Acute Cutaneous Barrier Perturbation Induces Maturation of Langerhans’ Cells in Hairless Mice

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Perturbation of the cutaneous water permeability barrier has been shown to result in many physiological events in keratinocytes, including upregulation of proinflammatory cytokine production. However, little is known about the effect of barrier disruption on Langerhans’ cells (LC). In the present study, we examined whether acute barrier perturbation by acetone treatment or tape stripping affects the phenotypes and functions of LC in male hairless mice. Both procedures resulted in a two- to five-fold increase in the expression of MHC class II antigens, B7-2 and intercellular adhesion molecule-1 on LC, whereas no significant changes were detected in levels of B7-1. Levels of intracellular interleukin-1β, as determined by flowcytometry, were also increased 1 h after acetone treatment or tape stripping. LC obtained from barrier-disrupted mice induced a significant increase in syngenic and allogeneic T-cell proliferation, compared to those from saline-treated mice. These results indicate that LC play a crucial role in maintaining cutaneous homeostasis against increasing exposure to external substances resulting from barrier disruption. Key words: acetone treatment; tape stripping; B7-2; interleukin-1β; antigen-presenting capacity.

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One of the most obvious functions of the skin is to prevent loss of fluids from the body (1). The cutaneous water permeability barrier is attributed to the extracellular lipid domains of the stratum corneum, which arise from the exocytosis of the lipid enriched contents of epidermal lamellar bodies (2, 3). Reduced skin barrier function is associated with several cutaneous disorders, such as atopic dermatitis and occupational hand eczema (4, 5).

It is now known that the epidermis serves as the site initiating immune responses, rather than just as a target tissue for these reactions (6). Langerhans’ cells (LC), as principal antigen-presenting epidermal cells, play an essential role in contact hypersensitivity reactions by taking up antigen, transporting it to lymph nodes, processing it if necessary, and presenting it to T-cells in an MHC-restricted manner (6–9). In atopic dermatitis, LC have been shown to take up and present house dust mite antigens to T-cells via high affinity receptor for IgE (10, 11). To better understand the pathogenesis of skin diseases in which the cutaneous permeability barrier is diminished, it is worth analyzing the influence of barrier abrogation on LC. However, there have been few studies on the effect of cutaneous barrier disruption on the function and phenotypes of LC (12). Perturbation of the cutaneous barrier caused by acetone treatment, tape stripping, or an essential fatty acid-deficient diet has been shown to stimulate production of proinflammatory cytokines by epidermal cells, most of which are keratinocytes (2, 13–15). Therefore, barrier disruption could affect LC directly and/or indirectly.

In this study, we investigated whether cutaneous barrier disruption influences the function and the phenotypes of LC, using hairless mice. In addition to MHC class II molecules, activation of T-cells by LC requires co-stimulatory molecules such as B7-1 and B7-2, which provide co-stimulatory signals by interacting with CD28 on T-cells (16). We examined the levels of expression of MHC class II, B7-1, B7-2 and intercellular adhesion molecule (ICAM-1) on LC after acute barrier disruption, since they increased in LC maturation induced by hapten painting and short-term culture (17). We then analyzed the effect of acute barrier perturbation on the levels of intracellular interleukin (IL)-1β, since LC-derived IL-1β is considered to be required for the initiation of primary immune responses in the skin (18, 19). We also investigated whether acute barrier perturbation affects antigen-presenting capacity, as determined by mixed dermal cell lymphocyte reactions.

MATERIAL AND METHODS

Animals
Male hairless BALB/c mice, aged 4 weeks, were obtained from Kyudoh Inc. (Kumamoto, Japan) and used at 8 weeks of age.

Acute barrier perturbation and occlusion
Acute perturbation of the cutaneous barrier was performed as previously described (2). Briefly, the right side of the truncal skin was treated with acetone-soaked cotton balls, by gently rolling the balls along the skin for 10 min. Alternatively, barrier abrogation was achieved by repeated applications of cellophane tape (eight to ten times). These procedures were terminated when the transepidermal water loss reached 8 mg/cm²/h, as measured with an electrolyte water analyzer (TM210, Kourage – Kanzawa Electronic, Germany). The left side of the truncal skin was treated with saline for 10 min or left untreated. Occlusion of the epidermis was accomplished as described elsewhere, by sliding mice into one finger of a latex glove so that the entire treated area was covered snugly with a water vapor-impermeable membrane immediately after completing barrier disruption (13). Six independent experiments were performed. In each experiment, groups of five mice were sacrificed by cervical dislocation at the indicated time and prepared for analyses.

Epidermal cell preparation
Truncal skin was treated with trypsin (type XI, Sigma Chemical Co., St. Louis, MO, U.S.A.) or Dispase (type II, Godo Syusel, Tokyo, Japan), as described previously (7, 17). The epidermal cells were suspended in PBS containing 1% fetal bovine serum (washing buffer). LC enrichment was carried out with lymphocyte M gradients (Cedarlane Laboratories Ltd., Ontario, Canada), as described (17). This procedure resulted in enrichment of LC to 5–8%.

Flowcytometry
Epidermal cell suspensions were first incubated with anti-CD32/16 monoclonal antibody (MoAb, 24G2) for 3 min and then treated with FITC-conjugated anti-I-Aα MoAb (AMS-32.1) and PE-conjugated...
anti-B7-1 MoAb (1G10), PE-conjugated anti-B7-2 MoAb (GL1), PE-conjugated anti-ICAM-1 MoAb (38K), or isotype-matched mouse, rat or hamster control antibodies for 30 min on ice. These MoAbs were purchased commercially from Pharmingen (San Diego, CA, U.S.A.). The stained cell populations were analyzed on a FACScan (Becton Dickinson, San Jose, CA, U.S.A.). Percentage mean fluorescence intensity (MFI) was calculated as follows (17):

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\% \text{ MFI} = \frac{\text{MFI of LC treated with acetone}}{\text{MFI of LC treated with saline}} \times 100\%
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**Intracytoplasmic IL-1β**

Two-color flow cytometry for intracellular IL-1β was carried out as described previously, with some modification (20). Epidermal cell suspensions enriched for LC by density gradient were first incubated with anti-CD32/16 MoAb for 3 min and then treated with FITC-conjugated anti-I-A^β MoAb for 30 min at 4°C. These cells were washed twice with washing buffer and fixed with 4% paraformaldehyde at 4°C for 15 min. The cells were washed again with washing buffer and cryopreserved at -70°C in PBS containing 1% bovine albumin and 10% dimethyl sulfoxide. Cryopreserved cells were thawed at 37°C, washed in washing buffer, and then the pellet was resuspended in PBS containing 1% fetal bovine serum and 0.1% saponin. The cell suspensions were incubated with hamster anti-IL-1β MoAb (Genzyme, Cambridge, MA, U.S.A.) for 30 min at room temperature. After washing with PBS containing 1% fetal bovine serum and 0.1% saponin, the cells were incubated with PE-labeled goat anti-hamster IgG antibody (Caltag Laboratories, Burlingame, CA, U.S.A.) for 30 min at room temperature. Samples were analyzed on a FACScan.

**Mixed epidermal cell-lymphocyte reaction (MECLR)**

LC-enriched populations of hairless BALB/c mice were incubated with 50 μg/ml of mitomycin C (Sigma) at 37°C for 30 min, washed four times and used as stimulator cells. Responder allogeneic and syngeneic T-cells were obtained from spleen cells of C3H/HeN and BALB/c mice, respectively, by a panning method with goat anti-mouse immunoglobulins (Saitakaku Corporation, Tokyo, Japan), as previously described (21). Various numbers of stimulator EC were co-cultured with responder T-cells (2 × 10^5/well) in triplicate wells in 96-well flat-bottom microtiter plates for 96 h. To estimate T-cell proliferation, the number of viable cells was assayed using a Cell Titer 96^TM^ AQueous Non-Radioactive Cell Proliferation Assay^TM^ (Promega, Madison, WI, U.S.A.) according to the manufacturer’s instructions (7). The absorbance of the formazan product at 490 nm (OD490) was measured with a microplate reader (Model 450, BIO-RAD, Hercules, CA, U.S.A.).

**Statistical analysis**

The Mann-Whitney U-test was used to compare results. A value of p < 0.05 was considered significant.

**RESULTS**

**Expression of MHC class II antigens and co-stimulatory molecules in LC**

We initially analyzed whether acute barrier perturbation by acetone treatment or tape stripping affects the phenotypes of LC by flow cytometry. The result of representative data from six different experiments is demonstrated in Fig. 1. As seen in Fig. 1A and 1B, acute barrier perturbation by acetone treatment or tape stripping resulted in approximately a three- and two-fold increase in the levels of MHC class II molecules and ICAM-1 after 12 h, respectively. The expression of B7-2 on LC was upregulated 6 h after barrier disruption and increased four- to five-fold after 12 h. The levels of MHC class antigens, B7-2, and ICAM-1 after 12 h of treatment, compared with those in saline-treated mice, are statistically significant. The level of B7-2 expression was still significantly higher than in saline-treated mice 48 h after barrier perturbation. However, B7-1 expression was not significantly affected by acute barrier disruption. These procedures did not affect the percentage of MHC class II-positive cells, as determined by flow cytometry (data not shown). Occlusion immediately after tape stripping

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Flowcytometric analysis of intracellular IL-1β

It has been reported that activated LC produce IL-1β but do not secrete it at detectable levels (22). In addition, it is difficult to obtain a highly purified LC population large enough to determine cytokine secretion by the ELISA system. Therefore, we analyzed the levels of IL-1β in the cytoplasm of LC by flowcytometry to determine whether acute barrier perturbation upregulates IL-1β production by LC. The result of representative data from six different experiments is demonstrated in Fig. 1A and 1B. LC treated with saline showed detectable levels of IL-1β in the cytoplasm, suggesting that LC produce IL-1β constitutively (data not shown). Intracellular IL-1β levels increased at 1–24 h after perturbation (2–2.5 fold increase) but had returned to control levels by 48 h; our data, obtained by indirect immunofluorescence, are not strictly quantitative. Occlusion after disruption did not block the upregulation of intracellular IL-1β levels (Fig. 1C).

MECLR

To determine whether acute barrier perturbation affects the antigen-presenting capacity of LC, we carried out both syngeneic and allogeneic MECLR, using LC harvested from the back skin at various time periods after barrier disruption. The result of representative data from six different experiments is demonstrated in Fig. 2. In terms of syngeneic and allogeneic T-cell stimulatory functions, LC from acetone-treated or tape-stripped skin showed significantly increased stimulatory activity compared with those from non-treated skin (Fig. 2).

![Graphs showing ALLOGENEIC and SYNGENEIC LC responses](image)

**Fig. 2.** Acute barrier perturbation upregulates the antigen presenting function of LC. Epidermal suspensions obtained 12 h after treatment with acetone (---○--), tape stripping (---■--) or saline (---□--) were enriched by density gradient centrifugation. Various numbers of LC were co-cultured with spleen T-cells of C57/HsN or BALB/c mice for 96 hr. T-cell proliferation is given as an absorbance value at 490 nm. *p < 0.05 versus control.

**DISCUSSION**

Our results show that acute barrier disruption by acetone treatment or tape stripping induces LC maturation. Acute barrier perturbation was achieved by chemical and physical means and resulted in an increase in TEWL. In addition, occlusion treatment after barrier disruption partly suppressed the upregulation of the antigen-presenting function of LC and the expression of MHC class II and co-stimulatory molecules on LC. Therefore, we speculate that the changes demonstrated in this study are induced by barrier disruption and are not non-specific effects of acetone or tape stripping.

Recent studies have shown that the levels of epidermal proinflammatory cytokines such as IL-1α, IL-1β, tumor necrosis factor (TNF)-α and granulocyte macrophage-colony stimulating factor (GM-CSF) are increased following acute barrier disruption (2, 13-15). Several studies have demonstrated that these cytokines can induce maturation of LC (17, 19, 23-25). The antigen-presenting function of LC, as determined by MECLR, is upregulated by IL-α, IL-1β and GM-CSF (24-26). Ozawa et al. (17) and Chang et al. (23) showed that in vivo or in vitro treatment with these proinflammatory cytokines induces an increase in the expression of MHC class I molecules and co-stimulatory molecules: ICAM-1, B7-1 and B7-2. Therefore, it is suggested that the upregulation of the accessory function of LC and the expression of these molecules on LC following barrier perturbation are induced in part by keratinocyte-derived proinflammatory cytokines (2, 13-15).

Interestingly, acute barrier disruption significantly upregulated the expression of B7-2 on LC, whereas the expression of B7-1 is not increased. B7-2 is the dominant co-stimulatory ligand during primary immune responses, whereas the level of B7-1 is upregulated later in immune responses (27). For example, MoAbs against B7-2 much more strongly inhibit allogeneic MECLR than those against B7-1 (28). The upregulation of the expression of B7-2 soon after acute barrier perturbation is a homeostatic response, since it causes an increase in exposure to external substances, including chemical and environmental allergens (12). It has been shown that the expression of B7-1 and B7-2 on LC is differentially regulated by several cytokines (16, 17, 23). Ozawa et al. (17) demonstrated that an intradermal injection of IL-1β and TNF-α increased the expression of MHC class II molecules, ICAM-1 and B7-2, but not B7-1. This result implies that the differential upregulation of the co-stimulatory molecule expression induced by acute barrier disruption is attributed to TNF-α derived from keratinocytes and IL-1β produced mainly by LC. We intend to investigate this further by treatment with MoAbs against proinflammatory cytokines before barrier perturbation.

In this study, occlusion immediately after barrier disruption partly blocked the increase in the expression of MHC class II antigen and co-stimulatory molecules. Wood et al. (13) have demonstrated that occlusion did not inhibit the increase in TNF-α, IL-1α and IL-1β mRNA levels or TNF-α protein levels induced by acute barrier perturbation. We also observed that occlusion did not inhibit the increase in the level of intracytoplasmic IL-1β induced by acute barrier perturbation. These facts suggest that the maturation of LC observed in this study is attributed to other factors which are suppressed by occlusion, for example, the increased exposure to external substances resulting from acute barrier disruption.

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The earliest event detected in the activation of mouse LC after hapten painting is an increase in the level of IL-1β mRNA and protein (18, 32). However, epicutaneous application of irritants or tolerogen does not induce these changes, suggesting that LC-derived IL-1β is required for initiation of the primary immune response in skin (18, 32). We detected increased levels of intracellular IL-1β 1 h after acute barrier disruption. This implies that LC participate in cutaneous homeostasis soon after barrier perturbation by self-activating, at least in part, in an autocrine fashion (19). The mechanisms by which the production of IL-1β by LC is stimulated during acute barrier disruption are unknown. Keratinocyte-derived proinflammatory cytokines, some of which are detected within 10 min after barrier perturbation, may induce the upregulation of IL-1β production by LC (15, 19, 33). Injection of IL-1α or TNF-α upregulates IL-1β production by LC but weakly so relative to hapten painting (19). This may explain why there is a much weaker increase in the level of intracytoplasmic IL-1β in LC of barrier-disrupted skin than in that of hapten-treated skin (unpublished observation). Alternatively, the increased exposure to external substances resulting from acute barrier disruption may cause upregulation of IL-1β production by LC.

In summary, we demonstrated that acute barrier perturbation induces LC maturation both phenotypically and functionally. It is likely that these reactions are a homeostatic response, since barrier disruption results in increased exposure to external substances, including chemical and environmental allergens. In addition, the excessive response of LC may contribute to the pathogenesis of several inflammatory skin diseases, such as atopic dermatitis and occupational dermatoses.

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