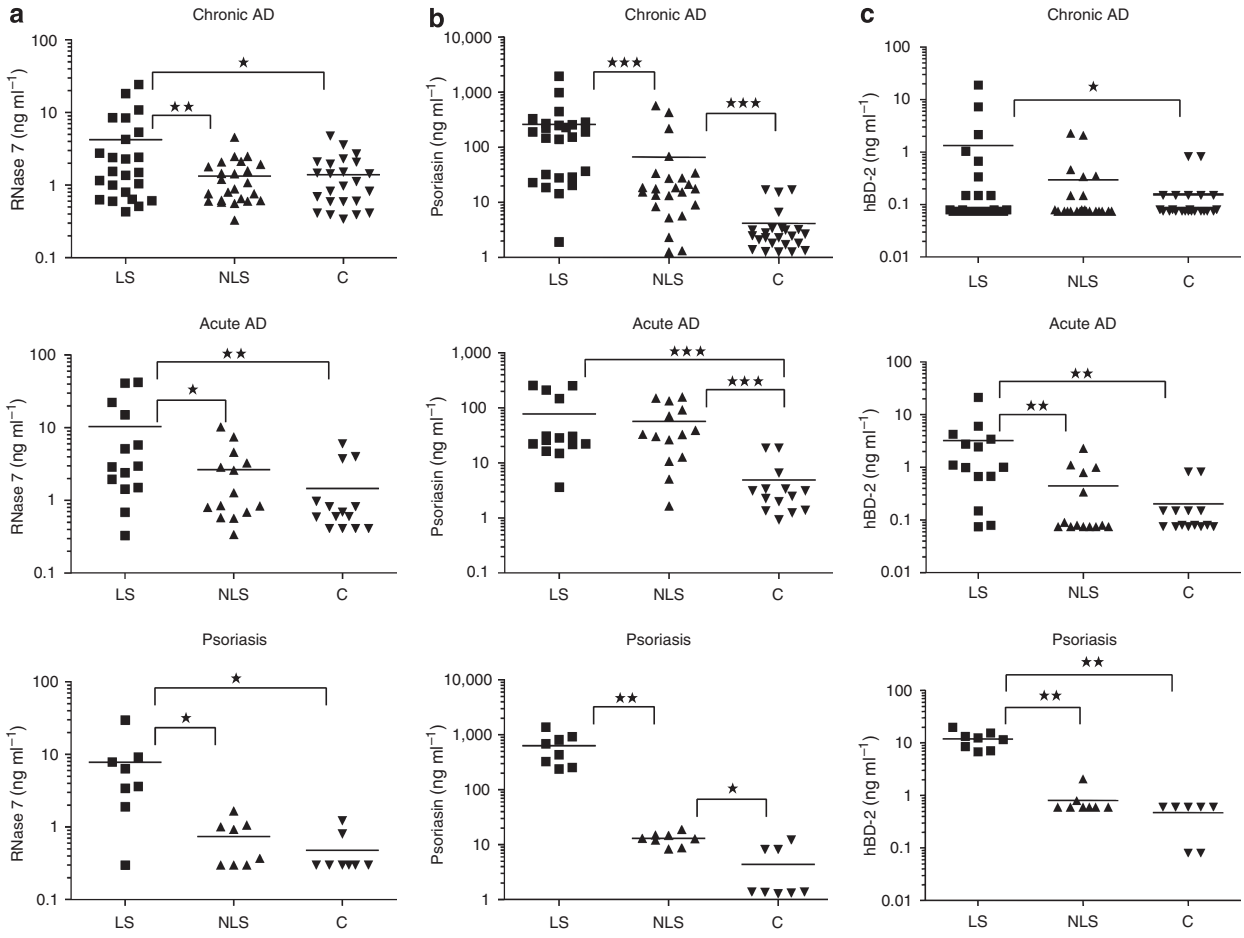

Figure 2. Enhanced secretion of antimicrobial peptides (AMPs) in non-lesional and lesional skin of chronic and acute atopic dermatitis (AD) and psoriasis patients. The *in vivo* secretion levels of (**a**) RNase 7, (**b**) psoriasin, and (**c**) human β-defensin-2 (hBD-2) on chronic and acute AD as well as psoriasis skin surface were analyzed by rinsing a defined skin area (0.55 cm^2) followed by ELISA-based determination of AMP concentrations in the skin-derived washing fluids. (**c**) Lesional skin (LS) and non-lesional skin (NLS) of untreated patients with chronic AD ($n=24$, left panel), acute AD ($n=14$, mean panel), and psoriasis vulgaris ($n=8$, right panel) were compared with matching normal controls. Bars indicate the mean concentration. Groups were compared using the Wilcoxon signed-rank test. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

AMP secretion does not correlate with *S. aureus* colonization or disease severity of AD

To analyze whether the expression of AMPs may correlate with *S. aureus* colonization on the skin of AD patients, we measured the colony-forming units (CFU) of *S. aureus* present in skin washing fluids and performed correlation analyses of skin-derived CFU with the according AMP levels.

First, we compared the CFU of *S. aureus* detected in the skin washing fluids of AD patients with the severity of AD symptoms measured by ''SCORing of Atopic Dermatitis'' (SCORAD). We found a statistically significant correlation between *S. aureus* CFU and SCORAD (Figure 3a). In addition, the *S. aureus* skin colonization rate was much higher in patients with acute AD (93%) when compared with chronic AD (46%). These results are consistent with published findings (Guzik *et al.*, 2005) and indicate the efficiency and validity of our method. In contrast to the correlation between severity of AD and *S. aureus* colonization, no significant correlation was detected between the presence and concentrations of AMPs (RNase 7, psoriasin, and hBD-2) on AD skin and *S. aureus* CFU found on the identical skin surface area (Figures 3b–d). In addition, there was no correlation between AMP levels and coagulase-negative *Staphylococci*, *Micrococci*, and *Corynebacteria* (data not shown). Furthermore, no statistically significant correlation between SCORAD and AMP secretion levels were detected (Figures 3e–g).

Enhanced AMP immunoreactivity and secretion in normal skin upon experimental barrier disruption

Both AD and psoriasis are characterized by a disturbed skin barrier function. Recently, we have reported an increased psoriasin expression upon superficial barrier disruption in AD (Gläser *et al.*, 2009a). To elucidate whether superficial barrier injury may also trigger the expression of RNase 7, hBD-2, and hBD-3, we performed barrier disruption of healthy skin by tape stripping until a transepidermal water loss of 40 g m^{-2} was reached. Immunohistochemistry of skin biopsies taken

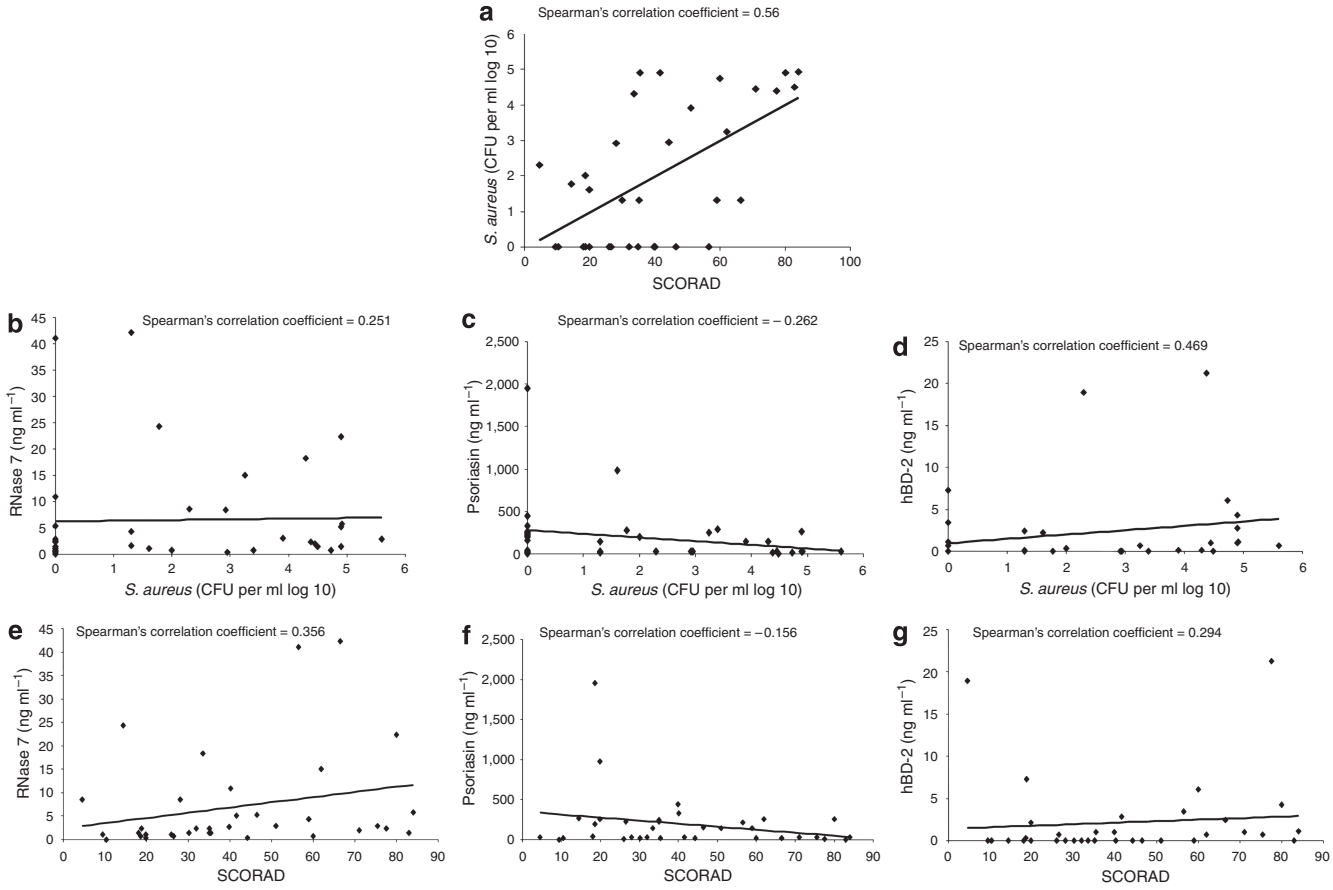

Figure 3. Antimicrobial peptide (AMP) secretion revealed no significant correlation with *Staphylococcus aureus* colonization or disease severity of atopic dermatitis (AD). (**a**) Spearman's rank correlation analysis of *S. aureus* colony-forming units (CFU) present in AD skin washing fluids ($n = 38$) with disease severity of AD measured by "SCORing of Atopic Dermatitis" (SCORAD). (**b–d**) Spearman's rank correlation analysis of *S. aureus* CFU present in AD skin washing fluids ($n = 38$) with the concentration of (**b**) RNase 7, (**c**) psoriasin, and (**d**) human β-defensin-2 (hBD-2). (**e–g**) Spearman's rank correlation analysis of disease severity of AD measured by SCORAD with the concentrations of (**e**) RNase 7, (**f**) psoriasin, and (**g**) hBD-2.

24 hours after tape stripping revealed an upregulation of hBD-3 expression in the uppermost epidermal layers when compared with untreated controls derived from corresponding localizations (Figure 4a). RNase 7 immunoreactivity was not induced by barrier disruption with and without occlusion, whereas hBD-2 immunoreactivity was induced only under occlusion after 24 hours (Figure 4a).

To analyze the influence of experimental barrier disruption on AMP secretion, we analyzed skin washing fluids at different time points after tape stripping. The secretion of RNase 7 increased significantly after 1 hour of barrier disruption independently of occlusion (Figure 4b). At 24 hours after barrier disruption, RNase 7 expression was still enhanced but the secretion levels differed only significantly from control samples when occlusion was applied (Figure 4b). In contrast to RNase 7, hBD-2 secretion was not induced after 1 hour (Figure 4c). Only after 24 hours under occlusion a significant induction of hBD-2 secretion was detected, confirming the immunostaining experiments (Figure 4c). The secretion levels of hBD-3 in all washing fluids were below the detection limit of the ELISA (1.25 ng ml^{-1}).

DISCUSSION

Patients suffering from psoriasis have an unexpected low incidence of cutaneous infection despite the presence of the disrupted skin surface in psoriatic lesions (Henseler and Christophers, 1995). This led to the idea that AMPs could have an important role in the first-line defense of this disease. Isolation of several AMPs from psoriatic lesional skin supported a role of AMPs in protecting psoriatic skin from infection (Harder *et al.*, 2007). In line with this hypothesis, our immunostaining experiments as well as the analysis of AMP secretion on the skin surface revealed an enhanced expression of psoriasin, RNase 7, hBD-2, and hBD-3 in psoriatic skin.

In contrast to psoriasis, the role of AMPs in AD is still emerging. The first studies reported a reduced expression of AMPs in lesional skin of AD, leading to the logical and straightforward hypothesis that an impaired expression and induction of AMPs may contribute to the increased susceptibility of atopic skin to infection, in particular with *S. aureus* (Boguniewicz and Leung, 2006; Lin *et al.*, 2007). However, this issue seems to be more complex as in these studies the

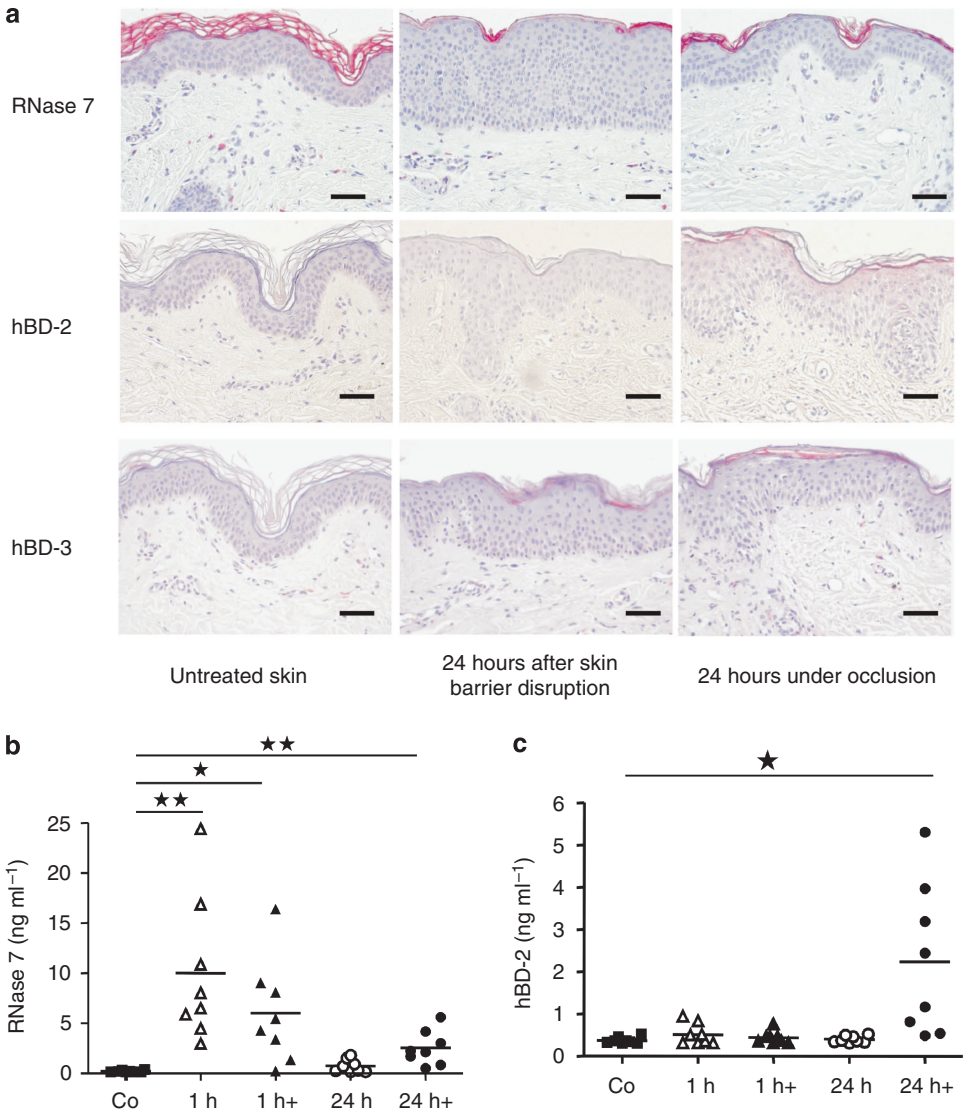

Figure 4. Enhanced antimicrobial peptide (AMP) immunoreactivity and secretion in normal skin upon superficial barrier disruption. Superficial barrier disruption was performed by tape stripping until a transepidermal water loss of 40 g m^{-2} h^{-1} was reached. (**a**) Expression of RNase 7, human β-defensin (hBD)-2, and hBD-3 was analyzed by immunohistochemistry in skin biopsies taken 24 h after tape stripping without and under occlusion in comparison with the untreated control at the same localization (forearm). One representative tissue sample is shown ($n=3$). Scale bar = 50 μM. (**b**, **c**) Secretion of (**b**) RNase 7 and (**c**) hBD-2 was analyzed using ELISA in washing fluids derived from a defined skin area (0.55 cm^2) before (Co) and after tape-stripping at 1 and 24 hours without and under occlusion (+). Bars indicate the mean concentration of samples ($n=8$). The Wilcoxon signed-rank test was used for comparison. *$P<0.05$; **$P<0.01$.

AMP expression was found reduced primarily in comparison to lesional psoriatic skin (Ong *et al.*, 2002; Nomura *et al.*, 2003; Howell *et al.*, 2005; de Jongh *et al.*, 2005; Guttman-Yassky *et al.*, 2008). Interestingly, Ong *et al.* (2002) detected using immunohistochemistry higher hBD-2 staining in several AD patients when compared with healthy controls, thus providing first evidence of an increased expression of antimicrobial peptides in AD skin. Therefore, we started to systematically analyze and evaluate the role of several major epidermal AMPs in a high number of chronic and acute AD patients with respect to lesional and non-lesional skin, disease severity, and bacterial burden, especially *S. aureus*. A comparison with tissues samples and skin washing fluids derived from healthy sex- and age-matched controls as well as from patients with psoriasis vulgaris was performed. In addition, we measured for the first time the *in vivo* AMP protein secretion in correlation with SCORAD and *S. aureus* colonization.

These analyses revealed an induction of all analyzed AMPs in lesional AD skin compared with healthy controls. The data indicate that the induction of AMPs is not generally impaired in the skin of patients suffering from AD. However, the absolute levels of AMP expression and secretion were lower than levels detected in psoriasis, which is in agreement with published results (Ong *et al.*, 2002; Nomura *et al.*, 2003; de Jongh *et al.*, 2005; Howell *et al.*, 2005). Whether these

quantitative differences may still explain the higher susceptibility of AD skin with infections, especially by *S. aureus,* is still emerging.

The lack of major AMP inducers in AD skin, such as IL-1, IL-17, and IL-22, and suppression of AMP induction by elevated levels of T-helper 2 cytokines, shown *in vitro* for IL-4, IL-10, and IL-13, may explain the lower AMP levels in AD when compared with psoriasis (Nomura *et al.*, 2003; Howell *et al.*, 2005). Interestingly, our study revealed that in patients with acute exacerbation of AD, secretion level of RNase 7 and hBD-2 were higher when compared with patients with chronic AD. In addition, hBD-3 was detectable in the lesional skin of six patients, five of whom were characterized by acute exacerbation of AD. These *in vivo* data indicate that elevated T-helper 2 cytokines, which have been associated with acute AD, are not sufficient enough to inhibit the induction of AMPs, such as RNase 7, hBD-2, and hBD-3, in the acute AD lesion.

To gain further insight into the role of AMPs in regulating *S. aureus* colonization in AD, we compared the secretion of various AMPs with cutaneous colonization by *S. aureus*. First, we verified a correlation of increased *S. aureus* colonization with a higher SCORAD level (Guzik *et al.*, 2005), confirming the reliability of our method to analyze *S. aureus* in the skin washing fluids. In contrast, we could not detect any significant correlation between the secretion of AMPs (RNase 7, psoriasin, hBD-2, and hBD-3) and *S. aureus* colonization. In addition, no significant correlation between AMP secretion and SCORAD was detected. These data, together with the observed upregulation of AMP in AD, support the hypothesis that the increased susceptibility of AD skin to *S. aureus* colonization and infection may not mainly be caused by reduced amounts of AMP. However, we cannot exclude that other AMPs not included in our study may have an important role regarding *S. aureus* colonization in AD. It has been shown, e.g., that the sweat-gland-derived AMP, dermcidin, is deficient in the sweat of AD patients (Rieg *et al.*, 2005). Another explanation could be the expression of yet-unknown factors inhibiting the antimicrobial activity of AMPs in AD patients.

In accordance with our data, a recent publication by Ballardini *et al.* (2009) reported enhanced expression of LL-37 in lesional AD skin. Using quantitative PCR as well as immunostaining they detected higher levels of LL-37 in lesional skin than in non-lesional skin, but did not find any correlation between LL-37 expression and severity of AD. In addition, Gambichler *et al.* (2008) also reported messenger RNA induction of AMPs in psoriasis and AD, although they did not differentiate between epidermis and deeper compartments of the skin and did not analyze AMP expression at the protein level.

The enhanced expression of AMPs in AD skin may protect the skin from colonization by different bacteria that are therefore not regularly found in AD. Psoriasin, e.g., as a potent *E. coli*-cidal AMP may prevent cutaneous *E. coli* infections in healthy as well as psoriatic and AD skin (Gläser *et al.*, 2005, 2009a; Harder and Schröder, 2005b). The *in vivo* relevance of psoriasin for preventing colonization and infection with *E. coli* has been shown by *in vivo* application of neutralizing antibodies (Gläser *et al.*, 2005). The clinical observation that *E. coli* does not represent a harmful germ in either psoriasis or AD patients underlines this concept. Furthermore, the induced expression of AMPs in AD skin may control a kind of stable *S. aureus* colonization rate in these patients who rarely develop severe deep tissue infections or sepsis despite the disturbed skin barrier (Hanifin and Rogge, 1977). Accordingly, a recent review of the literature revealed only a rare number of cases of invasive *S. aureus* infections associated with AD (Benenson *et al.*, 2005).

Because AD is characterized by a chronically impaired skin barrier function, we analyzed whether a disturbed barrier influences the expression of AMPs. Recently, it was shown that epidermal barrier disruption in mouse led to the enhanced expression of the antimicrobial peptides mouse β-defensin-3 and cathelin-related antimicrobial peptide (Aberg *et al.*, 2008). In our study we report for the first time that a barrier disruption of healthy human skin leads to a rapid secretion of RNase 7 already after 1 hour of superficial skin injury by tape stripping. As RNase 7 shows a potent broad spectrum of antimicrobial activity (Harder and Schröder, 2002), the fast release of RNase 7 during cutaneous injury may contribute to a rapid defense shield, protecting superficially wounded skin from microbial colonization.

Interestingly, expression of hBD-2 was not induced by skin barrier disruption alone, but only upon subsequent occlusion for 24 hours. This may indicate that occlusion triggers the expression of factors that selectively induce the expression of hBD-2 but do not induce other AMPs, such as RNase 7 and hBD-3. Prolonged occlusion of human skin may lead to hyperhydration, which causes irritation, inflammation, and cytokine induction (Warner *et al.*, 2003). This sequence of events is well known in occupational dermatology after wearing occlusive gloves for several hours and this phenomenon may explain our observation of enhanced hBD-2 expression after occlusion.

Several metabolic events, e.g. increase in lipid synthesis, are ameliorated by occlusion with an impermeable membrane after skin barrier disruption (Grubauer *et al.*, 1989). However, occlusion also leads to irritation and induction of cytokines (Wood *et al.*, 1994). It has been shown that induction of cytokines by skin barrier disruption in mouse skin is only partially reduced by occlusion. Irritation is even more pronounced after skin barrier disruption and occlusion in human skin (Welzel *et al.*, 1996). Therefore, the lacking effect of occlusion on the reduction of RNase 7 and hBD-2 expression after skin barrier disruption does not mean that skin barrier disruption is not the crucial factor. Nevertheless, we cannot rule out that tissue damage itself, which also releases cytokines, is an additional factor leading to increased AMP synthesis.

Although we could not quantify hBD-3 secretion above the detection limit of the ELISA in the skin washing fluids, the immunostaining experiments revealed an upregulation of hBD-3 after 24 hours of experimental barrier disruption. The induction of hBD-3 after barrier disruption probably occurs as a consequence of the induction of growth factors. Growth

factors regulate hBD-3 expression (Sorensen *et al.*, 2003) and may be crucially involved in the repair of the superficial wounds after skin barrier disruption without significant inflammation. Expression of growth factors, however, seems to be slightly reduced by occlusion after skin barrier disruption (Liou *et al.*, 1997). This may explain why hBD-3 expression is induced by skin barrier disruption alone but is not further enhanced after occlusion. Recently, it has been shown that sterile skin wounding induced by a scalpel led to the enhanced expression of hBD-3 after 4 days, a process that required the activation of the epidermal growth factor receptor (Sorensen *et al.*, 2006). Whether activation of the epidermal growth factor receptor is also required for the enhanced hBD-3 expression after superficial barrier disruption remains to be analyzed (Hirsch *et al.*, 2009). In a very recent paper it was shown that hBD-3 significantly promotes wound closure in *S. aureus*-infected diabetic wounds (Hirsch *et al.*, 2009).

Recently, hBD-3 has been reported to serve as an important AMP, participating in the antimicrobial activity of normal human skin against *S. aureus* (Kisich *et al.*, 2007). Our immunostaining experiments clearly indicate that hBD-3 is induced in non-lesional and lesional AD skin, denying a general expression defect of hBD-3 in AD. However, in the washing fluids hBD-3 was only hardly detectable in a few samples. Even in the barrier disruption experiments, no hBD-3 secretion above the detection limit of the ELISA (1.25 ng ml^{-1}) could be observed, suggesting that the skin secrets only low levels of hBD-3. However, given the strong expression of hBD-3 in the immunostaining experiments, it is also possible that hBD-3 is not efficiently elutable with aqueous buffer solutions because the highly cationic hBD-3 may bind to negatively charged structures present in the epidermis, which hampers an efficient release of hBD-3 into the washing fluids.

In summary, the expression and secretion of the AMPs, RNase 7, psoriasin, hBD-2, and hBD-3, is on one hand, as reported previously, reduced in the skin of AD patients when compared with psoriatic skin, and on the other, clearly induced in comparison to healthy skin. In addition, no correlation of AMP secretion and S. *aureus* colonization or severity of AD was detected. Therefore, the frequent superinfections of AD with *S. aureus* cannot be mainly explained by a reduced expression of these AMPs. Consequently, the functional role of AMPs in AD, especially with regard to the control of *S. aureus* colonization, needs further investigation.

MATERIALS AND METHODS

Patients and controls

In total, 38 patients (0.5–72 years; 21 males and 17 females) affected with atopic dermatitis, 8 patients with psoriasis vulgaris (5–64 years; 6 males and 2 females), and 34 healthy controls (0.7–65 years; 14 males and 20 females) were included in this study. All patients had no specific treatment within the past 2 weeks. For immunohistochemical analysis each patient was assigned to a healthy control matched in age, gender, and the site of skin biopsy. Severity of symptoms was measured using SCORAD according to the European Task Force (Kunz *et al.*, 1997). The study was approved by the University committee for ethical affairs, Kiel (AZ A 104/06), in accordance with the guidelines of the Declaration of Helsinki Principles. All participants included in this investigation provided written informed consent.

Immunostaining

Punch biopsies were taken in local anesthesia from lesional and non-lesional skin in AD patients ($n=4$; forearm, trunk, and buttock), psoriasis patients ($n=4$; forearm, trunk, and thigh), and from corresponding skin areas of sex- and age-matched healthy controls ($n=8$). Fixation of the tissue samples was performed in 4% paraformaldehyde, and tissue sections (5 μm) were deparaffinized and rehydrated for the immunostaining experiments. The slides were incubated for heat-induced antigen retrieval in 0.01 M citrate buffer (pH 6.0) at 90 °C for 80 minutes. Subsequently, slides were blocked with normal rabbit serum (1:75, Dako Cytomation, Glostrup, Denmark) for 20 minutes and immunohistochemical staining was performed at room temperature using the antibodies and protocol as previously described (Gläser *et al.*, 2009b). In addition, quantitative scoring of AD immunostainings was performed (see Supplementary Table S1 online).

Collection of skin-derived washing fluids

Washing fluids of standardized skin (0.55 cm^2) areas from lesional and non-lesional skin of patients with untreated AD (total $n=38$; chronic AD $n=24$ and acute AD $n=14$) and psoriasis vulgaris ($n=8$) as well as matched controls ($n=34$) were analyzed for AMP levels using ELISA. In detail, the active secretion of RNase 7, psoriasin, hBD-2, and hBD-3 in patients as well as in controls was analyzed by rinsing the skin with 900 μl of 10 mM sodium phosphate buffer pH 7.4 containing 150 mM NaCl (skin rinsing buffer). Samples were mixed with 100 μl skin rinsing buffer containing 10% (w v^{-1}) bovine serum albumin (Sigma, Deisenhofen, Germany) and supernatants were stored after centrifugation of samples in aliquots at −80 °C until further processing.

ELISA

Protein levels of RNase 7, psoriasin, hBD-2, and hBD-3 in the skin washing fluids were measured using ELISA as previously described (Gläser *et al.*, 2009b). The detection limit for ELISA was 0.3 ng ml^{-1} for psoriasin and RNase 7, respectively, 0.075 ng ml^{-1} for hBD-2, and 1.25 ng ml^{-1} for hBD-3.

Bacteriology

Skin washing fluids were used for analysis of bacterial colonization shortly before storing at −80 °C. Serial dilutions of the washing fluids were plated on blood agar plates (MHS plate, BioMèrieux, Marcy l'Etoile, France) as well as on selective agar plates for *S. aureus* (SAID plate, BioMèrieux). All plates were cultured at 37 °C for 24–48 hours. Growing bacteria were differentiated into *S. aureus, Staphylococcus epidermidis, Micrococcus,* and *Corynebacteria* by using Gram-stain and testing coagulase and catalase activity (BioMèrieux).

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 ($n=8$, 25–50 years, 6 females and 2 males) until a transepidermal water loss of 40 g m^{-2} h^{-1} monitored by

Tewameter TM210 (Courage and Khazaka, Cologne, Germany) was reached. Both forearms were treated as described above. Occlusion was performed after experimental skin barrier disruption on one arm for up to 24 hours to achieve an artificial reconstitution of the epidermal barrier (Harris *et al.*, 1997). Large Finn Chambers on Scanpor were used for effective occlusion (Epitest Oy, Tuusula, Finnland). *In vivo* washing fluids (10 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.4) derived from the untreated forearm and the pre-treated area were collected at different time points and the amounts of RNase 7, hBD-2, and hBD-3 were analyzed using ELISA as described above. In addition, punch biopsies were taken from the forearms before (control) and 24 hours after skin barrier disruption for immunohistological analysis of RNase 7, hBD-2, and hBD-3 expression ($n = 3$).

Statistics

Calculations were performed using Wilcoxon's signed-rank test and Spearman's rank correlation analysis.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Christel Martensen-Kerl, Vera Beck-Jendroschek, Angela Preschke, Claudia Neumann, and Kerstin Schultz for excellent technical assistance and Jürgen Hedderich for support with statistical analyses. This study was supported by grants of the Federal Ministry of Education and Research (BMBF, SkinStaph) given to R Gläser, J Harder, and U Meyer-Hoffert and the Deutsche Forschungsgemeinschaft (SFB 617 given to J Harder, E Proksch, J-M Schröder, and T Schwarz and SCH 625/4-1 given to T Schwarz). J Harder was supported by a Heisenberg-program of the Deutsche Forschungsgemeinschaft.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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