

Percutaneous application of peptidoglycan from *Staphylococcus aureus* induces an increase in mast cell numbers in the dermis of mice

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Summary

Background Atopic dermatitis (AD) is a chronic inflammatory skin disease with immunopathologic features that vary depending on the duration of the lesion. The dermis of lesional skin of AD patients shows an increased number of inflammatory cells such as mast cells, eosinophils and mononuclear cells and superficial *Staphylococcus aureus* colonization.

Objective The purpose of this study was to determine the effects of peptidoglycan (PEG) from *S. aureus* on mast cell induction in murine skin.

Methods PEG was applied to barrier-disrupted abdominal skin of mice every 5 days and the number of mast cells in the abdominal skin was counted 20 days after the first application. The cytokine response was investigated by RT-PCR and immunohistologic analysis.

Results The number of mast cells in the skin of mice treated with PEG was increased significantly compared with that of mice given phosphate-buffered saline. In addition, application of PEG to the abdominal skin increased the expression of mRNA for transforming growth factor- β_1 (TGF- β_1), which supports mast cell migration, but not that for IL-3 or stem cell factor, which support both mast cell proliferation and mast cell migration. Immunohistologic analysis demonstrated that levels of TGF- β_1 transcripts corresponded with those of protein synthesis in the epidermis. TGF- β_1 was found to be highly expressed in keratinocytes of the basal epidermis of PEG-treated skin. Furthermore, intraperitoneal injection of anti-TGF- β_1 antibodies neutralized the induction of mast cells into the skin.

Conclusion These results suggest that PEG may have the ability to induce an increase in mast cell numbers in the skin through TGF- β_1 production by epidermal keratinocytes. Skin inflammation might therefore be linked to colonization with *S. aureus* in AD patients.

Keywords atopic dermatitis, mast cell, peptidoglycan, *Staphylococcus aureus*, TGF- β_1

Submitted 27 July 2004; revised 10 November 2004; accepted 8 January 2005

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease with immunopathologic features that vary depending on the duration of the lesion. Chronic skin lesions of AD patients show an increased number of inflammatory cells such as mast cells, eosinophils and mononuclear cells in the dermis and superficial *Staphylococcus aureus* colonization [1]. *S. aureus* can be isolated from 75% to 90% of skin lesions of AD patients, whereas the rate of colonization of healthy individuals is only around 5% [2]. In a previous study, the rate of detection of *S. aureus* on the lesional skin of AD patients was found to be higher than that on non-lesional skin of AD patients [3]. Furthermore, the cell count of *S. aureus* on the lesional skin of AD patients was also significantly higher than that on non-lesional skin. However, there was no

significant difference in the rate of detection of superantigen exotoxin-producing *S. aureus* between lesional and non-lesional skin of AD patients.

Gram-positive bacterial cell walls are composed of highly cross-linked peptidoglycan (PEG) decorated to a variable extent with teichoic acid polymers. The latter are also linked to plasma membrane phospholipids, of which lipoteichoic acid (LTA) and PEG are the major surface components [4, 5]. Gram-positive bacteria contain no lipopolysaccharide, and LTA and/or PEG are thought to be the major inflammatory products in their cell walls. Therefore, it was hypothesized that LTA and/or PEG from *S. aureus* may be pathogenic factors in patients with AD. In previous studies, LTA and PEG were found to induce IL-5 production by peripheral blood mononuclear cells from patients with AD [6], and intradermal injection of LTA induced localized AD-like inflammation associated with an increased number of mast cells, eosinophils and mononuclear cells in the dermis of allergen-sensitized mice [7]. However, it is unclear whether another component of the Gram-positive bacterial cell wall, PEG, has the ability to induce AD-like inflammatory cells in

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the dermis. In this study, therefore, the percutaneous route was chosen as a physiologically possible antigen-invasion route, and the effects of percutaneous PEG-application on mast cell induction, which is one of the histologic features of AD inflammation, were investigated in mouse skin.

Materials and methods

Peptidoglycan

PEG derived from *S. aureus* was obtained from Fluka (Buchs SG, Switzerland). PEG was reconstituted in phosphate-buffered saline (PBS), pH 7.4, at a concentration of 1 mg/mL and sonicated for 1 h before use.

Mice

Female, specific-pathogen-free, BALB/c mice were obtained from Tokyo Laboratory Animals Science Co. Ltd. (Tokyo, Japan), and used when they were 6–8 weeks of age. The mice were housed in plastic cages in a clean, air-conditioned room at 24 °C and allowed free access to a standard laboratory diet and water. All procedures performed on the mice were in accordance with the Animal Care and Use Committee of Meiji Pharmaceutical University, Tokyo, Japan.

Percutaneous application of peptidoglycan

Percutaneous application of PEG was performed as described previously [7]. Briefly, the skin of each mouse was barrier-disrupted by repeatedly (eight times) applying adhesive cellophane tape to shaved abdominal skin and then pulling it off. PEG solution (100 µL; 1 mg/mL in PBS) was applied topically to a 10 cm² area of barrier-disrupted skin on days 0, 5, 10 and 15. PEG-non-challenged control mice were treated by applying 100 µL PBS to barrier-disrupted skin in the same manner.

Histologic analysis

Paraffin-embedded cross-sections (3 µm) of PEG-treated abdominal skin fixed in 10% phosphate-buffered formalin were stained with haematoxylin–eosin (HE) and toluidine blue (TB), respectively. The number of mast cells was counted in 20 high power fields (HPFs; × 400) and expressed as cells/HPF with calculation of the mean. The final results were shown as means ± SD of six independent experiments.

Reverse transcription-polymerase chain reaction

To determine the expression levels of IL-3, stem cell factor (SCF) and transforming growth factor-β₁ (TGF-β₁) mRNA in the skin, total RNA was extracted from PEG-treated abdominal skin 20 days after the first application by the single-step method with TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA). cDNA was then synthesized from 8 µg total RNA using a First-strand cDNA synthesis kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). PCR was performed using the following primers: β-actin (540 bp): 5' primer, 5'-GTGGGCGCTCTAGGCACCAA-3' and 3' primer, 5'-CTCTTTGATGTCACGCACGATTT C-3'; IL-3 (489 bp): 5' primer, 5'-GTTCTTGCCAGCTCTA

CCACCAGC-3' and 3' primer, 5'-CACGGTTCCACGGT TAGGAGAGAC-3'; SCF (508 bp): 5' primer, 5'-GAGATC TGCGG GAATCCTGTGAC-3' and 3' primer, 5'-CATTC CTAAGGGAGCTGGCTGC-3'; TGF-β₁ (406 bp): 5' primer, 5'-CGGGGCGACCTGGGCACCATCCATGAC-3' and 3' primer, 5'-CTGCTCCACCTTGGGCTTGCGACC-CAC-3'. Each PCR was performed using a GeneAmp PCR System 9700 (Perkin-Elmer Co., Norwalk, CT, USA) with a 25 µL reaction mixture comprising 1.5 µL cDNA (corresponding to 800 ng total RNA starting material), 200 µM deoxynucleotide triphosphate mixture, 400 nM of each PCR primer and 25 U/mL Ex Taq DNA polymerase (Takara, Shiga, Japan). The reaction conditions were as follows: one 4-min cycle at 94 °C, 35 cycles comprising 45 s at 94 °C, 45 s at 61 °C and 2 min at 72 °C, followed by one 7-min cycle at 72 °C. The PCR products were then separated on a 2% agarose gel containing ethidium bromide.

Immunohistologic analysis

Immunohistochemical staining for TGF-β₁ was performed on sections of PEG-treated abdominal skin. The skin specimens were embedded and frozen with OCT compound (Miles, Elkhart, IN, USA) at –80 °C. Frozen sections, 6 µm thick, were fixed in cold acetone (–20 °C) for 10 min, treated with blocking buffer (10% normal goat serum in PBS), and incubated with a mouse anti-TGF-β₁ monoclonal antibody (1D11, IgG1) (R & D systems, Minneapolis, MN, USA) at a concentration of 5 µg/mL, followed by biotinylated goat anti-mouse immunoglobulin (1:500; Dako, Carpinteria, CA, USA). After being washed with PBS, the skin sections were incubated with peroxidase-conjugated streptavidin (Dako). Endogenous peroxidase activity was blocked by incubation for 30 min with 0.3% H₂O₂ in PBS. Endogenous biotin was blocked by sequential incubation with avidin (Vector Laboratories, Burlingame, CA, USA) and biotin (Sigma, St Louis, MO, USA). The tissue sections were stained by incubation with 3, 3'-diaminobenzidine tetrahydrochloride (Sigma) and treated with methyl green solution for visualization of nuclei.

Purification of keratinocytes

Keratinocytes were separated as described by Tada et al. [8]. Briefly, mouse skin was treated with dispase (3000 U/mL; Godo Shusei, Tokyo, Japan) in RPMI 1640 medium with L-glutamine (Sigma) containing 10% fetal calf serum (Sigma), 25 mM HEPES (Sigma), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco RBL, Grand Island, NY, USA) (RPMI 10) for 3 h at 37 °C. The epidermis was separated from the dermis and incubated in RPMI 10 containing 0.025% deoxyribonuclease I (Sigma) for 20 min at room temperature. An epidermal cell suspension was obtained by vigorous pipetting of the epidermal sheets. The epidermal cell suspension was then treated with a mouse anti-mouse I-A^d monoclonal antibody (040–220, IgG2a) (1:600; Meiji Dairies Co., Tokyo, Japan) in RPMI 10 for 45 min on ice. The cells were then incubated in plates precoated with a goat anti-mouse IgG antibody (1:100; Sigma) for 45 min at 4 °C. Floating cells were collected as keratinocytes and adherent cells were used as Langerhans cells.

Neutralization of mast cell induction with anti-transforming growth factor- β_1 antibodies

Percutaneous application of PEG to mice was performed as described above. Three hours after each percutaneous application (four times), 100 μ L of mouse anti-TGF- β_1 monoclonal antibody (1D11, IgG1) (1 mg/mL in PBS; R & D systems) was injected intraperitoneally. A mouse IgG monoclonal antibody (MOPC 21, IgG1) (Sigma) was also

used as an isotype-matched control antibody. Twenty days later, the PEG-treated skin was cut out, fixed and stained for counting mast cell numbers as described above.

Statistical analysis

The data are expressed as means ($\pm$ SD). Differences between means were analyzed using the Student's *t*-test with a two-tailed test of significance. Differences at $P < 0.05$ were considered to be statistically significant.

Results

Effects of peptidoglycan on mast cell induction in skin

Preliminary experiments were carried out to examine mast cell induction in skin after percutaneous application of PEG to mice. A 10 cm² area of barrier-disrupted abdominal skin was treated with 100 μ g PEG every 5 and 20 days after the first application the skin was removed and processed for detection of mast cells. Figure 1 shows the number of mast cells in skin sections from each group of mice ($n = 6$). The number of mast cells in the skin of PEG-treated mice was significantly higher than that of non-treated mice and PBS-treated mice; percutaneous application of PBS to the skin did not induce mast cells. In PEG-treated mice, all mast cells were observed in the dermis, with more than 50% of mast cells observed in the upper layer of the dermis (Fig. 2). These results indicate that percutaneous application of PEG can induce an increase in mast cell numbers in the dermis of mice. Furthermore, there was slight thickening and eosinophilic infiltration in the dermis at the site of application of PEG.

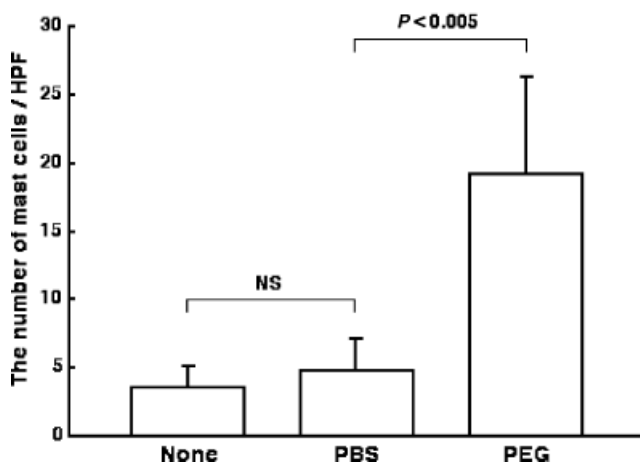


Fig. 1. Quantification of mast cells in the dermis of mice treated with peptidoglycan (PEG). Mice were treated topically with 100 μ L PEG solution (1 mg/mL) on a 10-cm² area of barrier-disrupted abdominal skin every 5 days. Control mice were treated percutaneously with 100 μ L phosphate-buffered saline (PBS). Skin biopsies were taken 20 days after the first application, stained with toluidine blue and the mast cell number was counted. The results are expressed as means $\pm$ SD ($n = 6$) of the number of mast cells per high power field (HPF).

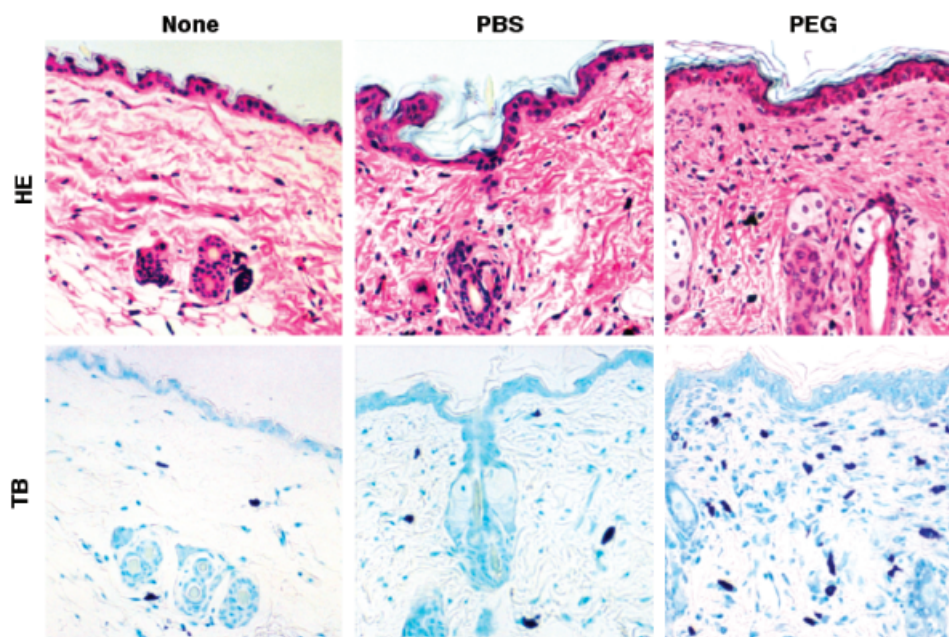


Fig. 2. Mast cells in the dermis of mice treated with peptidoglycan (PEG). Mice were treated topically with 100 μ L PEG solution (1 mg/mL) on a 10-cm² area of barrier-disrupted abdominal skin every 5 days. Control mice were treated percutaneously with 100 μ L of phosphate-buffered saline (PBS). After 20 days, skin sections were stained with haematoxylin-eosin (HE) and toluidine blue (TB) and observed at $\times 100$. The data shown are the representative results of four independent experiments.

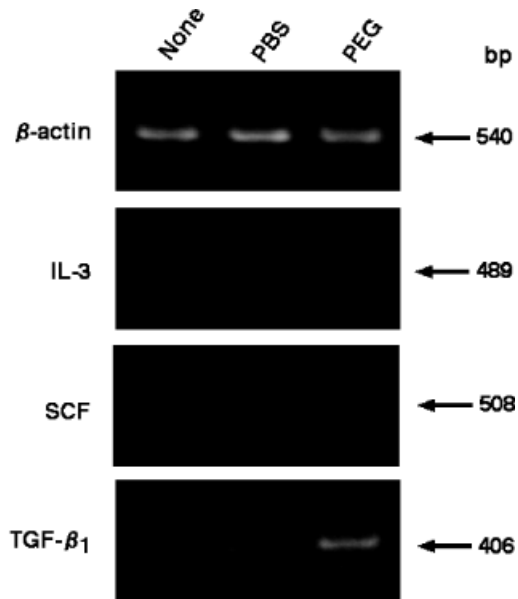


Fig. 3. Effect of peptidoglycan (PEG) on transforming growth factor- β_1 (TGF- β_1) mRNA expression in the skin. Mice were treated topically with 100 μ L PEG solution (1 mg/mL) on a 10-cm² area of barrier-disrupted abdominal skin every 5 days. Control mice were treated percutaneously with 100 μ L phosphate-buffered saline (PBS). After 20 days, cytoplasmic RNA was extracted from the skin of each mouse, reverse-transcribed and amplified by PCR using β -actin, IL-3, stem cell factor and TGF- β_1 primer sets. The data shown are the representative results of four independent experiments.

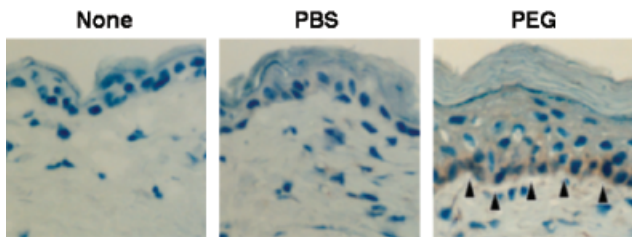


Fig. 4. Immunohistochemical staining in the skin after application of peptidoglycan (PEG). Mice were treated topically with 100 μ L PEG solution (1 mg/mL) on a 10-cm² area of barrier-disrupted abdominal skin every 5 days. Control mice were treated percutaneously with 100 μ L phosphate-buffered saline (PBS). After 20 days, skin sections were treated with monoclonal antibody to transforming growth factor- β_1 (TGF- β_1), stained with a biotinylated anti-IgG monoclonal antibody, and observed at $\times 200$. The arrowheads indicate the basal cell layers. The data shown are the representative results of four independent experiments.

Production of transforming growth factor- β_1 in the skin of peptidoglycan-treated mice

To determine whether IL-3, SCF and TGF- β_1 , which support mast cell proliferation and/or mast cell migration, are produced in the skin of PEG-treated mice, each mRNA expression in the skin 20 days after PEG-application was investigated by RT-PCR. Figure 3 shows that the skin from PEG-treated mice expressed TGF- β_1 mRNA, but not IL-3 or SCF mRNA. The skin from non-treated mice and PBS-treated mice did not express any mRNA. Immunohistologic analysis of skin biopsies was then carried out to determine whether expression of mRNA for TGF- β_1 in skin from PEG-treated mice was correlated with protein synthesis. Immuno-

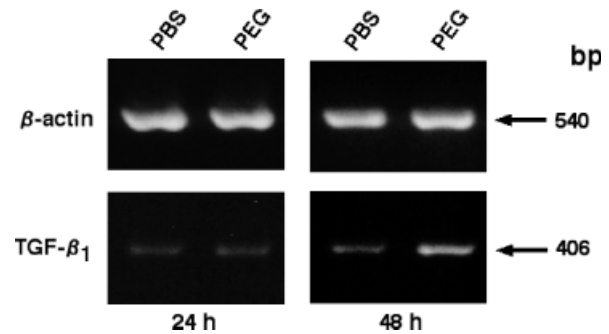


Fig. 5. Effect of peptidoglycan (PEG) on transforming growth factor- β_1 (TGF- β_1) mRNA expression in keratinocytes. Keratinocytes (2×10^6 /mL) from the mouse epidermis were treated with or without 10 μ g/mL PEG for 24–48 h. Cytoplasmic RNA was extracted, reverse-transcribed and amplified by PCR using β -actin and TGF- β_1 primer sets. The data shown are the representative results for four independent experiments.

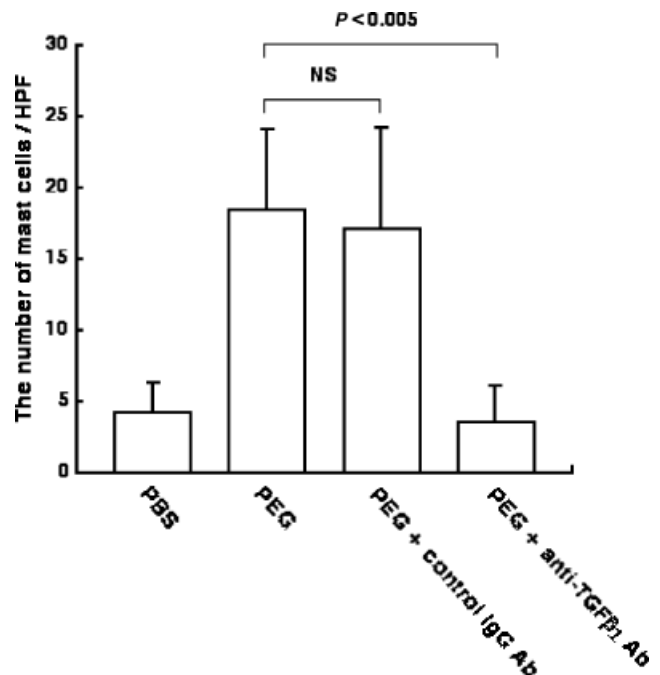


Fig. 6. Neutralization of mast cell induction in peptidoglycan (PEG)-treated skin of mice. Mice were treated topically with 100 μ L PEG solution (1 mg/mL) on a 10-cm² area of barrier-disrupted abdominal skin. After 3 h, 100 μ L of a monoclonal antibody (1 mg/mL) to transforming growth factor- β_1 (TGF- β_1) was injected intraperitoneally. An IgG monoclonal antibody was also used as an isotype-matched control antibody. PEG-treatment of abdominal skin and intraperitoneal antibody-injection was performed every 5 days. Control mice were treated percutaneously with 100 μ L PBS. Skin biopsies were taken 20 days after the first application, stained with toluidine blue, and the mast cell number was counted. The results are expressed as means $\pm$ SD ($n = 6$) of the number of mast cells per high power field (HPF).

histologic analysis was carried out on skin 20 days after PEG-application. Figure 4 shows that the skin of PEG-treated mice, but not of normal mice or PBS-treated mice induced cells positive for TGF- β_1 in the epidermis. TGF- β_1 -positive staining was observed mainly in the basal cell layer of the epidermis and lower levels of TGF- β_1 were observed in the whole epidermal site of PEG-treated mice. Since TGF- β_1 production was observed in the basal cell layer of the epidermis, it was thought that the source cells for TGF- β_1 would be keratinocytes. Therefore, keratinocytes were

separated from epidermal cells of a normal mouse and stimulated *in vitro* with PEG. Figure 5 shows that keratinocytes purified from the epidermis spontaneously expressed TGF- β_1 mRNA and that expression was augmented 48 h after *in vitro* stimulation with the optimum concentration of PEG. However, the increase in TGF- β_1 mRNA expression by PEG-stimulation was not observed in epidermal Langerhans cells (data not shown). These results suggest that TGF- β_1 is produced from PEG-stimulated epidermal keratinocytes.

Neutralization of peptidoglycan-induced mast cell infiltration with anti-transforming growth factor- β_1 antibodies

The appearance of mast cells after application of PEG to the skin and treatment with anti-TGF- β_1 antibodies was investigated to assess the role of TGF- β_1 in mast cell induction in the dermis. Intraperitoneal injection of anti-TGF- β_1 antibodies or control IgG antibodies was carried out 3 h after each PEG-application and 20 days after the first application the skin was removed and processed for detection of mast cells by TB staining. As shown in Fig. 6, complete inhibition of PEG-induced mast cell infiltration was seen in mice treated with the anti-TGF- β_1 antibodies, whereas it was not observed in mice treated with the control IgG antibodies ($n = 6$). These results indicate that mast cells infiltrate into the dermis through TGF- β_1 production by epidermal keratinocytes.

Discussion

A high incidence of chronic skin colonization with *S. aureus* is a prominent feature in patients with AD. Although many attempts have been made to characterize the role of *S. aureus* on the skin of AD patients, most studies have focused on the role of staphylococcal exotoxins in AD [9]. However, 50% of *S. aureus* strains isolated from AD patients are not capable of producing superantigens [3, 10, 11] and, therefore, the role of these strains in the skin lesions of AD patients is not fully understood. A previous study demonstrated that intradermal injection of a cell wall component of *S. aureus*, LTA, could induce localized AD-like inflammation, which was characterized by infiltration of mast cells, eosinophils and mononuclear cells into the dermis [7]. Therefore, *S. aureus* strains that do not produce toxins that act as superantigens would also be capable of causing inflammation of AD lesions.

Mast cell numbers have been shown to increase in the lesional skin of AD patients [12–14], and these mast cells release inflammatory chemical mediators, such as histamine, leukotrienes and prostaglandins, on IgE-mediated stimulation with specific allergens. Furthermore, mast cells produce a wide variety of cytokines, suggesting their active participation in the late-phase reaction and the chronic stage of inflammation [15, 16]. The purpose of this study was to determine the effects of another cell wall component, PEG, on mast cell induction in murine skin. PEG was applied via the percutaneous route since this is a physiologically possible route for antigen invasion. The data from the study show that skin of mice given PEG contained an increased number of mast cells compared with skin of mice treated with PBS. It seems that this result is specific to PEG, since superantigenic

exotoxins such as staphylococcal enterotoxin B and toxic shock syndrome toxin-1 did not induce an increase in mast cell numbers in the dermis of mice (data not shown). As the density of *S. aureus* in AD patients exceeds 1×10^7 organisms/cm² of their skin lesions, it seems $10 \mu\text{g PEG/cm}^2$ is a physiologically possible amount [17]. In addition, application of PEG to abdominal skin of mice increased the levels of expression of mRNA for TGF- β_1 , but not IL-3 or SCF, in the skin. Immunohistologic analysis demonstrated that levels of TGF- β_1 transcripts corresponded with those of protein synthesis. Furthermore, intraperitoneal injection of anti-TGF- β_1 antibodies neutralized mast cell induction in the skin. TGF- β_1 is a member of the TGF- β superfamily that exerts a wide range of biological functions [18]. In the murine system, IL-3 and SCF induce not only mast cell proliferation [19, 20], but also mast cell migration [21, 22], and TGF- β_1 also mediates more potent mast cell chemotaxis [23]. A previous report demonstrated that intraarticular injection of TGF- β_1 leads to inflammatory synovitis with a large number of mast cells [24]. This fact, and our present study, suggests that localized TGF- β_1 production in the skin would induce mast cell migration.

TGF- β_1 production was observed mainly in the basal cell layer of the epidermis showing that differentiating and proliferating keratinocytes produced TGF- β_1 . Indeed, *in vitro* experiments using keratinocytes from normal murine skin suggested that PEG would directly stimulate keratinocytes and induce TGF- β_1 production. The similar TGF- β_1 mRNA expression was also observed in PEG-stimulated normal human keratinocytes (data not shown). Since PEG is well known as a toll-like receptor 2 (TLR 2) agonist [25], signaling through TLR2 would be associated with TGF- β_1 production. The results of RT-PCR using a TLR2 primer set predicted the existence of TLR2 in mouse and human keratinocytes (data not shown), and it has been already confirmed that PEG used in this study is a specific TLR2-stimulant [26]. In PEG-treated murine skin, mast cells were observed mainly in the upper dermis, and mast cells in the skin of AD patients were also frequent in the superficial dermis [13]. These observations suggest that TGF- β_1 produced predominantly in the basal cell layer of the epidermis may play an important role in the induction of chemotaxis of mast cells into the upper dermis of the skin of AD patients. Furthermore, the fact that mast cells were not observed in the epidermis suggests that TGF- β_1 produced in the epidermis would be unable to make mast cells migrate beyond dermoepidermal junction.

Previous reports have not fully explained the importance of *S. aureus* in mast cell induction in the lesional skin of AD patients. The present results suggest that skin colonization with *S. aureus* may play a critical role in perpetuating skin tissue inflammation through mast cell infiltration induced not only by LTA, but also by PEG, which are common cell components in superantigen exotoxin-producing and -non-producing *S. aureus* strains. As the skin of most AD patients shows superficial *S. aureus* colonization and barrier disruption because of reduced ceramide levels [27], PEG and LTA would be expected to penetrate continuously into the skin. Therefore, antimicrobial treatment in a subgroup of AD patients, irrespective of whether they show clinical signs of superinfection, may be considered as a new therapeutic strategy for AD.

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