METHODS

mRNA extraction of purified keratinocytes from murine ear skin

The hairs on murine ear skin were removed with depilatory cream. After removing hairs, the ears were split into dorsal and ventral halves, and the cartilage was removed. Then the skin was floated on 0.25% trypsin/EDTA for 30 minutes at 37°C and separated into epidermis and dermis. Single epidermal cell suspension (EC suspension) was done by means of vigorous trituration of the epidermal sheet. Because EC suspensions are mixtures of keratinocytes, Langerhans cells, and γδ T cells, we purified keratinocytes by removing Langerhans cells and γδ T cells from the EC suspension with the autoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) using magnetic microbeads coated with anti-mouse CD45 antibody. The purity of keratinocytes is greater than 99%. Then we extracted RNA from keratinocytes with the RNeasy mini kit (Qiagen).

Real time RT-PCR primer

The primer sequences of CXCL2 were 5′-GCC TAT CGC CAA TGA GC-3′ (forward) and 5′-TGG ACA ATT TTC TGA ACC AAG-3′ (reverse).

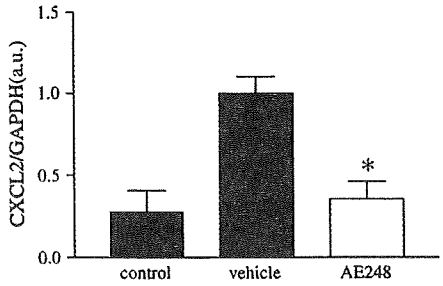

FIG E1. Real-time RT-PCR analysis on mRNA expression of CXCL2 in the purified keratinocytes in hapten-challenged ears of vehicle- or AE248-treated mice (n = 3 per group). Results are expressed as means ± SEMs. *P < .05 versus the vehicle-treated group. *GAPDH,* Glyceraldehyde-3-phosphate dehydrogenase.

ORIGINAL ARTICLE

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.

Journal of Investigative Dermatology (2009) **129**, 705–713; doi:10.1038/jid.2008.304; published online 9 October 2008

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

Received 30 January 2008; revised 12 July 2008; accepted 17 July 2008; published online 9 October 2008

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).

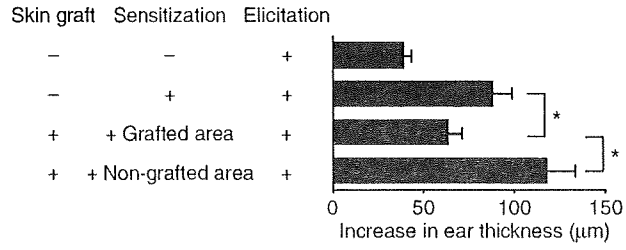

Figure 1. CHS responses in mice sensitized with PCl through grafted or non-grafted skin. Mice were sensitized with PCl on the grafted back skin or non-grafted abdominal skin 7 days after skin grafting. PCl was challenged on each earlobe 5 days after sensitization. The change in ear thickness was measured 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,

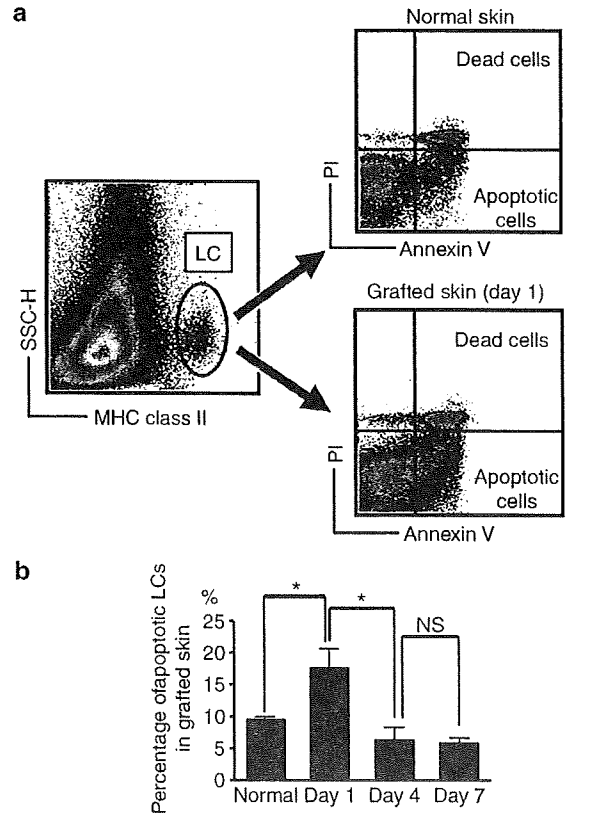

Figure 2. Numerical and morphological alterations and apoptosis of LCs in grafted skin. (a) Apoptosis of Langerhans cells in the grafted skin. EC suspensions from the 1-day grafted or non-grafted skin were stained with FITC-conjugated MHC class II, APC-conjugated Annexin V and PI. Apoptotic LCs were 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.

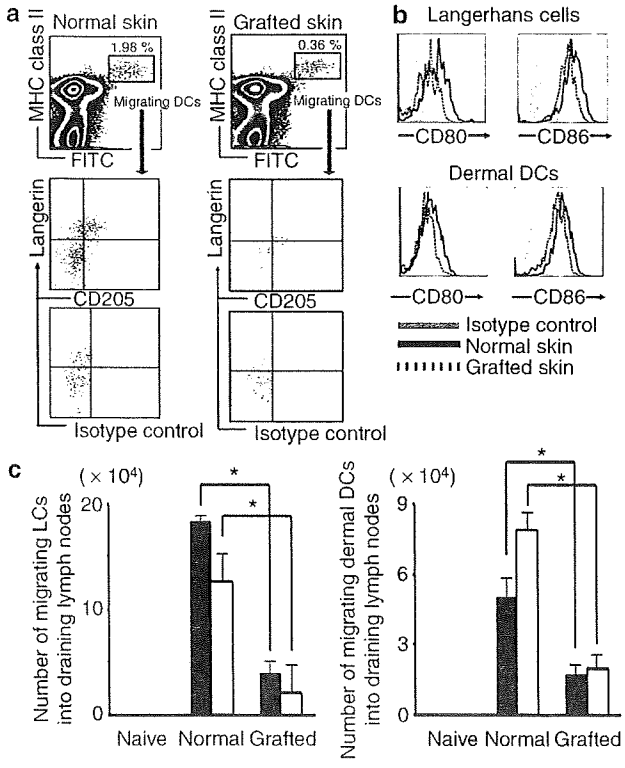

Figure 3. Expression of co-stimulatory molecules and number of migrating DCs in grafted mice. (**a**) Draining lymph node cells were taken from mice painted with FITC on the grafted or normal skin and stained with anti-MHC class II, CD205 and Langerin mAbs. CD205$^+$ cells are virtually the same population 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 PCI (day 7) on the dorsal grafted skin or untreated. As control, mice without skin grafts were sensitized with PCI 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 PCI 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 PCI 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

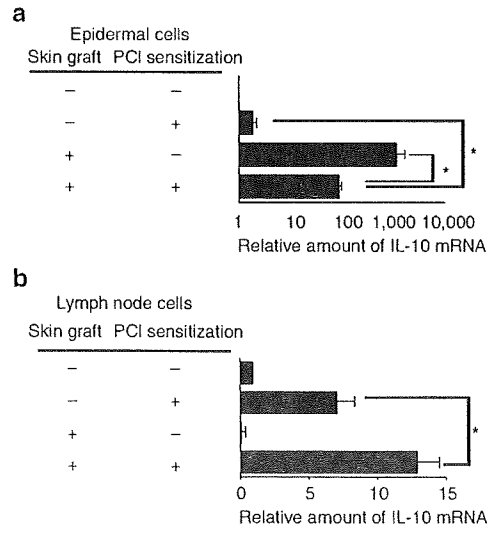

Figure 4. IL-10 expression in ECs and lymph node cells from grafted mice. (**a**) Epidermal sheets were prepared from grafted (7 days after operation) or non-grafted skin that was painted with PCI or left untreated. IL-10 mRNA expression was measured by real-time PCR. (**b**) Draining lymph node cells 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. *$P<0.05$.

PCI 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 PCI painting. Skin grafting alone did not augment IL-10 production by lymph node cells (Figure 4b). PCI 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 PCI-painted grafted skin produced less IL-10 than the PCI-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 PCI.

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 PCI through grafted skin. We performed an adoptive transfer study to evaluate this possibility. Donor mice were sensitized with PCI 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

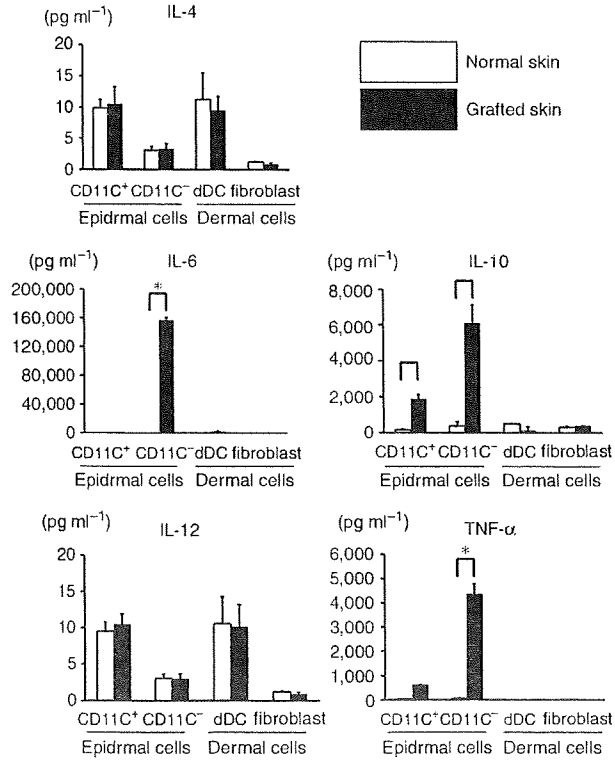

Figure 5. Cytokine production by ECs and dermal cells. EC suspensions were obtained from grafted or non-grafted skin, and fractionated to $CD11c^+$ cells (70–80% LCs) and CD11c cells (LC-depleted cells, mainly keratinocytes) with auto-MACS using CD11c microbeads. Dermal cell suspensions were centrifuged 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. *$P<0.05$.

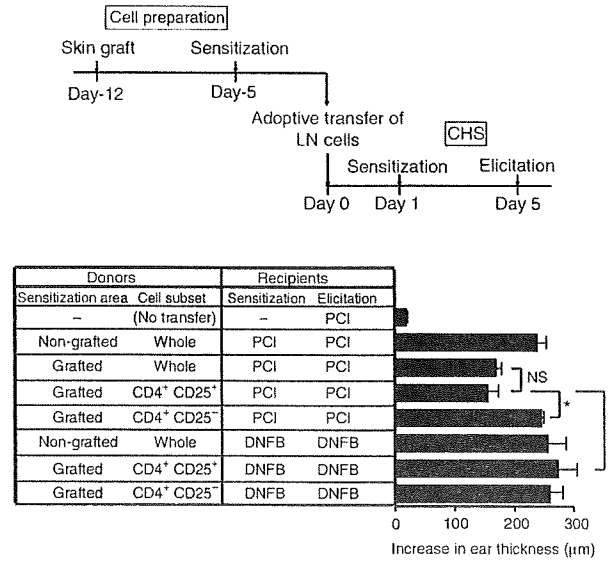

Figure 6. Transfer study of $CD4^+CD25^+$ cells from donor mice sensitized through grafted skin. Mice were sensitized with PCI on the grafted (7 days after operation) or non-grafted area. Five days after sensitization, draining lymph nodes and spleen cells were taken from the mice. Whole cells, $CD4^+CD25^+$ 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 (PCI 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.

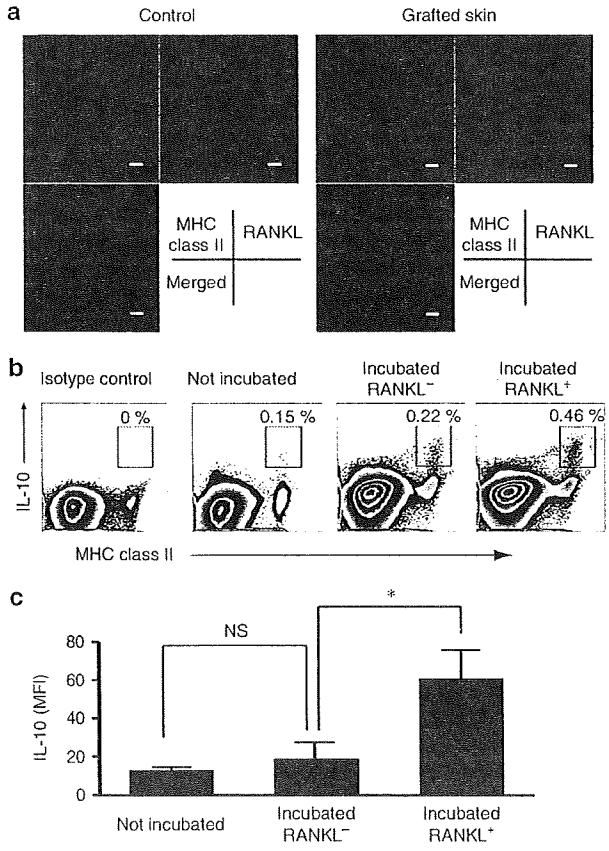

Figure 7. Induction of RANKL in grafted skin and stimulation of LCs to produce IL-10 by RANKL. (a) The grafted or non-grafted skin was stained with antibodies to MHC class II and RANKL. Red, MHC-class II; blue, RANKL. Merged image is shown in purple. (b, c) EC suspensions were cultured with or without 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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Novel mutation p.Gly59Arg in *GJB6* encoding connexin 30 underlies palmoplantar keratoderma with pseudoainhum, knuckle pads and hearing loss

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Accepted for publication
31 January 2009

Key words
Bart–Pumphrey syndrome, Clouston syndrome, gap junction, pseudoainhum, Vohwinkel syndrome

Conflicts of interest
None declared.

DOI 10.1111/j.1365-2133.2009.09137.x

Summary

Background Connexins, components of the gap junction, are expressed in several organs including the skin and the cochlea. Mutations in connexin genes including GJB2 (Cx26), GJB3 (Cx31), GJB4 (Cx30.3), GJB6 (Cx30) and GJA1 (Cx43) are responsible for various dermatological syndromes and/or inherited hearing loss, frequently showing overlapping phenotypes.

Objectives To clarify the spectrum of clinical phenotypes caused by connexin mutations.

Methods We report a 32-year-old Japanese woman with mild palmoplantar keratoderma (PPK) with severe sensorineural hearing loss, knuckle pads and pseudoainhum of her toes.

Results Direct sequencing revealed no mutation in GJB2, but a novel heterozygous missense mutation p.Gly59Arg in GJB6. Electron microscopy revealed no apparent morphological abnormality of gap junctions in the patient's lesional epidermis.

Conclusions The patient harboured the novel GJB6 missense mutation p.Gly59Arg in the first extracellular loop of Cx30. Mutations in glycine 59 of Cx26 are associated with PPK–deafness syndrome, and the similar phenotype here supports the observed heteromeric channel formation; the dominant nature of the mutation suggests an effect on gap junctions similar to that of the comparable mutation in Cx26.

Gap junctions are cell-to-cell connecting structures containing clusters of intercellular channels that allow intercellular passage of ions and molecules of up to 1 kDa. These channels are oligomeric assemblies of family members of related proteins called connexins. Six connexin polypeptides assemble into a connexon, a hemichannel that interacts with its counterpart in an adjacent cell membrane to form a complete intercellular channel.[1] All the connexins in an individual connexon may be of the same type (homomeric), or heteromeric connexons may be formed by oligomerization between different connexins. Connexin 26 (Cx26) and connexin 30 (Cx30) are known to form heteromeric connexon hemichannels.[2]

Connexins are expressed in several organs including the skin and the cochlea. Mutations in connexin genes including GJB2 (Cx26), GJB3 (Cx31), GJB4 (Cx30.3), GJB6 (Cx30) and GJA1 (Cx43) are responsible for several hereditary skin disorders associated with hearing loss.[3] Cx30 mutations are typically associated with Clouston syndrome[4] in which palmoplantar keratoderma (PPK) is only occasional and not usual, although cases resembling keratitis–ichthyosis–deafness (KID) syndrome have also been reported.[5] Various mutations affecting Cx26 cause PPK–deafness syndrome.[6] PPK–deafness syndromes are typically GJB2 associated. However, as Cx26 and Cx30 interact, one might expect more phenotypic overlap as exemplified by the report of Jan et al.[5]

Here, we report a Japanese woman with clinical features resembling those of Vohwinkel syndrome and Bart–Pumphrey syndrome. We found a novel heterozygous missense mutation p.Gly59Arg in GJB6. As far as we know, this is the first reported patient with PPK with pseudoainhum, knuckle pads and hearing loss, harbouring a GJB6 gene mutation.

Patients and methods

Patient

The patient was a 32-year-old Japanese woman. She was diagnosed with congenital sensorineural hearing loss when she

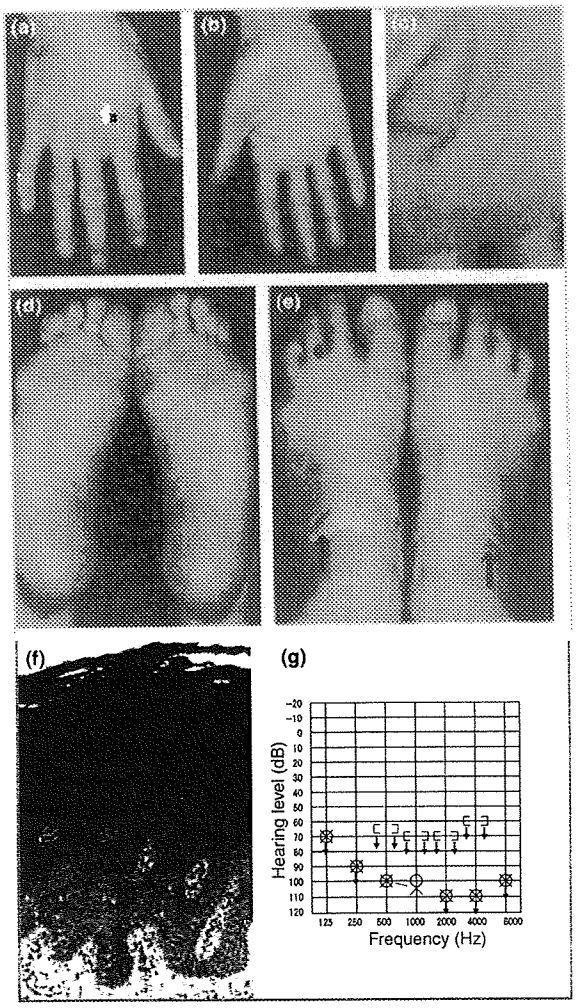

Fig 1. (a–e) Clinical features of the patient's skin. (a) Knuckle pads on the dorsa of the fingers of the patient; (b–d) diffuse palmar and plantar hyperkeratosis without honeycomb features; (d, e) amputation due to constriction bands on the fifth toe of each foot; extensive hyperkeratosis was seen on the ankle joints. (f) Skin biopsy from the sole revealed marked orthohyperkeratosis with hypergranulosis (haematoxylin and eosin; original magnification × 100). (g) Patient's pure tone audiogram showed pronounced sensory hearing loss: air conduction indicated by cross/round marks (cross, left ear; round, right ear); bone conduction indicated by bracket marks ([, right ear;], left ear); arrows pointing downwards indicate the loudest tone that was not heard.

was 3 years old. She also had diffuse PPK without a honeycomb hyperkeratosis appearance and hyperkeratotic plaques over the knuckles (Fig. 1a–c). An audiogram obtained at 24 years of age showed pronounced sensorineural hearing loss (Fig. 1g). At the age of 26 years, the fifth toe on each foot was surgically amputated due to pseudoainhum (Fig. 1d,e). Her fingers did not show mutilation. Extensive hyperkeratosis was seen in areas exposed to mechanical stress, such as the extensor (ventral) aspect of her ankle joints (Fig. 1e) probably because of folding the legs in the Japanese sitting or kneeling style. She had no features of ichthyosis on her trunk or extremities. Her hair, teeth and nails were normal and she had no ocular involvement. There was no family history of skin disorders or auditory dysfunction, or consanguinity in her family. All members of the family, including her parents and her elder sister, were generally healthy and were without PPK or hearing loss.

Mutation detection

After fully informed, written consent, peripheral blood samples were obtained from the patient and genomic DNA was extracted (Qiagen, Hilden, Germany). The entire coding region and exon/intron boundaries of *GJB2* and *GJB6* were amplified by polymerase chain reaction (PCR) using the specific primers described previously.[7,8] PCR products were sequenced and mutation was confirmed by enzyme digestion with *Btg*ZI restriction enzymes (New England Biolabs, Ipswich, MA, U.S.A.). Reference cDNA for GJB6 was cDNA sequence GenBank accession number NT_009799.

Morphological observations

A skin biopsy was taken from the right sole of the patient with fully informed consent. The biopsy specimen was processed for routine histological analysis and for ultrastructural observations as previously described.[9]

Results

Mutation analysis

Analysis in GJB2 revealed no mutation, although a common polymorphism p.Val27Ile was found. We identified a heterozygous 175G>C transversion in GJB6 (Fig. 2a). This novel nucleotide alteration leads to the replacement of glycine 59 (neutral, hydrophilic residue) with a positively charged hydrophilic arginine residue (p.Gly59Arg) in the first extracellular loop. The mutation introduces a single *Btg*ZI restriction site in the gene. We confirmed the presence of the mutation in the patient's genomic DNA by restriction enzyme *Btg*ZI digestion (Fig. 2b). This nucleotide change was not detected in 100 unrelated, healthy Japanese individuals (200 alleles).

Histological evaluation of the patient's skin

A biopsy specimen from the patient's plantar skin revealed compact orthohyperkeratosis with hypergranulosis and acanthosis in the epidermis (Fig. 1f).

Electron microscopic findings

Ultrastructurally, keratinocytes in the epidermis of the patient's plantar skin assembled gap junctions showing normal morphology with a typical pentalaminar structure, 20 nm in width (data not shown).

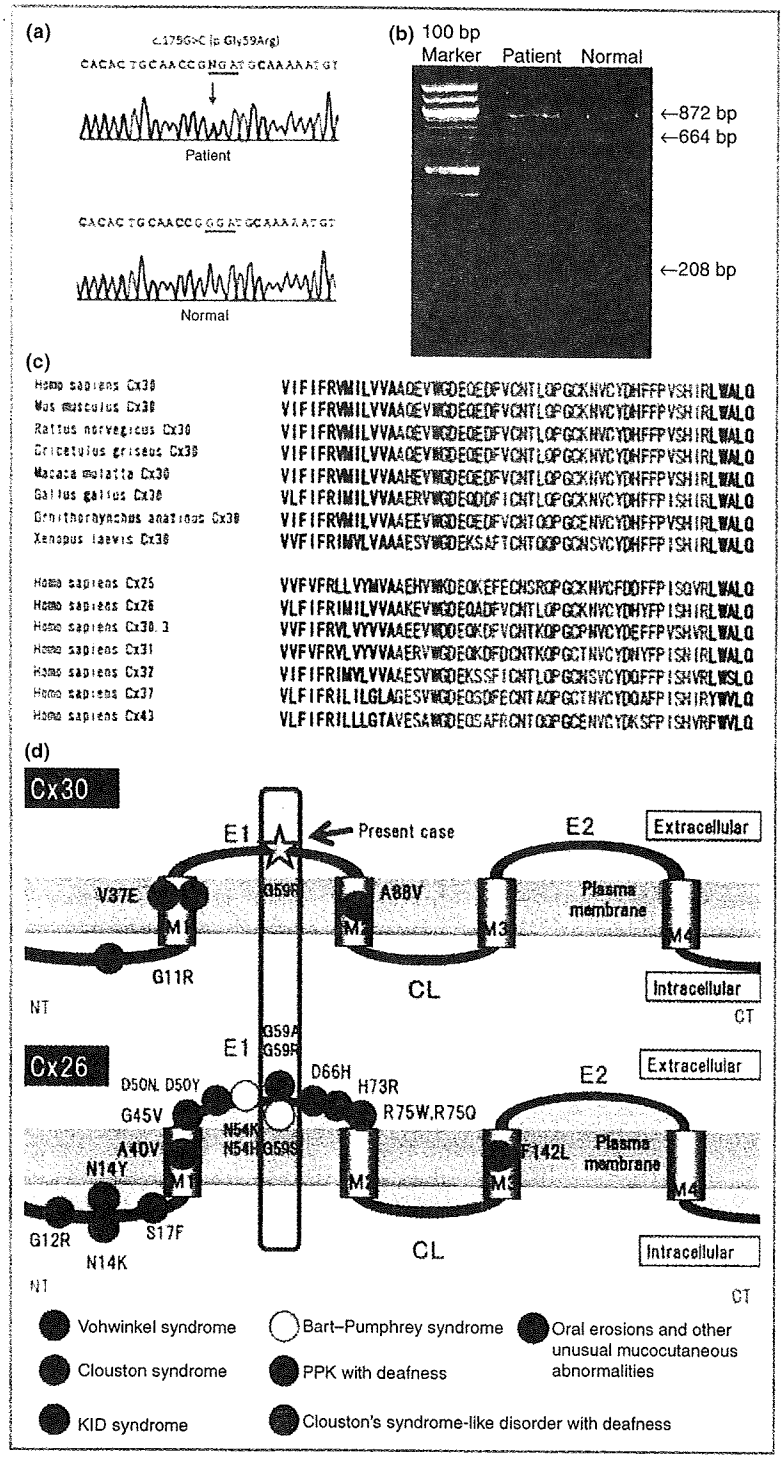

Fig 2. (a) Sequence chromatograms of GJB6 from the patient (upper) showed the heterozygous transition c.175G>C at codon 59 (p.Gly59Arg). (b) Confirmation of the presence of the mutation p.Gly59Arg in the patient by BtgZI restriction digestion. An 872 bp polymerase chain reaction fragment from the mutant allele was digested into 664 bp and 208 bp fragments, whereas that from the wild-type allele was not cut. Thus, 872 bp, 664 bp and 208 bp bands were seen in the patient harbouring the heterozygous p.Gly59Arg mutation, although only a 872 bp band was detected in the normal control. (c) Comparison of amino acid sequences of Cx30 from diverse species and other members of the human connexin family. Glycine residue at codon position 59 (red) is located in the centre of the first extracellular domain (blue) and is highly conserved in diverse species and other members of the human connexin family. (d) Cx30/Cx26-associated syndromes and reported causative mutations in Cx30/Cx26. M1–M4, transmembrane domains 1–4, respectively; E1 and E2, extracellular domains 1 and 2, respectively; CL, cytoplasmic loop; PPK, palmoplantar keratoderma; KID, keratitis–ichthyosis–deafness.

Discussion

We herewith report, as far as we know, the first case of PPK–deafness caused by a mutation affecting the E1 domain of Cx30. Glycine 59 in Cx30 is located in the first extracellular loop (Fig. 2c,d), which exhibits high sequence conservation in homologous proteins from different species (Fig. 2c). The first extracellular loop is thought to be essential for the

interaction between a connexon and its counterpart in an adjacent cell to form a complete intercellular channel. Three mutations in Cx26, p.Gly59Ala, p.Gly59Trp and p.Gly59Ser, occur at glycine 59 which is orthologous to glycine 59 in Cx30. These were reported in syndromes comprising sensorineural hearing loss and PPK[6,10] (Fig. 2d).

Cx30 and Cx26 form heteromeric junctions both in the skin and the inner ear and functional data suggest a dominant interaction between the two.[2,11] From these facts, we think it reasonable to speculate that missense mutations in the first extracellular loop domain in either Cx26 or Cx30 can lead to similar phenotypes. However, this hypothesis does not explain why the phenotypes look so similar or why, for example, p.Gly11Arg in Cx30 does not lead to KID syndrome whereas p.Gly12Arg in Cx26 does. There may be a genetic background effect which contributes to the phenotype in each patient.

The GJB2 keratoderma/deafness phenotypes are almost all caused by mutations clustering in the first extracellular loop domain.[12] The Cx26 mutation p.Gly59Arg results in a diffuse (although more severe) keratoderma, as does this mutation in Cx30 which is shown in the present patient. In contrast, mutations in the N-terminal cytoplasmic region in Cx26 are associated with KID syndrome and similar phenotypes. Likewise, p.Gly11Arg and p.Val37Glu in Cx30 in the N-terminal cytoplasmic region underlie KID-like syndrome or Clouston syndrome. We do not know the exact function of the N-terminal domain. There are two hypotheses: the first is that the N-terminus is involved in connexon assembly;[13] the second is that the N-terminal domain works as a plug in a vestibule of the connexon hemichannel, which physically blocks the channel (plug gating mechanism).[14] It was suggested that the mutation p.Asn14Tyr in Cx26, which is associated with a KID phenotype, causes the channel to be locked in a closed position by the plug.[15] Thus, if a similar phenomenon happens with the Cx30 mutation in a heteromeric channel composed of Cx30 and Cx26, Cx30 mutations in the N-terminus may lead to similar KID-like phenotypes. The present patient harbouring the Cx30 mutation in the first extracellular loop domain showed a phenotype distinct from KID syndrome.

Light microscopic observation of the lesional skin showed orthohyperkeratosis with hypergranulosis without any specific findings for our present patient. The mutation we found did not affect connexin morphology. Likewise, some Cx26 mutations in the E1 domain were examined for their effects on connexin morphology and apparently did not affect it.[11,16]

In conclusion, we present a novel GJB6 missense mutation p.Gly59Arg in a patient who had PPK with pseudoainhum, knuckle pads and hearing loss. The present case expands the clinical spectrum of GJB6 mutations and shows that, in PPK–deafness cases where we do not find GJB2 mutations, we should check GJB6. Furthermore, our results suggest an interaction between Cx30 and Cx26 E1 domains.

Acknowledgments

We thank Ms Kaori Sakai, MS, Ms Megumi Sato and Ms Akari Nagasaki for their technical assistance on this project. We also thank Prof. James R. McMillan and Dr Heather Ann Long for proofreading this manuscript. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to M.A. (Kiban B 20390304).

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日皮会誌：119（7），1219—1224，2009（平21）

2. 遺伝カウンセリング

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要　約

遺伝カウンセリングとは遺伝性疾患の患者・家族に対して，臨床遺伝学的診断にもとづき，適切な情報を提供し，支援する対話行為である．医師（カウンセラー）は患者・家族（クライアント）に傾聴し，共感的に受容することから対話を始め，非指示的に望ましい行動変容を起こさせることを目標とする．このために実際必要なことは　1. 正確な診断　2. 最新の情報の収集と提供　3. 遺伝子検査の必要性の検討　4. 遺伝子検査の結果とその意味の説明　5. 継続的なサポートである．

はじめに

この20年足らずの間に遺伝性疾患の解明は飛躍的に進み，メンデル遺伝形式をとる単一遺伝子病のほとんどの責任遺伝子が解明されてきている．遺伝子を解析し，遺伝子変異を同定することにより，その疾患の予後の予測，次子の再発危険率が推定することが可能となり，疾患によっては出生前診断，着床前診断が可能な場合もある．しかし，遺伝子は究極の個人情報であり，その取り扱いには注意を要する．遺伝性非ポリポーシス大腸癌や遺伝性乳がん・卵巣がんなどの遺伝子情報は保険会社にしてみればのどから手が出るほど欲しい情報となりうる．また，遺伝子異常を排除することは優生思想につながるとの批判もあり，高い倫理感に立脚し施行されなければならない．遺伝子診断を受けた患者さんがその将来を悲観して自殺するという事例も起こりうる．患者さんは遺伝子変異を受け継いでいるか否かを知らないでいる権利もあることを忘れてはならない．このように遺伝子診断は一般生化学検査などとは大きく異なり，医師側の判断のみで施行してはならない．クライアント（相談者：通常患者本人やその家族である）の自立的な判断のもと，行われなければならない．遺伝カウンセリングとは「遺伝性疾患の患者・家族またはその可能性のある人に対して，生活設計上の選択を自らの意思で決定し行動できるように臨床遺伝学的診断を行い，医学的判断に基づき適切な情報を提供し，支援する医療行為」である[1]．つまり，遺伝病またはそのリスクのあるクライアントに対し，遺伝病を正しく理解し，受容し，好ましい行動ができるよう，正しい情報を提供し，クライアントをサポートするコミュニケーション行為である[2]．

遺伝病に関わるすべての医師が遺伝カウンセリングに精通しているべきであるが，実際の医療現場では短い診察時間，多くの患者数の中，クライアントの自発的判断を促すきめ細かな介入は困難なことが多く，2005年には認定遺伝カウンセラーが誕生し，資格を有する専門職が養成されるようになった．しかし，遺伝カウンセリング自体が保険適応になっていないこともあり，その必要性とその対価につき十分な社会認知がされているとは言えない．現実にはそれぞれの医療現場で医師により，遺伝カウンセリングも行われているケースが多いであろう．本稿では遺伝性皮膚疾患の遺伝カウンセリングに必要な理論と技術を概説した後，皮膚科医として表皮水疱症を例として具体的な行動につき論じたい．

1. カウンセリングの理論とテクニック

カウンセリングは本来専門職が医療を実践する医師とは別の立場でクライアントに寄り添い，共感的な理解を示し，情報を提供し，自発的な判断を促すべきものである[3]．しかし，現実には各科の医師がカウンセリングも行っており，遺伝病を扱う医師はカウンセリングの理論，テクニックを知っておくことは重要である．医師のための遺伝カウンセリング技術に関して，千代博士が他誌に詳しく連載している[2)~6)]ので是非ご一読されることをおすすめする．ここでは氏の論文にもとづき重要と思われる事項を解説する．

1）カウンセリングの目標

人間は自分がこうありたいと思っている像と現実の自分との間にギャップが生じるとストレスが発生し，神経症など精神・身体の異常となって発現してくるとされている[4]．これを自己不一致の状態といい，それを解消し自己一致の状況に変容することが治療である．カウンセラーはクライアントにけして変容を強要して

はならず，あくまでその行動変容のための決断を手助けをするのである．遺伝カウンセリングの目標は医療現場でクライアントに好ましい行動変容を起こすことである．まず始めに重要なことは医師がクライアントに拒絶されないことであろう．遺伝病の多くの場合にあてはまる治療法がないという重い現実は，患者さんにとって医師の介入は全く無意味であるような誤解をもたれてしまいがちであり，詐欺まがいの民間療法へ傾倒してゆく危険を生み出す．医師とクライアントとの良好な関係作りはカウンセリングを行うため必須の条件である．つぎに，具体的な到達目標は個々の事例により若干異なるが，表皮水疱症の場合にたとえていえば，疾患の原因，症状を正しく理解すること，自分を卑下したり，将来を悲観しないこと，水疱ができないために守るべきことを良く理解すること，水疱に対処する具体的手段をもつこと，現時点では根治療法が無いことを受け入れること，将来的には遺伝子治療が実用化される可能性があり，希望を持つこと，遺伝に対して正しく理解し，冷静に把握できること，特定疾患に対する補助など社会的援助が受けられること，家族も上記のことを良く理解し，協力がえられること等々である．

2）カウンセリングの環境

カウンセリングを行うためにはまずそれなりの環境が必要である[5]．皮膚科一般外来の喧噪の中，たとえ30分時間を割いたとしても多くの患者を待たせている状況や，医師が他の指示を色々出しながらの状況ではカウンセラーとクライアントの共感は得られないであろう．静かな診察室と，十分な時間が必要である．長すぎる待ち時間もクライアントの自発的発言意欲をそぎ，指示を待つ態度にさせる原因となりうる．また，診察時間も可能な限り30分なり1時間なりあらかじめ設定し，クライアントが時間を自由に使える雰囲気があると良い．

3）カウンセラーに求められるもの

ロジャースのカウンセリングの3条件といわれるものがある．それは1. クライアントの絶対的受容　2. カウンセラーの自己一致　3. コミュニケーションスキルである[4]．クライアントに「この人は私の気持ちをよくわかってくれる人だ」と感じさせることはクライアントの抱えている思いも寄らない誤解や理解不足を感知するために，また，こんなことを聞いたら恥ずかしいとか，馬鹿だと思われるなどコミュニケーションの障害になる垣根を取り払うために重要である．そのためにはクライアントの思いをまずはすべて受け止めることから始まる．たとえ理解不足や誤解に基づく誤った言動があったとしても即座に否定したり，誘導したりしてはいけない．まずはクライアントの思考過程を理解し，共感的態度を示しながら，何が誤解の源泉なのかをクライアントと協調して見つけてゆく姿勢を明らかにすることが重要である．これは医療面接一般にも必要といわれている傾聴であり，共感的態度である．後述のコミュニケーションスキルを身につけておくこともクライアント受容に役立つ．

クライアントは自己不一致があるからカウンセリングを受けるのであるが，カウンセラー自身に自己不一致があっては好ましい行動変容へのサポートはできない．クライアントの絶対的受容をすることは，カウンセラーも，ともに不一致になることとは異なる．クライアントの不一致を受容し，非指示的な方法で誘導するにはカウンセラーに揺るぎのない確固とした自己一致が必要なのである．

コミュニケーションスキルは必ずしも話し上手であることを意味しない[6]．傾聴することだけでもクライアントは自由に思いを述べ，言葉に落とし込むことにより，自らの問題点をあぶり出すことができ，みずから解決の糸口を見つけることができるかもしれない．こうして得た解答が正しい方向であればもうほとんど介入する必要はないわけである．正しくない方向の場合には，正しい部分だけを支持し，好ましくない部分はその先に待つ矛盾や有害事象などをともに想像してゆくことにより再考を促す．何がクライアントにとって引っかかっているかが傾聴のみではわからない場合，適宜質問を行うが，「はい」「いいえ」で答える閉鎖的な質問は最小限にし，開放的な質問でクライアントに話させるように心がける．時々，クライアントの話を繰り返し，まとめながら意見の確認と明確化を行うとさらに良いであろう．ほかに細かいスキルとして，アイコンタクトをほどよくとること，相手の態度に言外の意識を読み取ること，専門用語を避けること（ネットで詳しく勉強をしてきたインテリジェンシーの高いクライアントは逆に意識して専門用語も使用する），時計を見たり目をそらしたり拒絶的な態度をとらないことなどがスムーズなコミュニケーションのために必要である．また，一定の好ましい方向性が出た際には結論を支持し勇気づけることが行動変容の持続のために力

表 1 表皮水疱症の主要病型分類

大病型	主要病型	責任蛋白・遺伝子	臨床像
単純型	Weber-Cockayne 型	K5, K14	手足に限局した浅い水疱
	Köbner 型	K5, K14	躯幹四肢の浅い水疱
	Dowling-Meara 型	K5, K14	全身の疱疹状水疱
	筋ジストロフィー合併型	プレクチン	遅発性筋ジストロフィーを合併
接合部型	Herlitz 型	ラミニン 5	全身の重症なびらん，致死的
	non-Herlitz 型	ラミニン 5，XVII 型コラーゲン	脱毛，爪の変形，歯の変形，軽度の水疱びらん
	幽門閉鎖症合併型	α6β4 インテグリン	幽門閉鎖症にて致死的
栄養障害型	優性型	VII 型コラーゲン	四肢に限局した深い水疱，びらん，爪の変形
	Hallopeau-Siemens 劣性型	VII 型コラーゲン	全身の深いびらん，偽合指症，食道狭窄，貧血
	non-Hallopeau-Siemens 劣性型	VII 型コラーゲン	四肢に限局した深い水疱，びらん，爪の変形

となることも忘れてはならない．

2. 遺伝性皮膚疾患における遺伝相談の実際

さて，前述のような傾聴，共感的理解を通じて患者さんが疾患を受容して好ましい行動を起こしてゆくことが遺伝病の診療に要求されることである．このために実際必要なこと[7]は 1. 正確な診断 2. 最新の情報の収集と提供 3. 遺伝子検査の必要性の検討 4. 遺伝子検査の結果とその意味の説明 5. 継続的なサポートである．実際の診療の場を表皮水疱症を例にして提示してみる．

1) 正確な診断

遺伝カウンセリングを行うためには，正確な診断が必要であることはいうまでもない．まずはきっちりとした家系図を作成することにより，遺伝型を明らかにする[8]．また，遺伝形式や責任遺伝子によりサブタイプがある場合はそこまでの詳しい診断を下す必要がある．たとえば表皮水疱症の場合，接合部型には 4 種の原因遺伝子があり，合併症状や予後が全く異なる（表 1）．

図に提示した症例は正常な非近親婚の両親の第 1 子で，生下時より四肢を中心に水疱・びらんの形成を認めた．図 1a のように手指には小びらんがあり，稗粒腫の形成が見られる．爪甲は萎縮しているが脱落はしていない．指の癒合はなく，粘膜に症状はない．皮膚生検では表皮下水疱を認めた．患者皮膚を基質とし，各種基底膜分子に対するモノクローナル抗体を用いた蛍光抗体法により各分子の基底膜部における発現を見たところ，VII 型コラーゲンの発現に若干の減弱が見られ（図 1b，c），他の分子は正常に発現が見られた．電顕的には水疱は基底板の真皮側に形成され，係留線維は低形成で細く短いものが多く観察された（図 1d）．以上により栄養障害型表皮水疱症の確定診断がえられた．VII 型コラーゲンの発現が見られることと，症状が軽症であることから，最重症型である Hallopeau-Siemens 型は否定される．しかし，軽症の劣性型か，優性型の突然変異体かは遺伝子診断以外の方法では，確定することはできない．

2) 最新情報の収集

診断が確定した次の段階は最新情報の収集である．自分の専門とする疾患でない場合にはまず始めに信頼のおける成書（Andrews'や Rook，最新皮膚科学体系など）の記述を読み，全体像を把握する．その後 OMIM（Online Mendelian Inheritance in Man）の Web site（http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim）をチェックし，疾患により記載の新鮮度にばらつきがあるものの，原因遺伝子に関する重要論文はそこで容易に見つけることができる．また，GeneReviews のサイト（http://www.geneclinics.org/profiles/all.html）は最新の情報にあふれ記述も体系立っており，非常に重宝する．ネットにつながるパソコンさえあれば遺伝病の summary をどこでも見ることができる．

以上の情報源から疾患の位置づけ，症状の頻度や程度，年齢による変化，生命や身体機能的予後，発症の原因・メカニズム，そして次子や患者の子への遺伝の可能性につき十分把握しておく．

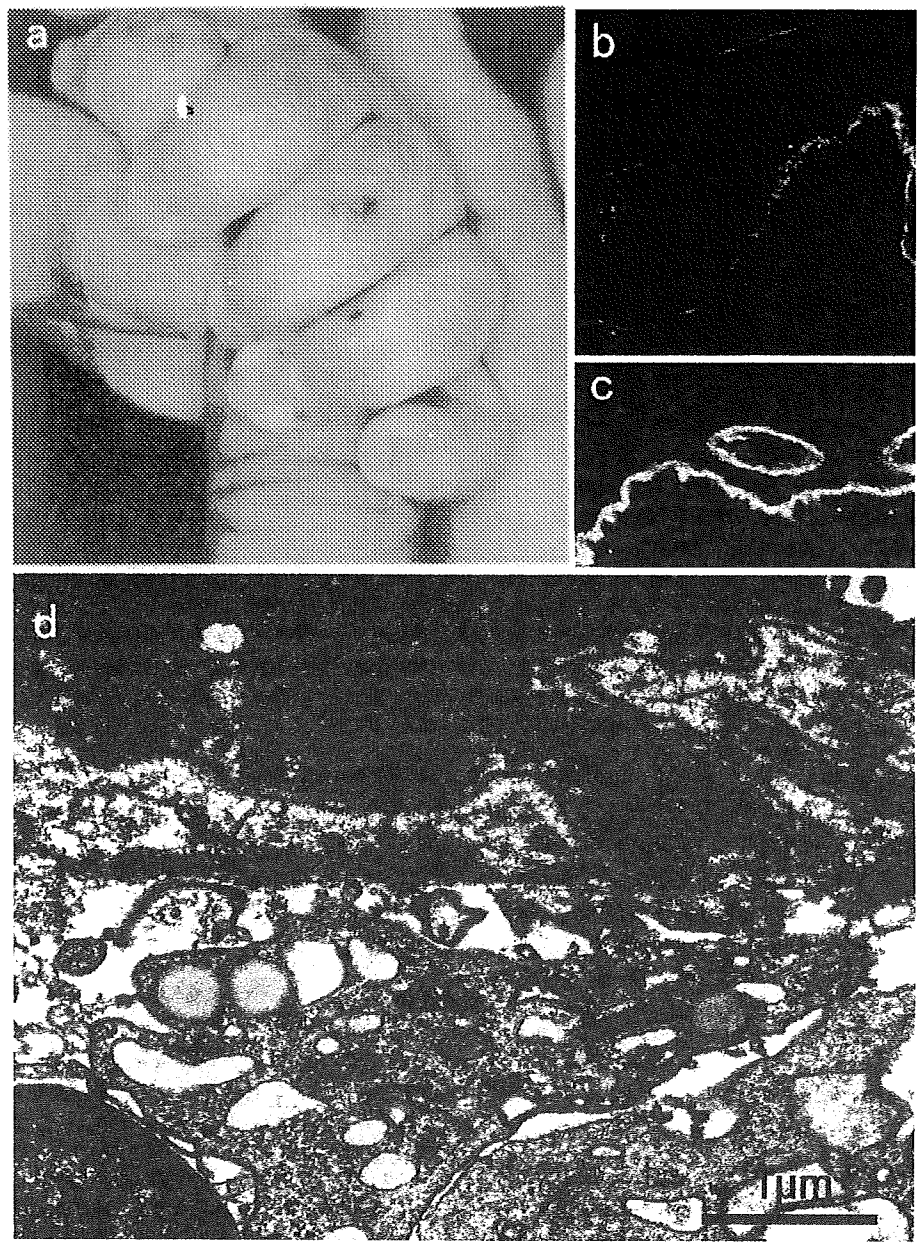

図 1
a. 患者の臨床症状．手指伸側に小びらんがあり，稗粒腫の形成がみられる．中指の爪甲は萎縮しているのが確認できる．b. 患者の表皮基底膜部におけるVII型コラーゲンの発現（LH7.2抗体）正常（c）と比べると若干蛍光の減弱がみられる．d. 患者皮膚の電顕像．水疱は真皮側に形成され，係留線維は細く短いものが多い．

3）遺伝子検査の必要性の検討

遺伝子診断をするかどうかの判断をクライアントとともに考えることは，想像以上に重要なことである．Weber-Cockayne型の単純型表皮水疱症はほとんどが優性遺伝であり，症状も軽症である．家系図が優性遺伝性を示唆しているのであれば，通常は確定診断のた

めの遺伝子検査をする必要はない．また，提示した症例のような家族歴のない栄養障害型の場合，遺伝子検査を行う以外，遺伝形式を同定するすべがない．

では，常染色体劣性遺伝病であるチロジナーゼ活性が欠損する眼皮膚白皮症 Ia 型の患者を兄弟に持つ非罹患者が，結婚にあたって自分が保因者かどうか知りたい，といった場合はどうであろうか？このクライアントが保因者である確率は 2/3 である．この家系の罹患者がチロジナーゼ遺伝子にもつ変異が同定されていることを前提条件として，保因者診断が技術的には可能である．しかし，検査希望の根源が，本人からではなく，パートナーおよびその家族の希望である場合も少なくない．遺伝子異常は「知らないでいる権利」があることを忘れてはならない．クライアントの自らの意思で保因者診断を受けたいのかどうかを十分に議論する必要がある．結果をパートナーに知らせることにより，婚約が破棄される可能性があることまでクライアントが認識しているかどうかもう一度確認すべきである．仮にクライアントが保因者であったとしても，パートナーが保因者である確率は多くても 1/100 程度であり，二人の間に罹患児が生まれる確率は 1/400 である．1/400 のリスクのため，パートナーの家族から結婚反対の意見が出されることは不当であると考えられるが，そのときの対応もふくめ，検査施行の判断を最終的にはクライアントにゆだねるべきであろう．

それではパートナーの方も保因者診断をしてくれないか，という考えも出るかもしれない．しかし，家系内に罹患者がなく，原因遺伝子変異が特定されてない場合，たとえチロジナーゼ遺伝子の全エクソンおよびその周囲のイントロンの配列が正常であったとしても，保因者である可能性を完全に否定することはできない．罹患者であっても遺伝子変異が二つのアレルに同定される確率は 90% に満たないのである．

このように遺伝子検査を行うべきかどうか，その必要性についてクライアントとともに十分検討して，しっかりとした動機をもってから遺伝子検査に臨むべきである．その後に，あらためて検査の概要，目的，遺伝情報の取り扱い，遺伝子変異がわからない可能性もあること，同意や同意撤回の自由などを明記した文書による説明を行い，文書による同意をとることが必要である．

4) 遺伝子検査の結果説明

遺伝子検査についての動機付けがしっかりなされていれば結果説明は比較的スムーズに進むものである．最も重要なことは事実を正確に，クライアントが理解できるように説明することである．

前述の症例では VII 型コラーゲンをコードする遺伝子 *COL7A1* の全 118 エクソンを調べた結果，エクソン 112 にナンセンス変異，エクソン 46 にミスセンス変異が確認された．前者は父方変異でこれまで報告例のないものであったがナンセンス変異であり，正常コントロール 100 例に認められないことから病的変異であると考えられた．後者は母方変異で，そのホモ接合体は軽症の栄養障害型表皮水疱症を発症していることがわかっており，病的変異である．劣性遺伝の仕組みや遺伝子異常から発症に至るまでのメカニズムを図示しながら詳しく説明を行った．

次に子供が生まれるときは発症する確率が 1/4，保因者となる確率が 1/2 であること，次子が発症した場合，病気の重症度は同程度となる可能性が高いことを説明した．将来保因者の子供に病気が発症する可能性は一般集団の保因者確率は 1/200 以下であり，きわめて低いことも併せて説明した．

さらに，保因者である両親に対してもフォローが必要である．ほとんどのケースでご両親は少なからず自責の念や恥の意識を持たれるものである．劣性遺伝子変異を持っていることは特殊なことではなく，正常に見える人でも確率的に 4 個以上の何らかの劣性遺伝病の保因者であることがわかっている．今回の発病は両親が異常だからというわけではなく，組み合わせによる偶然のなせる業であることをよくお話しする．十分に傾聴し，誤解の無いようカウンセリングする必要がある．また，遺伝子治療や再生医療は日進月歩，進歩しており，将来的にはよりよい対症療法や根治的治療が開発される可能性が高く，希望をもち疾患に対処してゆくように指導することも大切である．

5) 継続的なサポート

臨床医による遺伝カウンセリングの場合，診断の確定と予後予測だけで終了するわけではない．日常のケアは生涯にわたって続くため，通院しやすい皮膚科専門医のいる病院・医院などに十分な情報提供とともに依頼し，病診連携により患者の利便性をはかるべきである．連携元の遺伝相談外来には年に 1〜数回定期的に通院していただき，最新の情報や年齢とともに変化する悩み，保育園や学校への必要な情報提供を行い，また，疾患の治療に関する新しい情報があるときには