

Microanalysis of an antimicrobial peptide, β -defensin-2, in the stratum corneum from patients with atopic dermatitis

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Summary

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Conflicts of interest

None declared.

Background Antimicrobial peptides, such as defensin and cathelicidin, have recently been reported to play important roles in host defence and in cutaneous innate immunity. Although β -defensin-2 has been reported to be downregulated in the skin of patients with atopic dermatitis (AD), little is known about its role in the colonization of *Staphylococcus aureus* in the stratum corneum of patients with AD. A precise evaluation of these peptides in the stratum corneum as an antimicrobial barrier against *S. aureus* colonization has not yet been performed.

Objectives To compare β -defensin-2 levels in the skin of patients with AD and healthy controls.

Methods We developed a microanalytical technique to measure β -defensin-2 in the stratum corneum using a combination of immunoprecipitation and Western blotting.

Results β -Defensin-2 in the stratum corneum was significantly higher in AD lesional skin compared with healthy control skin. The β -defensin-2 content in AD lesional skin also increased in proportion to the severity of the disease. Counting bacterial colonies revealed higher populations of *S. aureus* on lesional and non-lesional skin surfaces of patients with AD compared with healthy controls. Comparison of *S. aureus* colony numbers and β -defensin-2 levels demonstrated a positive correlation ($r = 0.342$, $P = 0.004$, $n = 67$) between both factors.

Conclusions Collectively, these findings suggest that β -defensin-2 is induced in response to bacteria, injury or inflammatory stimuli and is not associated with vulnerability to *S. aureus* colonization in the skin of patients with AD.

The stratum corneum of the skin of patients with atopic dermatitis (AD) is highly susceptible to colonization by various bacteria, including *Staphylococcus aureus*. Because the frequency of bacterial colonization, even in nonlesional skin of patients with AD, is significantly higher than in other types of dermatitis,¹ the defence system of the skin against bacterial invasion appears to be significantly disrupted in AD. Little is known about the defence mechanisms involved although the decreased secretion of IgA through the production of sweat in patients with AD has been attributed to a vulnerability to *S. aureus* colonization.² Further, a slight change towards an alkaline pH in the skin of patients with AD compared with skin of healthy subjects has been implicated in the high incidence of *S. aureus* colonization.¹ In contrast, although there is no direct evidence, the constitutive barrier disruption seen in the

stratum corneum of patients with AD has been thought to be associated with their predisposition to *S. aureus* colonization. This concept may lead to an important association between sphingolipid metabolites and the defence mechanism against *S. aureus* colonization because the barrier disruption has been ascribed to decreased levels of ceramides³ in the stratum corneum which appear to reflect altered sphingolipid metabolism in AD skin. In this respect, we have already reported the possibility that vulnerability to bacterial colonization in the skin of patients with AD is associated with reduced levels of a natural antimicrobial lipid, sphingosine, which results from decreased levels of the ceramide substrate and from diminished activities of its metabolic enzyme, acid ceramidase.⁴ On the other hand, antimicrobial peptides, such as defensin and cathelicidin, have recently been reported to play

important roles in host defence and in cutaneous innate immunity.⁵ Although β -defensin-2 was reported to be down-regulated at the mRNA and protein levels in the skin of patients with AD compared with patients with psoriasis but not with healthy controls,⁶ little is known about its role in the colonization of *S. aureus* in the skin of patients with AD. A precise evaluation of these peptides in the stratum corneum as an antimicrobial barrier against *S. aureus* colonization has not yet been performed, especially in comparison with healthy controls where there is no colonization of *S. aureus*. As human β -defensin-2 peptides are localized in the Malpighian layer of the epidermis and/or in the stratum corneum⁷ and are stored within lamellar bodies of keratinocytes in the spinous layer of the epidermis,⁸ it is likely that β -defensin-2 is primarily localized in intercellular lipid lamellar spaces of the stratum corneum. The antimicrobial functions of the stratum corneum play a key role in blocking the colonization of *S. aureus* in healthy skin. Therefore, a precise comparison of the level of β -defensin-2 with the colonization of *S. aureus* might provide a reasonable clue to the antimicrobial role of β -defensin-2 in the stratum corneum.

Previous studies were unable to determine precisely the levels of β -defensin-2 due to the nonspecific procedures used which consisted of direct immunoblotting or ion-exchange chromatography.^{5,6} In this study, we developed a microanalytical technique to measure β -defensin-2 in the stratum corneum precisely using a combination of immunoprecipitation and Western blotting, and we used that method to compare β -defensin-2 levels in the skin of patients with AD and healthy controls.

Materials and methods

Diagnosis

In total, 100 patients with AD (age range 15–41 years), 76 healthy non-AD volunteers with low serum IgE levels ($< 250 \text{ U mL}^{-1}$) (age range 20–50 years), 15 patients with psoriasis (age range 20–75 years) and one patient with contact dermatitis were enrolled in this study. The diagnosis of AD was made according to the criteria of Hanifin and Rajka.⁹ The severity of AD was evaluated by the criteria reported by Rajka and Langeland¹⁰ and was classified as mild, moderate or severe. Serum IgE levels of the patients ranged from 7670 to 53 300 IU mL⁻¹ (mean 18 254). Among 100 patients with AD, 48, 35 and 17 patients were classified as having mild, moderate and severe AD, respectively. Sixty-three and 65 patients were enrolled in the studies on *S. aureus* colonization and β -defensin-2 quantitation, respectively, of whom 28 overlapped. In the study on distribution of β -defensin-2 through the stratum corneum layers, 10 patients with AD were enrolled: six, three and one with mild, moderate and severe disease, respectively. Among 76 healthy control subjects, 66 and 27 subjects were enrolled in the studies on *S. aureus* colonization and β -defensin-2 quantitation, respectively, of whom 17 overlapped. All patients were referred by previous doctors

who described their treatment schedules. None had taken oral steroids, and none had evident systemic disease. Measurements in this study were performed after informed consent had been obtained. This study was approved by the Ethics Committee for Human Research at the Tokyo Women's Medical University.

Measurement of *Staphylococcus aureus* colonies on the skin surface

Semiquantitation of *S. aureus* was carried out by the contact-plate method.¹ The contact-plate method used in this study is not as efficient in collecting bacteria on the skin surface as the scrubbing technique: the log of colony-forming units (CFU) values obtained using the contact-plate method is smaller by a factor of approximately 2 than the log CFU values obtained using the scrubbing technique.¹ However, there is a close correlation between both methods in terms of relative values,¹ which indicates that the contact-plate method provides a reliable estimation of the relative degrees of colonization. In brief, stamp plates supplemented with nutrients adequate to support growth of *S. aureus* were applied for 5 s once on the skin surface and were then incubated for 48 h at room temperature. Numbers of colonies were counted, expressed as CFU per 20 cm², and were then classified into one of the following three groups: group 0, skin sites with no colonies; group $< 10^2$, skin sites with fewer than 100 colonies; group $> 10^2$, skin sites with more than 100 colonies.

Preparation of stratum corneum

Specimens of stratum corneum were obtained from normal forearm skin of healthy individuals and from uninvolved and involved skin of patients with AD, psoriasis or contact dermatitis by tape-stripping four times (Protect-label/B type, 40–60 mm; Iuchiiseidou Corp., Tokyo, Japan) in the same region. Such tapes were stored frozen at -80°C until used. Informed consent was obtained from each patient prior to sampling.

Extraction, immunoprecipitation and immunodetection of human β -defensin 2

Quantitation of human β -defensin 2 was carried out as follows. Human β -defensin-2 was extracted from three tapes, excluding the first strip, with 15 mL extraction buffer (Tris-buffered saline containing 0.03% Tween 20; Sigma, St Louis, MO, U.S.A.), overnight at 4°C . Each extract was cleared by filtration through a polytetrafluoroethylene (PTFE) membrane filter (Mitex; Millipore Co., Bedford, MA, U.S.A.). The membranes, which trapped corneocytes released from the tapes, were kept together with the tapes for later analyses.

Antihuman β -defensin-2 antibody (rabbit IgG; Peptide Institute Inc., Osaka, Japan) was added to extracts at a 1 : 15 000 dilution. Each extract was incubated with gentle agitation for 24 h at 4°C . Ten microlitres of a suspension of Protein G immobilized on magnetic beads (Protein G-Dynabeads, Oslo, Norway) was added to immunoprecipitates with the extracted

β -defensin-2, and incubated with gentle agitation for another 24 h at 4 °C. The immunobeads were then collected and washed twice with the extraction buffer.

After heating at 95 °C for 5 min in Tricine sample buffer (Bio-Rad Laboratories, Hercules, CA, U.S.A.), the immunobeads were loaded on 16.5% sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis gels (ReadyGelJ Peptide; Bio-Rad Laboratories). Gels were electrophoresed in Tris/Tricine/SDS buffer (Bio-Rad Laboratories) and were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Co.).

Membranes were blotted using a highly sensitive indirect chemiluminescence immunodetection method (ECL Advance; Amersham Biosciences Co., Piscataway, NJ, U.S.A.) employing the primary antihuman β -defensin 2 antibody (Peptide Institute Inc.) and a secondary horseradish peroxidase-labelled antirabbit IgG antibody (Amersham Biosciences Co.), according to the manufacturer's instructions. Briefly, after blocking for 2 h, membranes were incubated overnight with the primary antibody at a 1 : 30 000 dilution at 4 °C. The membranes were then treated with the secondary antibody at a 1 : 45 000 dilution for 60 min at room temperature followed by visualization with the chemiluminescence substrate and exposure to X-ray film. The intensity of each β -defensin-2 band was quantitated by digital densitometry. The standard curve was prepared using recombinant β -defensin (4.3 kDa; Bachem, Bubendorf, Switzerland) according to the procedure described above. In this quantitation, minimum detectable levels were 0.2–0.5 ng and the yield was 56% in comparison with Western blotting without immunoprecipitation.

Quantitation of stratum corneum protein

Membranes and tapes reserved from the above-mentioned β -defensin-2 extraction procedure were subjected to corneocyte isolation. Firstly, the membranes and tapes were treated in glass vials with 15 mL hexane, which facilitates the release of corneocytes adhering to the tapes by dissolving the adhesive, for 20 min at room temperature. The entire liquid was then filtered through a PTFE membrane (Mitex) to collect the released insoluble material. The first membrane from the β -defensin-2 extraction and the second membrane from the hexane treatment were used together to extract keratin protein with 0.25 mL 50 mmol L⁻¹ Tris buffer containing 8 mol L⁻¹ urea, 1% SDS, 100 mmol L⁻¹ 2-mercaptoethanol and 1 mmol L⁻¹ dithiothreitol. After overnight extraction at room temperature, extracts were used for a reducing agent-compatible, detergent-compatible protein quantitation (RC DC Protein Assay; Bio-Rad Laboratories) following the manufacturer's instructions. Bovine serum albumin was used for the protein concentration standard.

Statistics

Data were analysed using one-way ANOVA with Tukey's post hoc multiple comparison test, Spearman's rank correlation coefficient, and Pearson's correlation coefficient. $P < 0.05$ was considered significant.

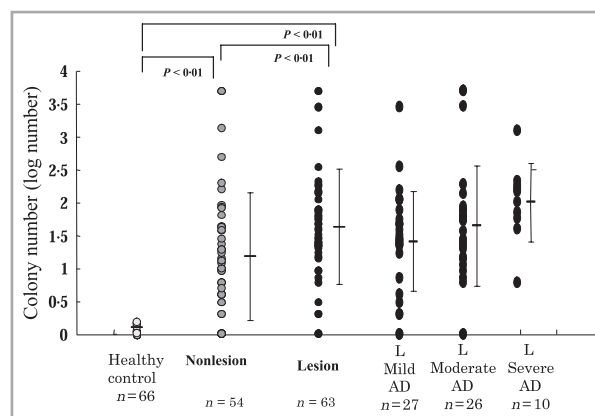


Fig 1. Comparison of *Staphylococcus aureus* colonization of atopic dermatitis (AD) skin with healthy control skin and its relation to AD severity. Results are shown as mean $\pm$ SD. L, lesional skin. Semiquantitation of *S. aureus* was carried out by the contact-plate method¹ as described in Materials and methods. Numbers of colonies counted were expressed as colony-forming units per 20 cm².

Results

Comparison of colonization of *Staphylococcus aureus* between lesional and nonlesional atopic dermatitis skin

Skin hydration resulting from infiltrating serum or blood components is believed to be a key factor for stimulating the colonization of *S. aureus*. To determine whether inflammation contributes to the colonization of *S. aureus*, we compared its colonization between lesional and nonlesional AD skin. The colonization of *S. aureus* was significantly higher in nonlesional AD skin compared with healthy control skin and was further significantly increased in AD lesional skin (Fig. 1). To determine whether the severity of AD is associated with the colonization of *S. aureus*, we compared the colony numbers of *S. aureus* with the severity of AD. There was a positive relationship in the lesional skin between the colonization and the severity of AD (Fig. 1). This suggests that the disease severity predominantly triggers the defence mechanism against *S. aureus* colonization.

Distribution of β -defensin-2 through the stratum corneum layers

To determine the distribution of β -defensin-2 through the stratum corneum layers, we measured levels of β -defensin-2 in five pools (each consisting of three successive tape strips through the stratum corneum layers, a total of 15 strips, excluding the first strip) obtained from uninvolved skin of patients with AD and from healthy controls. Representative Western blots of stratum corneum extracts obtained from the nonlesional skin of patients with AD and from healthy controls are shown in Figure 2. While there was a slight but nonsignificant increase in β -defensin-2 content depending on the depth of the stratum corneum layers in the healthy control skin, the

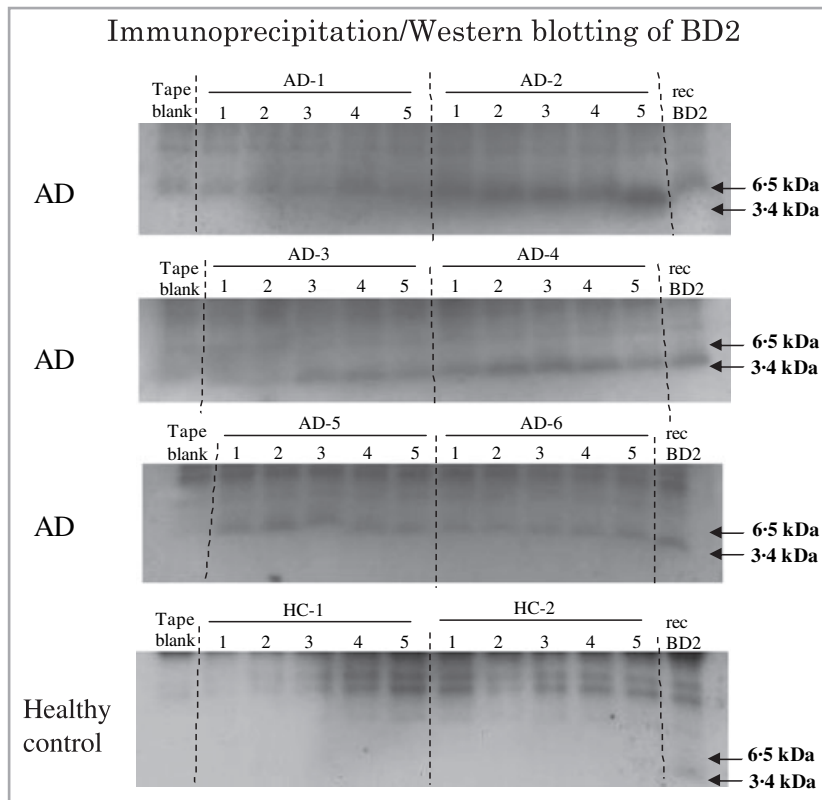


Fig 2. Representative Western blotting following immunoprecipitation of stratum corneum extracts obtained from nonlesional skin of patients with atopic dermatitis (AD) and healthy control (HC) skin. BD2, β -defensin-2; recBD2, recombinant BD2 (4.3 kDa). Numbers 1–5 represent each of five tape pools consisting of three successive tape strips through the stratum corneum layers: a total of 15 strips, excluding the first strip.

nonlesional skin of patients with AD had significantly higher levels of β -defensin-2 in each of three strips (except for pool 4) than the healthy control skin with no substantial difference through the stratum corneum layers (Fig. 3). This indicates that there is no substantial difference in the depth-dependent distribution of β -defensin-2 content within the stratum corneum layers of AD skin and of healthy control skin.

Comparison of β -defensin-2 levels between lesional and nonlesional skin of patients with atopic dermatitis

To determine whether the frequent colonization of bacteria on the skin of patients with AD is somehow related to qualitative or quantitative alterations in the antimicrobial peptide, β -defensin-2, we measured levels of β -defensin-2 in the upper stratum corneum. We measured three tape strips obtained from uninvolved and involved skin of patients with AD ($n = 56$ and $n = 65$ for nonlesional and lesional skin, respectively), patients with psoriasis ($n = 10$ and $n = 15$) and contact dermatitis ($n = 1$) and healthy controls ($n = 27$), as the three strips represent the mean levels of β -defensin-2 in the total stratum corneum layers (Fig. 3). Representative Western blots of stratum corneum extracts are shown in Figure 4. Comparison of healthy control skin, nonlesional and lesional AD skin and nonlesional and lesional psoriatic skin revealed that the content of β -defensin-2 (expressed per three strips) significantly increased in the lesional skin of patients with AD and psoriasis compared with the healthy controls (Fig. 5). In contrast, there was no significant difference between healthy control skin and nonlesional AD skin (Fig. 5). Quantitation of protein content in

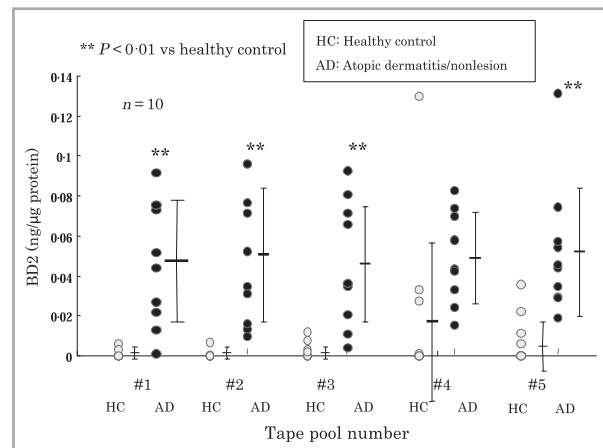


Fig 3. Distribution of β -defensin-2 through the stratum corneum layers of nonlesional atopic dermatitis (AD) skin and healthy control (HC) skin. Results are shown as mean $\pm$ SD. BD2, β -defensin-2. Numbers 1–5 represent each of five tape pools consisting of three successive tape strips through the stratum corneum layers: a total of 15 strips, excluding the first strip. Pool 1, $P = 0.0013$; pool 2, $P = 0.0015$; pool 3, $P = 0.0020$; pool 4, $P = 0.0553$; pool 5, $P = 0.0018$ between AD and HC.

each set of three strips revealed mean $\pm$ SD protein levels of 12.03 ± 9.83 ($n = 27$), 17.16 ± 13.32 ($n = 56$), 22.73 ± 26.7 ($n = 65$), 7.14 ± 3.52 ($n = 10$) and 69.13 ± 60.53 μ g ($n = 15$) for healthy controls, AD nonlesional skin, AD lesional skin, psoriasis nonlesional skin and psoriasis lesional skin, respectively. Another comparison by β -defensin-2 levels

Fig 4. Immunoprecipitation/Western blotting of β-defensin-2 in the stratum corneum of patients with atopic dermatitis/psoriasis/contact dermatitis (CD) and healthy controls. BT, blank tape; NL, nonlesional skin; L, lesional skin; recBD2, recombinant β-defensin-2 (4.3 kDa). Human β-defensin-2 was extracted from three tapes excluding the first strip. The extracts were subjected to immunoprecipitation followed by Western blotting as described in Materials and methods.

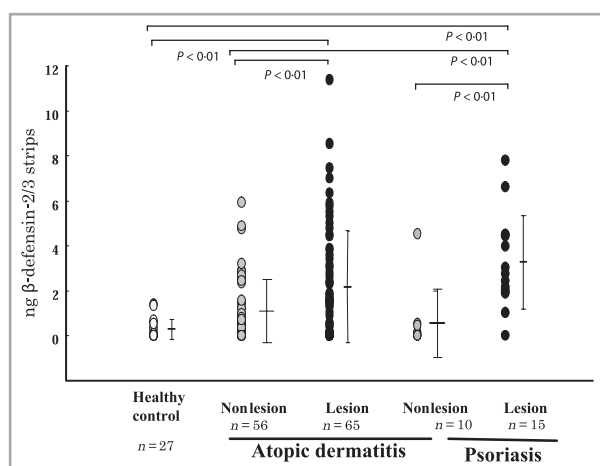
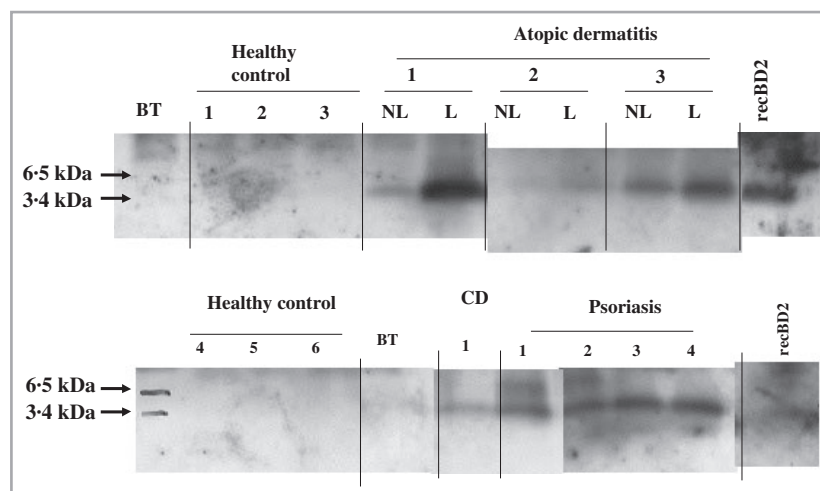


Fig 5. β-Defensin-2 content expressed per three strips in the stratum corneum of patients with atopic dermatitis/psoriasis and healthy controls. Results are shown as mean ± SD. Human β-defensin-2 was extracted from three tapes excluding the first strip. The extracts were subjected to immunoprecipitation followed by Western blotting as described in Materials and methods. β-Defensin-2 content was determined using a standard curve and expressed per three strips.

(expressed as $\text{ng } \mu\text{g}^{-1}$ stratum corneum protein) demonstrated significantly increased levels of β-defensin-2 in the lesional skin of patients with AD and psoriasis compared with healthy controls although there was no significant difference between healthy control skin and nonlesional AD skin (Fig. 6). The content of β-defensin-2 in lesional AD skin was significantly higher than in nonlesional skin.

Relationship between the level of β-defensin-2 and the severity of atopic dermatitis

We examined whether the severity of AD is associated with the content of β-defensin-2 in the stratum corneum. Comparisons between AD skin with different severity of disease revealed that the level of β-defensin-2 in lesional skin increases in proportion to the severity of AD with significant

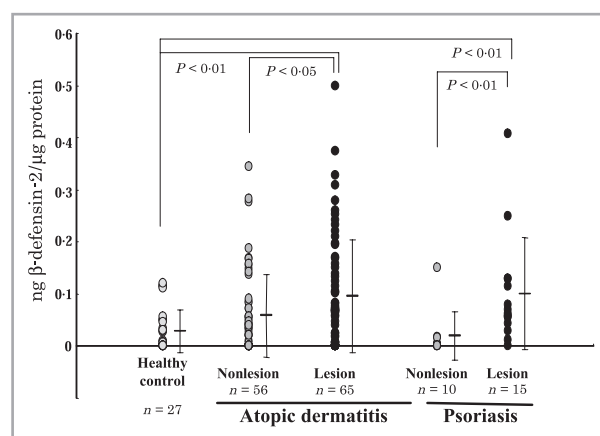


Fig 6. β-Defensin-2 content expressed per μg protein in the stratum corneum of patients with atopic dermatitis/psoriasis and healthy controls. Results are shown as mean ± SD. Human β-defensin-2 was extracted from three tapes excluding the first strip. The extracts were subjected to immunoprecipitation followed by Western blotting as described in Materials and methods. β-Defensin-2 content was determined using a standard curve and expressed per μg protein after quantitation of stratum corneum protein as described in Materials and methods.

differences between healthy controls and the moderate group and between healthy controls and the severe group (Fig. 7), but not between healthy controls and the mild group. Comparison between the same severity of AD revealed a significant or a slight difference in the level of β-defensin-2 between the lesional and nonlesional skin in the moderate and severe AD groups. This suggests that the disease severity predominantly triggers the defence mechanism by stimulating the production of β-defensin-2 in the epidermis.

Correlation between the content of β-defensin-2 and the colonization of *Staphylococcus aureus*

As the increased level of β-defensin-2 in the stratum corneum is associated with inflammation and/or disease severity, it is

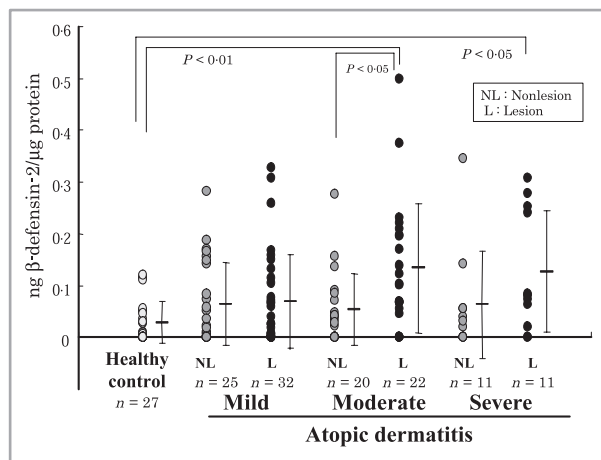


Fig 7. β -Defensin-2 content in the stratum corneum of patients with atopic dermatitis (AD) in relation to AD severity. Results are shown as mean $\pm$ SD. The severity of AD was evaluated by the criteria reported by Rajka and Langeland¹⁰ and was classified as mild, moderate or severe.

plausible that the production of β -defensin-2 is primarily triggered by the colonization of *S. aureus* to be secondarily stimulated rather than by an innate mechanism against *S. aureus*. When AD groups were classified according to different colony numbers, there was a weak correlation between increased levels of β -defensin-2 and increased numbers of *S. aureus* on the skin surface (Fig. 8). Comparison of the increased colonization and the increased β -defensin-2 levels revealed a distinct correlation between these two parameters in both lesional and non-lesional skin (Fig. 9a, $n = 67$, $r = 0.342$, $P = 0.004$) and in only lesional skin (Fig. 9b, $n = 38$, $r = 0.427$, $P = 0.006$) of the same patients with AD.

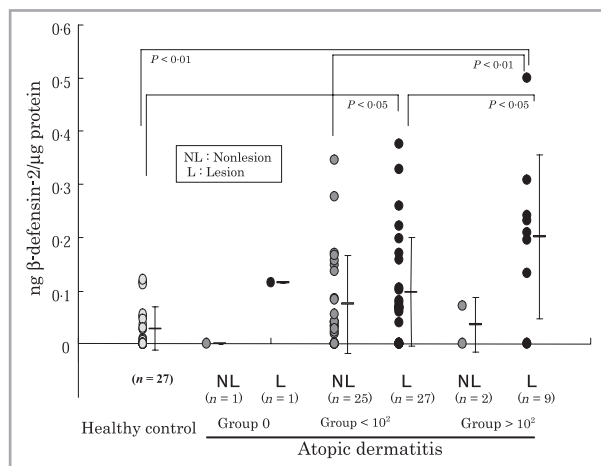


Fig 8. β -Defensin-2 content in the stratum corneum of patients with atopic dermatitis in relation to *Staphylococcus aureus* colonization. Results are shown as mean $\pm$ SD. Numbers of *S. aureus* colonies were counted, expressed as colony-forming units per 20 cm² and were then classified into one of the following three groups: group 0, skin sites with no colonies; group $< 10^2$, skin sites with fewer than 100 colonies; group $> 10^2$, skin sites with more than 100 colonies.

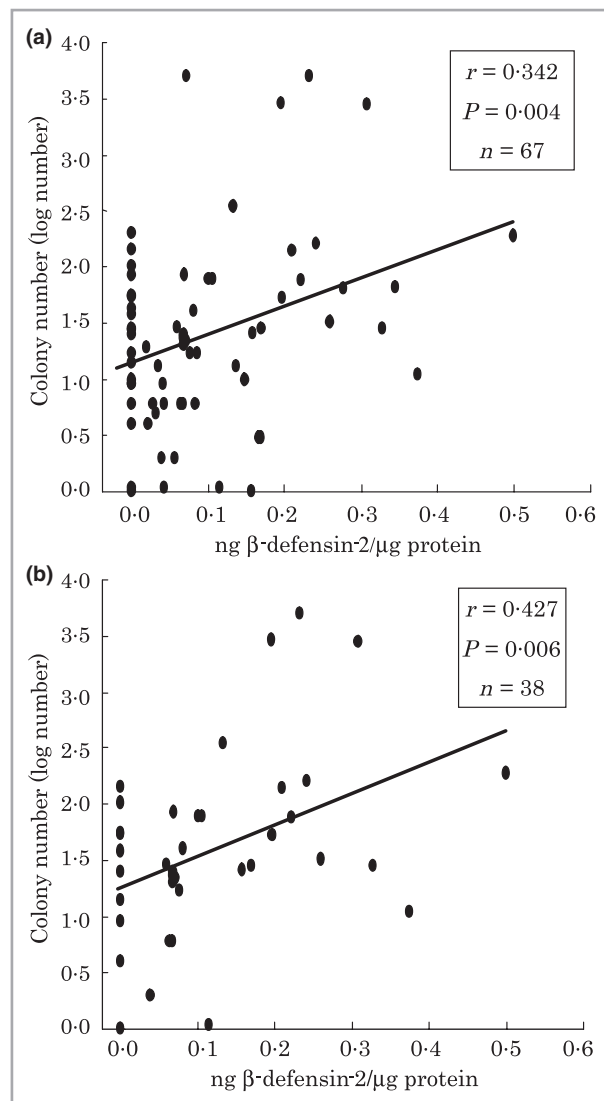


Fig 9. Relationship between β -defensin-2 content in the stratum corneum (three strips) of patients with atopic dermatitis and *Staphylococcus aureus* colonization. (a) Lesional + nonlesional skin; (b) lesional skin. Human β -defensin-2 was extracted from three tapes excluding the first strip. The extracts were subjected to immunoprecipitation followed by Western blotting as described in Materials and methods. β -Defensin-2 content was determined using a standard curve and expressed per μ g protein after quantitation of stratum corneum protein as described in Materials and methods. Semiquantitation of *S. aureus* was carried out by the contact-plate method¹ as described in Materials and methods. Numbers of colonies counted were expressed as colony-forming units per 20 cm².

Discussion

Antimicrobial peptides such as defensins have recently been reported to play key roles in host defence and in cutaneous innate immunity.^{11,12} Consistently, human keratinocytes are stimulated to produce such antimicrobial peptides in response to bacteria, injury or inflammatory stimuli.^{13–15} Thus, it is plausible that if a significant alteration or deficiency of

antimicrobial peptides occurs in the epidermis, which is the primary host defence system of the skin, skin diseases with a high vulnerability to infection by skin surface bacteria, such as *S. aureus*, would develop. In this connection, Leung's group⁶ recently reported that a deficiency in the expression of antimicrobial peptides may account for the susceptibility of patients with AD to skin infections with *S. aureus*. They based that on the evidence that the expression of β -defensin-2 is significantly downregulated in the AD epidermis at both the mRNA and protein levels compared with psoriatic skin but not healthy control skin. However, if one wishes to examine the role of antimicrobial peptides in the host defence mechanism against *S. aureus* infection, the situation in healthy control skin, where there is no infection, should be compared with that in the skin of patients with AD. A comparison between AD skin and psoriatic skin does not necessarily account for the host defensive role of antimicrobial peptides such as β -defensin-2 in the skin of healthy controls recognized as an uninfected site.

Several studies have demonstrated that there is a high frequency of colonization by *S. aureus* in the stratum corneum of AD skin,^{1,4} which may reflect the high incidence of infection by *S. aureus* seen as impetigo in AD. Thus, it is likely that the defence mechanism against transient pathogens in the epidermis, as well as their colonization in the stratum corneum (which occurs efficiently in healthy skin), is significantly attenuated in AD skin. For this reason, we have developed a microanalytical technique to measure β -defensin-2 in the stratum corneum using a combination of immunoprecipitation and Western blotting. We could then determine if substantial quantitative changes in β -defensin-2 levels occur in the stratum corneum of AD skin, compared with the skin of healthy subjects.

This study revealed that levels of β -defensin-2 (expressed as ng μg^{-1} protein) in the upper stratum corneum is significantly increased (by approximately 3.0-fold) in the lesional skin of patients with AD compared with healthy controls. When the level of β -defensin-2 was compared in three tape strips of the stratum corneum, the lesional AD skin contained a significantly higher level of β -defensin-2 than the healthy control skin, although there was no significant difference in the amounts of stratum corneum in the three strips between the nonlesional AD skin and the healthy control skin. Furthermore, analysis of the distribution of β -defensin-2 through the stratum corneum layers demonstrated that while there was a slight but nonsignificant increase in β -defensin-2 content (depending on the depth of the stratum corneum layer) in the healthy control skin, each layer in the nonlesional skin of patients with AD had a significantly higher level of β -defensin-2 than the healthy control skin, with no substantial difference among each depth through the stratum corneum layers. This strongly suggests a distinct increase in β -defensin-2 content in the lesional and nonlesional stratum corneum of patients with AD. This upregulated production of β -defensin-2 is associated with increased colonization of the skin by *S. aureus* which is significantly higher in AD skin compared with

healthy controls. This indicates the possibility that the colonization of AD skin by *S. aureus* directly triggers the increased level of β -defensin-2 because it is known that *S. aureus* itself induces the expression of human β -defensin.¹⁶ In contrast to the report by Leung's group,⁶ which focused on the host defence mechanism against bacterial infection but not against colonization in AD skin, the observed abundance of β -defensin-2 in the present study may not account for the high incidence of *S. aureus* colonization seen on the AD skin. Therefore, the increased levels of β -defensin-2 and its relevance for increased colonization of AD skin suggest that β -defensin-2 does not play any significant role as an innate defence mechanism against the proliferation of *S. aureus* in the stratum corneum of healthy control skin.

Direct contact of cells with *S. aureus* or primary inflammatory cytokines such as interleukin (IL)-1 has been reported to stimulate the production of antimicrobial peptides, including β -defensin-2, via toll-like receptors by various epithelial cells, including keratinocytes.¹⁷ β -defensin-2 by itself induces IL-18 secretion through p38 and ERK MAPK activation in primary human keratinocytes.¹⁸ Based on such evidence, a close positive correlation between the content of β -defensin-2 and the colonization of *S. aureus* in the upper stratum corneum, as observed in this study, suggests that the *S. aureus* colonization primarily occurs due to the lack of defence mechanisms other than a β -defensin-2 deficiency. It is likely that the *S. aureus* colonization itself results in the stimulation of β -defensin-2 production in living epidermal keratinocytes, which in turn reflects the increased content of β -defensin-2 in the stratum corneum. Analysis using reverse transcription-polymerase chain reaction has already revealed that although the expression level of mRNA encoding β -defensin-2 in the lesional skin of patients with AD is significantly diminished compared with the skin of patients with psoriasis, there is no significant difference in the faint levels of immunostaining between patients with AD and healthy controls.⁶ Our finding that the content of β -defensin-2 in the lesional stratum corneum of patients with AD is significantly increased to a level similar to that of psoriatic skin compared with healthy control skin is not in agreement with Leung's group's data⁶ on the gene expression described above. In their study, the protein expression of β -defensin-2 was analysed by immunohistochemistry or direct blotting using an antibody to β -defensin-2. Thus, their methods are not reliable for precise detection of β -defensin-2. Therefore, there had been no precise way to determine the protein level of β -defensin-2 in AD skin. Our combined method using immunoprecipitation and Western blotting allows us to determine the precise protein level of β -defensin-2 in the stratum corneum for the first time.

As for the discrepancy between the mRNA and protein expression levels of β -defensin-2, it should be noted that frequently available drugs, such as corticosteroids, used to treat AD have been reported to have a potent capacity to enhance the expression of β -defensin-2 in contrast to ciclosporin which rather downregulates its expression.¹⁹ Thus, this discrepancy may result from different clinical situations in which

the skin derived from patients with AD used in our study had been more frequently exposed to corticosteroid treatment compared with the patients with AD used in the study by Leung's group,⁶ although the amounts of corticosteroid used and the duration of the therapy could not be determined. Even in the nonlesional AD skin, where corticosteroid therapy had not been applied in our study, there was a slight, but not significant, increase in the expression of β -defensin-2. Thus, the question of whether the observed increase in β -defensin-2 can be ascribed to corticosteroid treatment remains unresolved.

In conclusion, in the involved and uninvolved stratum corneum of patients with AD, β -defensin-2 levels are not downregulated but rather are upregulated compared with healthy controls. Further, *S. aureus* colonization is not inversely correlated with levels of β -defensin-2 in the involved stratum corneum of AD skin. These findings suggest that β -defensin-2 is induced in response to bacteria, injury or inflammatory stimuli and is not associated with the vulnerability to *S. aureus* colonization in the skin of patients with AD.

Acknowledgments

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