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IL-10-Producing Langerhans Cells and Regulatory T Cells Are Responsible for Depressed Contact Hypersensitivity in Grafted Skin

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Although skin grafting is a common surgical technique, the immunological state of grafted skin remains unelucidated. An experimental model has shown that the development of murine contact hypersensitivity (CHS) is depressed when mice are sensitized with a hapten through full-thickness grafted skin. We explored the immunological mechanisms underlying this hyposensitization, focusing on the fate of Langerhans cells (LCs). When FITC was applied to grafted skin, FITC-bearing LCs were capable of migrating to the draining lymph nodes. Epidermal cell suspensions isolated from the grafted skin produced a high amount of IL-10 as assessed by real-time PCR. Adoptive transfer of immune lymph node cells from the sensitized mice suppressed the CHS response of recipients in an antigen-specific manner. $CD4^+CD25^+$ but not $CD4^+CD25^-$ T cells purified from lymph node cells were responsible for this suppression. Finally, we detected high expression of receptor activators of nuclear factor κ-B ligand (RANKL) in the grafted skin, and found that recombinant RANKL stimulated LCs to produce IL-10. These findings suggest that the hyposensitization of CHS through the grafted skin is not attributable merely to the reduction of LC number but that IL-10-producing LCs exert a downmodulatory effect by inducing regulatory T cells.

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INTRODUCTION

Skin grafting is frequently employed in dermatology and plastic surgery. Skin grafts from the same individual can be successfully implemented 7 days after an operation, and they survive throughout the patient's life. Full-thickness skin grafts exhibit a well-matched appearance, whereas thin-thickness grafts match poorly in texture. Although there have been many cosmetic studies on skin grafting, little is known about the immunological state or the fate of immunocompetent cells in grafted skin. In particular, the function of Langerhans cells (LCs) in grafted skin is poorly understood. Clinical observations have suggested impaired immunity in grafted skin (Doiurnon *et al.*, 2001), and the lack of dermatitis on the graft is empirically known; therefore, some immunological alterations are thought to take place in the graft. Only one study has demonstrated that murine contact hypersensitivity (CHS) to a hapten cannot develop upon sensitization through full-thickness grafted skin (Yasuda *et al.*, 1996).

LCs are the major immunocompetent cells in the skin (Katz *et al.*, 1979; Stingl *et al.*, 1980) and have a positive (Silberberg-Sinakin and Thorbecke, 1980) or, in certain conditions, suppressive (Kaplan *et al.*, 2005) role in the development of CHS. They take up external antigens, migrate to draining lymph nodes, and present the antigenic determinant to naive T cells in the context of major histocompatibility complex (MHC) molecules. Thus, LCs are critical in sampling and presenting antigens in the skin. Recent studies have disclosed an immunoregulatory role of LCs. These epidermal dendritic cells (DCs) may exert a suppressive effect when they produce IL-10 (Kang *et al.*, 1998; Flacher *et al.*, 2006). This is consistent with the observation that IL-10 production by pulmonary DCs is critical for the induction of tolerance (Akbari *et al.*, 2001). Recently, it has been shown that receptor activators of nuclear factor κ-B ligand (RANKL) produced in UV light-irradiated epidermis mediate immunosuppression by modulating LCs (Loser *et al.*, 2007).

In this study, we explored the fate and functional alterations of LCs in full-thickness grafted skin using a murine CHS experimental system. Our results suggest that the immunological tolerance induced by sensitization through

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Abbreviations: CHS, contact hypersensitivity; DC, dendritic cell; dDC, dermal DC; EC, epidermal cell; LC, Langerhans cell; mAb, monoclonal antibody; MACS, magnetic cell sorting; MHC, major histocompatibility complex; PE, phycoerythrin; PCl, picryl chloride; Treg, regulatory T cell; TNF, tumor necrosis factor; RANKL, receptor activator of nuclear factor κ-B ligand

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the grafted skin is mediated by IL-10-producing LCs after the induction of $CD4^+CD25^+$ regulatory T (Treg) cells. Over-expressed RANKL in the keratinocytes of grafted skin may stimulate LCs to produce IL-10.

RESULTS

Poor development of CHS in mice sensitized with PCl through grafted skin

To confirm the previous observation by Yasuda *et al.* (1996) and to further examine whether the suppression of CHS by sensitization through skin grafting is a local or systemic phenomenon, mice were sensitized with picryl chloride (PCl) through grafted dorsal skin or non-grafted abdominal skin after skin graft implementation (on day 7 after operation). When PCl was applied to the grafted area for sensitization, the ear swelling challenge response was significantly lower than that of the positive control without skin graft (Figure 1). In contrast, sensitization of the skin-grafted mice through the non-grafted abdominal area did not abrogate CHS response. Similar data were obtained from three independent series of experiments. Thus, the depressed CHS response occurred only when sensitization was performed through the grafted local area.

Numerical alteration and apoptosis of LCs in grafted skin

LCs are critical for CHS, as they serve as antigen-presenting cells and migrate to the draining lymph nodes (Romani *et al.*, 2003). We therefore investigated the change in number of LCs in the grafted skin along with the draining lymph nodes. Epidermal sheets were taken from the grafted skin on days 7 and 14 after operation and stained with phycoerythrin (PE)-labeled anti-I-A monoclonal antibody (mAb). The number of LCs was lower in the grafted skin (200–300 mm^{-2}) on day 7 than in the untreated control skin (800–1,000 mm^{-2}). Moreover, the number of LCs in grafted skin returned to normal on day 14 (data not shown). These results indicate that the LC number was reduced at the time of sensitization. Morphologically, LCs in the grafted skin exhibited a round appearance. This reduction in LC number was not due to the migration of LCs from the skin, as the number of DCs was not increased in the regional lymph nodes (data not shown). Epidermal cell (EC) suspensions from normal skin or grafted skin were assessed for apoptosis by flow cytometry. The fraction of apoptosis in LCs was demonstrated as the Annexin-V-positive propidium iodide-negative subset in the grafted skin (Figure 2a). The percentage of apoptotic LCs was significantly increased on day 1 after grafting, but returned to the baseline on day 4 (Figure 2b).

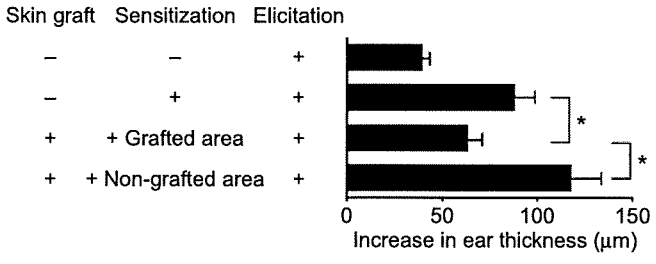

Figure 1. CHS responses in mice sensitized with PCl through grafted or non-grafted skin. Mice were sensitized with PCl on the grafted back skin or non-grafted abdominal skin 7 days after skin grafting. PCl was challenged on each earlobe 5 days after sensitization. The change in ear thickness was measured 24 hours later. Data are representative of three independent experiments. Each group consisted of more than four mice. *$P<0.05$.

Phenotypes and numbers of migrating LCs and dermal DCs in grafted skin

LCs are capable of migrating from the epidermis into the lymph nodes on sensitization (Kabashima *et al.*, 2003). The migratory ability of LCs in grafted skin was examined with FITC, which is not only a hapten but also a cell-tracking marker. On day 7 after skin grafting, FITC was applied to the grafted area. Draining lymph node cells were taken 24 hours later and labeled with allophycocyanin-labeled anti-I-A mAb, anti-mouse CD205 rat IgG, followed by PE-conjugated anti-rat IgG mAb. $FITC^+$ MHC class II^+ cells were defined as migrating DCs from the skin. CD205 is expressed by LCs as well as by dermal DCs (dDC; Henri *et al.*, 2001). In fact,

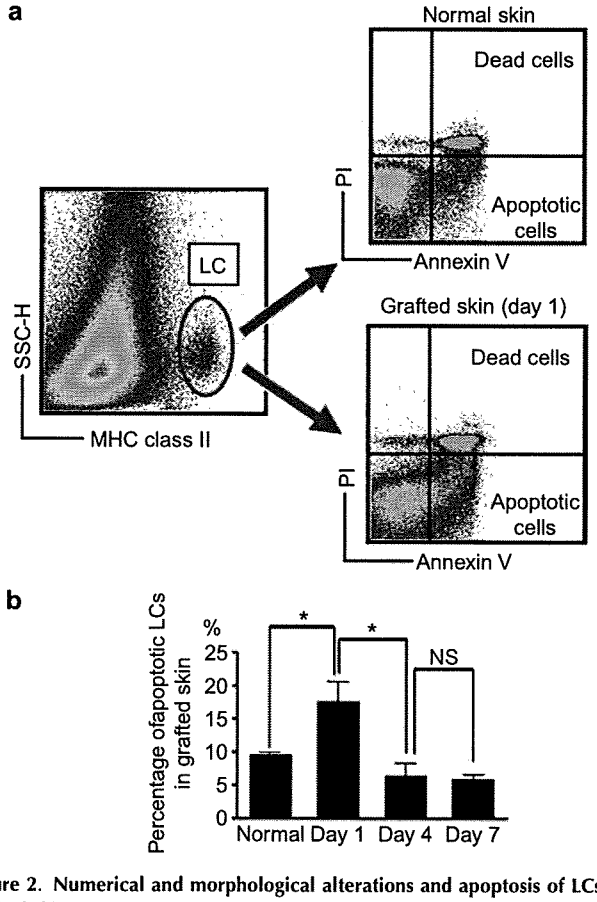

Figure 2. Numerical and morphological alterations and apoptosis of LCs in grafted skin. (**a**) Apoptosis of Langerhans cells in the grafted skin. EC suspensions from the 1-day grafted or non-grafted skin were stained with FITC-conjugated MHC class II, APC-conjugated Annexin V and PI. Apoptotic LCs were defined as MHC class II^+ Annexin V^+, but PI^-, whereas necrotic cells were double positive. (**b**) Percentage of apoptotic LCs after grafting procedure. Day 0 represents normal skin and day 1, 4, or 7 shows the day after grafting. Data are representative of three independent experiments. Each group consisted of five mice. *$P<0.05$.

migrating DCs were divided into a CD205$^+$ Langerin$^+$ subset and a CD205$^-$ Langerin$^-$ subset. Therefore, the vast majority of FITC$^+$ MHC class II^{hi} CD205$^+$ cells represent LCs, whereas FITC$^+$ MHC class II^{hi} CD205$^-$ cells are mostly dDCs (Figure 3a).

To determine whether migrating LCs/dDCs in grafted skin retained their antigen-presenting capacity, migrating DCs were stained with PE-conjugated mAbs to CD80 and CD86. CD80 and CD86 were present on both LCs and dDCs. Thus, LCs/dDCs in grafted skin retained their antigen-presenting capacity for naive T cells.

Compared to the control mice painted with FITC on normal skin, the mice sensitized at the skin-grafted site had reduced numbers of both FITC$^+$ MHC class II^{hi} CD205$^+$ and FITC$^+$ MHC class II^{hi} CD205$^-$ populations in the draining lymph nodes (Figure 3c). From three independent series of experiments, the number of LCs and/or DCs in the lymph nodes of grafted skin-sensitized mice was approximately one-fourth that of normal skin-sensitized mice (Figure 3c). Considering that the grafted skin originally had about one-fourth the LCs of normal skin, the migratory ability of LCs in grafted skin was virtually the same as that in normal skin.

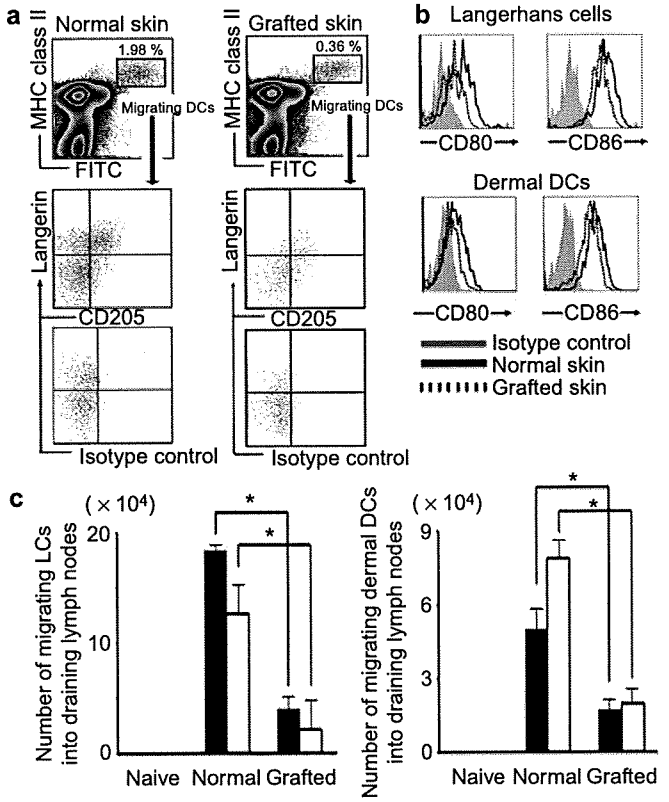

Figure 3. Expression of co-stimulatory molecules and number of migrating DCs in grafted mice. (**a**) Draining lymph node cells were taken from mice painted with FITC on the grafted or normal skin and stained with anti-MHC class II, CD205 and Langerin mAbs. CD205$^+$ cells are virtually the same population as Langerin$^+$ cells. (**b**) After being stained with anti-CD205, CD80, and CD86 mAbs, lymph node cells were subjected to flow cytometric analysis to assess the expression of co-stimulatory molecules. (**c**) Numbers of FITC$^+$ MHC class II^{hi} CD205$^+$ cells (mainly LCs) and FITC$^+$ MHC class II^{hi} CD205$^-$ cells (mainly dDCs) migrating from the skin to the draining lymph nodes. The numbers are calculated based on flow cytometric analysis gated as seen in Figure 3a. Each group consisted of more than four mice. *$P<0.05$.

Increased IL-10 expression in grafted skin

It is well known that UV B (UVB) radiation has immunosuppressive effects on normal cutaneous processes (Elmets *et al.*, 1983; Yagi *et al.*, 1996) and this UV-induced immunosuppression is associated with upregulation of anti-inflammatory Th2 cytokines, IL-4 and IL-10 (Rivas and Ullrich, 1992; Shreedhar *et al.*, 1998). Among cytokines, IL-10 is critical for CHS suppression (Simkin *et al.*, 2000), and both LCs (Takashima, 1995; Flacher *et al.*, 2006) and keratinocytes (Rivas and Ullrich, 1992) are possible candidates for the IL-10 source. To address the involvement of IL-10, mice receiving skin grafts (day 0) were either sensitized with PCl (day 7) on the dorsal grafted skin or untreated. As control, mice without skin grafts were sensitized with PCl on the dorsal skin or untreated. EC suspensions were obtained from the dorsal skin of these four experimental groups (day 8). As shown in Figure 4a, whereas the normal skin had little or no ability to elaborate IL-10 irrespective of PCl sensitization, the grafted skin expressed high levels of IL-10 mRNA, as assessed by real-time PCR. Relative amounts of mRNA were measured by the $\Delta\Delta C_t$ method (Atarashi *et al.*, 2007). Although both grafted skin samples with and without subsequent PCl painting yielded IL-10 mRNA, the nonsensitized epidermis was more productive; it may be that IL-10 produced by keratinocytes was reduced in proportion to

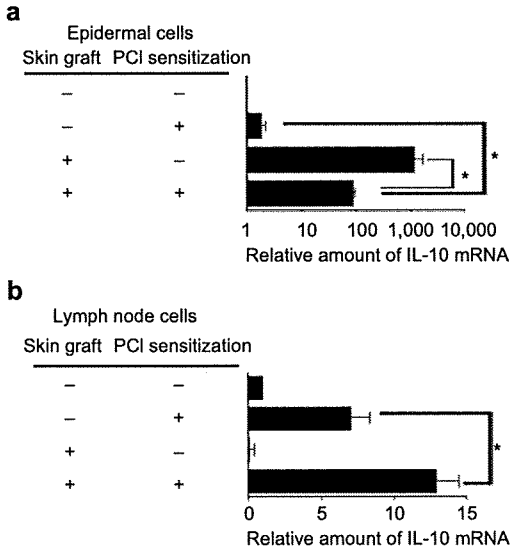

Figure 4. IL-10 expression in ECs and lymph node cells from grafted mice. (**a**) Epidermal sheets were prepared from grafted (7 days after operation) or non-grafted skin that was painted with PCl or left untreated. IL-10 mRNA expression was measured by real-time PCR. (**b**) Draining lymph node cells were obtained 5 days after sensitization of mice through grafted or non-grafted skin. IL-10 mRNA expression was measured by real-time PCR. The relative amounts of mRNA expression were calculated using the $\Delta\Delta C_t$ method. Each group consisted of more than four mice. Data are the mean ± SD of three independent experiments. *$P<0.05$.

PCl sensitization or, alternatively, IL-10-producing LCs emigrated on sensitization.

Along with ECs, we examined the expression of IL-10 mRNA in the draining lymph nodes. Lymph node cells were prepared from mice receiving skin grafts and/or subsequent PCl painting. Skin grafting alone did not augment IL-10 production by lymph node cells (Figure 4b). PCl sensitization on the grafted skin dramatically enhanced the production of IL-10. This increase of IL-10 was considered to be derived from LCs or proliferative T cells in the draining lymph nodes. As the PCl-painted grafted skin produced less IL-10 than the PCl-non-painted grafted skin (see Figure 3a), we postulate that IL-10-producing LCs emigrated from the grafted skin to the lymph nodes on sensitization with PCl.

LCs as a source of IL-10 in grafted skin

To dissect the cytokine-producing populations in grafted skin, EC and dermal cell suspensions were prepared from grafted (day 7) or normal skin. EC suspensions and dermal cell suspensions were fractionated to $CD11c^+$ (containing 70–80% LCs or dDCs and <0.01% T cells) and $CD11c^-$ (containing mainly keratinocytes or fibroblasts and <0.05% DCs) subsets with magnetic cell sorting (MACS). As shown in Figure 5, both $CD11c^+$ and $CD11c^-$ fractions produced a high amount of IL-10, indicating that not only keratinocytes but also LCs from the grafted skin were stimulated to produce IL-10. In contrast, $CD11c^-$ cells secreted more IL-6 in grafted skin than normal skin, suggesting that keratinocytes in the graft produced IL-6. Both $CD11c^+$ and $CD11c^-$ cells produced higher amounts of tumor necrosis factor-α (TNF-α) in the grafted skin than in the normal skin. Surgical trauma induces an early hyperinflammatory response, which is characterized by proinflammatory TNF-α, IL-1, and IL-6 cytokine release (Menger and Vollmar, 2004). IL-6 has a crucial role in the neutrophil and macrophage infiltration in the wound healing process (Lin *et al.*, 2003). Thus, this increase of IL-6 and TNF-α is considered to be a hyperinflammatory response of the wound healing process.

Induction of $CD4^+CD25^+$ regulatory T cells by sensitization through grafted skin

The presence of IL-10-producing LCs in the epidermis and lymph nodes raised the possibility that Treg cells were induced in mice sensitized with PCl through grafted skin. We performed an adoptive transfer study to evaluate this possibility. Donor mice were sensitized with PCl on the grafted skin, and lymph nodes and spleen cells were taken from the mice 5 days later (Figure 6, top). $CD4^+CD25^+$ and $CD4^+CD25^-$ subsets were purified from the pooled cells by

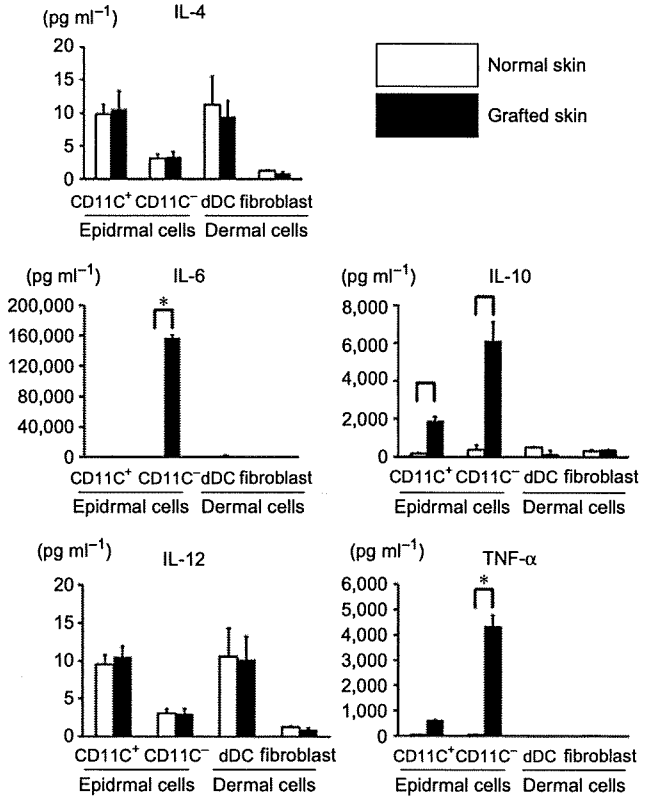

Figure 5. Cytokine production by ECs and dermal cells. EC suspensions were obtained from grafted or non-grafted skin, and fractionated to $CD11c^+$ cells (70–80% LCs) and $CD11c^-$ cells (LC-depleted cells, mainly keratinocytes) with auto-MACS using CD11c microbeads. Dermal cell suspensions were centrifuged with Ficoll-Paque, followed by auto-MACS, to fractionate dDCs and other cells (mainly fibroblasts). Each subset was cultured for 24 hours. The amounts of IL-4, IL-6, IL-10, IL-12, and TNF-α in the culture supernatants were quantified using cytometric beads array. Data are the mean ± SD of three independent experiments. Each group consisted of more than four mice. *P<0.05.

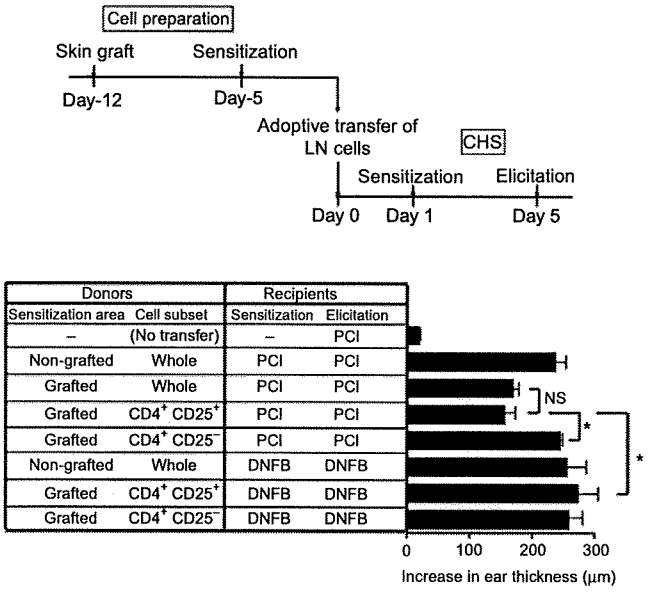

Figure 6. Transfer study of $CD4^+CD25^+$ cells from donor mice sensitized through grafted skin. Mice were sensitized with PCl on the grafted (7 days after operation) or non-grafted area. Five days after sensitization, draining lymph nodes and spleen cells were taken from the mice. Whole cells, $CD4^+CD25^+$ sorted cells, or $CD4^+CD25^-$ sorted cells were transferred into syngeneic naive mice (5×10^6 for each mouse). Then, the recipients were sensitized on the dorsum and challenged on the ears with hapten (PCl or DNFB) as indicated in the figure. Change in ear thickness was measured 24 hours later. Each group consisted of more than four mice. Data are representative of three independent experiments. *P<0.05.

MACS. Whole unfractionated cells or cells of each subset were transferred into syngeneic naive recipients (5×10^6 for each mouse), which were then sensitized and challenged with PCl. The transfer of $CD4^+CD25^+$ T cells decreased the CHS response of the recipients, whereas $CD4^+CD25^-$ T cells were not suppressive (Figure 6, bottom). When recipient mice were sensitized and challenged with another hapten dinitrofluorobenzene, such suppression was not observed, suggesting that the immunosuppression was antigen specific. Thus, the depressed sensitization through the grafted skin was associated with the appearance of $CD4^+CD25^+$ Treg cells.

Augmentation of IL-10 production by LCs exposed to RANKL

It has been recently reported that LCs express receptor activators of nuclear factor κ-B, that UVB irradiation upregulates cutaneous RANKL, and that RANKL activates DCs as well as Tregs in the skin (Loser *et al.*, 2006). We hypothesized that the grafted skin expresses RANKL and activates LCs to produce IL-10. When the grafted skin was stained with anti-RANKL and MHC class II antibodies, we found that ECs of the grafted skin expressed RANKL (Figure 7a, RANKL, blue; LCs, red). Notably, RANKL was strongly expressed around LCs (merged purple), suggesting that receptor activators of nuclear factor κ-B/RANKL interactions might be important for the induction of hyposensitization in grafted skin. To identify the function of receptor activator of nuclear factor κ-B/RANKL in the skin immune system, we tested the ability of LCs to produce IL-10 when they were exposed to recombinant RANKL. EC suspensions were cultured with or without recombinant RANKL (R&D Systems, McKinley, MN) for 24 hours, and stained with anti-MHC class II antibody. Then, the cells were permeabilized and stained with anti-IL-10 antibody. We found that the addition of RANKL increased the fraction of IL-10-positive LCs (Figure 6b) and the mean fluorescence intensity of IL-10 in LCs (Figure 6c). These findings indicated that RANKL expressed by keratinocytes of grafted skin stimulates LCs to produce IL-10.

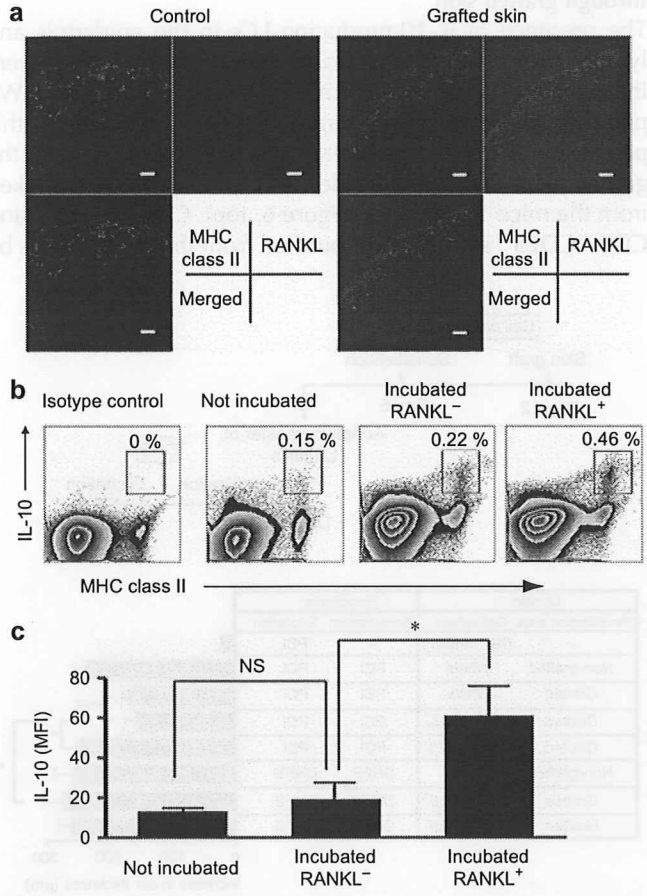

Figure 7. Induction of RANKL in grafted skin and stimulation of LCs to produce IL-10 by RANKL. (**a**) The grafted or non-grafted skin was stained with antibodies to MHC class II and RANKL. Red, MHC-class II; blue, RANKL. Merged image is shown in purple. (**b**, **c**) EC suspensions were cultured with or without recombinant RANKL for 2 days. Cells were fixed, permeabilized, stained with IL-10 and MHC class II antibodies, and analyzed by flow cytometry (**b**). The mean fluorescence intensity of IL-10 in MHC class II + LCs existing in EC suspensions. Bars indicate the mean + SD. Data are representative of three independent experiments. Each group consisted of more than four mice. Scale bar = 50 μm $^*P<0.05$.

DISCUSSION

This study addressed the immunological mechanism underlying impaired sensitization through grafted skin. CHS was depressed only when mice were immunized with hapten through grafted skin, and even skin graft-bearing mice fully developed CHS when sensitized through non-grafted skin. Therefore, the induction of immunosuppression is local, whereas its effects are specific. The local immunological condition of the grafted skin is responsible for impaired induction. The fate of LCs in the graft seems to be a key to resolving the mechanism. Mainly because of the apoptotic death of LCs, the number of LCs in freshly implemented skin was up to one-fourth that of normal skin. However, when FITC was applied to the grafted skin, FITC-bearing LCs were present in the draining lymph nodes, again at a cell number one-fourth that of normal skin-sensitized mice. Thus, LCs were capable of migrating efficiently from the grafted skin to the draining lymph nodes. As LCs can serve as both positive and negative antigen-presenting cells depending on the surrounding milieu (Silberberg-Sinakin and Thorbecke, 1980; Kaplan *et al.*, 2005), not only the numerical but also functional changes of LCs determine CHS development. We further investigated whether the hyposensitization of CHS through grafted skin was attributable merely to the reduction of LC number, or whether some function of LCs was altered.

DCs in peripheral tissues, such as epidermal LCs, remain immature in the steady state, and express small quantities of MHC class II and co-stimulatory molecules and produce low levels of immunostimulatory cytokines. During the process of antigen capture/presentation and migration into T-cell areas of regional lymph nodes, maturation of DCs simultaneously occurs, as they express high amounts of these surface molecules and cytokines (Inaba *et al.*, 1997; Huang *et al.*, 2000; Lutz and Schuler, 2002). Recent studies have revealed that peripheral tolerance is induced by immature DCs (Steinman *et al.*, 2000; Lutz and Schuler, 2002) or partially

by mature DCs that express MHC class II, CD80 and CD86 molecules but lack secretion of IL-12, IL-6, and TNF-α (Groux, 2003). On the basis of their migrating ability, it seemed that nonapoptotic, live LCs in the grafted skin were functionally mature.

Given that surgical trauma is one of the injuries that induce a hyperinflammatory response, the skin graft employed in this study likely leads to the production of proinflammatory cytokines such as TNF-α and IL-6. However, the skin graft is not merely surgical trauma, because living skin is applied to the raw surface. More specifically, the intact ECs produce high amounts of IL-6 and TNF-α (see Figure 5), suggesting that skin grafting induces inflammatory cytokine production more vigorously than simple trauma. In wound healing after skin grafting, the exaggerated inflammatory response should be downregulated in due course. Among various factors that suppress the inflammatory response, IL-10 is one of the most important candidates, because it is a potent inhibitor of the activation of monocytes/macrophages and of the expression of TNF-α and other proinflammatory mediators (Denys *et al.*, 2002). In fact, IL-10 was produced at a high level by keratinocytes in grafted skin. We explored the possibility that LCs have a polarized cytokine production pattern in the graft, which leads to peripheral tolerance. In this scenario, IL-10 is a strong candidate as a skewing cytokine.

LCs in grafted skin exhibited a rounder and less dendritic appearance than those in normal skin. Along with this morphological change, LCs in grafted skin expressed IL-10 at a higher level than those in non-grafted skin, suggesting that IL-10-producing LCs have an important role in depressed CHS. Many studies have shown that IL-10 is an essential cytokine in depression of CHS (Annacker *et al.*, 2001; Girolomoni *et al.*, 2004; Ghoreishi and Dutz, 2006). Similarly, IL-10 production by pulmonary DCs is critical for the induction of tolerance (Akbari *et al.*, 2001). Besides LCs, keratinocytes also secrete IL-10, which causes CHS suppression when overexpressed by certain stimuli such as UVB radiation (Schwarz *et al.*, 2004; Ghoreishi and Dutz, 2006). Keratinocyte-derived IL-10 might further condition LCs to be regulatory as well as suppress the injury-associated inflammation.

Conversely, the adoptive transfer of $CD4^+CD25^+$ T cells from the tolerant mice induced CHS suppression, whereas $CD4^+CD25^-$ T cells had no effect. This suggests that $CD4^+CD25^+$ Treg cells as well as IL-10-producing LCs participate in the skin graft–induced suppression of CHS. At present, at least four types of Treg cells can be identified based on the expression of cell-surface markers, secretion of cytokines, and suppression mechanisms (Groux, 2003). Recent accumulating evidence has indicated that Treg cells have a key role in peripheral tolerance (Takahashi *et al.*, 1998; Shevach, 2001; Taylor *et al.*, 2001; Thorstenson and Khoruts, 2001; Zhang *et al.*, 2001) under the influence of immature or maturing DCs (Min *et al.*, 2003; Roelofs-Haarhuis *et al.*, 2003).

The relationship between IL-10-producing LCs and Treg cells remains partly elucidated. In UVB-induced tolerance, intravenous transfer of Treg cells suppresses CHS, and this phenomenon is dependent on host-derived IL-10 (Ghoreishi and Dutz, 2006). Other groups have reported that Treg cells regulate the expansion of peripheral $CD4^+$ T cells with IL-10 (Annacker *et al.*, 2001). These findings have suggested that IL-10 is essential for Treg cells for successful downmodulation, and the source of IL-10 is a constituent of the immunological milieu, such as epithelial cells, or Treg cells *per se*. However, we found that LCs are the IL-10 producer, raising the possibility that IL-10-producing LCs are an inducer of Treg cells. In addition, IL-10 released from LCs also might inhibit effector T cells concerned with CHS.

A group of investigators have found that RANKL, which is expressed in the keratinocytes of inflamed skin, controls Treg cell numbers by activation of DCs (Loser *et al.*, 2007). In this study, we showed that both epidermal $CD11c^+$ LCs and $CD11c^-$ cells (keratinocytes) produce high amounts of representative proinflammatory cytokine TNF-α in the grafted area, which might lead to the expression of RANKL on keratinocytes. Our finding that LCs exposed to recombinant RANKL produced a high level of IL-10 suggests that RANKL from keratinocytes in the grafted skin can induce IL-10-producing LCs at the initiation stage of immunosuppression. The reduction in LC number may cause impaired sensitization, but in accordance with recent observations (Kaplan *et al.*, 2005; Kissenpfennig *et al.*, 2005), the altered function of LCs is more likely involved in the depression of CHS.

Our study is clinically relevant in two aspects. First, grafted skin provides a specialized immunological status, in which T cells do not respond well to external stimuli such as contactants, as a result of skewed function and a reduced number of LCs. In this context, contact dermatitis cannot easily develop in the grafted skin. Second, the skin graft may be used for the induction of antigen-specific peripheral tolerance by application of antigen through the grafted skin. This strategy may have great potential for controlling allergic diseases and autoimmune disorders. Further investigation of this skin graft-associated immunosuppression may develop safe and effective methodologies for tolerance induction.

MATERIALS AND METHODS

Mice

BALB/c (7- to 10-week-old) female mice were obtained from Kyudo Co. Ltd (Kumamoto, Japan). Mice were maintained on a 12-hour light/dark cycle under specific pathogen-free conditions. Protocols were approved by the Institutional Animal Care and Use Committee of the University of Occupational and Environmental Health.

Preparation of skin graft

One day before skin grafting, the back of each mouse was clipped and hairs were removed with depilatory cream (Shiseido Cosmetic Co., Tokyo, Japan). A 25 × 20 mm area of full-thickness back skin was resected under intraperitoneal anesthesia with ketamine and dolmicam. The same skin was grafted onto the back of each mouse. The graft was sutured and fixed with a tie-over dressing. The dressing was removed on day 7 after operation.

Contact hypersensitivity

For contact sensitization, 25 μl of PCl solution (0.5% w/v in acetone-olive oil mixed at 4:1) was painted on the grafted or non-grafted area. For elicitation, 10 μl of 0.2% PCl solution was painted on each earlobe of the PCl-sensitized mice on day 5 after sensitization. In some experiments, 50 μl of dinitrofluorobenzene (Nacalai Tesque Co., Tokyo, Japan) solution (0.5% w/v in acetone–olive oil mixed at 4:1) was painted on the abdomen, and elicited with 10 μl of 0.3% dinitrofluorobenzene solution on each earlobe 5 days after sensitization. The thickness of each ear was measured with a micrometer 24 hours after elicitation. Swelling was expressed as the increase in ear thickness.

Culture medium

RPMI 1640 (Gibco BRL Life Technology, Grand Island, NY) was supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10^{-5} M sodium pyruvate, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1% nonessential amino acids, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (all from Gibco BRL Life Technology).

Immunohistochemistry

For immunofluorescence analysis, epidermal sheets were separated from the dermis with 0.5 M ammonium thiocyanate, fixed in acetone for 5 minutes at −20 °C, and stained with PE-conjugated anti-I-A^d mAb (BD Biosciences, San Diego, CA). In some experiments, the back skin of mice was frozen in Tissue-Tek OCT compound 4583 (Sakura Finetechnical Co. Ltd, Tokyo, Japan). Cryostat sections (10 μm) were fixed in acetone and stained with PE-conjugated anti-I-A^d mAb, biotin-conjugated anti RANKL mAb (R&D Systems) followed by staining with allophycocyanin-Cy7 conjugated streptavidin. Images were viewed with a Zeiss confocal microscope and processed with an LSM Image Browser (Zeiss).

Preparation of EC and dermal cell suspensions, and purification for LCs and dDCs

Skin sheets were floated in 0.2% trypsin in phosphate-buffered saline (pH 7.4) for 1 hour at 37 °C as described previously (Tokura *et al.*, 1994). The epidermis was separated from the dermis with forceps in phosphate-buffered saline supplemented with 10% fetal calf serum. EC suspensions were prepared by pipetting and filtration through nylon mesh. Dermal cells were obtained from normal or grafted skin from which the epidermis had been removed. Samples were minced and incubated for 2 hours at 37 °C in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with collagenase XI (4,830 U ml^{-1}; Sigma, Tokyo, Japan), hyaluronidase (260 U ml^{-1}; Sigma), DNase (0.1 mg ml^{-1}; ICN, Costa Mesa, CA), and 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Sigma). The obtained cells were filtered through a 40 μm filter. dDCs and other cells (mainly fibroblasts) were fractionated from the dermal cell suspensions with Ficoll-Paque (GE Healthcare UK Ltd) For enrichment of LCs and dDCs, EC suspensions and derma cell suspensions after Ficoll-Paque fractionation were purified for $CD11c^+$ and $CD11c^-$ subsets using anti-CD11c mAb and auto-MACS (Miltenyi Biotec, Gladbach, Germany). The purity of $CD11c^+$ cells was 70–80% (Supplementary information, Figure S1), containing less than 0.01% $CD3^+$ T cells as determined by flow cytometric analysis. The $CD11c^-$ cells from EC and dermal cell suspensions were mainly keratinocytes and fibroblasts, respectively, containing less than 0.05% DC subsets.

Flow cytometry

Cells were immunostained with various combinations of fluorescence-conjugated mAbs and analyzed with three-channel FACSCanto flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc, Ashland, OH). The expression of cell-surface and intracytoplasmic cytokines were analyzed using antibodies to PE-conjugated anti-CD11c, CD80, CD86 and PE-conjugated anti-rat IgG, purified anti-mouse CD205 (DEC205) rat IgG, PerCP-conjugated anti-CD45R mAbs, PE-conjugated anti-MHC class II, biotin-conjugated anti-IL-10, and PE-Cy7-conjugated streptavidin. Antibodies were purchased from e-Bioscience (San Diego, CA). All mAbs were used at 1–5 μg 10^{-6} cells, and each incubation was performed for 30 minutes at 4 °C, followed by two washes in phosphate-buffered saline supplemented with 5% fetal calf serum and 0.02% sodium azide. Viable cells were identified by 7-AAD uptake. Intracytoplasmic IL-10 was detected in permeabilized cell suspensions using BD Cytofix/Cytoperm Plus Kit (BD Biosciences).

Apoptosis analysis

EC suspensions from control or grafted (1, 4, and 7 days after) skin were stained with FITC-conjugated MHC-class II mAb for 30 minutes on ice and stained with allophycocyanin-conjugated Annexin V and propidium iodide (Invitrogen), according to the manufacturer's protocol. Apoptosis in LCs was analyzed by a FACScant using FlowJo software (Tree Star Inc) as described earlier (Goldszmid *et al.*, 2003).

Cutaneous DC migration into draining lymph nodes

Mice were painted with 200 μl of 2% FITC (Sigma-Aldrich, St Louis, MO), and axillar and inguinal lymph nodes were taken 24 hours later. Single-cell suspensions were prepared and subjected to flow cytometric analysis.

Real-time PCR

Total RNA was extracted from axillary and inguinal lymph nodes and EC suspensions with the SVTotal RNA isolation system (Promega, Madison, WI) according to the manufacturer's protocol. Murine IL-10 gene expression was quantified in a two-step reverse transcription–PCR. cDNA was reverse transcribed from total RNA samples using the TaqMan RT reagents (Applied Biosystems, Foster, CA). Target gene expression was quantified using TaqMan Gene Expression Assay (Applied Biosystems) in the ABI PRISM 7000 sequence detection system (Applied Biosystems). The probe was synthesized with VIC as the reporter dye and Tamra as the quencher dye. The forward primer, reverse primer, and TaqMan probe were purchased from Applied Biosystems. As an endogenous control for these PCR quantification studies, glyceraldehyde-3-phosphate dehydrogenase gene expression was measured using the TaqMan rodent GAPDH control reagents (Applied Biosystems). Results represented normalized IL-10 mRNA amounts relative to skin-grafted groups using the $\Delta\Delta C_t$ method.

Measurement of cytokine amounts in culture supernatants

$CD11c^+$ and $CD11c^-$ cells were purified from EC and dermal cell suspensions. Cells of each subset (2×10^6 cells per 1.5 ml well) were

cultured in medium for 24 hours in 24-well plates (Corning Glass Works, Corning, NJ). The concentration of IL-4, IL-6, IL-10, IL-12, and TNF-α in culture supernatants was measured using a cytometric beads array system (BD Biosciences) according to the manufacturer's protocol.

Purification and adoptive transfer of Treg cells

Axillary and inguinal lymph nodes were harvested from mice. They were meshed through a cell strainer into RPMI 1640 containing 2% fetal calf serum to prepare single-cell suspensions. To purify $CD4^+CD25^+$ cells or $CD4^+CD25^-$ cells, a mouse Treg isolation kit (Miltenyi Biotec) was used according to the manufacturer's protocol. Briefly, $CD4^-$ cells were depleted with the biotin-labeled antibody cocktail. Subsequently, the $CD4^+$ fractions were magnet-separated with CD25-PE mAb. The positive fraction contained $CD4^+CD25^+$ cells with more than 95% purity, and the flow-through fractions from magnet columns were used as $CD4^+CD25^-$ fraction.

Statistic analysis

All data were statistically analyzed using Student's *t*-test. A *P*-value of less than 0.05 was considered to be significant. Bar graphs were presented as mean ± SD of the mean value.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Figure S1. Purification of DCs.

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Short Communication

Congenital woolly hair without *P2RY5* mutation

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Abbreviations: PCR, polymerase chain reaction

Key words: P2RY5, congenital woolly hair, mutation, autosomal recessive, G-protein coupled receptor

Congenital woolly hair is a disorder with structural defects of the hair shafts. Curled hairs are noticed at birth or soon after birth and often improve with age. Some cases of woolly hairs are associated with systemic or other skin diseases. Congenital woolly hair without any associated disorder is inherited in an autosomal dominant or autosomal recessive manner. Recently, mutations in *P2RY5* gene, encoding a G-protein coupled receptor, have been shown to be responsible for autosomal recessive woolly hair. Here, we report the second Japanese case of congenital woolly hair, showing no *P2RY5* gene mutation.

Sir,

Congenital woolly hair is a disorder with structural defects of the hair shafts. Curled hairs are present at birth or noticed soon after birth. Woolly hair occasionally appears as a part of certain syndromes such as Naxos disease or Carvajal syndrome.[1] However, the most of the patients show only woolly hair without any associated disorder. This pure type of congenital woolly hair is inherited in an autosomal dominant or autosomal recessive manner. Recently, mutations in *P2RY5* gene, encoding a G-protein coupled receptor, have been shown to be responsible for autosomal recessive woolly hair.[2] Here, we report a case of congenital woolly hair whose grandfather's brother showed similar woolly hairs. A gene analysis disclosed that the patient had no mutation in *P2RY5*.

Case Report

A five year old Japanese girl presented with woolly hairs. Tightly curled hairs were noticed at birth over the whole scalp. Her hairs were incapable of growing long. She had never her hairs cut by barbers. On examination, her hairs were very fine, thin and lightly colored (Fig. 1A and B). A light microscopic study of her easily plucked hairs revealed a dystrophic root of the hair without root sheath components. A possible differential diagnosis may include pili trianguli et canaliculi, which shows fragile hairs with ultrastructural changes

Figure 1A. Tightly curled and short hairs in the occipital region.

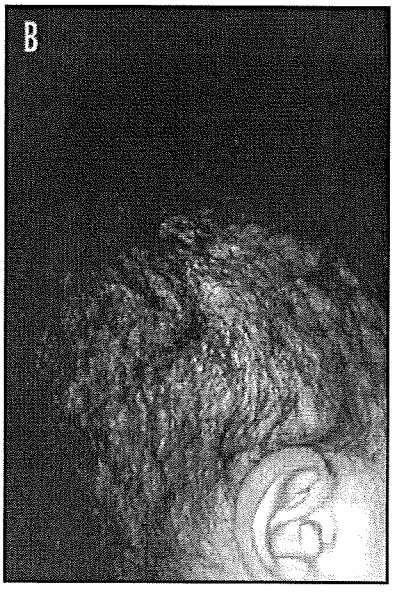

Figure 1B. Tightly curled and short hairs in the temporal region.

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consisting of tonofilament-desmosomal detachment and tonofilament clumping within inner root sheath cells.[3] However, their hair shafts appear normal under light microscopic examination.[3] Pili torti is an inherited hair disorder and another candidate for differential diagnosis, but our case lacked the flattening or twist of the hair shafts through 180 degrees around the axes under light microscope. She had no abnormality in nail, limb or skeletal development. Her sweating and teeth formation were normal. She had no mental retardation or cardiomyopathy. Her palms and soles showed no hyperkeratosis. The hairs of her parents and her younger brother were normal. However, a younger brother of her grandfather had suffered from woolly hairs since his birth (Fig. 1C). There was no family history of consanguineous marriage. Based on the clinical findings and family history, we diagnosed the condition as hereditary congenital woolly hair, that is inherited in an autosomal recessive manner, or as a sporadic type of congenital woolly hair.

After obtaining informed consent from the patient, genomic DNA was extracted form the patient's blood (Qiagen; Hilden, Germany). The coding exons and flanking regions of *P2RY5* were amplified by polymerase chain reaction (PCR) using the specific primers described previously.[2] PCR products were purified using QIAquick gel extraction kit (Qiagen) and directly sequenced as described.[4] We could identify no mutation in exons and flanking regions of *P2RY5*. Unfortunately, we could not obtain informed consent from the patient's affected kindred for genetic evaluation.

Discussion

To our knowledge, there has been only one case report of Japanese congenital woolly hair.[5] That patient had a family history of consanguineous marriage and the patient's sister showed similar tightly curled hairs, suggesting an autosomal recessive inheritance. Genetic evaluation was not performed for the patient.

P2RY5 is a seven-pass transmembrane protein and a member of G-protein coupled receptor for oleoyl-L-α-lysophosphatidic acid.[6] An immunofluorescence study revealed that P2RY5 is expressed in Henle's and Huxley's layers of the inner root sheath of the hair follicles.[2] The inner root sheath surrounds and supports the hair shaft. A mutation in *P2RY5* expressed in inner root sheath may lead to defects in transmitting signals from oleoyl-L-α-lysophosphatidic acid via P2RY5 into intracellular cyclic AMP concentration. Therefore, inner root sheath cells may be unable to normally proliferate, differentiate and sustain hair shafts. Lack of mutation in *P2RY5* in our patient may suggest the existence of another mutation in another molecule concerned with the signaling cascades for P2RY5.

In summary, our patient had no mutation in *P2RY5* and may be classified as having another type of congenital woolly hair caused by an unidentified gene.

Acknowledgements

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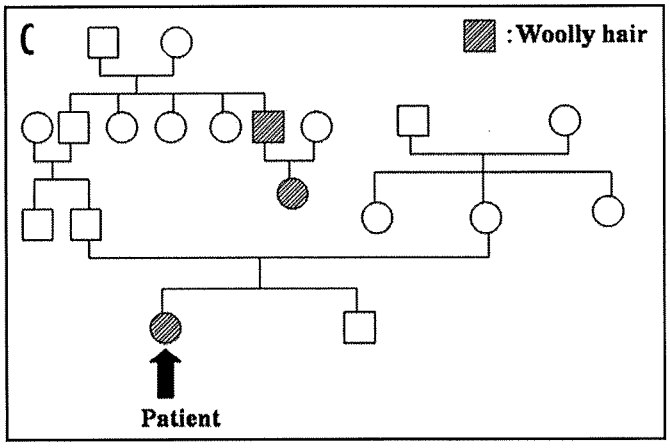

Figure 1C. (C) Genealogy of the disorder in the patient's family.

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In the light of our patient's history, symptoms and clinical presentation we diagnosed AGEP following the intake of moxifloxacine. After discontinuation of the antibiotic and intravenous administration of corticosteroids (100 mg prednisolone daily) and antihistaminics (30 mg diphenhydramine hydrochloride twice a day), the rash cleared with typical generalized desquamation within one week. A consecutive patch test with moxifloxacine remained negative. A provocation test with the drug was not performed.
Recent reports suggest an association between the administration of moxifloxacine and elevated liver enzymes, potentially ending in hepatitis fulminans and Stevens-Johnson-Syndrome or TEN (toxic epidermal necrolysis). In this regard our patient remained without relevant pathological findings. AGEP is usually caused by a large variety of drugs, including antibiotics (ß-lactams, pristinamycine, co-trimoxazole, metronidazole), antifungal agents (nystatin, terbinafine, fluconazole, amphotericin B), carbamazepine, hydroxy-chloroquine, azathioprine, diltiazem, nimesulide, non-ionic contrast media and others [1, 3]. Occasional cases of acute pustular drug reactions after the intake of other quinolone antibacterial agents have already been reported [4-6]. Our case is the first description in the literature of AGEP after treatment with moxifloxacine. ■

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Cisplatin-induced acral erythema

Chemotherapy-induced acral erythema (CAE) is a cutaneous reaction associated with the use of various systemic chemotherapeutic agents, usually administered at high doses. Since 1982, when CAE was first described, it has been reported under a variety of names, such as palmoplantar erythema, palmoplantar erythrodysaesthesia syndrome and hand-foot syndrome. CAE tends to occur in patients with malignancies, especially those receiving cumulative high-dose chemotherapy with concomitant irradiation and bone-marrow transplantation. The incidence of acral erythema during chemotherapy is about 2% [1] and the main agents responsible are fluorouracil and doxorubicine, followed by docetaxel, pacritaxel, methotrexate, vinorelvine, gemcitabine, cytarabine and cyclophosphamide [1, 2]. We report a patient who developed an erythematous eruption on the palms after systemic administrations of cisplatin, and review the literature of cisplatin as the causative agent of CAE.
A 35-year-old woman was diagnosed as having cervical cancer. After hysterectomy, a combination therapy with irradiation (30 Gy/total) and cisplatin (30 mg/m^2) was started. Cisplatin was administered intravenously through her left cephalic vein. At night on the initial day of the treatment, she felt a burning pain in her reddish swollen forearm. The swelling subsided within a day by topical application of betamethasone 1 g per day. However, on the 6th day of chemotherapy, she again developed swelling on her left upper limb and was referred to us.
On physical examination, her left forearm was swollen and slightly reddish. There was symmetrical erythema on the bilateral palms extending to the palmar surface of the fingers *(figures 1A, B)*. The eruption consisted of multiple, salmon-pink, faintly demarcated, non-tender lesions. No eruption was noted on the dorsum of hands and fingers. Laboratory data revealed no leucocytosis or peripheral eosinophilia. We did not perform any skin tests for ethical reasons, because of the cytotoxic properties. Drug-

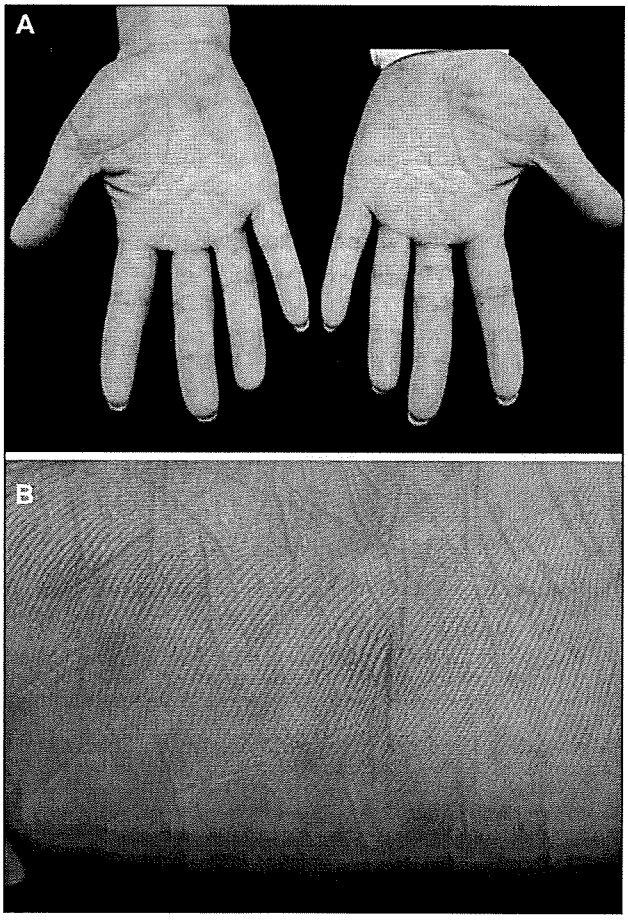

Figure 1. Acral erythematous eruption on both palms **(A)**, showing multiple, demarcated, salmon-pink lesions **(B)**.

induced lymphocyte stimulation testing was tried, but it was difficult to evaluate its antigenicity, since lymphocytes were incapable of surviving during culture with cisplatin. A tentative diagnosis of drug eruption was made; she stopped taking cisplatin and her eruption was completely resolved in a week without any treatment.

A biopsy specimen from a palmar lesion revealed a perivascular mild infiltrate of lymphocytes and a few eosinophils in the dermis with mildly acanthotic epidermis. There were no epidermal necrotic cells or dermal inflammatory cells invading into the eccrine glands.

Although cisplatin is widely used for the treatment of various tumors, cisplatin-induced acral erythema has been reported in only four cases [2-4]. All patients developed symmetrical erythema of the palms and fingers and/or soles, and the eruptions were resolved by discontinuing cisplatin. However, there are clinical and histological differences among these cases. First, acral erythema occurred only one day after the initiation of chemotherapy in our case, while it appeared 3 months after the initiation in another patient [2]. Second, the lesional sites were slightly different between the cases. Third, the infiltrates consisted of lymphocytes [2], or neutrophils [4]. Finally, lichenoid or vesicular changes in the epidermis were present in two cases [2, 4] but not in our case.

The occurrence of CAE depends on various factors, which may contribute the typical localization to the palms and soles, including elevated drug concentration in eccrine glands, rapid cell proliferation, regional temperature gradient, gravitational forces, and vasculature anatomy [5]. Thus, it is likely that CAE is a toxic but not allergic reaction. Because the onset of CAE was soon after administration, our case also supports some toxic mechanism underlying CAE.

It may be noted that three out of the four reported cases are Japanese, and some ethnic genetic background might influence on the susceptibility of CAE to cisplatin. The polymorphisms for certain drug transporter genes, such as Multi drug resistance 1, might underlie the ethnic variation [6]. ■

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Successful treatment of cutaneous leishmaniasis by photodynamic therapy and cryotherapy

Cutaneous leishmaniasis (CL) sometimes brings a therapeutic problem. The treatments of Old World cutaneous leishmaniasis include cryotherapy [1], heat therapy, itraconazole [2], pentavalent antimony compounds, amphotericin B and other antimycotic drugs, topical and intralesional application of paramomycin, and in recent years also photodynamic therapy (PDT) [3-5]. Lesions of CL heal spontaneously over several months to 2-3 years, and therapy is not always essential. Nevertheless, treatment is indicated in spreading multiple lesions and when the lesion is located on the face and exposed areas of the extremities.

A 39-year-old man was referred to us with multiple lesions similar to furunculosis, which had occured 3 months previous without healing after topical steroid therapy. He was a keen diver and the lesions developed 4 weeks after his last return from vacation in Croatia. The examination revealed a healthy man with 9 slightly tender nodules and plaques of bright pink-reddish colour, sized 1 to 4 cm on the forearms, neck, and thigh. They periodically developed some blistering on the surface *(figure 1A)*. The diagnosis of cutaneous leishmaniasis was confirmed by a biopsy specimen. *Leishmania* bodies were present in the macrophages as well as extracellularily. No microorganisms were seen in PAS staining and in sections stained to show acid-fast organisms. A culture was not performed.

The following treatment was introduced: Five lesions were treated with PDT whereas the other 4 lesions were treated with cryotherapy. Before initiation of PDT, the crusts and scales were mechanically removed. Then, 20% ALA in gel (hydrochloride form) was applied on the lesions in a 1 mm thick layer. The areas were covered with an occlusive dressing for 3 hours after which the gel was washed off using 0.9 saline solution. Each lesion was illuminated with non-coherent red light with an emission spectrum of 580 to 680 nm (Medeikonos, Sweden), with a total light dose of 75 J/cm^2 and a light intensity of 88-123 mW/cm^2. The PDT procedure was repeated once a week for six-weeks.

The remaining 4 lesions were treated simultaneously with a hand-held liquid nitrogen spray unit. The liquid nitrogen was applied directly on the lesion from a distance of 2-3 cm for a freezing time of 30 seconds and a thaw of 60 seconds. Double freeze-thaw cycle treatment per session was used. The procedure was performed once a week for five weeks in total.

The lesions treated with the PDT method improved after 4 sessions and after 6 sessions they were healed with only minimal pigmentation and minor central scarring *(figure 1B)*. The lesions treated with cryotherapy healed after 5 applications with slight pigmentation and minor scar formation in the centre of the lesions, too *(figure 1C)*. No biopsies from clinically healed skin were performed after completion of the treatment. In a 12 month follow-up period no signs of reccurence occured.

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CLINICAL REPORT

Therapeutic Effectiveness of Various Treatments for Eosinophilic Pustular Folliculitis

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Eosinophilic pustular folliculitis is a rare dermatosis. Recently, in addition to oral indomethacin, other treatments have been applied for eosinophilic pustular folliculitis. The aim of this study was to assess the effectiveness of various therapies encompassing conventional to newly applied drugs for eosinophilic pustular folliculitis. Twenty patients with eosinophilic pustular folliculitis seen in our department were investigated. The effectiveness of each treatment was assessed by a severity score index. Eleven patients were treated with oral indomethacin, and the severity scores of all patients were decreased after the treatment. Oral cyclosporine was markedly effective in all 11 patients treated, and topical tacrolimus ointment alleviated eosinophilic pustular folliculitis in 3 of 7 with one patient showing a remarkable reduction in the severity score. In addition to indomethacin or other oral non-steroidal anti-inflammatory drugs, oral cyclosporine and topical tacrolimus may be beneficial choices when patients have been resistant to previous treatments. ***Key words: eosinophilic pustular folliculitis; indomethacin; cyclosporine; tacrolimus.***

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Eosinophilic pustular folliculitis (EPF), first reported in 1965 (1), is a rare dermatosis. It is clinically characterized by recurrent, pruritic, erythematous patches consisting of follicular papules and sterile pustules with peripheral blood eosinophilia. It affects the face, trunk and extremities, occasionally extending to the palms and soles. While some Western patients with EPF are positive for human immunodeficiency virus (HIV) and exhibit papules mainly on the trunk (2, 3), almost all Japanese patients are HIV-negative and have annularly configured lesions mostly on the face and upper back. Histopathologically, EPF shows predominant infiltration of eosinophils into hair follicles (4–6). Although the aetiology has not been fully elucidated, a Th2-mediated, interleukin (IL)-5-induced mechanism has been proposed (4–6). IL-5 is known to be a growth and differentiation factor for eosinophils and may also be involved in the migration of eosinophils to the follicles (4, 6).

Among several treatment options for EPF, indomethacin has been used as one of the first-line treatments since 1984, when oral administration of this non-steroidal anti-inflammatory drug (NSAID) was reported to exert a therapeutic effect on EPF (7–10). Other treatments capable of improving EPF include oral and topical corticosteroids (11, 12), oral minocycline (13), roxithromycin (14) and aminodiphenyl sulfone (DDS) (15–17). More recently, systemic Th1-skewing reagent interferon-γ (IFN-γ) (4, 6) and immunosuppressive agents, including oral cyclosporine (6,18) and topical tacrolimus (5, 19), have been used for the treatment of EPF.

Over the past 10 years, we have seen 20 patients with EPF and treated them with various modalities. To evaluate further the efficacy of each therapy, we performed a retrospective study with the use of our records and photographs. We assessed changes in the intensity of skin eruption before and after treatment by using the severity score index composed of erythema, papules, pustules, pruritus, and number of involved sites.

MATERIALS AND METHODS

Patients

We included all patients who had been diagnosed and recorded as EPF in our hospital, and analysed them retrospectively. Twenty patients with EPF, who were seen from 1998 to 2007 in the Department of Dermatology of our University Hospital, were enrolled in this study (Table I). The patients consisted of 6 men and 14 women, mean age 38 years (age range 23–69 years). The diagnosis of EPF was made based on the clinical appearance, symptoms and histological findings (20–22). Peripheral eosinophil counts were examined in 13 patients, and 5 of them had eosinophilia (> 500/μl). Skin biopsy was performed in 20 patients with the typical result of follicular infiltration of eosinophils, including one patient diagnosed as EPF by a dermatologist in another private clinic prior to referral to us. Although the eosinophil counts in cases 3 and 15 were more than 1000/μl, they did not show any other organ symptoms seen in hyper-eosinophilic syndrome, and eosinophils infiltrated mainly around hair follicles in the biopsy specimens. This study included one HIV-positive patient (case 5).

Assessment score

To assess the effect of each treatment, we used a "severity score index for EPF", which we determined as follows. Based

Table I. *Patients enrolled in this study*

			Treatments							
			Systemic					Topical		
Pat. no.	Age/Sex	Eos/μl	Indomethacin	Loxoprofen	Cyclosporine	IFN-γ	DDS	Indomethacin	Tacrolimus	Combination with
1	24/F	208	PR							s-steroid
2	45/F	165	PR							MC
3	30/F	1123	PR							
4	45/M	609	PR							
5	69/M	ND	PR							t-steroid, MC
6	64/F	ND	PR							MC
7	49/F	256	PR				NR			s-steroid, MC
8	29/M	492	PR							t-steroid, MC
9	45/F	417	PR							t-steroid, RXM
10	23/F	340		PR	PR			NR	NR	s-steroid, RXM
11	24/M	ND	PR						PR	
12	26/M	667			PR	PR		PR	NR	s-steroid, RXM
13	25/F	294			CR				PR	
14	35/F	768		NR	PR				NR	t-steroid, MC, RXM
15	41/M	1555		NR	PR			NR	NR	RXM
16	31/F	235		PR				PR	CR	RXM
17	40/F	ND			PR					
18	44/F	ND								RXM
19	35/F	ND	PR							
20	43/F	ND						PR		

Eos: eosinophil counts per μl; s-steroid: systemic steroid, MC: oral minocyclin; t-steroid: topical steroid; RXM: oral roxithromycin; DDS: dimethyldiphenyl sulfone; IFN-γ: systemic interferon-γ; ND: not done; PR: partial response, defined as the severity score of post-treatment lower than that of pre-treatment; CR: complete response, defined as the severity score of post-treatment lowered to 0; NR: no response, defined as the severity score of post-treatment equal to or higher than that of pre-treatment.

on the essential features of EPF, we chose five items, three from eruption elements (erythema, papules and pustules), one from symptoms (pruritus), and the number of involved sites. We scored the former four items from 0 to 3 points (0, none; 1, mild; 2, moderate; and 3, severe). When the patient had several areas of lesions, we selected the most severe area for scoring. The number of involved sites was assessed from 0 to 4. In the patient enrolled, "4" was the highest number of the affected sites, and "0" was for patients completely improved after therapy. The final assessment was made with the sum of the above values (from 0 to 16), and we scored them before and after each treatment with revision of the records and photographs of the patients.

Therapies and evaluation

When a single therapy was applied, the assessment was performed before and after a given treatment. In some patients, two or more monotherapies were administered in a sequential fashion, and the efficacy of each therapy was evaluated just before and after treatment. The duration of each therapy was 1–4 weeks. In the case of combined therapies, the patients were administered with both the evaluated and non-evaluated drugs. The non-evaluated drug(s) continued without any change in dose for at least one week prior to the beginning of the evaluated drug. The duration of treatment with the evaluated drugs was 1–24 weeks, except for cases 5 and 8 who had been treated intermittently for one and 4 years, retrospectively. The evaluated drugs included oral indomethacin (25–50 mg daily for 1–8 weeks), another oral NSAID (loxoprofen, 60–120 mg daily for 1–8 weeks), topical indomethacin (1% [w/w] twice daily for 2–12 weeks), oral cyclosporine (100–150 mg daily for 2–12 weeks), and topical tacrolimus (0.1%). The non-evaluated drugs included oral corticosteroids (prednisolone 10 mg daily and betamethasone 1–2 mg daily), and topical corticosteroids (twice daily for 3–24 weeks), oral minocycline (100–200 mg daily) and oral roxithromycin (300 mg daily). We could not assess the therapeutic effect of these non-evaluated drugs, because they were mostly used together with the above-evaluated drugs, including indomethacin, cyclosporine, or tacrolimus. Oral DDS (75 mg daily) and IFN-γ (2×10^6 Japanese reference unit [JRU] daily) were used for a single patient.

RESULTS

The changes in the severity score before and after treatments are shown in Fig. 1, and the number and percentage of patients showing complete or partial responses are summarized in Table II. The follow-up period was from one to 8 months.

Eleven patients were treated with oral indomethacin. Among them 4 patients received indomethacin monotherapy and the rest were given indomethacin in combination with other drugs (Table I). Indomethacin improved the skin lesions of all 11 patients, as the

Table II. *Therapeutic effectiveness of each drug for eosinophilic pustular folliculitis*

Drug	Patients successfully treated[a] *n* (%)
Systemic administration	
Indomethacin	11/11 (100)
Loxoprofen	2/4 (50)
Cyclosporine	6/6 (100)
Topical application	
Indomethacin	3/5 (60)
Tacrolimus	3/7 (43)

[a]Number of patients showing complete or partial response.

mean score was remarkably reduced from 10.2 to 3.7 (Fig. 1A). Four patients were treated with another oral NSAID, loxoprofen sodium. The mean severity score was changed from 8.5 to 6.0, without statistical significance ($p=0.11$), but it is notable that 2 of 4 patients were improved by this treatment (Fig. 1B). Oral cyclosporine was greatly effective in all 6 patients treated, as the mean severity score was reduced from 8.3 to 3.3 (Fig. 1C). Topical indomethacin did not statistically alter the severity score (Fig. 1D). Seven patients were treated with topical tacrolimus. In one patient (case 16) among them, whose biopsied skin specimen from the face showed a dense infiltrate of eosinophils around hair follicles and sebaceous glands, confirming the diagnosis of EPF (Fig. 2C), the eruption disappeared completely with topical tacrolimus treatment (Fig. 2 A, B), and in the other 2 patients (cases 11 and 13), their severity scores were reduced by this drug. However, the overall scores in all 7 patients were not significantly decreased (Fig. 1E). IFN-γ and DDS were used in a single patient, respectively, and each patient showed a partial response or no response; the severity score indices were changed from 15 to 7 and from 3 to 3, respectively.

DISCUSSION

Oral indomethacin is a conventional treatment for EPF (9). The present study further confirmed its effectiveness, since the severity scores were reduced in all 11 patients. Four patients responded well to indomethacin alone and the rest did so in combination with other drugs. However, it should be noted that some of the 9 patients who were not treated with oral indomethacin in this study had already received this drug in other clinics without a good therapeutic response. This exclusion of potential low responders from this study might yield a biased result in its effectiveness. Another oral NSAID, loxoprofen, was also effective in 2 of 4 patients, but the overall efficacy seemed to be limited. Although the mechanism underlying NSAID efficacy remains unclear, it has been proposed that this inhibitor of cyclooxygenase reduces a certain factor that attracts eosinophils into the hair follicles (23), and indomethacin decreases the expression of major eosinophil chemokine receptor CCR3 on eosinophils (24). In addition, the action of NSAIDs depends on the activity of cellular kinases, but the dependency differs among NSAIDs. For example, indomethacin is capable of acting peroxisome proliferator-activating receptor γ (PPARγ), whereas other NSAIDs are incapable (25). It might influence the differential effectiveness for EPF between indomethacin and other NSAIDs. Topical indomethacin partially improved EPF in 3 out of 5 patients, but the overall severity score was not statistically decreased. Moreover, 2 out of 3 patients improved with topical indomethacin were treated with roxithromycin or systemic steroid in combination, further suggesting its partial contribution to the therapeutic effect.

Cyclosporine is a potent generalized suppressor of cytokine production by T lymphocytes, and a couple of reports documented successful treatment of EPF with systemic cyclosporine (6, 18). Our study demonstrated that cyclosporine is a powerful drug for EPF in patients refractory to other treatments. Since the doses used in this study were low (100–150 mg daily, corresponding to 1.7–3.0 mg/kg/day), cyclosporine is considered to

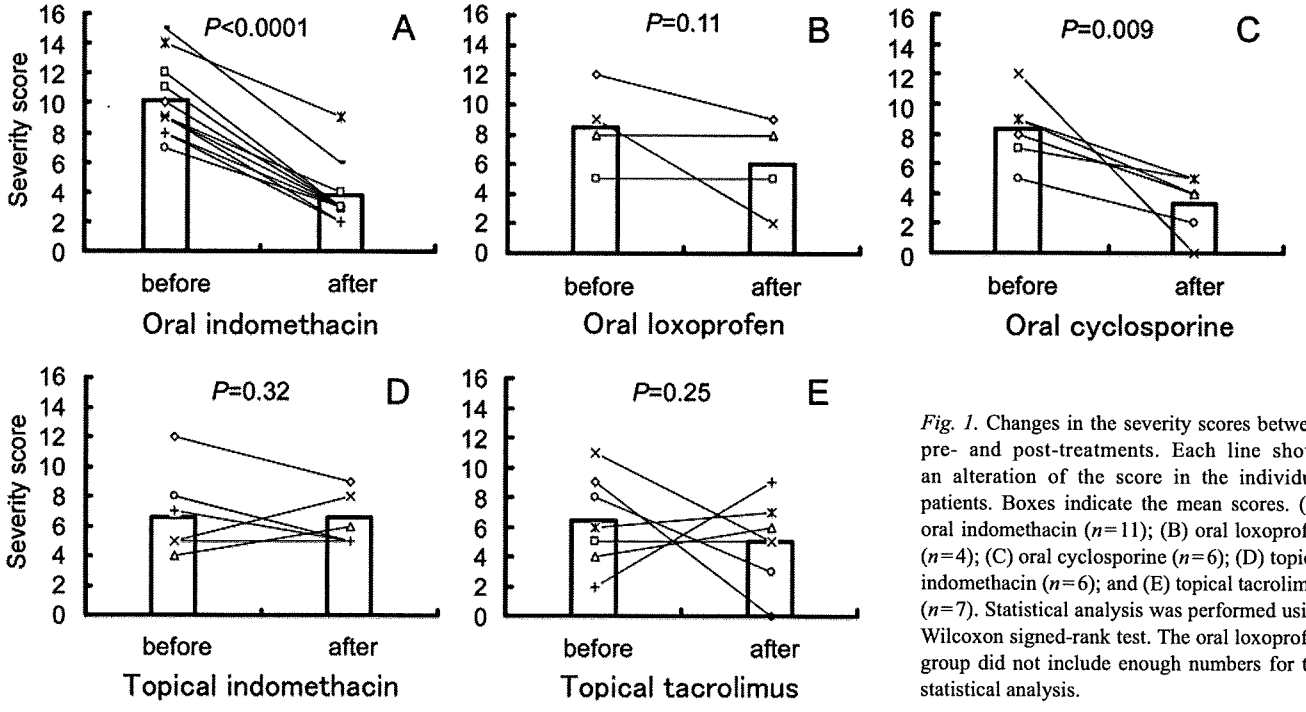

Fig. 1. Changes in the severity scores between pre- and post-treatments. Each line shows an alteration of the score in the individual patients. Boxes indicate the mean scores. (A) oral indomethacin ($n=11$); (B) oral loxoprofen ($n=4$); (C) oral cyclosporine ($n=6$); (D) topical indomethacin ($n=6$); and (E) topical tacrolimus ($n=7$). Statistical analysis was performed using Wilcoxon signed-rank test. The oral loxoprofen group did not include enough numbers for the statistical analysis.

exert a clinical efficacy without side-effects in most patients. Another immunosuppressive calcineurin inhibitor, tacrolimus, has been established as an alternative to topical corticosteroids for inflammatory skin diseases (26), such as atopic eczema. Tacrolimus inhibits T-cell activation and resultant release of both Th1 and Th2 cytokines (27, 28), including IL-2, IL-4, and IL-5. There have been several reports of successful treatment with topical tacrolimus for EPF (5, 29, 30). Our study suggests that topical tacrolimus has a therapeutic effect in limited patients; it occasionally exerts a positive effect, presumably when the patient's skin condition allows the drug to penetrate. It is also notable that this study included only two cases that show complete responses to the calcineurin inhibitors, one treated with oral cyclosporine and the other with topical tacrolimus. This may provide not only a good therapeutic indication of these immunosuppressants but also an important clue for elucidation of the mechanism of EPF.

EPF is an uncommon disease with prominent symptoms and often refractory to treatments. Lack of comprehensive reports on therapies for EPF may have yielded some confusion on the therapeutic approaches. The number of patients enrolled here is limited and the study is retrospective. However, we found that the severity score index is a useful means of assessment of EPF therapies. By using this score, it is suggested that oral cyclosporine is highly effective as well as oral indomethacin. It is recommended that oral indomethacin serves as the first choice for treatment of EPF. When it is ineffective or unavailable because of its side-effects, oral cyclosporine is a candidate for second-line treatment. Topical indomethacin and tacrolimus can be used in combination with the systemic drugs. Further assessment of combination therapy is required in future studies.

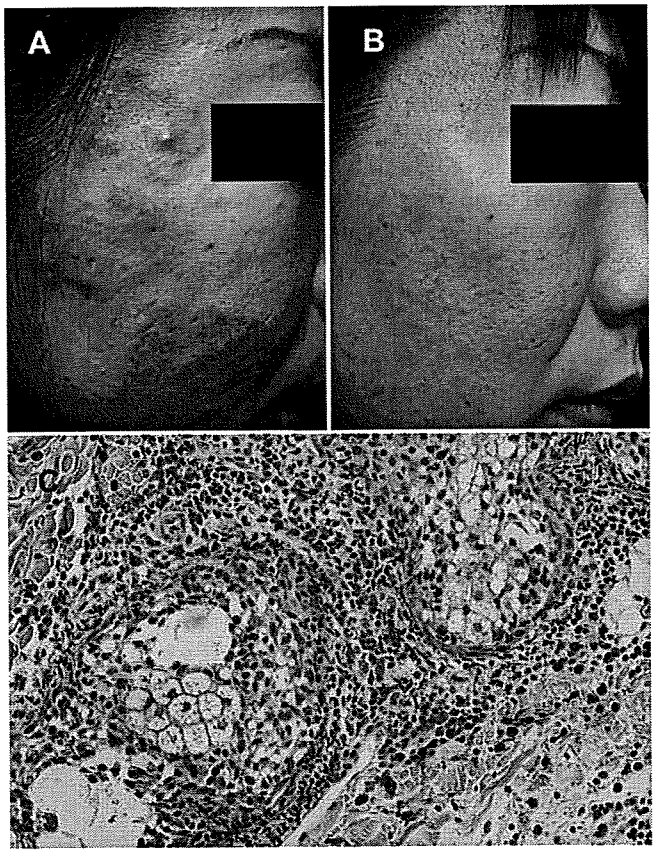

Fig. 2. A patient showing remarkable improvement with topical tacrolimus treatment. Clinical pictures (A) pre-treatment (severity score: 12) and (B) post-treatment (severity score: 0). The patient was a 31-year-old woman who presented with 4-month history of an annular eruption on her face and multiple red and brownish plaques accompanied with papules and pustules on her trunk and extremities. She was initially treated with oral loxoprofen sodium 120 mg daily, oral roxithromycin 300 mg and topical indomethacin twice a day. Two weeks later, the redness became mild and pustules disappeared, but the eruption and pruritus remained at level 9 of the severity score. Topical tacrolimus twice a day was added to her regime for another 24 weeks. All plaques and pruritus disappeared completely (severity score: 0). (C) A biopsied skin specimen from the face (H&E stain ×200), showing a dense infiltrate of eosinophils around hair follicles and sebaceous glands.

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Skin application of ketoprofen systemically suppresses contact hypersensitivity by inducing CD4$^+$ CD25$^+$ regulatory T cells

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ABSTRACT

Background: Ketoprofen (KP) is a widely used nonsteroidal anti-inflammatory drug that inhibits prostaglandin biosynthesis. We have previously shown that topical KP treatment at the sensitizing site inhibits the development of contact hypersensitivity (CHS) to picryl chloride (PCl).
Objective: We investigated the mechanism underlying the KP-induced immunosuppression of CHS by application of KP.
Methods: We analyzed the CHS responses to the non-sensitizing site and subsequent sensitization with PCl, and by transfer of the draining lymph node cells (LNCs) from KP-tolerated mice to recipient mice. Changes in the Foxp3 expression of LNCs from KP-phototreated skin were also examined by real-time PCR.
Results: Topical application of KP to not only the sensitizing but also non-sensitizing site suppressed CHS response. The immunosuppression was transferred with LNCs from mice treated with PCl plus KP, but not from mice treated oxazolone plus KP. In this transfer study, the CD4$^+$ CD25$^+$ subset of LNCs exerted the suppressive effect, while CD25$^+$ cell-depleted LNCs lost the inhibitory ability. CTLA-4 blocking with a specific antibody, but not IL-10 blocking, abrogated the activity of CD4$^+$ CD25$^+$ cells. Moreover, Foxp3 mRNA expression was remarkably increased in LNCs from PCl and KP-treated mice.
Conclusion: The immunosuppression of CHS by topical application of KP is systemic and hapten-specific. Treg cells play an important role in the suppressive effect by KP.

1. Introduction

Contact hypersensitivity (CHS) is a delayed-type cutaneous reaction in which various immunocompetent cells, including epidermal Langerhans cells (LCs), dermal dendritic cells (dDCs), keratinocytes and T cells, and their cytokines and chemokines are involved [1,2]. Ketoprofen (KP) is a nonsteroidal anti-inflammatory drug (NSAID) that inhibits prostaglandin (PG) biosynthesis and widely used topical NSAID to remove pain. We have previously shown that *in vivo* application or *in vitro* addition of KP inhibits the maturation of LCs [3]. As a result, topical application of KP to the sensitizing site inhibits CHS responses to hapten. Aspirin, a representative NSAID, has an inhibitory effect on the *in vitro* maturation of LCs, but aspirin did not suppress CHS response to hapten. Thus, KP is unique in this suppressive ability, and in addition to the topical modulation of LCs, another mechanism might exist in KP-induced immunosuppression. In this respect, it remains unclear whether KP induces systemic or local immunosuppression and how KP application induces the immunological tolerance in CHS.

Regulatory T cells (Treg) prevent from harmful immune responses to self and nonself antigens in a dominant manner [4–7]. In mouse models, the responses of CHS are reduced by Treg cells-inducing treatments such as irradiation of skin with UVB, skin graft, and oral application of antigen. Treg cells can be classified into two major categories: thymus-derived natural Treg cells and those induced in the periphery [8,9]. The principal subset of natural Treg cells is CD4$^+$ cells that constitutively express CD25 [10,11]. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is an important molecule that is expressed on the surface of Treg cells and exhibits the ability to ligate the B7 family molecules (CD80 and CD86) like CD28 [6,7]. Unlike CD28, however, cross-linking of CTLA-4 downregulates interleukin (IL)-2 production and cell cycle progression, and therefore, blockade of CTLA-4 reduces CD4$^+$

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