these diseases, whereas serum granulysin in DIHS might be released against virus-infected cells. This speculation is consistent with the present data that show the duration of DIHS manifestation to coincide with the timing of elevated serum granulysin levels. Recently we developed a rapid immunochromatographic test to detect high serum granulysin level in 15 minutes[6]. We expect that monitoring of serum granulysin by the rapid test might contribute to the early diagnosis of DIHS as well as of SJS/TEN. In conclusion, serum granulysin might help early diagnosis and predict disease prognosis.

Figure Legend

Fig 1. Granulysin levels of healthy controls, and of ODSRs patients and DIHS patients at different stages of the diseases.

In DIHS patients, we examined the concentration of granulysin for three terms: day 1 to 10, day 11 to 20, and after day 21. The granulysin level was elevated from day 1 to 20, compared to those for ODSRs and normal controls (*:$p<0.01$).

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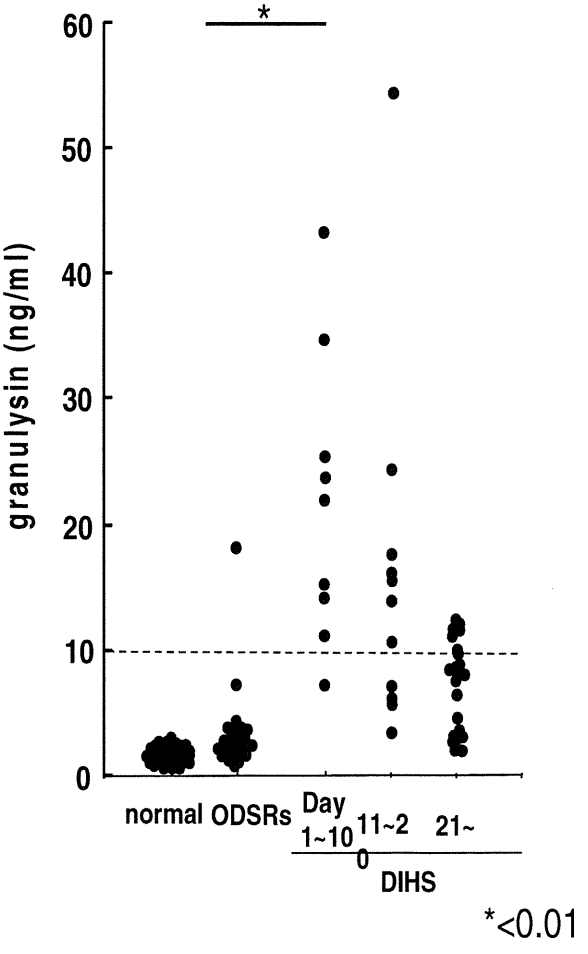
Fig 1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Eur J Dermatol 2012 (epub ahead of print)

CORRESPONDENCE

Intraepidermal neutrophilic IgA pemphigus successfully treated with dapsone

A 25-year-old woman presented with a 2-month history of erythematous, intensely itchy macules and vesicles on the extremities and trunk. Before onset, she was in good health and took no medication. Physical examination revealed pinkish or reddish, edematous, well-demarcated erythema *(figure 1A)*. The lesions tended to coalesce, forming annular patterns, some of which had vesicles around the margins, forming a sunflower-like configuration. The oral cavity and genital area were unaffected. Histopathological findings of a pustule revealed intraepidermal blisters with neutrophil infiltrates without prominent acantholysis *(figure 1B)*. Laboratory examinations, including serum immunoglobulins, and ELISA for anti-desmoglein 1 and 3 were within normal ranges. Chest X-ray, electrocardiogram, and blood tests revealed no other related diseases and monoclonal gammopathy. DIF of the erythematous lesion revealed IgA deposition in the intercellular space throughout the epidermis *(figure 1C)*. IIF revealed circulating IgA autoantibodies binding to the cell surfaces of the entire epidermis of normal human skin (titer: 64×). Immunoblot analysis using epidermal extracts from normal human skin and recombinant desmocollin 3 showed no specific bands for either IgA or IgG antibodies. These findings led to the diagnosis of IEN-type IgA pemphigus. Treatment was initiated with topical corticosteroids, achieving only a slight effect; dapsone (50 mg per day) was therefore started. The pruritus and lesions improved but the symptoms recurred after four weeks. For that reason the dose was raised to 75 mg dapsone and the itchness subsided within a few days. Two weeks later, only pigmented macules with no active lesions were observed. The titer of IIF also decreased from 64× to 16×.

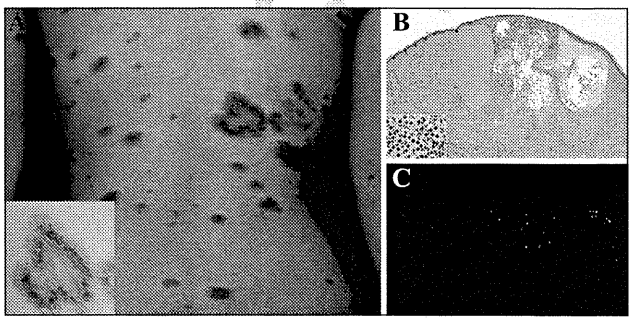

Figure 1. A) Pinkish and reddish edematous erythema with vesicles around the margins are scattered on the trunk. **B)** Histopathological findings of a pustule reveal intraepidermal blisters with neutrophil infiltrates. **C)** Direct immunofluorescence of the perilesional skin biopsy specimen reveals IgA deposits on the keratinocyte cell surfaces.

IgA pemphigus is a distinct group of auto-immune intraepidermal blistering diseases that present with vesiculopustular eruption, neutrophil infiltration with or without acantholysis. IgA autoantibodies that target keratinocyte cell surfaces and desmosomal components in the epidermis have been detected in DIF and IIF [1]. IgA pemphigus is divided into two major subtypes: the IEN type, and the SPD type. While SPD-type IgA pemphigus shows subcorneal pustules, the IEN type is characterized by pustule formation, mainly in the middle or lower epidermis.

In DIF, SPD-type IgA pemphigus involves cell surface IgA binding only in the upper epidermis, whereas IEN-type IgA pemphigus shows binding throughout the epidermis [2]. Desmocollin 1 has been identified as an autoantigen in SPD-type IgA pemphigus, suggesting that it plays an important role in the pathogenesis of this disease subtype [3]. Although autoantibodies against desmogleins [4] and desmocollins [5] have been reported in some cases of IEN-type IgA pemphigus, the specific autoantigen remains unidentified. In our case, we were also unable to detect specific autoantibodies using immunoblot analysis. Interestingly, a case with clinical and histological features compatible with SPD-type IgA pemphigus, but for which anti-desmocollins antibodies were not detected, was diagnosed as IEN-type IgA pemphigus [6]. That report suggested that the subtypes of IgA pemphigus might be considered to be divided by autoantigens.

In contrast to the common types of pemphigus, like pemphigus vulgaris, treatment for some cases of IgA pemphigus does not require corticosteroid or other immunosuppressive therapy. These cases of IgA pemphigus are well controlled using only anti-inflammatory treatments, such as dapsone, colchicine or isotretinoin [1]. Dapsone may be useful in treating IgA pemphigus due to its effect in suppressing neutrophilic infiltration. However refractory cases require plasmapheresis or cyclophosphamide. In the present case, oral administration of dapsone quickly caused the symptoms to subside. In IgA pemphigus, it is important to make the correct diagnosis and to choose a suitable therapy to avoid the side effects by the prolonged use of systemic corticosteroids. ■

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Conversion from human haematopoietic stem cells to keratinocytes requires keratinocyte secretory factors

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Summary

Background. Recent studies have reported that bone-marrow-derived stem cells (BMSCs), including haematopoietic stem cells (HSCs) and mesenchymal stromal cells, differentiate in order to regenerate various cellular lineages. Based on these findings, it is known that BMSCs can be used clinically to treat various disorders, such as myocardial infarction and neurotraumatic injuries. However, the mechanism of HSC conversion into organ cells is incompletely understood. The mechanism is suspected to involve direct cell–cell interaction between BMSCs, damaged organ cells, and paracrine-regulated soluble factors from the organ, but to date, there have been no investigations into which of these are essential for keratinocyte differentiation from HSCs.

Aim. To elucidate the mechanism and necessary conditions for HSC differentiation into keratinocytes *in vitro*.

Methods. We cultured human (h)HSCs under various conditions to try to elucidate the mechanism and necessary conditions for hHSCs to differentiate into keratinocytes.

Result. hHSCs cocultured with mouse keratinocytes induced expression of human keratin 14 and transglutaminase I. Only 0.1% of the differentiated keratinocytes possessed multiple nuclei indicating cell fusion. Coculture of hHSCs with fixed murine keratinocytes (predicted to stabilize cellular components) failed to induce conversion into keratinocytes. Conversely, keratinocyte-conditioned medium from both human and mouse keratinocytes was found to mediate hHSC conversion into keratinocytes.

Conclusions. Human HSCs are capable of differentiation into keratinocytes, and cell fusion is extremely rare. This differentiating is mediated by the plasma environment rather than by direct cell–cell interactions.

Introduction

Recent studies have reported that bone marrow-derived stem cells (BMSCs), including haematopoietic stem cells (HSCs) and multipotent mesenchymal stromal cells (MSCs), differentiate into various cellular lineages.[1–3] Based on these findings, BMSCs have been used to treat several disorders in animal models, including myocardial infarction, Parkinson disease and neurotraumatic injuries.[4–6] We previously used a murine bone-marrow transplantation model to show that HSCs can differentiate into functional keratinocytes *in vivo*.[7,8] Cutaneous T-cell-attracting chemokine (CTACK)/CCL27 was found to be the major regulator involved in the migration from bone marrow of keratinocyte precursor cells, which expressed CCR10, the receptor for the CTACK ligand.[7] In addition, we reported that bone marrow-derived MSCs also convert into keratin14-positive keratinocytes *in vivo* and *in vitro*.[9]

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The factor or mechanism governing the differentiation of HSCs into injured organ cells is not fully understood. Possible mechanisms include direct cell–cell interaction between peripheral haematopoietic progenitor cells and damaged organ cells, and involvement of paracrine-regulated soluble factors from the organ. Requirement of feeder cells such as 3T3 cells when culturing keratinocytes implies that direct cellular interactions play a major role in keratinocyte differentiation, proliferation and homeostasis. Previous papers have reported that use of secretory factors from damaged liver tissue enables HSCs to take on many of the characteristics of liver cells.[10]

We cultured human (h)HSCs under various conditions to elucidate the mechanism and necessary conditions of hHSC differentiation into keratinocytes.

Methods

The study was approved by the ethics committee of Hokkaido University Graduate School of Medicine, and volunteers signed consent forms approved by the Hokkaido University Graduate School of Medicine and the Hokkaido Red Cross Blood Centre Committee for the Protection of Human Subjects.[11] All animal procedures were conducted according to guidelines provided by the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol.

Cells

Human peripheral blood CD34-positive cells, which are considered to be hHSCs, were collected. Recombinant human granulocyte colony-stimulating factor (G-CSF; Chugai Pharmaceutical Co. and Kyowa Hakko Pharmaceutical Co., Tokyo, Japan) was administered to the healthy subjects. Mobilized hHSCs were then isolated from peripheral blood using immunomagnetic beads with an antibody against CD34 as described previously.[11,12]

Pam 212, a murine keratinocyte cell line, was derived from spontaneously transformed BALB/c keratinocytes. Murine dermal fibroblasts were obtained from the dorsal skin surface of C57BL/6 mice. Normal human epidermal keratinocytes (NHEKs; Cambrex, East Rutherford, NJ, USA) were used as controls.

Coculture of hHSCs

To distinguish the differentiated HSC-derived keratinocytes and cocultured keratinocytes, we chose hHSCs and murine keratinocytes Pam 212 to coculture. PAM 212 and murine dermal fibroblasts were grown separately on eight-well culture slides to 80% confluence in DMEM (Invitrogen, Grand Island, NY, USA), and NHEKs were grown in Konjac glucomannan medium (KGM, Lonza Walkersville, Walkersville, MD, USA). Each cell was washed twice with phosphate-buffered saline (PBS) and then 1×10^5 hHSCs were added to each well and cocultured for 5 days in RPMI medium (Invitrogen). Each experiment was repeated three times.

Paraformaldehyde fixation of keratinocytes

In coculture with fixed cells, Pam 212 cells or NHEKs were grown on eight-well culture slides to 80% confluence, and then fixed with 0.5% paraformaldehyde for 15 min at 25 °C. Each well was washed twice with PBS, and 1×10^5 hHSCs per well were cultured with fixed keratinocytes for 5 days with RPMI.

Preparation of conditioned medium

Pam 212 cells or NHEKs at 80% confluence were washed twice with PBS, and cultured for 48 h with RPMI. The conditioned media were centrifuged at 2500 ***g*** for 10 min, filtered through 0.22-μm filters (Millipore, Billerica, MA, USA), and stored at − 80 °C until use. These conditioned media were then used to culture 1×10^5 hHSCs on eight-well culture slides with the conditioned media collected from the Pam 212 cells or NHEKs.

Culture with secreted factors

The hHSCs (1×10^5) were plated onto eight-well slides as before, and cultured in keratinocyte basal medium (Invitrogen) containing 0.5 nmol/L bone morphogenetic protein-4 (R&D Systems, Minneapolis, MN, USA), keratinocyte growth factor (KGF; Invitrogen) or interleukin-1-induced growth factor (IGF; Invitrogen). After 48 h of culture, hHSCs were stained to investigate their differentiation into keratinocytes.

Immunocytochemistry

Skin samples were embedded in optimal cutting temperature compound (Sakura Finetek Japan, Tokyo, Japan), then cut on a cryostat into 5 μm sections, which were placed onto microscope slides. The slides were used for indirect immunofluorescence using the following primary antibodies: human cytokeratin (CK)5 (catalogue no. RCK103) and human CK14 (LL002) (both Santa Cruz Biotechnology, Santa Cruz, CA, USA), human transglutaminase 1 (B.C1) and human involucrin (rabbit polyclonal (both Biomedical Technologies, Stoughton,

MA, USA), human N-cadherin (GC4; Sigma-Aldrich, St. Louis, MO, USA), anti-pankeratin goat polyclonal, human α6-integrin (GoH3) and human HLA-ABC (G46-2.6) (all BD Biosciences Pharmingen, San Jose, CA, USA), and human nuclei (235-1; Millipore).

Fluorescence staining was investigated using a confocal laser scanning fluorescence microscope (Laser Scanning Confocal Imaging System MRC 1024; Bio-Rad, Richmond, CA, USA). Cells that stained positive to human (human nuclei or HLA-ABC) and keratinocyte markers were counted as positive for bone marrow-derived keratinocytes.

Statistical evaluation of results

Statistical analysis of differences in the means for each experimental group was carried out using the Student *t*-test, with significance set at $P < 0.05$.

Results

Human nuclei-positive and cytokeratin-positive cells are derived from human haematopoietic stem cells

No cells stained positively with antibodies against pankeratin, human CK5 or CK14, human transglutaminase I or human involucrin. Furthermore, culture of hHSCs in RPMI medium for 5 days did not result in any cells positive for any of these five antibodies. Spontaneous conversion of hHSCs to keratinocytes seldom happened.

Next, we investigated whether coculture with keratinocytes mediates hHSC differentiation into keratinocytes. Using a specific antibody against human nuclei,[13] we detected human nuclei+/cytokeratin+ cells (hNCs) after coculture of hHSCs and Pam 212 cells for 5 days (Fig. 1a). We also found human nuclei+/cytokeratin 14+ cells and human cytokeratin 5+ cells (Fig. 1b,c). These hNCs expressed HLA-ABC as a human origin marker (Fig. 1d). Furthermore, hNCs expressed human transglutaminase 1 and involucrin as other specific markers of keratinocytes (Fig. 1e). The number of hNCs increased relative to the coculture time, from $0.03 \pm 0.002\%$ at 6 h to $0.46 \pm 0.11\%$ at 48 h (Fig. 1f). However, coculture for 5 days did not introduce any colonies of hHSCs or hNCs.

Human haematopoietic stem cells cultured with fixed keratinocytes do not express keratinocyte markers

To determine whether the hNCs were generated through cell fusion or true differentiation, we examined whether multinucleate cells could result from cell fusionh between HSCs and keratinocytes.[14] Most hNCs were microscopically uninucleate, and only 0.1% of hNCs were binucleate, with human and nonhuman (presumed mouse) nuclei (Fig. 2a). To exclude the possibility of cell fusion at the initial transition of hHSCs to keratinocytes, hHSCs were cultured with 0.5% paraformaldehyde-fixed Pam 212 cells or NHEKs. It was predicted that the fixed cells would stabilize the cellular components, rendering the live hHSCs resistant to fusion. This method has been shown to prevent fusion of live cells with fixed cells, while not disrupting receptor-mediated recognition and association of these cell types.[15] This procedure failed to convert hHSCs to hNCs (Fig. 2b), suggesting that hHSCs seldom fuse with keratinocytes, and that the cell-surface molecules of keratinocytes do not induce hHSC differentiation into keratinocytes.

Human haematopoietic stem cells cultured with keratinocyte-conditioned media mediate their differentiation into keratinocytes

To clarify the potential role of the secretory factors released by keratinocytes, we treated hHSCs with the keratinocyte-conditioned medium from Pam 212 cells or NHEKs; interestingly, both types of medium induced hHSC conversion into keratinocytes (Fig. 2c). The number of hNCs in 10^4 hHSCs after 48 h in culture was 35 (0.35%) in NHEK-conditioned medium and 9 (0.09%) in Pam-212-conditioned medium (Table 1). The greater number in NHEK-conditioned medium compared with Pam-212-conditioned medium implies an association with species-specific factors. However, there were no significant differences between cultures with NHEK-conditioned medium and those with Pam-212-conditioned medium. Furthermore, 40 (0.40%) hNCs were detected in culture with fixed NHEKs in NHEK-conditioned medium, and 29 (0.29%) in culture with fixed Pam 212 cells in Pam-212-conditioned medium. Fixed keratinocytes seemed to accelerate the conversion of hHSCs with keratinocyte-conditioned medium, but the difference was not significant. In addition, as a control of cell type for the conditioned media, fibroblast-conditioned media never induced hHSCs to convert into keratinocytes.

To explore any additional effects of keratinocyte surface molecules on differentiation, we added blocking antibodies against keratinocyte surface molecules during culture. We chose human α6-integrin and human N-cadherin as the surface molecules, as these molecules are expressed on various stem cells, including epidermal

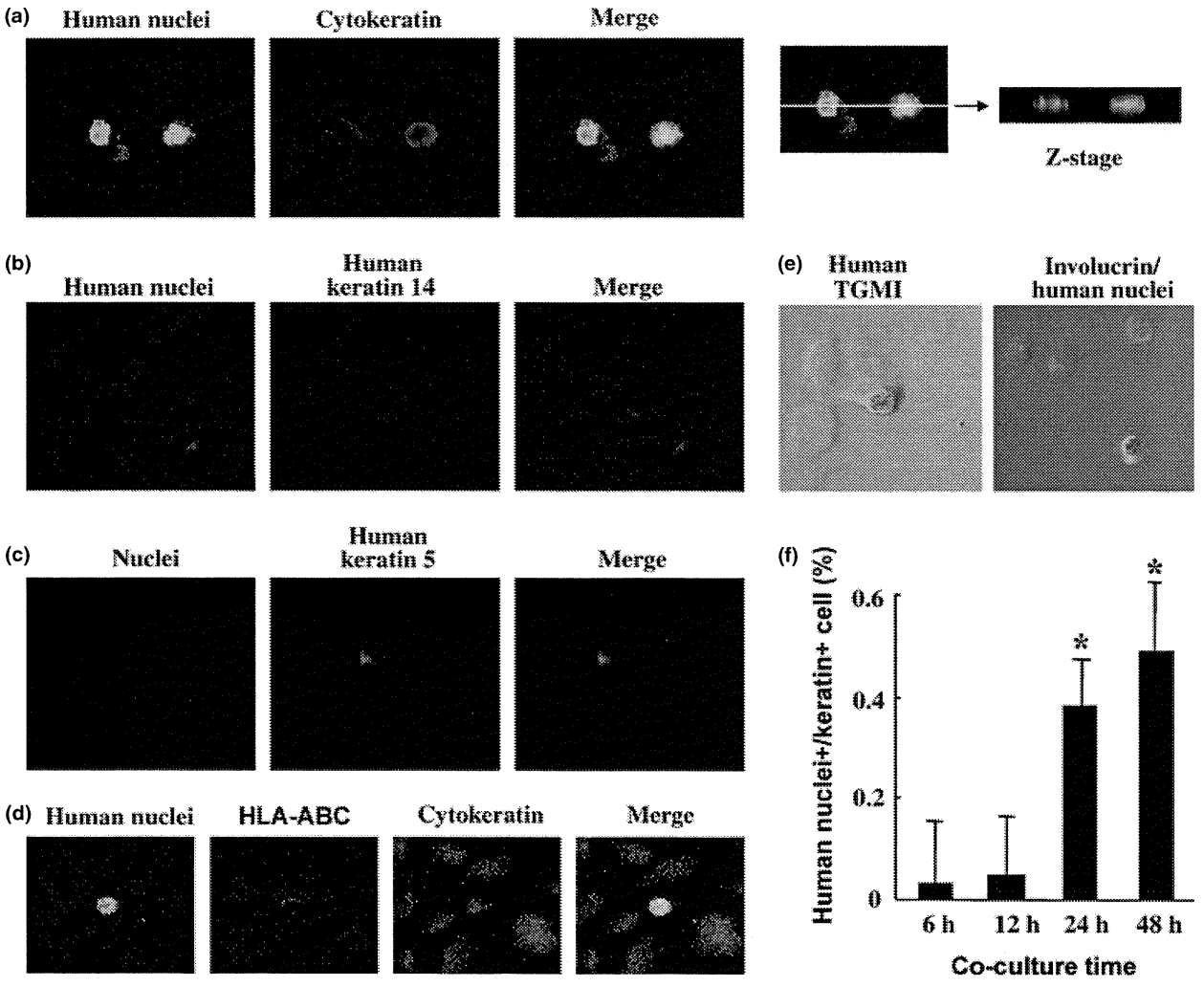

Figure 1 Coculture of human haematopoietic stem cells (hHSCs) and a mouse keratinocyte cell line, Pam212. (a) Expression of human nuclei (green) and cytokeratin (red) after 2 days in coculture, and cross-sectional analysis of the same cells (Z-axis). (b) Expression of human nuclei (green) and human cytokeratin 14 (red) after 2 days in coculture. (c) Expression of human cytokeratin 5 (green) and nuclei (propidium iodide staining, red) after 2 days in coculture. (d) Expression of human nuclei (green), human leucocyte antibody-ABC (red) and cytokeratin (blue). (e) Left: expression of human transglutaminase I (green) with transmission after 2 days in coculture; right: expression of involucrin (green) and human nuclei (red) with transmission after 2 days in coculture. (f) Percentages of hHSCs expressing keratin after 6, 12, 24 and 48 h in culture. $^*P < 0.05$ vs. 6 h.

stem cells, playing an important role in differentiation.[16,17] Blocking antibodies during coculture of hHSCs and fixed NHEK with NHEK-conditioned medium did not influence the keratinocyte conversion (data not shown).

It is possible that the humoral induction of keratinocyte differentiation is mediated by a specific growth factor such as KGF and IGF.[18] However, we did not observe hNCs with exposure of hHSCs to KGF or IGF, which are secreted exclusively from keratinocytes (data not shown). These findings suggest that soluble factors other than KGF and IGF in keratinocyte supernatant may mediate HSC differentiation.

Discussion

We have shown that hHSCs differentiate into keratinocytes in the presence of factors secreted from keratinocytes, without cell fusion. In this study, hHSCs converted into keratinocytes when cocultured with keratinocytes. By contrast, hHSCs cocultured with fixed keratinocytes were found never to convert into

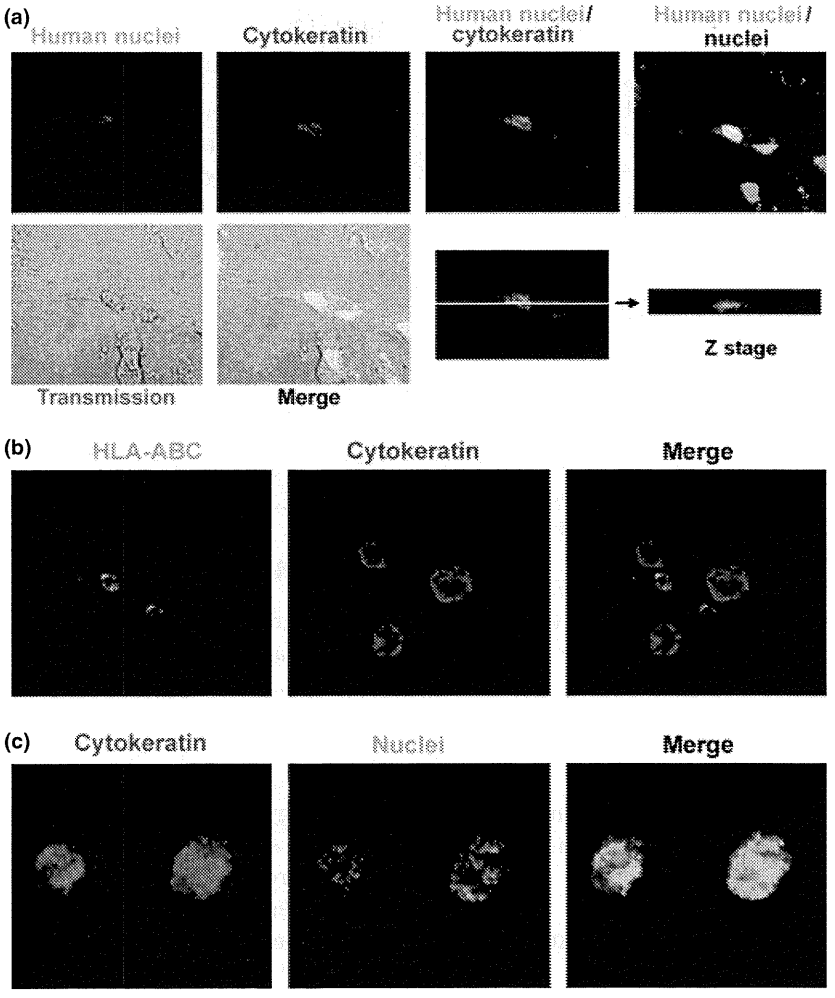

Figure 2 Coculture of human haematopoietic stem cells (hHSCs) and Pam212 cells fixed with 0.5% paraformaldehyde, and culture of hHSCs in conditioned medium of Pam 212 cells. (a) Expression of human nuclei (green), mouse nuclei (blue) and cytokeratin (red) after 2 days in coculture of hHSCs and fixed Pam212 cells, and analysis of the same cells by Z-axis or transmission. (b) Expression of human leucocyte antibody-ABC (green) and cytokeratin (red). (c) Expression of cytokeratin (blue) and human nuclei (green) in culture of hHSCs in the conditioned medium of Pam 212 cells.

Table 1 Frequency of cytokeratin-positive cells derived from human haematopoietic stem cells (hHSCs).

Treatment	CK-positive cells, *n**
Coculture with Pam 212 cells	49
Coculture with fixed Pam 212 cells	0
Coculture with mouse fibroblasts	0
Culture in Pam 212 CM	9
Culture with fixed Pam 212 in Pam-212 CM	29
Culture in NHEK CM	35
Culture with fixed NHEKs in NHEK CM	40

CK, cytokeratin; CM, conditioned medium; NHEK, normal human epidermal keratinocyte. *In 10^4 hHSCs.

keratinocytes, and hHSCs cultured with keratinocyte-conditioned medium expressed keratinocyte-specific markers. These data support the existence of factors secreted from keratinocytes or the existence of relatively paraformaldehyde-sensitive cell-surface molecules that induce hHSCs to differentiate into keratinocytes.

We did not observe differentiation after exposure of hHSCs to the growth factors KGF or IGF, which suggests that other soluble factors might mediate HSC differentiation. Indeed, a previous report on hepatocyte differentiation showed that the specific growth factors hepatocyte growth factor and fibroblast growth factor 4 failed to

mediate such conversion.[10] Further investigation is required to identify specific soluble factors that affect differentiation of HSCs to keratinocytes.

Recently Mortier *et al.*[19] succeeded in generating a skin equivalent model from human cord-blood cells, which contains heterogeneous cells including hHSCs and MSCs. Although the origin of the induced keratinocytes was not investigated, we propose that most of these cells are mesenchyme-derived, as our observation showed that purified hHSCs seldom convert to keratinocytes.

Murine BMSCs can contribute to the regeneration of injured adult tissues of various organs, including brain, liver and heart tissue, after bone-marrow transplantation.[1,3,20] These unexpected events were initially attributed to BMSC transdifferentiation, supporting the emerging idea of extended plasticity of adult stem cells. The alternative hypothesis of spontaneous cell fusion has also been proposed as the primary cause of unexpected cell-fate switches of BMSCs into various cell lineages.[21,22]

We found that the number of fused multinucleate cells (which are unlikely to undergo further cell division) in the skin was very low. Conversely, Fujino *et al.*[23] reported the observation of fused functional hepatocytes after hHSC injection into immunodeficient mice. Taking these results into consideration, it is likely that both cell fusion and conversion from HSCs play some role in the repair of damaged tissue.

Previously, we reported that CTACK/CCL27 accelerates skin regeneration via accumulation of BMDCs.[7] Furthermore, bone-marrow transplantation improves type XVII collagen-knockout epidermolysis bullosa (EB) mice, in which the deficient type XVII collagen, a cutaneous structure protein produced by keratinocytes, was restored by BMSCs.[8] Because there have been ethical and safety concerns in using embryonic stem cells and induced pluripotent stem cells, therapies using HSCs are thought to be safer.[24] In the near future, stem-cell therapies might be a candidate for the treatment of severe EB, for which there is no effective treatment other than palliative care.[25]

Conclusion

When exposed to skin tissue, hHSCs are capable of taking on many characteristics of the skin cell types, and this is mediated by the plasma environment rather than by direct cell–cell interactions, including the specific gene and/or protein expression and function of the cells.

Learning points

- It is known that HSCs have the potential for conversion into keratinocytes.
- Several mechanisms, including direct cell–cell interaction between HSCs and damaged skin, and involvement of paracrine-regulated soluble factors from the organ, have been suggested; however, there have been no reports identifying the precise mechanism involved.
- In this study, we found that the conversion of HSCs into keratinocytes is mediated by the plasma environment rather than by direct cell–cell interactions.

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ORIGINAL ARTICLE

Lipocalin-type prostaglandin D synthase as a marker for the proliferative potential of melanocyte-lineage cells in the human skin

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ABSTRACT

Melanocytes in the human epidermis actively produce and secrete various substances, thereby contributing to the maintenance of the skin homeostasis. Lipocalin-type prostaglandin D synthase (L-PGDS) that catalyzes the formation of prostaglandin D_2 (PGD_2) may be one of such secreted molecules. Once secreted, L-PGDS functions as a transporter for lipophilic ligands, including all-trans retinoic acid (RA). L-PGDS, therefore, may possess pleiotropic functions in the skin through PGD_2 and RA. We aimed to identify the cell types that express L-PGDS in human skin and to explore the role of L-PGDS in the growth potential of melanocyte-lineage cells. Immunohistochemical analysis for L-PGDS expression was performed with the tissue sections that were prepared from five malignant melanomas, six nevus cell nevi and one Spitz nevus. Normal skin tissues adjacent to the excised melanoma tissues were also analyzed. L-PGDS is expressed in epidermal melanocytes but its expression is undetectable in keratinocytes. Moreover, L-PGDS is undetectable in most benign nevus cells, which may reflect the marginally accelerated proliferation of nevus cells. In contrast, L-PGDS is overexpressed in malignant melanomas, although the frequency of L-PGDS-positive cells was variable (15–50%), depending on the specimens. Lastly, RNA interference analysis against human L-PGDS was performed with short interfering RNA. Knockdown of L-PGDS expression with short interfering RNA in cultured cells suggests that L-PGDS may restrict cell proliferation through RA. In conclusion, L-PGDS expression may contribute to the restricted proliferation of epidermal melanocytes, but conversely its overexpression may reflect the dysregulated proliferation of melanoma cells.

Key words: **lipocalin-type prostaglandin D syntheses, melanocyte, melanoma, nevus, retinoic acid.**

INTRODUCTION

Melanocytes in the human epidermis actively produce and secrete various substances, including melanin, thereby playing an important role in the maintenance of skin homeostasis.[1] As evident from the most symbolic example, melanin, melanocytes are responsible for protection against solar radiation. Accordingly, the development and survival of melanocytes have attracted much attention. Among many molecules that influence melanocyte development, microphthalmia-associated transcription factor (Mitf) has been considered a key molecule.[2–4] Homozygous Mitf-mutant mice, black-eyed white $Mitf^{mi-bw}$, lack melanocytes in the skin and inner ear and thus exhibit the complete white coat color and deafness.[5] $Mitf^{mi-bw}$ mice also exhibit augmentation of ventilatory responses to hypoxia and hypercapnia,[6] which may reflect a poorly characterized neuroendocrine function of melanocytes.[1] We have identified lipocalin-type prostaglandin D synthase (L-PGDS) that is not expressed in the newborn skin of $Mitf^{mi-bw}$ mice.[7]

Lipocalin-type prostaglandin D synthase catalyzes the isomerization of prostaglandin (PG) H_2 to produce PGD_2.[8] Importantly, L-PGDS is also secreted into various body fluids,[8] such as plasma and cerebrospinal fluid, and binds lipophilic ligands, including retinoic acid (RA), with high affinities.[8,9] It has been reported that PGD_2 is a potent inhibitor of the proliferation of human melanoma cells.[10,11] We have reported that L-PGDS mRNA is expressed in cultured human epidermal melanocytes, but is not expressed in human melanoma cell lines, as judged by reverse transcription polymerase chain reaction (RT–PCR).[7,12] It is therefore conceivable that L-PGDS may negatively regulate the proliferation of melanoma cell lines. In fact, we reported that L-PGDS enhanced the growth-inhibitory effect of RA on melanocytes,[12] although forced expression of L-PGDS itself did not influence the proliferation of human melanoma cell lines. In

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this connection, RA was shown to inhibit the proliferation of melanoma cells[13] and the ultraviolet-B-induced melanogenesis.[14]

Cutaneous melanoma is characterized by its high proliferative potential and poor prognosis,[15] while nevus cell nevi and Spitz nevi are benign melanocytic lesions that show marginally enhanced proliferation.[16] In the present study, we explored the cell types that express L-PGDS in human skin. We also analyzed melanomas and benign nevi to explore the link between L-PGDS expression and the proliferative potential of melanocyte-lineage cells.

METHODS

Cell culture

Human melanoma cell lines that stably express L-PGDS tagged with hexahistidine were established from 624 mel human melanoma cells.[12] L-PGDS#1 cells, one of such transformants, and mock transformants were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. The secreted L-PGDS protein tagged with hexahistidine was collected from the media of L-PGDS#1 cells using a Ni-column, and subjected to western blot analysis with anti-L-PGDS antibody (Novus Biologicals, Littleton, CO, USA). Normal human epidermal melanocytes were obtained from KURABO (Osaka, Japan) and cultured in Medium 154S (KURABO) containing human melanocyte growth supplement (KURABO). The human retinal pigment epithelium (RPE) cell lines, ARPE-19[17] and D407,[18] were cultured as detailed in the respective original reports.

Western blot analysis

Whole cell extracts were prepared from the cells by the method of Schreiber *et al.*[19] and then subjected to western blot analysis (100 μg/lane) using anti-L-PGDS antibody (Novus Biologicals) or anti-α-tubulin antibody (NeoMarkers, Fremont, CA, USA), as described previously.[20] L-PGDS#1 cells or mock transformants were cultured in 10 mL of medium on a 10-cm dish for 4 days. Each conditioned medium was subjected to Ni-column,[12] and the proteins bound to the Ni-column were eluted with 200 μL of elution buffer (1 mol/L imidazole, 500 mmol/L NaCl and 20 mmol/L Tris–HCl pH 7.9). An aliquot (2 μL) of each eluate was used for western blot analysis.

Ethics

The present study with the human skin tissues was performed with the approval of the Ethical Committee of Yamagata University School of Medicine. The tissue sections were prepared from five malignant melanomas, six nevus cell nevi and one Spitz nevus. In some cases, normal skin tissues adjacent to the excised melanoma tissues were also analyzed.

Immunohistochemistry

The isolated skin tissues were soaked at 4°C for 3 days in the Bouin's fixative, containing a saturated aqueous solution of picric acid, 40% formaldehyde and acetic acid (15:5:1 by volume), adjusted to pH 3.5–4.0 with NaOH. The tissues were paraffin-embedded and were cut into 6-μm sections for immunostaining. The sections were deparaffinized, hydrated and immersed in methanol containing 0.3% hydrogen peroxide for 30 min to block the endogenous peroxidase activity. Unmasking was performed with heat treatment at 95°C for 20 min. The tissue sections were incubated with Protein Block (DakoCytomation, Glostrup, Denmark) for 5 min at room temperature as a blocking procedure, and then with rabbit polyclonal anti-L-PGDS antibody overnight at 4°C. The sections were incubated with a biotinylated antirabbit immunoglobulin G antibody (DakoCytomation) for 10 min at room temperature and with streptavidin–biotin–peroxidase (SAB) complex (Nichirei Biosciences, Tokyo, Japan) for 10 min at room temperature, and visualized with 3-amino-9-ethylcarbazole. After incubation with each reagent, the sections were washed in distilled water three times. All sections were counterstained with hematoxylin–eosin.

RNA interference

RNA interference (RNAi) analysis against human L-PGDS was performed with short interfering RNA (siRNA). The three different siRNA against human L-PGDS were L-PGDSi-1 (5′-UAU UGU UCC GUC AUG CAC UUA UCG G-3′), L-PGDSi-2 (5′-AGG CGG UGA AUU UCU CCU UUA ACU C-3′) and L-PGDSi-3 (5′-AUC CAC AGC GUG UGA UGA GUA GCC A-3′), as described previously.[12] Negative control RNAi was designed according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). L-PGDS#1 cells were cultured for 16 h after plating in 96-well plates (1×10^3 cells/well), and then transfected with each L-PGDSi or negative control RNAi by Lipofectamine RNAiMAX protocol (Invitrogen). After incubation with each RNAi for 6 h, RA or vehicle was added to the culture medium. Cells were then incubated for 4 days and harvested for RNA preparation using TRI Reagent (Sigma, St Louis, MI, USA). Total RNA was subjected to RT–PCR.[12] Cell proliferation was assessed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Nakalai, Kyoto, Japan). MTT values, obtained at an optical density of 570, reflect the number of viable cells.

All data are mean ± standard deviation of at least three independent experiments. A two-tailed Student's *t*-test was used for comparison between the two groups. Differences between mean values were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Specificity of anti-L-PGDS antibody

Lipocalin-type prostaglandin D synthase and its reaction product, PGD_2, were accumulated in culture media of normal human epidermal melanocytes and a transformed cell line, L-PGDS#1, that expresses tagged L-PGDS.[12] L-PGDS#1 cells actively secrete the tagged L-PGDS into the culture medium, although the tagged L-PGDS was undetectable in the whole cell extracts.[12] We performed western blot analysis to confirm the specificity of the anti-L-PGDS antibody used in the present study. We also included ARPE-19 human RPE cells as a positive control for L-PGDS expression,[21] and mock transformed cells[12] and D407 human RPE cells as negative controls.[21] L-PGDS was detected as a faint band in whole cell extracts of normal human epidermal melanocytes, but not detectable in whole cell extracts of mock transformants (mock) and L-PGDS#1 cells (Fig. 1). L-PGDS was also detected in the whole cell extracts of ARPE-19 cells, but not in those of D407 RPE cells.[21] Subsequently,

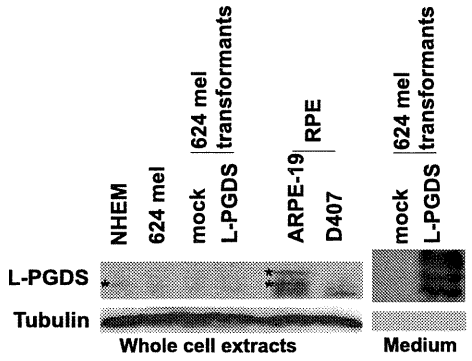

Figure 1. Specificity of anti-lipocalin-type prostaglandin D synthase (L-PGDS) antibody. Shown are the western blots of the whole cell extracts (left) and the fractionated culture media (right). Each lane contained whole cell extracts (100 μg protein) prepared from normal human epidermal melanocytes (NHEM) and various cell lines (left). The cell lines used were 624 mel human melanoma cells, mock transformants (mock), L-PGDS-expressing transformants, L-PGDS#1 (L-PGDS), and human retinal pigment epithelium (RPE) cell lines, D407 and ARPE-19. Asterisks indicate L-PGDS or L-PGDS with different glycosylation, detected in the whole cell extracts of ARPE-19 cells. Also shown are the fractionated conditioned media of L-PGDS#1 cells and mock transformants (right blot). Bottom blots show the expression levels of α-tubulin as internal control for whole cell extracts and negative control for fractionated culture media.

the secreted tagged-L-PGDS was enriched from the culture medium of L-PGDS#1 cells and subjected to western blot analysis with anti-L-PGDS antibody. L-PGDS was detected as multiple bands in the enriched fraction of the conditioned medium of L-PGDS#1 cells but not in the fraction derived from the medium of mock-transformed cells. The multiple bands may reflect the different degree of glycosylation.[12] These results verify the specificity of the anti-L-PGDS antibody that was used in the immunohistochemical study.

Expression of L-PGDS in human epidermal melanocytes and malignant melanoma

By immunohistochemical analysis, we examined the expression profile of L-PGDS in the human skin. The tissue specimens, containing the normal skin portion adjacent to the primary melanoma, were derived from one patient (#10708). Immunoreactive L-PGDS was detected as faint signals in epidermal melanocytes (Fig. 2a), which is consistent with the detection of L-PGDS in whole cell extracts of normal human epidermal melanocytes (Fig. 1). In contrast, we were unable to detect L-PGDS expression in keratinocytes (Fig. 2a), as expected from our earlier report that showed the lack of L-PGDS mRNA in human primary keratinocytes.[7]

We also analyzed the expression of L-PGDS in primary melanoma and metastatic melanoma of the same patient (#10708), showing that immunoreactive L-PGDS was detected as strong signals in malignant melanoma cells (Fig. 2b,c), compared to melanocytes (Fig. 2a). Moreover, no noticeable difference was detected in the frequency of L-PGDS-positive cells and the signal intensity of immunoreactive L-PGDS between primary melanoma and metastatic melanoma. Apparently, malignant melanoma consists of at least two types of melanoma cells, depending on the expression level of L-PGDS.

L-PGDS is overexpressed in human malignant melanomas

The expression of L-PGDS in melanoma cells seems to contradict the lack of L-PGDS expression in human melanoma cell lines, as judged by western blot analysis and RT-PCR.[7,12] Accordingly, to confirm the expression of L-PGDS in melanoma cells, we analyzed malignant melanomas excised from four other patients with different stages of melanoma. The clinical features of the patients are summarized in Table 1. The primary melanomas of advanced stages from two patients and the primary melanoma of an earlier stage from one patient are shown (Fig. 3). The primary melanomas of advanced stages showed positive immunoreactivity, with the variability in the signal intensity and the number of L-PGDS-positive

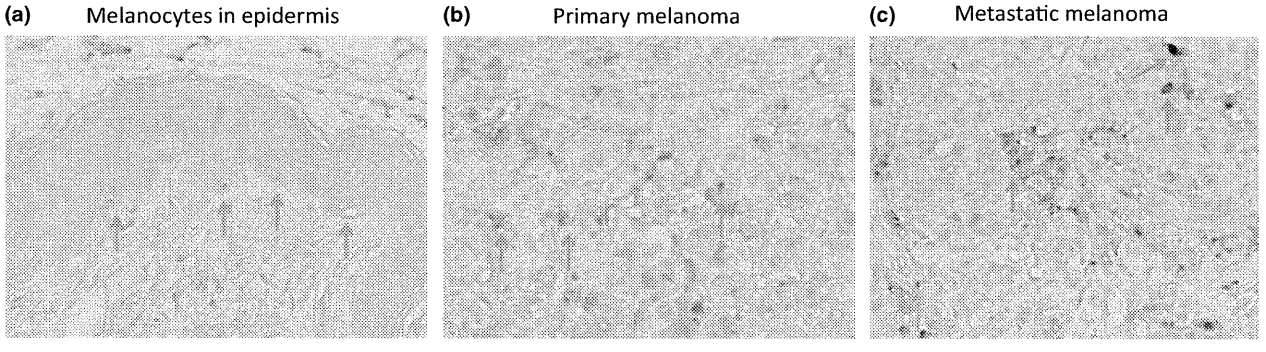

Figure 2. Expression of lipocalin-type prostaglandin D synthase (L-PGDS) in human epidermal melanocytes. The skin sections were derived from the excised malignant melanomas (patient #10708). The skin sections treated with anti-L-PGDS antibody are shown (original magnification ×400). (a) The normal skin portion, adjacent to the melanoma, was used for epidermal melanocytes. Arrows indicate L-PGDS-positive melanocytes in the epidermis. For comparison, the melanoma tissue sections of the same patient are also shown in (b) (primary melanoma) and (c) (metastatic melanoma). Note that only a few L-PGDS-positive melanoma cells are indicated with arrows. The clinical features of the patient are summarized in Table 1.

Table 1. Features of patients with malignant melanomas

Patient no.	Age	Sex	Stage	Original lesion	Metastasis lesion	Immunoreactivity with anti-L-PGDS antibody
10708	63	Female	IV	Occiput	Brain, lung, skin	Strong (Fig. 2b,c)
103006	61	Male	IIIC	Face	Lymph node	Strong (Fig. 3a)
103485	67	Female	IB	Right sole	None	Weak (not shown)
94466	83	Male	IIIA	Left back	Lymph node	Strong (Fig. 3b)
102565	69	Male	IIB	Face	None	Weak (Fig. 3c)

Immunoreactivity for lipocalin-type prostaglandin D synthase (L-PGDS) is arbitrarily classified as strong or weak. The relevant immunostaining data are presented in the indicated Figures. Occiput, posterior portion of the head.

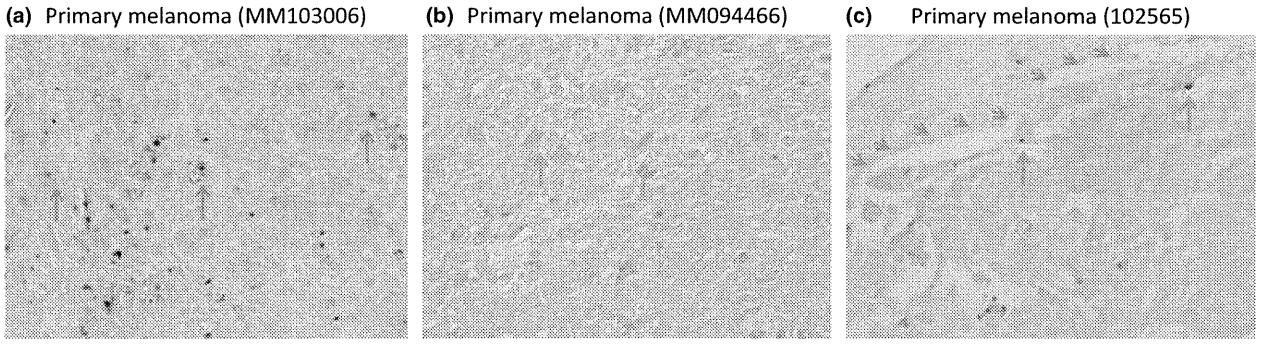

Figure 3. Overexpression of lipocalin-type prostaglandin D synthase (L-PGDS) in human melanoma cells. Primary melanomas excised from three different patients are shown (original magnification ×400): (a) #103006, (b) #094466 and (c) #102565. The clinical features of the patients are summarized in Table 1. The skin sections were treated with anti-L-PGDS antibody. (a,b) Melanomas of advanced stages. (c) Melanomas of an earlier stage, in which green arrows indicate the border between the epidermis (left upper corner) and the melanoma cells that invaded to the dermis.

cells (Fig. 3a,b). L-PGDS expression was detected mainly in the cytoplasm. Approximately 15–50% of malignant melanoma cells showed positive staining. In contrast, the frequency of L-PGDS-positive cells was lower in primary melanoma of the earlier stage (Fig. 3c, Table 1).

To evaluate whether L-PGDS expression in malignant melanomas is correlated with patient outcome, we analyzed the relationship between L-PGDS expression and clinicopathological variables (Table 1). Overexpression of L-PGDS seems to be correlated with advanced tumor stages, lymph node metastasis and distant metastasis, despite a small number of melanoma specimens. In other words, the intensity of immunoreactive L-PGDS and the number of L-PGDS-positive cells may correlate to the progression of malignant melanoma.

L-PGDS is undetectable in most benign nevus cells

Lastly, we examined the expression of L-PGDS in nevus cell nevi from six patients. These nevi are localized in the epidermis and/or the dermis. Nevus cells sometimes show a slightly accelerated proliferative rate,[16] thus providing a good model for evaluating the role of L-PGDS in cell proliferation. Nevus cell nevi of the junctional type (Fig. 4a,b) and the intradermal type (Fig. 4c,d) are shown, excised from separate patients. Expression of L-PGDS was detected as faint signals in a small population of nevus cells that are located in the periphery of the nevi (Fig. 4a,b). In fact, L-PGDS was not detected in most nevus cells (Fig. 4c,d). We also analyzed the nevus cell nevi from four other patients, confirming that L-PGDS is expressed only in a small population of nevus cells that are located near the periphery (data not shown). Moreover, the frequency of L-PGDS-positive cells was consistently lower in nevus cell nevi, compared to malignant melanomas.

We also analyzed the expression of L-PGDS in Spitz nevi that are sometimes morphologically indistinguishable from malignant melanoma.[16] However, Spitz nevi usually show the symmetrical growth patterns of nevus cells (Fig. 4e), suggesting their restricted proliferative potential. L-PGDS is expressed in Spitz nevus cells that are located near the periphery of the nevi (Fig. 4f). The frequency of L-PGDS-positive cells was lower in Spitz nevi, compared to malignant melanomas. These results indicate that the frequency of L-PGDS-positive cells is lower in benign nevi than malignant melanoma. It is therefore conceivable that L-PGDS may be a helpful marker for making differential diagnosis of malignant melanoma from benign nevi in routinely processed specimens.

L-PGDS may restrict cell proliferation

The expression of L-PGDS in malignant melanomas is in contrast to the lack of L-PGDS expression in human melanoma cell lines.[12] We initially hypothesized that the loss of L-PGDS expression might confer the growth advantage on a given melanoma cell line, because

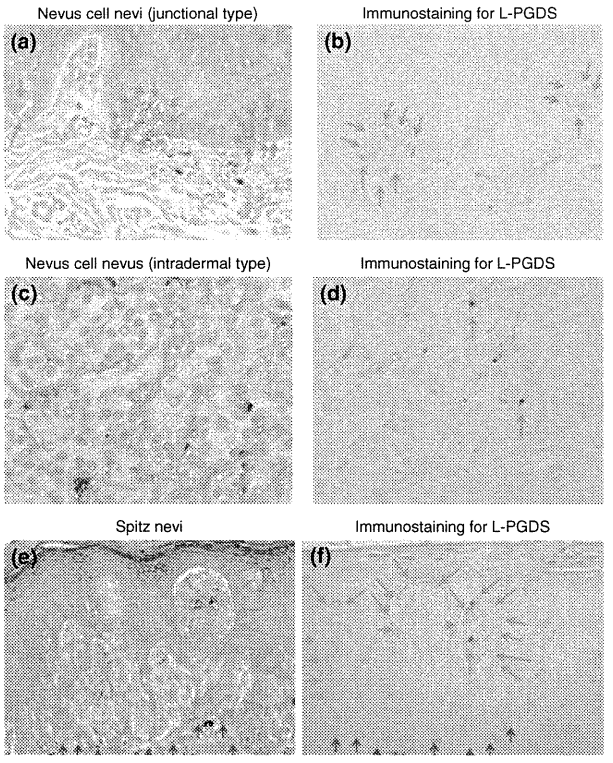

Figure 4. Expression of lipocalin-type prostaglandin D synthase (L-PGDS) is undetectable in most benign nevus cells. (a,c,e) Skin sections stained with hematoxylin–eosin and (b,d,f) those treated with anti-L-PGDS antibody. (a,b) Junctional nevus cell nevi (original magnification ×200). Green arrows indicate the margins of nevi. (c,d) Intradermal nevus cell nevus (×400). Nevus cells in the dermis are shown. Note that L-PGDS expression is undetectable in most of nevus cells. (e,f) Spitz nevi (×200). Green arrows indicate the margins of Spitz nevi. Red arrows indicate the border between the epidermis and the dermis.

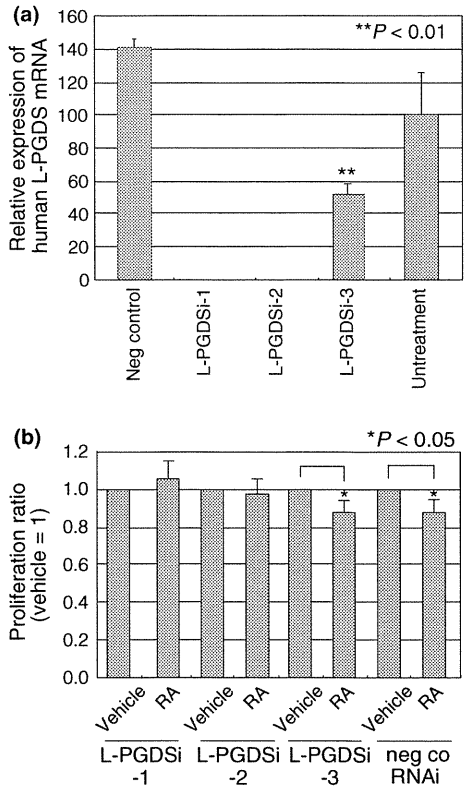

Figure 5. Lipocalin-type prostaglandin D synthase (L-PGDS) as a potential modulator for cell proliferation. (a) Effects of siRNA on the expression levels of L-PGDS mRNA. L-PGDS#1 cells were treated for 4 days with negative control RNA interference (RNAi) or each of stealth RNAi: L-PGDSi-1, L-PGDSi-2 and L-PGDSi-3. Total RNA was subjected to real-time polymerase chain reaction. L-PGDS mRNA expression was undetectable in the cells treated with L-PGDSi-1 or L-PGDSi-2. L-PGDSi-3 reduced the expression of human L-PGDS mRNA by approximately 50%. The data are means ± standard deviation of more than four independent experiments. **Indicates statistically significant difference compared to vehicle, $P < 0.01$. (b) Effects of L-PGDS knockdown on the retinoic acid (RA)-mediated inhibition of cell proliferation. L-PGDS#1 cells were transfected for 16 h with negative control RNAi or each RNAi against L-PGDS, and then treated with vehicle (ethanol) or 10 μmol/L RA for 4 days. Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each MTT value reflects the number of viable cells. Proliferation ratio is shown as the ratio to each MTT value of vehicle treatment. *Indicates statistically significant difference compared to vehicle, $P < 0.05$. Note that RA inhibited the proliferation of cells treated with L-PGDSi-3, in which L-PGDS expression was retained. In contrast, RA treatment caused no inhibition on the proliferation of cells treated with L-PGDSi-1 or -2, in which L-PGDS expression was undetectable.

PGD_2 inhibited the proliferation of human melanoma cell lines, including 624 mel melanoma cells.[12] However, using L-PGDS-expressing transformed cells, derived from the 624 mel melanoma cell line, we showed that expression of L-PGDS itself did not influence the proliferation of melanoma-derived cells.[12] Instead, we showed that the treatment with RA (10 μmol/L) for 4 days decreased the proliferation of L-PGDS-expressing transformed cells by activating a cyclin-dependent kinase inhibitor, $p21^{Cip1}$, whereas RA exerted no noticeable influence on the proliferation of mock transformed cells and 624 mel melanoma cells that lack L-PGDS expression.[12]

Accordingly, to explore the role of L-PGDS in the control of cell proliferation, we performed RNAi against L-PGDS using an L-PGDS-overexpressing cell line, L-PGDS#1. We used three different siRNA, L-PGDSi-1, L-PGDSi-2 and L-PGDSi-3, each of which targets L-PGDS mRNA. L-PGDS mRNA expression was undetectable in L-PGDS#1 cells that were treated with L-PGDSi-1 or L-PGDSi-2, while it was reduced by approximately 50% with L-PGDSi-3, compared with negative control RNAi or untreated control (Fig. 5a). Importantly, knockdown of L-PGDS expression with L-PGDSi-1 or L-PGDSi-2 abolished the RA-mediated proliferation inhibition (Fig. 5b). In contrast, RA inhibited the proliferation of L-PGDS#1 cells treated with L-PGDSi-3, in which L-PGDS expression was retained (Fig. 5a). Likewise, RA inhibited the proliferation of L-PGDS#1 cells that were treated with control RNAi. These results suggest that L-PGDS may enhance the effect of RA. It is therefore

conceivable that L-PGDS may restrict cell proliferation depending on the cellular microenvironments.

In conclusion, L-PGDS is expressed in epidermal melanocytes, but its expression is undetectable in keratinocytes that actively proliferate. In addition, the expression of L-PGDS is undetectable in most benign nevus cells, which may account for the marginally accelerated proliferation of nevus cells, compared to melanocytes. We therefore suggest that L-PGDS in itself may restrict the proliferative potential of melanocytes, thereby contributing to the regulation of epidermal pigmentation. On the other hand, L-PGDS is overexpressed in malignant melanomas, compared to epidermal melanocytes and benign nevus cells, with respect to the intensity of immunostaining for L-PGDS and the frequency of L-PGDS-positive cells. Thus, the overexpression of L-PGDS may reflect the dysregulated proliferation of malignant melanoma cells. L-PGDS is a useful marker for differentiating nevus cell nevus or Spitz nevus from malignant melanoma.

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Case of epidermolytic palmoplantar keratoderma with knuckle pads

Dear Editor,

Epidermolytic palmoplantar keratoderma (EPPK) (Online Mendelian Inheritance in Man 144200) is an autosomal dominant inherited genodermatosis first described by Vörner[1] in 1901. Its clinical features are well-demarcated thickening of the palms and the soles with a yellowish discoloration and an erythematous border. The gene responsible for EPPK is the keratin 9 gene (*KRT 9*),[2] which is exclusively expressed in the suprabasal keratinocytes of the palmoplantar epidermis.[3] Keratin 9 (K9) is member of the keratin interfilament superfamily and is essential for maintaining the cytoskeleton of epithelial cells.[4] Mutant K9 weakens the cytoskeleton and excessive hyperkeratosis occurs in response to mechanical friction.[5,6] Knuckle pads or knuckle pad-like keratosis may be seen in some patients with EPPK.[7–12] Though mechanical friction and/or a *KRT 9* mutation are suggested as its cause, its pathogenesis remains uncertain. Here, we report a Japanese patient with EPPK with knuckle pads and a unique distribution of keratotic erythema.

A 26-year-old Japanese man presented to our division due to thickened skin on the palms of his hands and soles of his feet. He was aware of this symptom since early childhood. His parents were not consanguineous. His mother, his sisters and his son had similar symptoms on their palms and soles. None of the affected family members showed knuckle pads on their hands. The patient had been working as an engineer for several years in the construction field. At first presentation, a diffuse thickening of his palms and soles with a yellowish discoloration was seen bilaterally (Fig. 1a,b). The border of hyperkeratosis was erythematous and well-circumscribed on the palmar side, though keratotic erythema was evenly spread onto the distal portion of the dorsal aspect of his fingers (Fig. 1c). Potassium hydroxide testing for dermatophytes from his palms and soles gave negative results. Knuckle pads were seen on the dorsal aspect of the proximal interphalangeal and metacarpophalangeal joints of both hands. The patient was right-handed, and the knuckle pads and thickening of the palm were more severe on his right hand than on his left hand. The knuckle pads of the right index and middle finger and the border of the keratotic erythema were continuous. No other areas of the skin or nails were affected. A biopsy from the right palm showed marked hyperkeratosis, thickening of the granular layer and granular degeneration (Fig. 2). We also suggested a biopsy from the knuckle pads and keratotic erythema, but the patient did not agree to it. After informed consent had been obtained, genomic DNA was extracted from the patient's peripheral blood cells, and the *KRT 9* status was analyzed. A heterozygous missense mutation c.488G>A in exon 1 leading to p.R163Q was detected (Fig. 3). This mutation has been reported previously.[2] We diagnosed this case as EPPK. Other members of the patient's family did not want to undergo a genetic analysis. Topical urea ointment and activated vitamin D_3 ointment were applied for the hyperkeratosis. The scales and thickening of the palms and soles were ameliorated somewhat and these agents were considered effective.

Keratins constitute the intermediate filament cytoskeleton of epithelial cells. K9 is exclusively expressed on the suprabasal keratinocytes of the palmoplantar epidermis.[3] Its molecular structure includes a head domain, an α-helical coiled-coil forming a rod domain and a tail domain. The rod domain consists of four α-helical segments (1A, 1B, 2A, 2B), and each segment is connected with a non-helical linker (L1, L12, L2). The α-helical segment has highly

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