lipid barrier in the stratum corneum (Akiyama, 2006).

As illustrated here, mutation analysis of the *ALDH3A2* gene is a highly sensitive method to confirm a diagnosis of SLS, which does not require a skin biopsy and can complement or replace FALDH enzymatic assays or analysis in SLS.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Novel Hairless *RET*-Transgenic Mouse Line with Melanocytic Nevi and Anagen Hair Follicles

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TO THE EDITOR

The c-*RET* proto-oncogene encodes a receptor-tyrosine kinase and glial cell line-derived neurotrophic factor ligands, including glial cell line-derived neurotrophic factor, neurturin, artemin, and persephin, have been reported to be ligands of RET (Takahashi, 2001). *RFP/RET* is a hybrid oncogene between c-*RET* and *RFP* that was isolated by NIH3T3 transfection assays (Takahashi *et al.*, 1985). Previously, we established a metallothionein-I/*RFP-RET* (*RET*)-transgenic mouse line (242) that spontaneously develops systemic skin melanosis without macroscopic tumors (Iwamoto *et al.*, 1991; Kato *et al.*, 1999). Generally, most hair follicles in adult wild-type mice are in telogen (Kato *et al.*, 2001). It is basically impossible to induce continuous anagen hair follicles in adult wild-type mice, although temporal anagen hair follicles are inducible by shaving hairs (Kato *et al.*, 2001). Interestingly, adult *RET*-transgenic mice have continuous anagen hair follicles with hyper melanin production (Kato *et al.*, 2001, 2004). Moreover, hair growth of adult transgenic mice was promoted compared with that of control C57BL/6 mice (Kato *et al.*, 2001). These results suggest that a continuous anagen phase of hair follicles plays an important role in hair growth.

We also established another *RET*-transgenic mouse line (304/B6) (Kato

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Epidermodysplasia verruciformis and generalized verrucosis: the same disease?

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Summary

We report a patient with epidermodysplasia verruciformis (EV) who had severe generalized verrucous skin lesions for 50 years without any immunological abnormality. Microscopic examination showed two histopathological features, including seborrhoeic keratosis and common warts. The detected human papilloma virus (HPV) types were found to be HPV 3, 50, 5, and 76, using a degenerate PCR method. EV and generalized verrucosis are distinguished by slight differences in clinical symptoms or HPV types, so there should be no apparent differential points common to both diseases. Therefore, we propose that an abnormal susceptibility specific to HPV, which is the most characteristic feature in EV, should be regarded as a differential point in these two diseases.

Epidermodysplasia verruciformis (EV) is most commonly described as an autosomal recessive disorder, which demonstrates generalized and persistent cutaneous infection with human papillomavirus (HPV). On the other hand, the term generalized verrucosis (GV) has been used for generalized warts in some reports, which were usually associated with immunocompromised conditions.

Report

A 63-year-old Japanese man was referred to our clinic with multiple, slowly growing, hyperkeratotic skin lesions over his feet and arms (Fig. 1a). He had first noticed the warty papules on his hands at the age of 10 years, and they became gradually more generalized and increased in both size and number. The lesions failed to respond to superficial radiotherapy and cryotherapy performed at other clinics. Six months prior to presentation, one of the papules on his right calf had started to grow rapidly. Physical examination revealed a 70 × 60 mm, dark-reddish, dome-shaped tumour with odour on the right calf (Fig. 1b). On the dorsa of the hands and feet, multiple, markedly papillomatous nodules with severe hyperkeratosis were present (Fig. 2a). There was no skin lesion on the other sun-exposed areas, including the patient's face and scalp. He had no past history of repeated infectious diseases implicating an immunocompromised condition, and no family history of recalcitrant warts.

For diagnostic and therapeutic purposes, the reddish tumour showing rapid growth on the right calf was surgically removed. Simultaneously, biopsies were performed from the hyperkeratotic erythematous plaque on the right forearm (Fig. 1c), and the nonhyperkeratotic pigmented macule on his upper back (Fig. 1d). Microscopic examination of sections of all the specimens, stained with haematoxylin and eosin, showed two distinct histopathological features. The first was an acanthotic epidermal layer with sharply defined nests of basaloid cells, which is seen in seborrhoeic keratosis (Fig. 3a,b). The other was the typical histopathological changes seen in common warts, showing papillomatosis,

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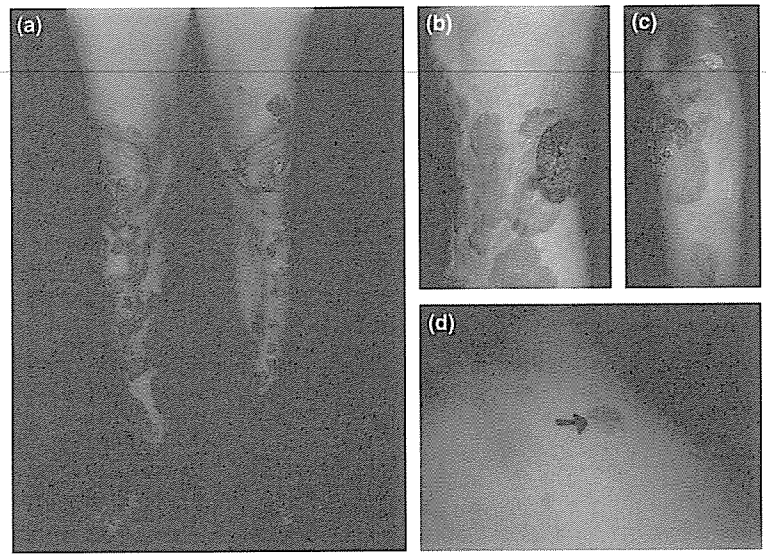

Figure 1 (a) Multiple hyperkeratotic skin lesions on the feet. (b) A 70 × 60 mm, dark-reddish, dome-shaped tumour with an odour on the right calf. HPV 3, 57 and 76 were detected. (c) Hyperkeratotic erythematous plaque on the right forearm. (d) The pigmented macule without hyperkeratosis on his upper back. HPV 3, 50, 57 and76 were detected.

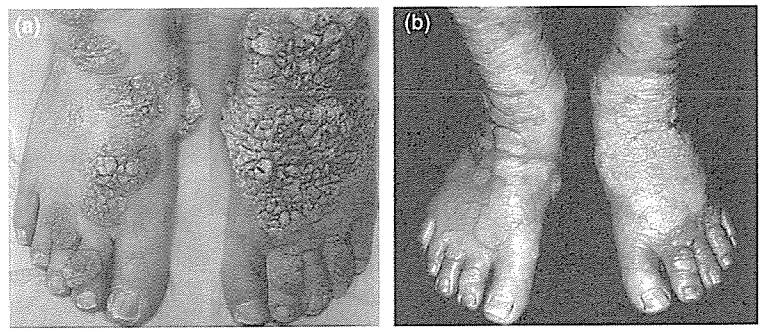

Figure 2 (a) Multiple markedly papillomatous nodules with severe hyperkeratosis were present on the dorsa of the feet; (b) improvement of the patient's cutaneous lesions was noted after 6 months of oral etretinate therapy.

hyperkeratosis and vacuolization of the cytoplasm of granular cells with coarse keratohyalin granules (Fig. 3c). Neither large keratinocytes showing clear cytoplasm, specific for EV, nor malignant changes were found. Laboratory evaluation revealed a normal, complete blood count and a normal absolute lymphocyte count [2000/mm^2, with 79% CD2 (normal range 60–80%), 56% CD4 (normal range 29–53%) and 15% CD8 (normal range 17–49%)]. All immunoglobulin levels were within the normal range. A serological test for human immunodeficiency virus was negative. Specimens from the pigmented macules and the large reddish tumour were investigated for human papilloma virus (HPV) typing, using a degenerating PCR method.[1] The HPV types detected in the specimens were HPV 3, 50, 57 and 76. Of these, HPV 76 belongs to species 3 of the genus Beta-papillomavirus, which is closely associated with EV.[2] HPV 3 and 57 belong to the Alpha-papillomavirus genus (species 2 and 4, respectively), and HPV 50 belongs to species 3 of the genus Gamma-papillomavirus. The patient started treatment with a combination of systemic etretinate, 50 mg/day, and topical maxacalcitol. All the skin lesions dramatically improved after 4 weeks of this treatment. His condition was relatively well controlled with this combination therapy at 6-month follow-up (Fig. 3b), but as the etretinate dose was reduced, his skin lesions relapsed.

Differences between EV and GV are so slight that a diagnosis is often difficult to make. In our case, several clinical features suggested against classic EV. Firstly, there was no parental consanguinity or familial occurrence of similar cutaneous lesions in our patient. Secondly, severely hyperkeratotic papillomatous nodules, which resemble common warts, were the predominant skin lesion in our case. These were different from the typical skin lesions, such as plane warts and

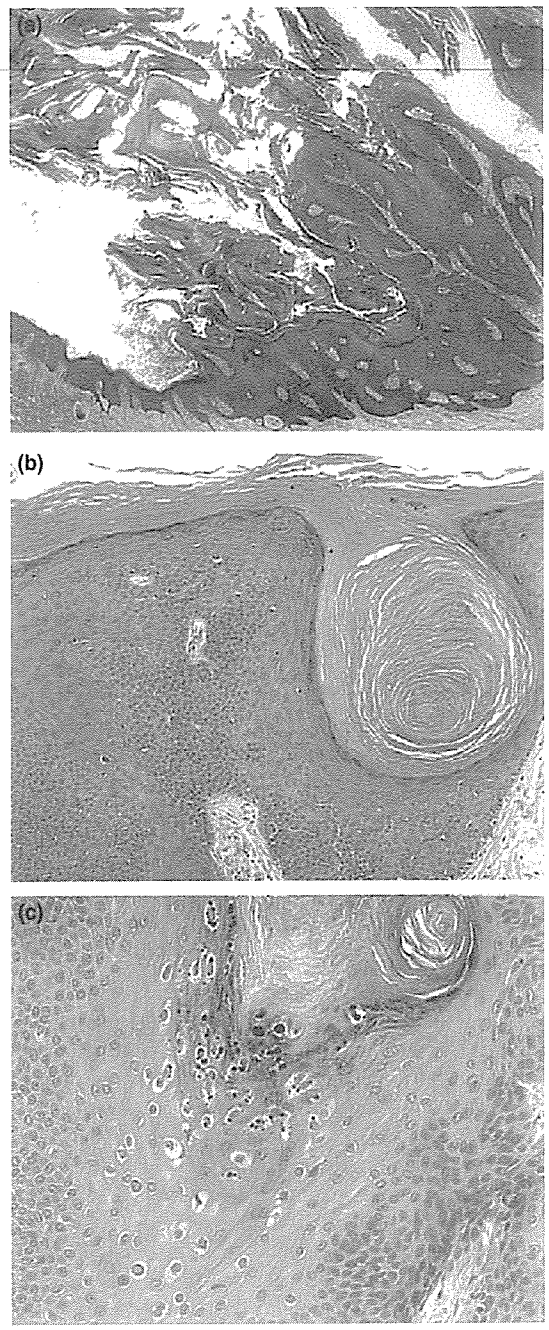

Figure 3 Histological findings (tumour on the right calf). (a) The tumour was exophytic, hyperkeratotic and papillomatous; (b) an acanthotic epidermal layer with sharply defined nests of basaloid cells; (c) vacuolization of the granular cell cytoplasm with coarse keratohyalin granules. Haematoxylin and eosin, original magnification (a) x 10; (b) x 40; (c) x 100.

pityriasis versicolor-like lesions, in an EV patient. Thirdly, histopathological studies showed the features of common warts or seborrhoeic keratosis rather than the characteristic EV lesion pathological features, such as large clear keratinocytes in the upper spinous layer and granular layers. Finally, the lesions in typical EV patients only demonstrate EV-specific HPVs;[3] however, in our patient, HPV typing revealed both EV-specific and non-EV-specific HPVs. Although these points fail to suggest that this case is classic EV, we thought it might be a unique variant of EV.

EV was originally described as an autosomal recessive disorder, characterized by a widespread and persistent cutaneous human papillomavirus infection without any apparent defects in both cellular and humoral immune systems, i.e., EV shows specific immunotolerance to HPVs. Patients with EV suffer from life-long, generalized verrucous skin lesions associated with various types of HPVs, some of which cause malignant transformation when infected with high-risk HPVs. Conversely, GV, in some reports, has been more typically associated with generalized warts, which are associated with immunocompromised conditions such as primary immunodeficiency, cyclic neutropenia and combined immune deficiency.[4–6] Our patient showed no history of repeated infections implicating an immunocompromised condition, therefore we propose that he had an abnormal specific susceptibility to HPV since early childhood. Previously, Requena *et al.* reported a patient with severe verrucosis similar to our case as GV.[4] They indicated that the mode of inheritance, typical clinical appearance including lesion distribution, characteristic histopathological findings and the presence of EV-specific HPV are critical points for the proper differential diagnosis of EV from GV. However, this is the only report that has demonstrated an immunocompetent patient with an abnormal susceptibility specific to HPV who was diagnosed as GV. EV and GV are distinguished by slight differences in clinical symptoms or HPV types, so there should be no apparent differential points common to both diseases. Therefore, we propose that an abnormal susceptibility specific to HPV, which is the most characteristic feature in EV, should be regarded as a differential point in these two diseases, both of which comprise systemic verrucosis.

Recently, EV-associated mutations were reported. Ramoz *et al.* reported nonsense mutations in two genes, *EVER1* and *EVER2*.[7] There is also one case report that demonstrates an *EVER1* mutation in an EV patient.[8] The *EVER1* and *EVER2* gene products show features of integral membrane proteins that are localized to the

endoplasmic reticulum. The function of these molecules is unknown, as is the relationship between the mutation and the abnormal HPV restricted susceptibility. Further follow-up studies and investigations of these gene products are required to clarify the underlying pathomechanisms resulting in the unique abnormal susceptibility to HPV, and to formulate a more precise diagnostic definition of EV.

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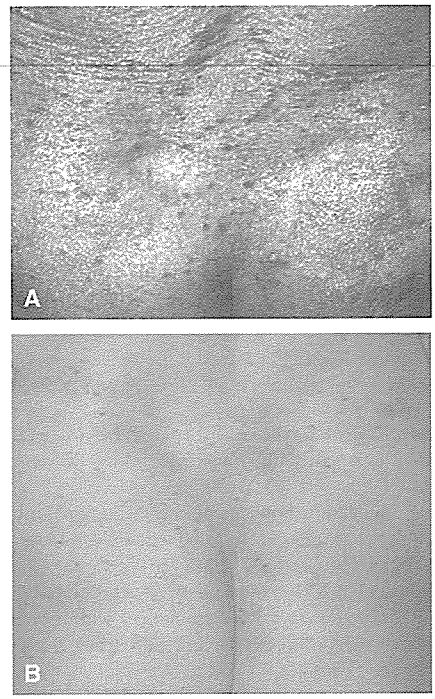

Fig 1. Buttocks with extensive psoriasis before (**A**) and 3 months after (**B**) etanercept therapy.

than 1,000,000 copies/mL viral RNA (normal < 1000). Considerable improvement of psoriasis was observed after only 2 weeks of treatment. Heartened by dramatic improvement of skin and joint disease by the second month of etanercept therapy, methotrexate was gradually withdrawn. Mild to moderate reactions at the injection site were alleviated by topical steroids. No other side effects or abnormal laboratory parameters were observed. After 5 and 12 months of etanercept therapy, the pretreatment HCV viral load remained unchanged. The patient continued to be free of significant papulosquamous activity during the follow-up period (Fig 1, *B*).

The discovery that tumor necrosis factor-alpha played an important role in the immunopathogenesis of both psoriasis and HCV[1-3] led us to pursue a trial of etanercept for a patient with intractable generalized psoriasis, psoriatic arthritis mutilans, and HCV. Etanercept in our patient effectively controlled skin and joint disease while eliminating a concomitant dependence on methotrexate therapy. This report is consistent with a recent case study of Zein[4] showing that etanercept is safe and effective as adjuvant therapy to interferon and ribavirin for the treatment of HCV.[4] Moreover, our findings amplify the conclusions of one other report in which etanercept was used successfully without complications in the setting of psoriasis and HCV.[5]

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Marked and restricted cutaneous pigmentation induced by selective intra-arterial cisplatin infusion

To the Editor: Super-selective intra-arterial high-dose chemotherapy has achieved remarkably good results in the treatment of head and neck cancer.[1] We report here a case of marked cutaneous pigmentation in the infusion area of super-selective intra-arterial cisplatin treatment.

We treated a buccal squamous cell carcinoma in a 66-year-old Japanese male with neoadjuvant chemotherapy followed by a radical operation. The chemotherapy consisted of intra-arterial infusion of 100 mg/m^2 cisplatin. Two days after chemotherapy, dark brown cutaneous pigmentation, different from the blue color of indigotin disulfonate, appeared around the tumor. The pigmentation was restricted to the infusion area for the super-selective

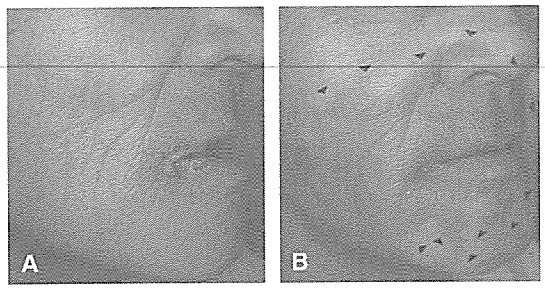

Fig 1. Cisplatin infusion chemotherapy induced cutaneous pigmentation restricted to the right cheek. **A**, Skin around the tumor before intra-arterial chemotherapy. **B**, Cutaneous pigmentation 10 days after the chemotherapy. Marked, dark-brown pigmentation was seen in the right cheek and the lips (*arrowheads*).

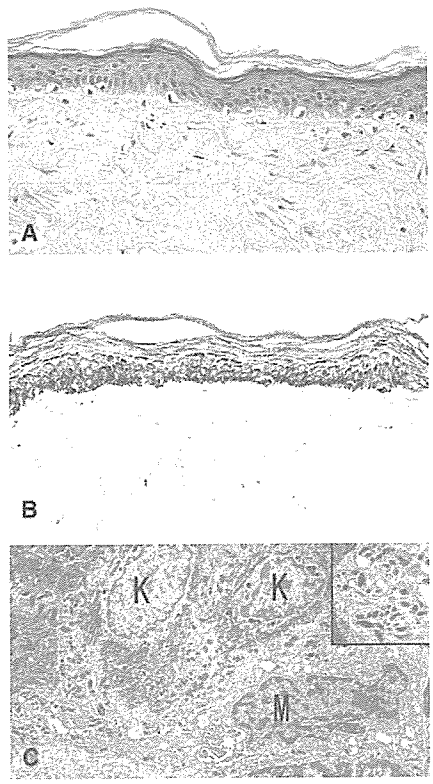

Fig 2. Skin biopsy specimen from the pigmented area on the right cheek. **A**, An increased number of melanocytes in the basal layer and basal melanosis were observed. **B**, Melanin staining clearly demonstrated the basal melanosis. **C**, Transmission electron microscopy demonstrated showed that melanocytes in the basal cell layer were producing a large number of melanosomes. *Inset*, Mature melanosomes were abundant in the cytoplasmic processes of a melanocyte. (**A**, Hematoxylin-eosin stain; **B**, Fontana-Masson stain; original magnifications: **A**, ×100; **B**, ×100; **C**, ×7000; *inset*, ×15,000.)

chemotherapy (Fig 1). No apparent inflammatory symptoms were seen in this region. A skin biopsy specimen showed that the number of melanocytes and amount of melanin pigment were increased in the basal layer (Fig 2, *A* and *B*). Neither inflammatory cell infiltration nor dermal melanocytes were detected. Electron microscopic observation revealed that enlarged melanocytes in the basal layer contained a large number of mature melanosomes in their cytoplasm (Fig 2, *C*). From these findings, the diagnosis of the cutaneous pigmentation caused by an intra-arterial cisplatin infusion was made. The cutaneous pigmentation gradually decreased in color and disappeared within 5 weeks.

Cutaneous complications with cisplatin include cutaneous allergic reactions, but marked cutaneous pigmentation induced by cisplatin has not yet been reported.[2,3] Although the exact mechanism of this hyperpigmentation has not yet been clarified, cutaneous pigmentation around an infusion area should be recognized as one of the possible adverse effect of intra-arterial cisplatin infusion therapy.

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Cutaneous metastases as the first manifestation of pleural malignant mesothelioma

To the Editor: Metastases of malignant mesothelioma (MM) are uncommon, but they have been reported

ORIGINAL ARTICLE

See related commentary on page 708

Fibroblasts Show More Potential as Target Cells than Keratinocytes in *COL7A1* Gene Therapy of Dystrophic Epidermolysis Bullosa

Maki Goto[1], Daisuke Sawamura[1], Kei Ito[1], Masataka Abe[1], Wataru Nishie[1], Kaori Sakai[1], Akihiko Shibaki[1], Masashi Akiyama[1] and Hiroshi Shimizu[1]

Dystrophic epidermolysis bullosa (DEB) is an inherited blistering skin disorder caused by mutations in the type VII collagen gene (*COL7A1*). Therapeutic introduction of *COL7A1* into skin cells holds significant promise for the treatment of DEB. The purpose of this study was to establish an efficient retroviral transfer method for *COL7A1* into DEB epidermal keratinocytes and dermal fibroblasts, and to determine which gene-transferred cells can most efficiently express collagen VII in the skin. We demonstrated that gene transfer using a combination of G protein of vesicular stomatitis virus-pseudotyped retroviral vector and retronectin introduced *COL7A1* into keratinocytes and fibroblasts from a DEB patient with the lack of *COL7A1* expression. Real-time polymerase chain reaction analysis of the normal human skin demonstrated that the quantity of *COL7A1* expression in the epidermis was significantly higher than that in the dermis. Subsequently, we have produced skin grafts with the gene-transferred or untreated DEB keratinocytes and fibroblasts, and have transplanted them into nude rats. Interestingly, the series of skin graft experiments showed that the gene-transferred fibroblasts supplied higher amount of collagen VII to the new dermal–epidermal junction than the gene-transferred keratinocytes. An ultrastructural study revealed that collagen VII from gene-transferred cells formed proper anchoring fibrils. These results suggest that fibroblasts may be a better gene therapy target of DEB treatment than keratinocytes.

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INTRODUCTION

Type VII collagen, a non-fibrillar collagen, is a major component of anchoring fibril loop structures beneath the epidermal basement membrane (Uitto *et al.*, 1992; Burgeson, 1993). Cloning of collagen VII cDNA demonstrated a primary sequence of 2,944 amino acids and the basic organization of the functional domains (Christiano *et al.*, 1994a). Subsequent genomic cloning has highlighted the structural organization of the collagen VII gene (*COL7A1*) (Christiano *et al.*, 1994b). This cloning information has enabled genomic DNA sequence analysis of *COL7A1* and has demonstrated that mutations within *COL7A1* are associated with the dystrophic forms of epidermolysis bullosa (DEB). DEB comprises a group of mechanobullous diseases characterized by cutaneous fragility with a tendency to form sub-basal lamina densa blisters (Christiano *et al.*, 1993; Pulkkinen and Uitto, 1999; Chen *et al.*, 2002a). In addition, targeted disruption of *COL7A1* in a mouse model demonstrated an almost identical phenotype to DEB in humans (Heinonen *et al.*, 1999). These results indicate that collagen VII is of critical importance for dermal–epidermal adhesion.

Approximately 300 distinct *COL7A1* mutations have been identified in DEB patients so far. Therapeutic introduction of *COL7A1* into skin cells is a promising treatment of DEB. Despite the relatively large size of *COL7A1*, the cDNA of which is still 9 kb makes gene transfer relatively problematic, and several methods including lentivirus- (Chen *et al.*, 2002b), retrovirus- (Baldeschi *et al.*, 2003) and ϕC31 integrase-based approaches (Ortiz-Urda *et al.*, 2002) have attempted to transfer *COL7A1* into keratinocytes. These studies used keratinocytes as target cells as collagen VII has been reported to be mainly synthesized and secreted by keratinocytes and to lesser extent by fibroblasts (Ryynanen *et al.*, 1992). However, application of gene-transferred DEB fibroblasts into the skin restored collagen VII expression in the dermal–epidermal junction (Ortiz-Urda *et al.*, 2003; Woodley *et al.*, 2003). In addition, using an intradermal injection of lentivirus with *COL7A1* induced the expression of collagen VII in fibroblasts and endothelial cells, resulting in collagen VII accumulation in the grafted DEB skin on the host animal (Woodley *et al.*, 2004).

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Abbreviations: DEB, dystrophic epidermolysis bullosa; FCS, fetal calf serum; VSV-G, G protein of vesicular stomatitis virus

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In this study, we have established a retroviral method to transfer *COL7A1* into DEB keratinocytes and fibroblasts. Next, we produced the skin grafts with gene-transferred keratinocytes or fibroblasts, and transplanted them into nude rats. Examination of collagen VII graft expression revealed that gene-transferred fibroblasts assembled more collagen VII in the form of anchoring fibrils beneath the basement membrane than gene-transferred keratinocytes. We conclude that fibroblasts are a more ideally suited target for *COL7A1* gene transfer than keratinocytes using retroviral gene therapy for the treatment of DEB.

RESULTS

Successful transfer COL7A1 using retroviral systems

We employed two retroviral vectors, pLIXN and pDON-AI, and full-length *COL7A1* cDNA was inserted into the retroviral vectors to generate plasmids termed pLI-COL and pDON-COL, respectively (Figure 1). Also, we created pDON(Δ) by removing the SV-40 promoter and *Neo* gene from pDON-AI and constructed a retroviral vector with *COL7A1* cDNA pDON(Δ)-COL (Figure 1). Several series of preliminary experiments demonstrated that retronectin (TAKARA) increased attachment of virus to keratinocytes and fibroblasts. Also, use of plasmid G protein of vesicular stomatitis virus (pVSV-G) (Pantropic System; Clontech, Palo Alto, CA) enabled concentration of viral particles by ultracentrifugation, resulting in an increase of transfer efficacy. After transfection of plasmids pLI-COL, pDON-COL, and pDON(Δ)-COL to 293 packaging cells, the culture media were collected. The virus titers (mean ± SD × 10^6/ml) of pLI-COL, pDON-COL, and pDON(Δ)-COL were 1.1 + 0.35, 1.6 + 0.46, and 2.7 + 0.55, respectively.

We transferred *COL7A1* into cultured DEB keratinocytes and fibroblasts using the retroviral system. Transfection experiments showed that retroviral methods using pLI-COL and pDON-COL failed to introduce *COL7A1* to DEB cells (data not shown). Plasmid pDON(Δ)-COL with the VSV-G system allowed a greater gene transfer after further concentration of the virus particles increased the transfer rate (Figure 2). Immunostaining revealed that the transfer rates in DEB keratinocytes and fibroblasts were almost the same (Figures 2 and 3a), and immunoblotting demonstrated that the amount of collagen VII in their culture media was also identical (Figure 3b). The average copy number per cell of the *COL7A1* cDNA was evaluated by Southern blot analysis of genomic DNA extracted from the transduced cells. The result indicated that the intensities of 7.2 kb band from the integrated cDNA were the same in treated keratinocytes and fibroblasts, suggesting that integration copies for keratinocytes and fibroblasts was almost equal (Figure 3c). The copy number was estimated 2–3 by comparing with a serial dilution standard (data not shown).

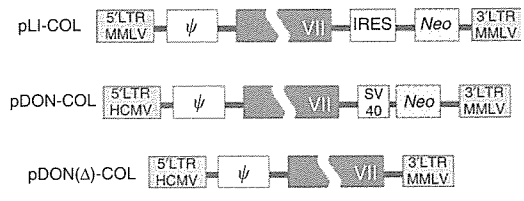

Figure 1. Schematic representation of retroviral *COL7A1* expression vectors. We employed two retroviral vectors, pLIXN (Clontech) and pDON-AI (Takara, Japan), and full-length *COL7A1* cDNA was inserted into the retroviral vectors to generate plasmids pLI-COL and pDON-COL, respectively. A pDON(Δ) vector was created by removing the SV-40 promoter and *Neo* gene from pDON-AI and *COL7A1* cDNA constructed retroviral vector made termed pDON(Δ)-COL. These vectors harbor long terminal repeat (LTR) derived from mouse moloney leukemia virus (MMLV) and human cytomegalovirus (HCMV). The internal ribosome entry site (IRES) enables expression of two unrelated reading frames from a single transcription unit. ψ: packaging signal.

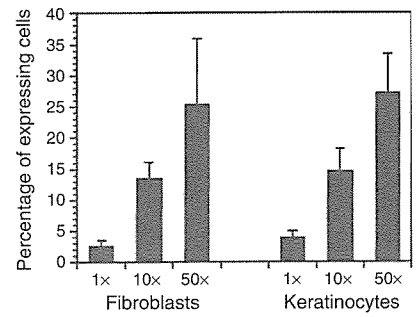

Figure 2. Successful gene transfer of *COL7A1* into DEB fibroblasts and keratinocytes using retroviral systems. *COL7A1* was transferred into DEB fibroblasts and keratinocytes using the retroviral systems. Immunostaining revealed that the transfer rates in DEB keratinocytes and fibroblasts were almost equal. The concentration of virus particles (by 10 or 50 times) using the VSV-G system improves the transfer rate. The values were represented the mean ± SD of six individual samples.

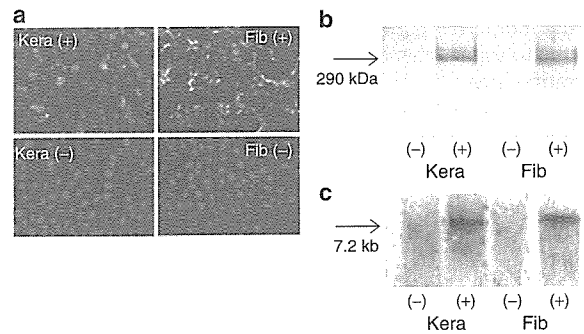

Figure 3. Corrective gene transfer of the *COL7A1* into DEB fibroblasts and keratinocytes. (**a**) Immnostaining showed that the gene-transfected DEB keratinocytes Kera (+) and fibroblasts Fib (+) expressed collagen VII, whereas no expression was found in either the untreated keratinocytes Kera (–) or fibroblasts Fib (–). Nuclei were counterstained with propidium iodide. (**b**) Western blot analysis demonstrated that the amount of transgene product in culture medium was almost the same between keratinocytes and fibroblasts. (**c**) Southern blot analysis of genomic DNA extracted from the transduced cells showed that the *COL7A1* cDNA integration copies for keratinocytes and fibroblasts were almost equal.

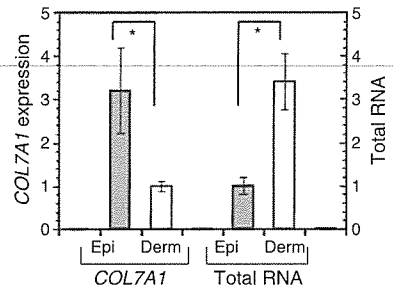

Figure 4. *In vivo COL7A1* expression in the epidermis is higher than that in the dermis. We measured *COL7A1* mRNA levels in the epidermis (Epi) and dermis (Derm) *in vivo*. Real-time PCR demonstrated that the *COL7A1*-specific signal (per RNA) of the epidermis was higher than that of the dermis. Comparison of the total RNA amounts from the epidermis and dermis in the same area of the excised normal skin showed that the amount from the dermis was higher than that from the epidermis. The *COL7A1* mRNA expression and total RNA amounts were expressed as an arbitrary scale. The values were represented the mean ± SD from three separate samples. $*P<0.01$, significant difference.

In vivo COL7A1 expression in epidermis is higher than that in dermis

To determine the *COL7A1* expression level in the epidermis and dermis *in vivo,* we separated the epidermis from the dermis, and measured *COL7A1* mRNA levels using real-time PCR. Real-time PCR demonstrated that the *COL7A1*-specific signal (per RNA) of the epidermis was higher than that of the dermis by 3.2-fold (Figure 4). Comparison of the total RNA amounts from the epidermis and dermis in the same area of the excised normal skin showed that the amount from the dermis was higher than that from the epidermis by 3.4-fold (Figure 4). Thus, the quantity of *COL7A1* expression in epidermis was significantly higher than that in dermis *in vivo*.

Gene-transferred fibroblasts can supply more collagen VII to the basement membrane zone than gene-transferred keratinocytes

We transplanted the gene-transferred DEB keratinocytes and fibroblasts into the wound of nude rats, and then observed *COL7A1* deposition 3, 6, and 9 weeks after transplantation. In the skin graft with gene-transferred keratinocytes and untreated fibroblasts, the *COL7A1* deposition was detectable in the basement membrane zone at 3 week and maintained this expression at least until 9 weeks (Figure 5). However, we found a greater accumulation of collagen VII in dermal–epidermal junction of the grafts using untreated keratinocytes and gene-transferred fibroblasts 3 weeks after transplantation. Furthermore, DEB fibroblasts transfected with *COL7A1* demonstrated more dermal–epidermal junction collagen VII staining than *COL7A1*-transfected DEB keratinocytes/untreated fibroblast (Figure 5) from 6 to 9 weeks. The grafts of DEB keratinocytes and fibroblasts as controls demonstrated no deposition (Figure 5). Semiquantification of *COL7A1* deposition in basement membrane zone in each point showed DEB fibroblasts with COL7A1 can supply higher amount of collagen VII to the basement membrane zone than DEB keratinocytes with *COL7A1* (Figure 6). Significant differences were found between the keratinocytes and fibroblasts samples at 3W and 9W points.

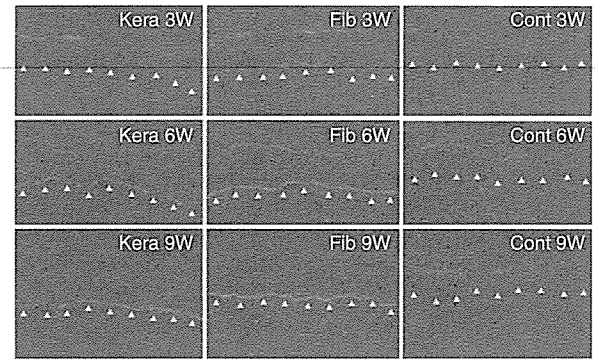

Figure 5. Gene-transferred fibroblasts can supply more collagen VII to the sub-basement membrane zone than gene-transferred keratinocytes. We transplanted gene-transferred DEB keratinocytes and DEB fibroblasts to nude rats wounded back skin, and observed *COL7A1* deposition by immunohistochemistry at 3, 6, and 9 weeks after transplantation. The skin graft with gene-transferred keratinocytes and untreated fibroblasts (Kera 3W, Kera 6W, Kera 9W) started dermal–epidermal junction collagen VII deposition at 3 week and maintained it until 9 weeks. A greater accumulation of collagen VII in dermal–epidermal junction of the grafts using untreated keratinocytes and gene-transferred fibroblasts was found 3 weeks after transplantation (Fib 3W). The DEB fibroblasts transfected with *COL7A1* demonstrated more dermal–epidermal junction collagen VII staining than *COL7A1*-transfected DEB keratinocytes/untreated fibroblast from 6 to 9 weeks (Fib 6W, Fib 9W). The control DEB keratinocyte and fibroblast cell grafts (Cont 3W, Cont 6W, Cont 9W) demonstrated no deposition. Arrowheads define the limit between the dermis and epidermis.

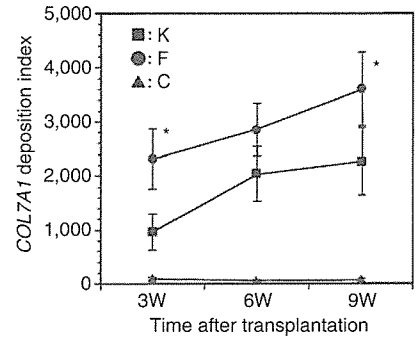

Figure 6. Semiquantification of *COL7A1* deposition in basement membrane zone. We transplanted gene-transferred DEB keratinocytes and DEB fibroblasts to nude rats wounded back skin, and observed *COL7A1* deposition by immunohistochemistry at 3, 6, and 9 weeks after transplantation. To semiquantify *COL7A1* deposition in basement membrane zone, we measured fluorescence intensity (arbitrary scale) in basement membrane zone at 10 areas at each point and the *COL7A1* deposition index was expressed as the mean ± SD from the 10 values. K: the skin graft with gene-transferred keratinocytes and untreated fibroblasts; F: the graft with untreated keratinocytes and gene-transferred fibroblasts grafts; C: the graft with untreated keratinocyte and untreated fibroblasts. $*P<0.01$, significant difference.

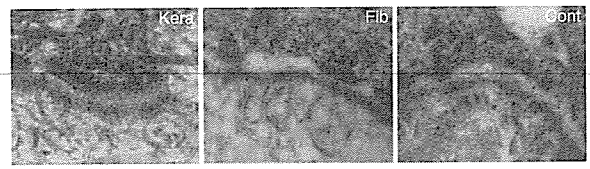

Figure 7. Collagen VII released from gene-transferred cells forms anchoring fibrils similar to normal skin. We examined the ultrastructural formation of anchoring fibrils in the grafts. The grafts with the gene-transferred keratinocytes (Kera) or the gene-transferred fibroblasts (Fib) demonstrated cross-banded, filamentous structures sometimes forming semicircular loops immediately beneath the lamina densa, corresponding to anchoring fibrils, whereas we failed to identify these filamentous structures in control without *COL7A1* transfection (Cont).

Collagen VII released from gene-transferred cells forms ultrastructurally normal anchoring fibrils

We examined the ultrastructural formation of anchoring fibrils in the graft. The grafts with both gene-transferred keratinocytes and fibroblasts demonstrated filamentous loop structures just beneath the lamina densa, which were corresponding to anchoring fibrils (Figure 7). We could not see any filamentous structures in control (untransfected) samples without *COL7A1*.

DISCUSSION

The developments in cloning the basement membrane protein genes have allowed the identification of the causative genes/proteins harboring the mutations responsible for this group of epidermolysis bullosa diseases (Uitto and Pulkkinen, 2001). We can now make good estimations about the prognosis and severity of these diseases with profound beneficial effects on genetic counseling and DNA-based prenatal diagnosis. However, patients most frequently desire an appropriate therapy for epidermolysis bullosa. Corrective transfer of the *COL7A1* gene back into the skin cells is a promising treatment of DEB.

The previous Northern hybridization study revealed a high level of *COL7A1* mRNA expression in cultured epidermal keratinocytes, whereas the expression was lower in cultured dermal fibroblasts (Ryynanen *et al.*, 1992). These results indicate that epidermal keratinocytes and dermal fibroblasts express the collagen VII, but also suggest that epidermal keratinocytes are the primary source of collagen VII in developing human skin. Many investigators have utilized keratinocytes as the target cells of DEB gene therapy. Several methods including viral- (Ghazizadeh and Taichman, 2000) and non-viral-mediated transduction (Vogel, 2000) have been reported for *in vivo* and *ex vivo* gene transfer into keratinocytes. *COL7A1* cDNA was recently transferred into cultured DEB keratinocytes using some methods including lentivirus- (Chen *et al.*, 2002b), retrovirus- (Baldeschi *et al.*, 2003) and ϕC31 integrase-based approaches (Ortiz-Urda *et al.*, 2002). The corrected DEB keratinocytes expressed the recombinant collagen VII and restored the *in vivo* synthesis of anchoring fibrils after implantation, demonstrating the feasibility of gene transfer using DEB keratinocytes. We also succeeded in transferring the *COL7A1* into *in vivo* keratinocytes using the naked DNA method (Sawamura *et al.*, 2002) although the *COL7A1* transfer efficacy was lower than the above *ex vivo* method.

Many gene therapy protocols have already utilized retroviral vectors for clinical practices. In this study, we also showed a retroviral vector could transfer the 9 kb *COL7A1* cDNA into DEB keratinocytes. Another group has succeeded in transducing *COL7A1* gene to keratinocytes using a retroviral plasmid containing the Neo selection gene (Baldeschi *et al.*, 2003). They showed that transduction efficacies to primary keratinocytes were 40 and 83–93% by retroviral vectors pLSRS-Ires-zero and pMSCV, respectively. However, the use of similar plasmids is not possible to efficiently introduce this gene into keratinocytes in our experiments. In our system, the efficacy was about 30%, which was lower than those in previous report. This study, as far as we know, has been the first to try a retronectin retroviral targeting system for keratinocytes. Retronectin is a recombinant peptide, which consists of three functional fibronectin domains and significantly enhances retrovirus-mediated gene transduction into mammalian cells. Our data showed that addition of retronectin increased transfer efficacy in keratinocytes by 3-fold (data not shown), indicating that retronectin is indeed efficient in this keratinocyte/retroviral system.

Some groups have succeeded in transferring this gene into keratinocytes as mentioned above. On the other hand, cutaneous injection of the DEB fibroblasts transduced using ϕC31 integrase-based approach also restores collagen VII deposition along the dermal–epidermal junction (Ortiz-Urda *et al.*, 2003). Also, gene-corrected DEB fibroblasts and normal human fibroblasts alone could supply type VII collagen deposition at the basement membrane zone *in vivo* (Woodley *et al.* 2003), and this implies a possibility that normal cultured human dermal fibroblasts are injected intradermally into recesive dystrophic epidermolysis bullosa patients' skin. Moreover, intradermal injection of lentiviral vector with *COL7A1* increased collagen VII expression in fibroblasts and endothelial cells, resulting in stronger deposition of collagen VII along the basement membrane zone anchoring fibrils as seen by electron microscopy (Woodley *et al.*, 2004). This study also introduced the *COL7A1* gene into DEB fibroblasts using the retroviral method and the consequent collagen VII assembly beneath the basement membrane of the fibroblast containing graft.

In this study, we compared dermal fibroblasts and epidermal keratinocytes as efficient target recipient cells for the collagen VII transgene product. After retroviral introduction of *COL7A1*, the transfer efficacy and the amount of collagen VII in the cultured keratinocytes media supernatant are almost the same as those of fibroblasts. Interestingly, a series of skin graft experiments first demonstrated that gene-transfected fibroblasts more efficiently assembled collagen VII into the dermal–epidermal junction than the gene-transferred keratinocytes. Previous Northern blotting analysis revealed higher level of *COL7A1* mRNA expression in cultured epidermal keratinocytes than fibroblasts (Ryynanen

et al., 1992). This study utilized real-time PCR technique and confirmed that the epidermis produced much more collagen VII than the dermis *in vivo*. If gene-transferred fibroblasts and keratinocytes express similar amounts of type VII collagen also *in vivo*, the fibroblasts may have a better ability to supply type VII collagen to the basement membrane than the keratinocytes.

It is evident that expression of recombinant collagen VII is driven by heterologous promoters, which escape the regulatory mechanisms that govern the expression of endogenous collagen VII in the different cell types. Also, keratinocytes have been preferred because of the possibility they offer of targeting stem cells and compared to keratinocytes, fibroblasts rapidly senesce *in vivo* (Krueger, 2000). However, fibroblasts are more robust and less susceptible to growth arrest and differentiation than epidermal keratinocytes (Ortiz-Urda *et al.*, 2003). Furthermore, genetically engineered fibroblasts have had their use explored for therapeutic applications including visceral and cutaneous implantation to supply gene products to circulation (Roth *et al.*, 2001). Given the above combined factors, it is proposed that fibroblasts may be potentially more feasible and a better target of DEB gene therapy than keratinocytes.

MATERIALS AND METHODS

Cell culture

Primary keratinocytes were isolated and grown in the presence of an irradiated 3T3 feeder layer (Rheinwald and Green, 1975). Briefly, keratinocytes, which were obtained from skin biopsy of a DEB patient and healthy controls, were cultured on feeder layers of mitomycin C-treated mouse 3T3 fibroblasts in DMEM: Ham's F-12 (3:1) supplemented with 10% fetal calf serum (FCS), 5 μg/ml insulin, 10 ng/ml epidermal growth factor, 0.4 μg/ml hydrocortisone, and 8 ng/ml cholera toxin. The DEB patient was diagnosed as the most severe subtype, and Hallopeau–Siemens type showed no *COL7A1* expression in the skin and harbored heterozygous premature stop codon mutations 1474del8 and 5818delC. Fibroblasts were also obtained from skin biopsy from the DEB patient and healthy controls, and were cultured in DMEM with 10% FCS. Packaging cells amphopack-293 and GP2-293 (Clontech, Palo Alto, CA) were maintained in DMEM with 10% FCS, 2 mM glutamine, and 2 mM sodium pyruvate.

Informed consents were obtained from all individual subjects in this study. The protocols were approved by the Ethical Committee at Hokkaido University Graduate School of Medicine. This study was conducted according to the Declaration of Helsinki Principles.

Intrinsic expression of collagen VII in control keratinocytes and fibroblasts

Human skin was obtained from normal volunteers, and treated with 10 mg/ml dispase for 3 hours at 37°C to separate the epidermis from the dermis. The epidermal and dermal sheets were minced and total RNA was extracted using an RNeasy RNA extraction kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized with reverse transcriptase (Life Sciences Inc., St Petersburg, FL) using an oligo-dT primer. Assays-on-Demand™ Products for *COL7A1* and *GAPDH* were purchased from Applied Biosystems (Foster City, CA). The 50 μl reaction in each well contained 1 μl of total cDNA, 300 nM sequence-specific primers, and 200 nM dual-labeled fluorogenic probe with 1 U of Taqman Universal PCR master mix (Applied Biosystems). A negative control PCR without template and a positive PCR control with a template of known amplification were included in each assay. The samples underwent the following stages: stage 1, 50°C for 2 minutes; stage 2, 95°C for 10 minutes; and stage 3, 95°C for 15 seconds, followed by 60°C for 1 minutes. Stage 3 was repeated 45 times. Gene-specific products were measured by means of an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA) continuously for 45 cycles. The *COL7A1*-specific signal was normalized by constitutively expressed *GAPDH* and expressed as arbitrary scale.

Construction of retroviral COL7A1 expression vectors and transfection

Human full-length *COL7A1* cDNA was constructed from several overlapping cDNA clones (Sawamura *et al.*, 2002). We employed two retroviral vectors, pLIXN (Clontech) and pDON-AI (Takara, Otsu, Japan), and full-length *COL7A1* cDNA was inserted into the retroviral vectors to generate plasmids termed pLI-COL and pDON-COL, respectively (Figure 1). Also, we created pDON(Δ) by removing the SV-40 promoter and *Neo* gene from pDON-AI and constructed a retroviral vector with *COL7A1* cDNA pDON(Δ)-COL (Figure 1). The recombinant retroviruses were produced by transfecting the retroviral plasmids into the amphotropic amphopack-293 packaging cells (Clontech) using calcium-phosphate coprecipitation. In addition, we tried VSV-G-pseudotyped retrovirus vectors. The retroviral plasmids and plasmid pVSV-G were cotransfected into pantropic GP2-293 packaging cells (Clontech). The viral particles were recovered from the cell culture medium 48 hours later and applied to keratinocyte or fibroblast cultures. To increase transfer efficacy, ultracentrifugation was performed to concentrate the VSV-G virus particles. The titer of the viral supernatant was determined by real-time quantitative PCR (Towers *et al.*, 1999).

Cells infection with retrovirus

Keratinocytes and fibroblasts were cultured to up to 60% of confluency and then infected with the viral suspensions in 5 μg/ml polybrene. To increase the virus-cell interactions, we coated the surface of the culture plates with 10 ng/ml retronectin (Takara; fibronectin fragment CH-296). After incubation for 24 hours at 32°C, we maintained the treated cells under fresh medium for another 24 hours until the transduction efficiency was assessed by immunofluorescence examination of the infected cells.

Immunostaining and immunoblot

Transfected cultured keratinocytes and fibroblasts were fixed with 2% parafromaldehye in phosphate-buffered saline, and were then incubated with the monoclonal antibody LH7.2 (1:100) against the NC1 domain of collagen VII (Chemicon, Temecula, CA) for 18 hours at 4°C. They were treated with secondary goat anti-mouse IgG antibodies conjugated with FITC (1:50) for 1 hour at 37°C, and preparations were examined under a fluorescence microscope. Nuclei were counterstained with propidium iodide (Dojindo Laboratories, Kumamoto, Japan). Subconfluent cell cultures were fed for 48 hours with serum-free medium supplemented with 50 μg/ml ascorbic acid. For SDS-PAGE analysis, the culture medium was

treated with Amicon Ultra-100,000 Centrifugal Filter Devices (Millipore, Bedford, MA) for concentration and desalting. The samples were separated on a 5% polyacrylamide gel under reducing conditions. Immunoblotting analysis was performed by treating with the LH7.2 monoclonal antibody (1:1,000) for 18 hours at 4°C, and then secondary goat anti-mouse IgG antibodies conjugated with peroxidase (1:2,000) for 1 hour at 37°C. The resultant complexes were processed for Phototope HRP Western Blot Detection System (Cell Signaling, Beverly, MA) according to the manufacturer's protocol.

Southern blot analysis

The average copy number per cell of the *COL7A1* cDNA was evaluated by Southern blot analysis (Baldeschi *et al.*, 2003). Briefly, genomic DNA was extracted from subconfluent cell cultures and digested with *Bgl*II and *Hind*III. Plasmid pDON(Δ)-COL was serially diluted with yeast genomic DNA at the final concentration ranging from 0.5 to 20 copies/cell. The digested DNA was electrophoresed on a 0.8% agarose gel and transferred to Zeta-Probe membrane (Bio-Rad, Hercules, CA). The 375 bp cDNA extending from exons 58 to 64 was generated by PCR amplification of *COL7A1* cDNA as a template. This fragment was designed to recognize a 7.2 kb band from the integrated cDNA, and also 1.0 and 0.3 kb bands from the intrinsic *COL7A1* gene. The membranes were hybridized with the 375 bp cDNA probe was labeled by random primed incorporation of digoxigenin-labeled 2′-deoxyuridine 5′-triphosphate using the DIG DNA Labeling Kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. After high stringency washes, blots were visualized using an enhanced chemiluminescence system.

Grafting of gene-transferred DEB cells

Gene-transferred and untreated DEB keratinocytes and DEB fibroblasts were cultured using the above methods. Fibroblasts (10^6) were seeded into a collagen sponge scaffold and maintained in DMEM with 10% FCS for 3 days. In nude rats (F344/N Jcl-rnu), the sites for transplantation were prepared by excising a 2 cm^2 area of dorsal epidermis and dermis, and then the collagen sponge (3 cm^2) containing the fibroblasts was placed into the skin wound. The confluent cultures of 10^6 keratinocytes were treated with dispase (1 nU/ml; Godo Shusei, Tokyo, Japan), and the floating epidermal sheet placed on the collagen sponge. Preliminary experiment showed that the number of fibroblasts was almost equal to that of keratinocytes when we applied the graft to the animal. An occlusive dressing was quickly placed over the graft to hold it in position and to prevent it from drying out and then the dressing was removed after 7 days. We prepared combinations of gene-transferred keratinocytes and untreated fibroblasts, of untreated keratinocytes and gene-transferred fibroblasts, and of untreated keratinocytes and fibroblasts as control. Skin biopsies were taken from the grafted skin at various time points and subjected to routine immunohistochemical staining using the LH7.2 monoclonal antibody and ultrastructural analysis. To semiquantify *COL7A1* accumulation in basement membrane zone, we converted color images to gray-scale images, and measured fluorescence intensity (arbitrary scale) in basement membrane zone at 10 areas using NIH Image software. The *COL7A1* deposition index was expressed as the mean±SD from the 10 values.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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confluency, and focal pagetoid scatter within the epidermis, resulting in possible diagnostic confusion with melanoma.[5] Even though superimposed LS makes interpretation more challenging, our patient's lesions demonstrated histologic features most in keeping with melanoma, including asymmetry, poor lateral circumscription, junctional confluency resulting in early pseudobullae, prominent pagetoid scatter, aberrant dermal growth, and dermal mitoses.[5] Hassanein et al[3] and Carlson et al[4] provided a hypothesis for the malignant transformation of melanoma in LS as a relational pattern of host immune response to melanoma, and local immune dysregulation in LS. We are unaware of any inherited association between melanoma and LS, and potential genetic contributions to LS have not yet been elucidated.

We report a case of vulvar melanoma with lymph node metastasis in a background of LS occurring in a child who is now alive 32 months after operation. The association of these two conditions requires further investigation. This case illustrates the need for awareness that melanoma, including vulvar melanoma, can and does occur in children.

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Multiple apocrine hidrocystoma showing plane pigmented macules

To the Editor: A 67-year-old Chinese man was referred to our hospital with pigmented asymptomatic eruptions on the face, which was thought to be suggestive of basal cell carcinoma at a previous clinic (Fig 1). He first noticed a small, pigmented lesion in his right nasal side wall 20 years ago. Afterward, similar pigmented macules appeared on the right wing of his nose. Physical examination revealed 4 slightly blackish pigmented macules on the right wing of his nose sized 7 × 6, 5 × 4, 3 × 3, and 2 × 2 mm.

Clinically, the lesions were thought to be basal cell carcinoma, melanocytic nevus, trichoblastic carcinoma, hemangioma, or apocrine hidrocystoma, and a punch biopsy was performed on a characteristic lesion. The biopsy specimen revealed several cystic structures in the papillary dermis. The cavities were lined by cuboidal apocrine secretory cells within flattened myoepithelial cells with some intraluminal papillary projections (Fig 2). Cellular atypia was not seen. From these findings, the diagnosis of multiple apocrine hidrocystoma was finally made. There was not enlarged superficial abnormal sebaceous gland implicating pre-existing nevus sebaceous. Because the patient declined surgical intervention, we performed carbon-dioxide laser therapy. Laser treatment decreased the pigmentation a little, but the patient did not wish to continue treatement. We have observed the patient carefully for a year, and these tumors have shown no remarkable change.

Apocrine hidrocystoma is benign cystic tumor that represents an adenomatous cystic proliferation of apocrine glands. It most commonly occurs as a solitary, dome-shaped, translucent papule around the eye.[1] Puncture of a lesion may result in extravasation of a straw-colored fluid. Although clinically atypical apocrine hidrocystoma showing facial nodules or giant solitary pigmented tumors has been reported, there has been no report of multiple apocrine hidrocystoma appearing as pigmented macules.[2-4] The current case is unique in that the

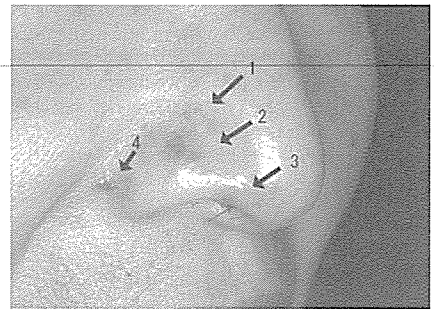

Fig 1. Clinical features: multiple blackish pigmented macules (*arrows 1-4*) on right side of nose.

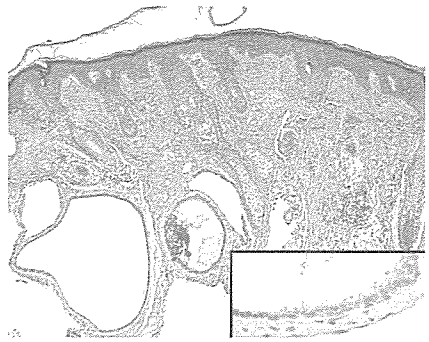

Fig 2. Histopathologic features: several cystic structures in papillary dermis. *Inset*, Cavities were lined by cuboidal apocrine secretory cells within flattened myoepithelial cells with some intraluminal papillary projections. (Hematoxylin-eosin stain; original magnifications: ×10; *inset*, ×100.)

tumors comprised multiple, plane, and nontranslucent macules. The diagnosis of this multiple apocrine hidrocystoma could be made after histopathologic observations. This case further suggests the marked clinical variation of apocrine hidrocystoma mimicking basal cell carcinoma.

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Penile condylomata? Traumatic neuromas!

To the Editor: Traumatic neuroma is a reactive process that leads to regeneration of an injured nerve forming a haphazard nodular proliferation of small nerve bundles. It is usually related to previous operation or trauma.[1] We describe a case of multiple traumatic neuromas clinically suggested to be condylomata acuminata.

A 29-year-old man from Liberia, Africa, had several painless, small papules on his penis, unrelated to local trauma. He claimed no sexual activity with partners during the last 2 years. Physical examination revealed few comedones on an uncircumcised penis and on scrotal skin and several grouped, firm, whitish, translucent papules, 1 to 2 mm in diameter, on the prepuce (Fig 1). A clinical diagnosis of condylomata acuminata was made, and a papule was excised with a 4-mm punch biopsy. Histologic examination of the hematoxylin and eosin—stained slide showed an epidermis with no sign of viral infection. A haphazard proliferation of nerve fibers organized in small fascicles supported by a fibrous stroma was present in the papillary dermis. It appeared to originate from a single nerve located in the reticular dermis (Fig 2). The histologic diagnosis was traumatic neuroma.

Clinical differential diagnosis should also include pearly penile papules,[2] but pearly papules are characterized by plump or stellate fibroblasts embedded in a vascularized connective stroma.

Two cases of traumatic neuroma in genital skin have been reported in association with local trauma.[3,4] Moreover, 3 cases, unrelated to trauma have been described to date.[5] In these latter cases, the authors described concurrent presence of multiple Meissner's corpuscles.[5] The pathogenetic mechanism of these lesions, given their acquired nature and also, in our case, their multiplicity, appears to be traumatic. On the other hand, the previous 3 reported cases were in young, sexually active men, and the authors hypothesized a correlation between the tumor and local microtraumas.[5]

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CASE REPORT

Successful treatment of severe recalcitrant erosive oral lichen planus with topical tacrolimus

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Keywords
oral lichen planus, tacrolimus, topical treatment

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Abstract

Oral lichen planus (LP) is a severe, painful form of LP, and is often resistant to topical corticosteroid therapy. Recently, open trials demonstrated that topical tacrolimus therapy was effective for the treatment of chronic erosive oral LP. We report two cases with severe recalcitrant erosive oral LP, who dramatically benefited from topical tacrolimus therapy. In case 1, a 64-year-old man presented with a 5-month history of painful erosions on his entire lower lip and buccal mucosa. Physical and histological examination confirmed a diagnosis of LP. He experienced rapid relief from pain and a dramatic improvement was obtained within 5 weeks of topical tacrolimus treatment. No significant irritation was observed and blood tacrolimus level was kept within a safe level (2.5 ng/mL). In case 2, a 68-year-old man developed painful erosions on his right lower lip and buccal mucosa 2 months before his arrival at our hospital. Histopathological analysis confirmed a diagnosis of oral LP. He experienced a rapid dramatic improvement of both lesions within 4 weeks of the start of tacrolimus application. No significant irritation or recurrence was observed. Thus, topical tacrolimus is suggested as a well-tolerated, effective therapy for oral LP.

Introduction

Oral lichen planus (LP) is a severe and painful form of LP, and is often resistant to topical corticosteroid therapy. Recently, some open trials demonstrated that topical tacrolimus therapy, which has been approved as a safe treatment for atopic dermatitis,[1,2] was effective for chronic erosive oral LP.[3,4] Here, we report two patients with severe recalcitrant erosive oral LP, who dramatically benefited from topical tacrolimus therapy.

Case 1

A 64-year-old man presented at our outpatient clinic with a 5-month history of painful erosions on his entire lower lip and buccal mucosa. Physical examination revealed irregular-shaped, blood-encrusted erosions with peripheral white streaks on his lower lip and buccal mucosa(fig. 1a). A biopsy specimen taken from his lower lip confirmed the diagnosis of oral LP (fig. 2a). Because he had been unsuccessfully treated with topical corticosteroids at several dermatological facilities, treatment with 0.1% topical tacrolimus, twice a day, was initiated. He experienced rapid relief from pain within a week of treatment. After 5 weeks of treatment, a dramatic improvement had been obtained on both his lips and buccal mucosa (fig. 1b). No significant irritation or recurrence was observed on continuous application during the 6-month follow-up period, and his blood tacrolimus level was kept within a safe level (2.5 ng/mL).

Case 2

A 68-year-old man developed a painful erosion on his lower right lip 2 months before his first arrival at our outpatient clinic. Physical examination revealed a 2.0 × 1.0 cm blood-encrusted erosion with peripheral white streaks on his right lower lip (fig. 1c). Race-form white streaks and irregular-shaped erosions were also present on his buccal mucosa. Histopathological analysis

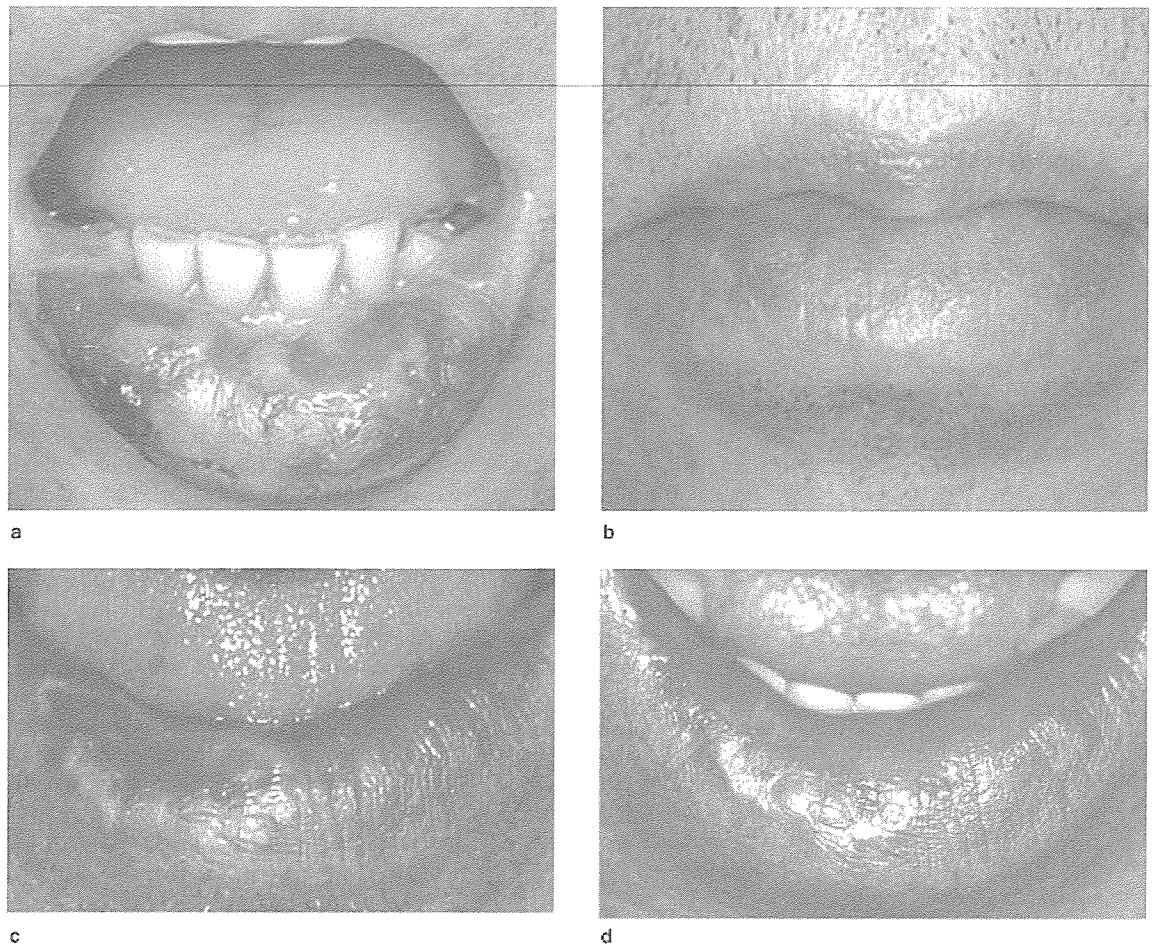

fig. 1 Clinical appearances of case 1 (a, b) and case 2 (c, d). (a) Irregular-shaped, blood-encrusted erosions with peripheral white streaks were observed on the entire lower lip. (b) After 5 weeks of treatment with topical tacrolimus, a dramatic improvement was observed. (c) A single 2.0 × 1.0 cm, blood-encrusted erosion with peripheral white streaks was present on the lower right lip. (d) A dramatic improvement was observed after 4 weeks of treatment with topical tacrolimus.

of a biopsy specimen taken from his lower lip confirmed a diagnosis of oral LP (fig. 2b). Because of his poor response to previous topical corticosteroid therapy, treatment with 0.1% topical tacrolimus, twice a day, was initiated. He experienced a rapid and dramatic improvement of both lesions within 4 weeks of treatment (fig. 1d). No significant irritation or recurrence was observed on continuous topical application of tacrolimus during the 6-month follow-up period.

Discussion

In contrast to cutaneous lesions, oral LP is often resistant to topical corticosteroid therapy.[5] Tacrolimus has powerful immunosuppressive activities by inhibiting T-cell production of proinflammatory cytokines.[6,7] Although the aetiology of LP is still unknown, cell-mediated immune reactions involving T lymphocytes are thought to play a central role. This may be one reason why topical application of tacrolimus could induce a rapid improvement in oral LP.[4] Although a minority of patients experience side-effects, such as a burning sensation and sore throat, these side-effects were not troublesome enough to discontinue topical tacrolimus application.[3] In our cases, no irritation was observed even after application onto the severely eroded lesions. There is also a concern that blood tacrolimus levels could increase during the treatment of these erosive lesions. According to previous reports, blood tacrolimus levels are not significantly different whether tacrolimus is used for oral LP or atopic dermatitis.[1,2,8] In contrast to the

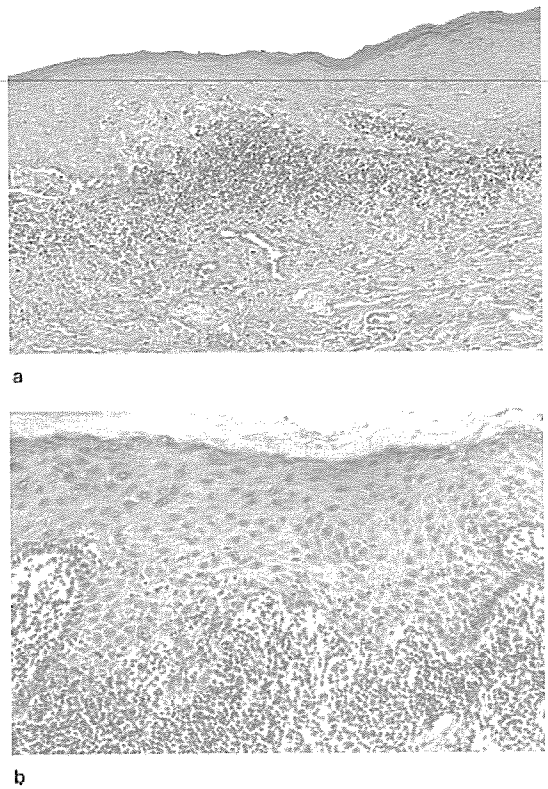

fig. 2 (a) Biopsy specimen of case 1 showing band-like infiltration of lymphocytes at the dermal–epidermal interface accompanied by vacuolar alteration of the basal keratinocyte layer and the presence of scattered necrotic keratinocytes. (b) Biopsy specimen of case 2 showing irregular acanthosis and focal hypergranulosis of the mucous epithelia accompanied by vacuolar alterations in the basal keratinocyte layer and a band-like infiltration of lymphocytes.

report by Olivier *et al.*,[4] the blood tacrolimus level in case 1 was minimally elevated (2.5 ng/mL). However, the tacrolimus level was still maintained within safe levels,[9,10] and was less than the recommended therapeutic range (5.0–20.0 ng/mL). Therefore, direct local effects may be the major contributing factors providing the therapeutic benefit observed in our case, although some systemic effects may partially have affected the clinical outcome.

Although flare-ups are commonly observed after discontinuing topical tacrolimus application,[3,4] and long-term side-effects still remain unknown, topical tacrolimus is suggested to be a well-tolerated, effective therapy for oral LP.

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Presence of Circulating CCR10+ T cells and Elevated Serum CTACK/CCL27 in the Early Stage of Mycosis Fungoides

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Abstract

Purpose: Mycosis fungoides (MF), a common type of cutaneous T cell lymphoma with an indolent clinical course, has the characteristic that malignant T cell clones are recruited into the skin from the early disease stages. The mechanisms of recruitment have been suggested from our knowledge of various chemokine-chemokine receptor interactions. Recently, CCR10 and CTACK/CCL27 were proposed to play a role in the recruitment of other types of cutaneous T cell lymphoma. We examined the expression of CCR10 in peripheral blood and serum CTACK/CCL27 levels in patients with MF.

Experimental Design: Eighteen patients with MF, six patients with atopic dermatitis, and nine healthy volunteers were enrolled in our investigation. We investigated the differences in CCR10+ CD4+ expression in peripheral blood mononuclear cells by flow cytometry. Serum CTACK/CCL27 levels were determined using a CTACK/CCL27 ELISA assay kit.

Results: The number of circulating CCR10+ CD4+ cells was significantly higher in MF peripheral blood than in controls, even during the early stages. In lesional MF skin, infiltrating tumor cells also showed extensive expression of CCR10. The serum level of CTACK/CCL27 was higher in patients with MF than normal controls, but no statistical difference was found compared with atopic dermatitis patients.

Conclusions: CCR10-CTACK/CCL27 interactions between circulating T cells and keratinocytes would seem to play an important role in the pathophysiology of MF from the early disease stages.

Mycosis fungoides (MF) is the most common cutaneous T cell lymphoma (CTCL), with an estimated incidence of 0.5 cases per 100,000 population per year in the western world (1). MF has a classically slow clinical course that progresses over years through the patch, plaque, and tumor stages, followed by lymph nodes and visceral involvement (2). In MF, although malignant T cells persist mainly in skin and only few cells circulate in peripheral blood, recent studies have revealed an aberrant T cell immunophenotype and circulating clonal cutaneous lymphocyte antigen (CLA)-positive T cells in patients' blood (3–5). It was also shown that CLA+ CD4+ T cells express the CC chemokine receptor 4 (CCR4), which is suspected of playing a role in skin-homing. CCR4 was originally discovered in memory T cells (6, 7). Furthermore, Sokolowska-Wojdylo et al. reported that another CC chemokine receptor associated with skin-homing, CCR10, is expressed in circulating clonal CLA+ CD4+ cells in Sézary syndrome (8). Another study showed that malignant T cells expressing CCR10 were infiltrated in skin tissues in Sézary syndrome, MF, and unspecified CTCL (9).

Cutaneous T cell attracting chemokine (CTACK/CCL27) is a skin-associated chemokine that attracts skin-homing memory T cells (10). CTACK/CCL27 is known to be the ligand for CCR10, and is mainly produced by activated keratinocytes in various diseases such as atopic dermatitis, psoriasis, drug reactions, and other inflammatory conditions (11, 12). CTACK/CCL27 is also expressed by dermal components and by the microvasculature, playing an important role in recruiting T cells into skin (13).

We hypothesized that CCR10-CTACK/CCL27 interactions play an early role in the pathophysiology of MF from the patch stage. In this report, we have examined the expression of CCR10 in CD4+ cells circulating in patients without apparent MF peripheral blood involvement. In addition, we have determined the concentration of CTACK/CCL27 protein contained in MF patients' serum.

Materials and Methods

Materials. The following monoclonal antibody was used in this study: CD4 was from BD Bioscience (San Jose, CA), CCR10 from Immuno Detect, Inc. (Fayetteville, NY), CTACK/CCL27 from R&D systems (Minneapolis, MN). DMEM was purchased from Invitrogen (Groningen, Netherlands), collagenase from Wako Pure Chemical (Osaka, Japan), Ficoll from Amersham Biosciences Corp. (Piscataway, NJ), human CTACK/CCL27 ELISA assay kit was from R&D Systems. All other chemicals were of reagent grade or higher.

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