Matsumoto T, et al.	Postoperative DAV-IFN-β Therapy Does Not Improve Survival Rates of Stage II and Stage III Melanoma Patients Significantly.	J Eur Acad Dermatol Venereol			in press
Sugiura K, Muro Y, Ishikawa R, Matsumoto T, et al.	Paraneoplastic pemphigus and antilaminin-332 mucous membrane pemphigoid in a patient with follicular dendritic cell sarcoma.	Arch Dermatol			in press

Ⅳ. 研究成果の刊行物・別刷

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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 ± 0.002% at 6 h to 0.46 ± 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

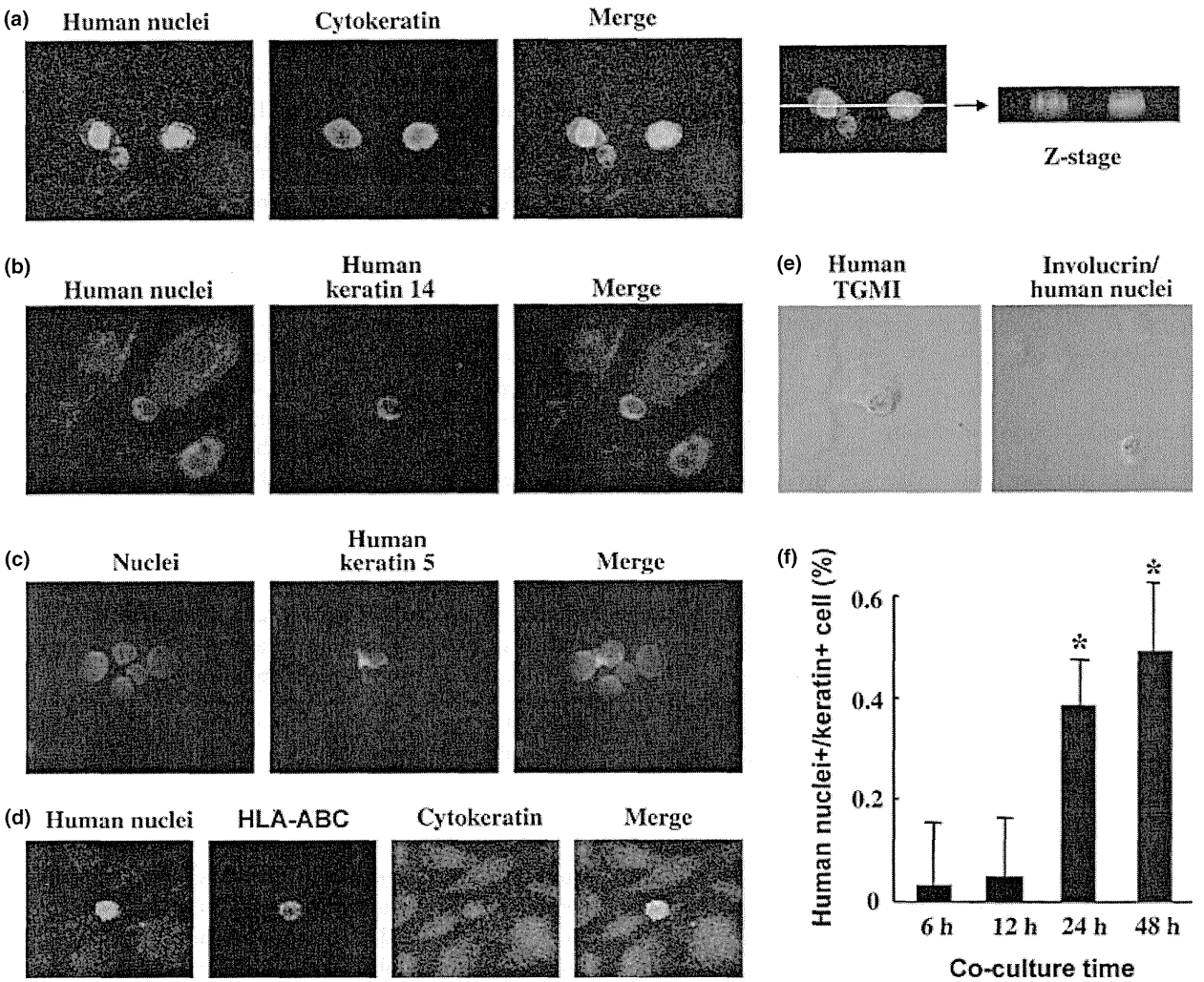

Figure 1 Coculture of human haematopoietic stem cells (hHSCs) and a mouse keratinocyte cell line, Pam212. (a) Expression of human nuclei (green) and cytokeratin (red) after 2 days in coculture, and cross-sectional analysis of the same cells (Z-axis). (b) Expression of human nuclei (green) and human cytokeratin 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

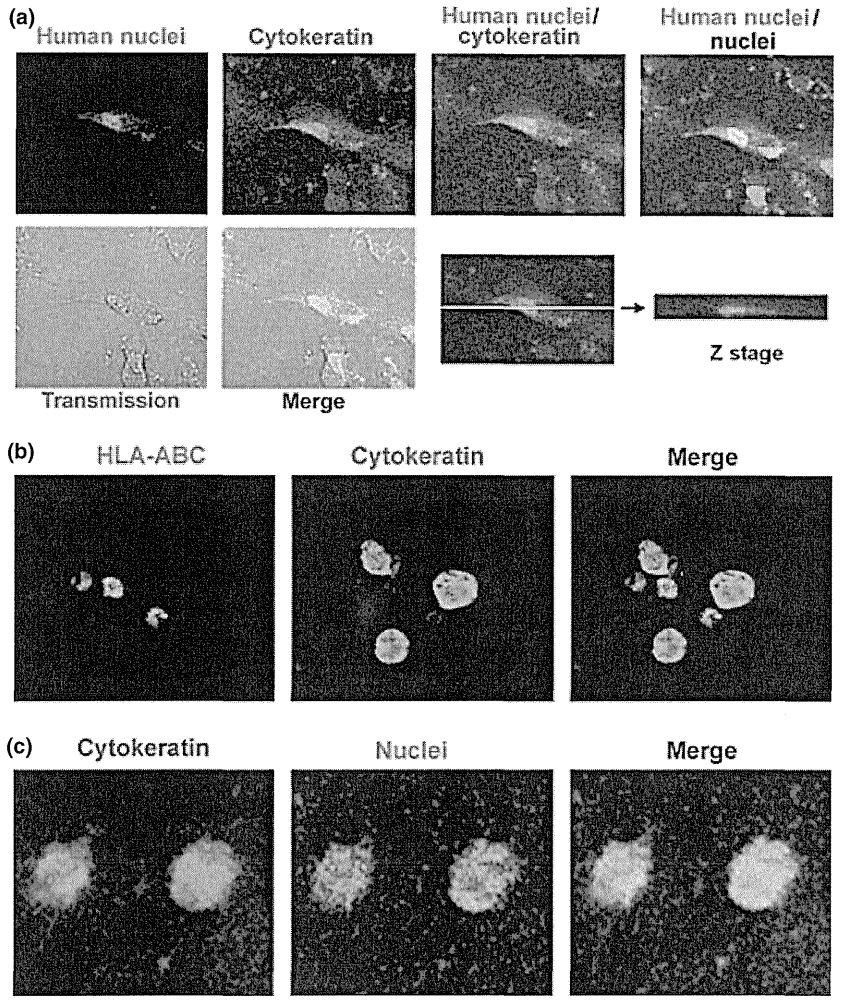

Figure 2 Coculture of human haematopoietic stem cells (hHSCs) and Pam212 cells fixed with 0.5% paraformaldehyde, and culture of hHSCs in conditioned medium of Pam 212 cells. (a) Expression of human nuclei (green), mouse nuclei (blue) and cytokeratin (red) after 2 days in coculture of hHSCs and fixed 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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Prolonged elevation of serum granulysin in drug-induced hypersensitivity syndrome

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MADAM, Drug-induced hypersensitivity syndrome (DIHS),[1] also known as drug rash with eosinophilia and systemic symptoms (DRESS),[2] has been established as a clinical entity in severe cutaneous adverse drug reactions. DIHS is characterized by the limited number of causative drugs, late onset, clinical similarity to infectious mononucleosis-like syndrome and prolonged clinical course due to relapse.[1]

Granulysin is a cytotoxic molecule produced against virus-infected cells, tumour cells, transplant cells, bacteria, fungi and parasites.[3] It plays an important role in the host defence against pathogens. A recent paper reported that granulysin is highly expressed in blisters of two other severe cutaneous adverse drug reactions: Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN).[4] In addition, we found that serum granulysin is more elevated in patients with early-stage SJS/TEN than in those with ordinary drug-induced skin reactions (ODSRs).[5]

This paper investigates the serum granulysin level of patients with DIHS. We assembled serum samples of patients with DIHS and analysed the correlation between granulysin concentrations with clinical manifestations and disease courses.

Sera of 15 patients with DIHS (10 men and five women; mean ± SD age 55·4 ± 19·9 years) were obtained from multiple institutions. All the patients had actively progressing reactions meeting the criteria for DIHS, as previously defined.[1] The disease onset (day 1) was defined as when the skin eruption appeared. Sera of patients with ODSRs (n = 24) and healthy controls (n = 31) were also obtained. ODSRs included maculopapular-type and erythema multiforme-type reactions. The granulysin concentrations of the serum samples were measured with an enzyme-linked immunosorbent assay as previously described.[6] In brief, serum samples were incubated on plates coated with RB1 antibody (MBL, Nagoya, Japan) and then reacted with biotinylated RC8 antibody (MBL). We performed assays in blind of the clinical features.

In serum samples taken from day 1 to day 10 (n = 9), eight samples (89%) showed elevated serum granulysin levels, over 10 ng mL^{-1} (mean ± SD 21·9 ± 12 ng mL^{-1}). In serum samples taken from day 11 to day 20 (n = 11), we detected elevated serum granulysin levels in seven (64%; mean ± SD 16·1 ± 14·8 ng mL^{-1}). Serum granulysin levels decreased gradually after day 21 (n = 20) [six elevated (30%); mean ± SD 7·6 ± 3·4 ng mL^{-1}] (Fig. 1). By day 20, the skin eruptions of all the patients with DIHS had disappeared. As we reported previously, no increase of granulysin level was detected in 31 healthy control subjects (mean ± SD 1·6 ± 0·6 ng mL^{-1}) and elevated granulysin was detected in only one of 24 patients with ODSRs (4%; mean ± SD 3·5 ± 3·4 ng mL^{-1}).[5]

To distinguish DIHS from ODSRs, the following clinical information is helpful: limited causative drugs; late onset after medication; manifestations similar to infectious mononucleosis such as fever, lymphadenopathy, hepatitis and haematological abnormalities. However, because of the diversity of ODSRs and similarity to viral exanthema, DIHS sometimes poses a diagnostic challenge. In addition, some patients have multiple organ failure. Therefore, early diagnosis and appropriate treatment is essential.

Unique mechanisms have been implicated in DIHS development, including detoxification defects leading to reactive metabolite formation and subsequent immunological reactions,[7]

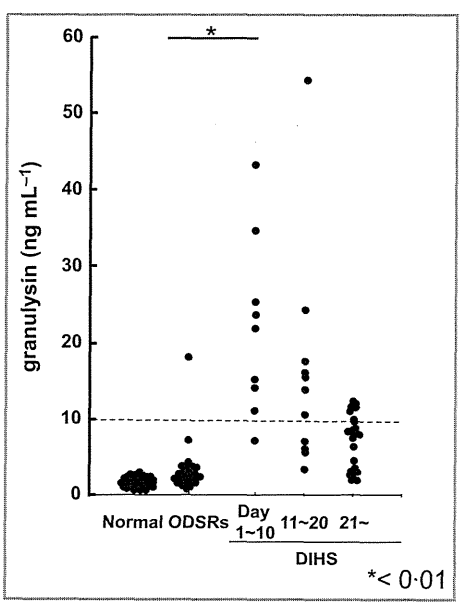

Fig 1. Granulysin levels of healthy controls, and of patients with ordinary drug-induced skin reactions (ODSRs) and drug-induced hypersensitivity syndrome (DIHS) at different stages of the disease. In patients with DIHS, we examined the concentration of granulysin for three periods: day 1–10, day 11–20, and day 21 onwards. The granulysin level was elevated from day 1 to 20, compared with levels for ODSRs and normal controls (*P < 0·01).

and reactivation of human herpesvirus (HHV).[8] In addition, it is increasingly apparent that there is a genetic predisposition to adverse drug reactions. Human leucocyte antigen-related genes have been identified as predictors of DIHS.[9]

In particular, the observation that HHV reactivation occurs during the acute phase of DIHS has led to suggestions of a pathogenic link. Shiohara *et al.*[8] identified early reactivation of HHV6 and Epstein–Barr virus (EBV), with later involvement of HHV7 and cytomegalovirus. The resulting expansion of virus-specific T cells might mediate the clinical disease. A recent paper showed that cutaneous and visceral symptoms of DIHS/DRESS are mediated by activated CD8+ T lymphocytes, which are directed against herpesviruses such as EBV.[10]

Granulysin exhibits potent cytotoxicity against a broad panel of microbial targets, including tumour cells, transplant cells, bacteria, fungi and parasites, damaging negatively charged cell membranes because of its positive charge.[3] Granulysin plays important roles in the host defence against pathogens and induces apoptosis of the target cells in a mechanism involving caspases and other pathways.[3] In the present study, we showed that granulysin levels in sera were significantly elevated in patients with DIHS compared with those with ODSRs. It is suggested that, in DIHS, activation of virus-specific cytotoxic T cells resulted in granulysin release in circulating blood. In contrast, granulysin was identified as the most highly expressed cytotoxic molecule in blisters of SJS/TEN resulting in massive keratinocyte apoptosis,[4] and we revealed that serum granulysin increased in early stage of SJS/TEN.[5] We speculated that granulysin is involved in SJS/TEN pathogenesis, inducing keratinocyte death in the early stage of 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 min.[6] We expect that monitoring of serum granulysin by this 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.

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The β9 Loop Domain of PA-PLA$_1$α Has a Crucial Role in Autosomal Recessive Woolly Hair/Hypotrichosis

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TO THE EDITOR

Membrane-associated phosphatidic acid–preferring phospholipase A$_1$α (PA-PLA$_1$α, also known as mPA-PLA$_1$α and lipase H (LIPH)) is an enzyme known to hydrolyze phosphatidic acid (PA) into 2-acyl lysophosphatidic acid (LPA) and free fatty acid (FFA; Sonoda *et al.*, 2002; Hiramatsu *et al.*, 2003). It has recently been shown that LPA, which is produced by PA-PLA$_1$α, works as a ligand for its receptor LPA$_6$, also known as P2Y5, and is speculated to regulate the proliferation and differentiation of inner root sheath cells of hair follicles (Inoue *et al.*, 2011). Recently, *LIPH* (MIM# 607365) and *LPAR6* (MIM# 609239), which encode PA-PLA$_1$α and LPA$_6$, respectively, were identified as causative genes for autosomal recessive woolly hair with associated hypotrichosis (ARWH/H; MIM# 604379, 278150; Kazantseva *et al.*, 2006; Pasternack *et al.*, 2008; Shimomura *et al.*, 2008). To our knowledge, we identified a previously unreported heterozygous missense mutation at the β9 loop domain of PA-PLA$_1$α, which is considered a crucial structure for substrate recognition (Sonoda *et al.*, 2002; Aoki *et al.*, 2007) in this study. To clarify the role of the β9 loop domain, the hydrolytic activity and LPA$_6$ activation ability of mutant PA-PLA$_1$α were evaluated.

A 3-year-old Japanese girl was seen at our hospital with woolly and sparse hair on her scalp without other abnormalities (Figure 1a). Her eyebrows and eyelashes were slightly sparse. Her parents were unrelated and nonconsanguineous, and had normal hair. Blood samples were collected for DNA extraction in accordance with standard methods, and an *LIPH* mutation search was performed as previously reported (Shimomura *et al.*, 2009). Direct sequencing analysis of all exons and intron–exon boundaries of the *LIPH* revealed that the patient was compound heterozygous for the two missense mutations c.619G>C (p.Asp207His)

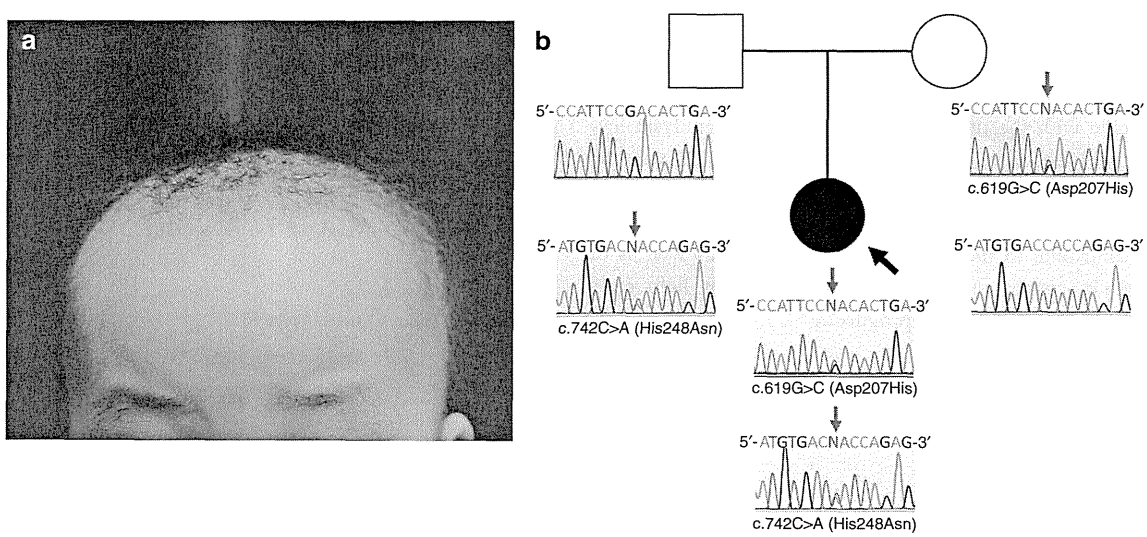

Figure 1. Clinical features and identification of mutations in the *LIPH* gene. (**a**) The affected individual has features of autosomal recessive woolly hair/hypotrichosis, which is characterized by sparse woolly hair on the scalp and slightly sparse eyebrows and eyelashes. There are no other abnormalities. (**b**) Family pedigree. The family history is consistent with autosomal recessive inheritance. Direct sequencing of the *LIPH* gene revealed that the patient had compound heterozygous missense mutations involving c.619G>C and c.742C>A. The complementary DNA (cDNA) nucleotides and the amino acids of the protein were numbered based on the previous sequence information (GenBank accession number; AY093498.1). Nucleotide numbering reflects cDNA numbering, with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to Human Genome Variation Society guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Abbreviations: a.a., amino acid; AP-TGFα, alkaline phosphatase–tagged transforming growth factor-α; FFA, free fatty acid; LPA, lysophosphatidic acid; PA, phosphatidic acid; PA-PLA$_1$α, membrane-associated phosphatidic acid–preferring phospholipase A$_1$α; PLA$_1$, phospholipase A$_1$; PS, phosphatidylserine; PS-PLA$_1$, phosphatidylserine-specific phospholipase A$_1$; TG, triacylglycerol; WT, wild type

and c.742C>A (p.His248Asn), which were segregated from her mother and father, respectively (Figure 1b). These mutations were verified by restriction enzyme digestions of the PCR products by *Hpy*188I and mutant allele-specific amplification analysis, respectively (Supplementary Figure S1 online). p.His248Asn, one amino acid (a.a.) of the catalytic triad, is known as a prevalent pathogenic mutation in the Japanese population (Shinkuma *et al.*, 2010).

To the best of our knowledge, c.619G>C (p.Asp207His) in *LIPH* is a previously unreported mutation and was not found in alleles from 100 normal unrelated individuals. Asp^{207} residue of PA-PLA$_1$α is conserved among diverse species, suggesting that Asp^{207} may have a critical role in enzyme activity (Supplementary Figure 2a online). To assess the role of Asp^{207} as a candidate for the ARWH/H, two distinct *in vitro* functional analyses were performed: for hydrolytic activity and for LPA$_6$ activation ability of PA-PLA$_1$α (Shinkuma *et al.*, 2010).

To investigate the molecular defects underlying the mutation, we synthesized p.Asp207His PA-PLA$_1$α expression constructs and compared the mutant protein expression with wild-type (WT) and with p.Ser154Ala, which was known as a loss-of-function mutation (Shinkuma *et al.*, 2010; Sonoda *et al.*, 2002). Immunoblot analysis using anti-PA-PLA$_1$α monoclonal antibody revealed that the transfection of p.Asp207His constructs into HEK293 cells resulted in the secretion of the 55-kDa mutant PA-PLA$_1$α at levels similar to those of WT and p.Ser154Ala (Figure 2a; Sonoda *et al.*, 2002). In addition, the same amounts of mutant PA-PLA$_1$α proteins were also recovered from the cell lysate (data not shown). These results indicate that there was no significant difference in protein amount between WT and mutant PA-PLA$_1$α.

Hydrolysis activity was determined by measuring FFA, which was concurrently produced from PA by PA-PLA$_1$α. Briefly, we added the supernatant from HEK293 cells transfected with WT, p.Ser154Ala, or p.Asp207His PA-PLA$_1$α to a medium containing 400 μM PA. After 3 hours of incubation at 37 °C, the amount of oleic acids, one kind

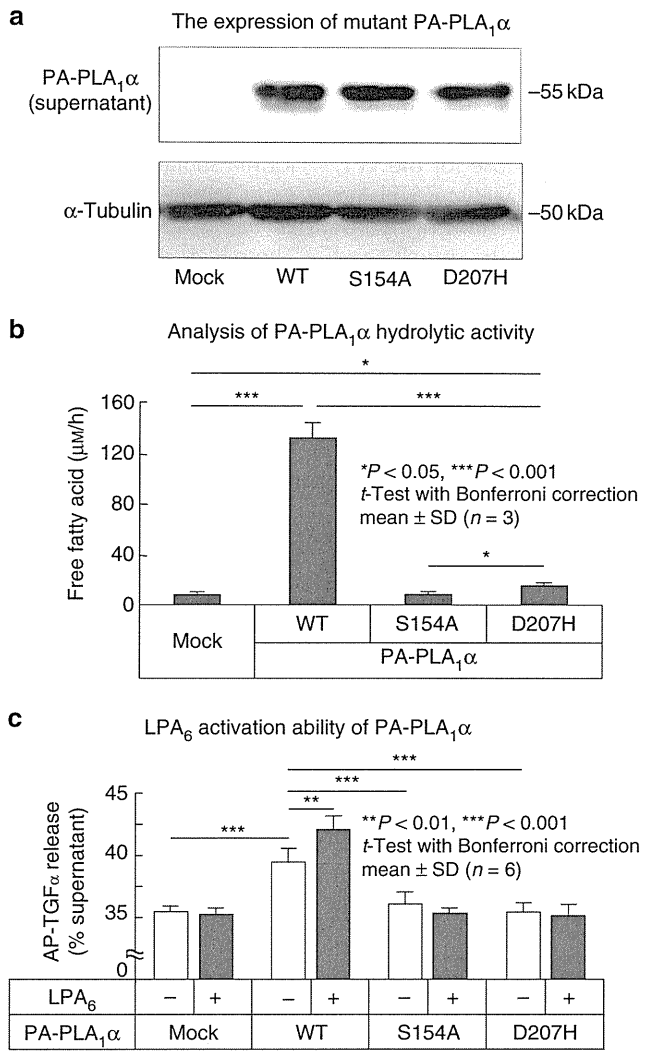

Figure 2. Hydrolytic activity and LPA$_6$ activation ability of p.Asp207His mutant membrane-associated phosphatidic acid-preferring phospholipase A$_1$α (PA-PLA$_1$α). (**a**) Expression of p.Asp207His mutant PA-PLA$_1$α in HEK 293 cells. HEK 293 cells were transfected with wild-type (WT), p.Ser154Ala (S154A), and p.Asp207His (D207H) *LIPH* cDNA, and the expression levels of PA-PLA$_1$α protein derived from the constructs in cell culture supernatant (upper panel) were compared. There were no significant differences in PA-PLA$_1$α protein expression levels among cells transfected with WT, S154A, and D207H. α-Tubulin expression was used as a standard for assessing the total amount of proteins from cell lysate (lower panel). (**b**) As PA-PLA$_1$α hydrolyzes the free fatty acid (FFA) from phosphatidic acid (PA), we monitored the levels of FFA to determine whether there were differences in PA-PLA$_1$α hydrolytic activity between the WT and each of the two mutants of PA-PLA$_1$α. After 3 hours of incubation of the supernatant from HEK293 cells expressing WT, S154A, or D207H PA-PLA$_1$α, with a medium including 400 μM PA, the levels of FFA hydrolyzed by D207H mutant PA-PLA$_1$α were significantly lower than those hydrolyzed by WT PA-PLA$_1$α; however, the D207H PA-PLA$_1$α slightly retained the function of hydrolytic activity compared with control S154A mutant and the empty vector (mock). WT = $P<0.001$ and D207H = $P<0.05$ versus mock; D207H = $P<0.001$ versus WT (one-way analysis of variance (ANOVA) with Dunnett's post test). (**c**) To monitor LPA$_6$ activation level by mutant and WT PA-PLA$_1$α, we used *p*-nitrophenyl phosphate as a substrate for cleavage of alkaline phosphatase-tagged transforming growth factor-α (AP-TGFα) and measured the amount of AP-TGFα released from the HEK293 cells. The amount of free AP-TGFα produced by LPA$_6$ mock-transfected (LPA$_6^-$) cells that were also transfected with WT PA-PLA$_1$α was significantly greater than that produced by LPA$_6^-$ cells transfected with an empty vector (mock PA-PLA$_1$α). This indicates that HEK293 cells act to shed AP-TGFα, an activity that might be mediated by intrinsic LPA receptors. The amounts of AP-TGFα released from LPA$_6$-transfected (LPA$_6^+$) cells expressing S154A or D207H mutant PA-PLA$_1$α and LPA$_6^+$ cells transfected with an empty vector (mock) are significantly lower than those released from LPA$_6^+$ cells expressing WT PA-PLA$_1$α. WT = $P<0.001$, S154A = not significant (NS) and D207H = NS versus mock (one-way ANOVA with Dunnett's post test).

of FFA, was measured using a NEFA C-Test Wako test kit (Wako Chemicals, Osaka, Japan; Shinkuma *et al.*, 2010). The quantities of oleic acids produced by the p.Asp207His PA-PLA$_1$α were markedly lower than those for the WT; however, the p.Asp207His PA-PLA$_1$α slightly retained the function of hydrolytic activity relative to mock and p.Ser154Ala (Figure 2b).

To perform the LPA$_6$ activation ability assay, alkaline phosphatase–tagged transforming growth factor-α (AP-TGFα; kindly provided by Dr Higashiyama, Ehime University, Japan; Tokumaru *et al.*, 2000), recombinant LPA$_6$, and PA-PLA$_1$α constructs (WT, p.Ser154Ala or p.Asp207His) were cotransfected to HEK293 cells. To measure the LPA$_6$ activation potency of mutant PA-PLA$_1$α, AP-TGFα release into conditioned media via a disintegrin and metalloprotease, which was triggered by the activation of LPA$_6$, was quantified using *p*-NPP as a substrate for AP (Shinkuma *et al.*, 2010; Inoue *et al.*, 2011). As previously reported, the free AP-TGFα from the LPA$_6$-untransfected (LPA$_6^-$) cells expressing the WT PA-PLA$_1$α was more abundant than that from the LPA$_6^-$ cells transfected with mock PA-PLA$_1$α, which indicated that the HEK293 cells had the ability to shed AP-TGFα mediated by intrinsic LPA receptor at some level (Figure 2c; Shinkuma *et al.*, 2010; Inoue *et al.*, 2011). AP-TGFα release from LPA$_6$-transfected (LPA$_6^+$) cells expressing the WT PA-PLA$_1$α was remarkably increased compared with mock or mutant PA-PLA$_1$α. There were no significant differences between the data obtained with cells expressing the mutant and mock PA-PLA$_1$α (Figure 2c). These data indicate that the p.Asp207His PA-PLA$_1$α results in the complete loss of LPA$_6$ activation activity. Despite the remaining slight hydrolytic activity, there was complete loss of LPA$_6$ activation ability and no significant difference in clinical features between this patient and other patients with *LIPH* mutations who were revealed as having complete loss of hydrolytic activity (Shinkuma *et al.*, 2010). These findings suggest that the p.Asp207His PA-PLA$_1$α retained slight hydrolytic activity, which was insufficient to activate LPA$_6$ and possibly leading to woolly hair.

Phospholipase A$_1$ (PLA$_1$) is an enzyme that hydrolyzes ester bonds of phospholipids (Aoki, 2004). PLA$_1$, such as phosphatidylserine (PS)-specific PLA$_1$ (PS-PLA$_1$), PA-PLA$_1$α, and PA-PLA$_1$β, form a subfamily in the pancreatic lipase gene family (Sato *et al.*, 1997; Sonoda *et al.*, 2002; Hiramatsu *et al.*, 2003). These PLA$_1$ enzymes distinctly differ from other lipases in that PLA$_1$ enzymes do not hydrolyze triacylglycerol (TG) and that they show strict substrate specificities and act specifically on PS and PA, respectively (Sato *et al.*, 1997; Sonoda *et al.*, 2002; Hiramatsu *et al.*, 2003). The lipase family has three a.a. residues that form the putative catalytic triad and has three surface loops called the lid, the β5 loop, and the β9 loop that cover the active site (Winkler *et al.*, 1990; Carriere *et al.*, 1998; Aoki *et al.*, 2007). A comparison of the a.a. sequences of PLA$_1$ and TG lipases, such as pancreatic lipase and lipoprotein lipase, revealed that PLA$_1$ enzymes have shorter lid and β9 loop than TG lipases (PLA$_1$: 12 a.a. and 13 a.a.; TG lipases: 22–23 a.a. and 18–19 a.a., respectively; Supplementary Figure 2b online; Aoki *et al.*, 2007). Therefore, the lid, the β5 loop, and the β9 loop are implicated in substrate specificity. In this study, we identified the p.Asp207His mutation at the β9-loop domain of PA-PLA$_1$α in the ARWH/H patient, and clarified that the mutant showed substantial abolition of hydrolytic activity and had no LPA$_6$ activation ability. These results confirm that the β9-loop domain of PA-PLA$_1$α has a crucial role in enzyme activity.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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A novel splice site mutation in *NCSTN* underlies a Japanese family with hidradenitis suppurativa

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Summary

Background Hidradenitis suppurativa (HS) is a chronic follicular occlusive disease with characteristic recurrent draining sinuses, skin abscesses and disfiguring scars, mainly involving the axilla, groin, perianal and perineal regions. While most HS cases are nonfamilial, familial cases showing autosomal dominant inheritance have been reported. Recently, loss-of-function mutations in the genes encoding γ-secretase have been identified as a cause of familial HS in the Chinese and British populations.

Objectives To identify mutations in the genes encoding γ-secretase in Japanese patients with familial and nonfamilial HS.

Methods Two affected and three unaffected individuals from a Japanese family with familial HS and nine patients with nonfamilial HS were recruited. We conducted mutation analysis of the γ-secretase genes in Japanese patients with familial and nonfamilial HS.

Results A novel splice site mutation in the nicastrin gene *NCSTN*, one of the six key component genes encoding γ-secretase, was identified in the patients with familial HS. Neither unaffected individuals in the family nor 100 ethnically matched control alleles carry this mutation. None of the nine patients with nonfamilial HS carry nonsense, frameshift or splice site mutations in this gene.

Conclusions A novel splice site mutation, c.582+1delG, in *NCSTN* was identified in the familial patients with HS. We also reveal for the first time that a γ-secretase gene mutation is not linked to the development of nonfamilial HS. These results would further pave the way to a better understanding of the contribution of γ-secretase and other genes to the pathogenesis of HS and to the development of a new therapeutic strategy for HS.

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Hidradenitis suppurativa (HS) is a chronic follicular occlusive disease characterized by recurrent draining sinuses, inflamed nodules and abscesses, with subsequent scarring and chronic seepage, mainly involving the intertriginous skin of the axilla and groin, and inframammary, perianal and perineal regions.[1] It has a profound impact not only on the quality of life of the patient, but also their prognosis, as it is associated with an increased risk of cutaneous squamous cell carcinoma (SCC).[2] Familial cases showing autosomal dominant inheritance have been reported in one-third of patients with HS.[3–10]

Recently, loss-of-function mutations in the presenilin 1 (*PSEN1*), presenilin enhancer 2 homolog (*PSENEN*) and nicastrin (*NCSTN*) genes, the genes encoding key components of the γ-secretase protein, were identified as a cause of familial HS in the Chinese population.[3] γ-secretase, an aspartyl protease that cleaves type 1 transmembrane proteins, consists of four essential protein subunits: one catalytic presenilin subunit, encoded by *PSEN1* and presenilin 2 (*PSEN2*), and three cofactor subunits, presenilin enhancer 2, nicastrin and anterior pharynx defective 1, encoded by *PSENEN*, *NCSTN* and the anterior pharynx defective 1A (*APH1A*) and anterior pharynx defective 1B (*APH1B*) genes, respectively.[3]

Here we conducted a mutation analysis of all six genes encoding γ-secretase in a Japanese family with familial HS.

Materials and methods

Blood samples were obtained from two affected and three unaffected members of a Japanese family with familial HS, and from nine Japanese patients with nonfamilial HS. The

diagnosis of HS was clinically determined by experienced dermatologists. A history of five or more painful or discharging nodules, cysts or abscesses in areas frequently affected by HS was required for the diagnosis.[9] Participants or their legal guardians gave written informed consent in compliance with the Declaration of Helsinki principles. The study was approved by the Medical Ethics Committee of the Hokkaido University, Sapporo, Japan.

We sequenced all exons and exon–intron boundaries of the γ-secretase genes. Briefly, genomic DNA isolated from peripheral blood was subjected to polymerase chain reaction (PCR) amplification, followed by direct automated sequencing using ABI PRISM 3130 genetic analysers (Applied Biosystems, Foster City, CA, U.S.A.).

A blood sample from the proband of the family with familial HS was available for reverse transcription (RT)-PCR analysis. Total RNA was extracted from peripheral lymphocytes using Ficoll-Paque PLUS (GE Healthcare, Buckinghamshire, U.K.) and then subjected to RT-PCR with the RNeasy Mini Kit (Qiagen, Hilden, Germany). The cDNA served as a template in quantitative real-time RT-PCR utilizing TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and TaqMan Gene Expression inventoried assay probe (Assay ID, Hs00950933_m1; Applied Biosystems). The assays were performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). *NCSTN* mRNA expression was normalized to that of the glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*), and then the relative expression level was determined. The *NCSTN* mRNA expression of the proband was compared with that of the control.

Results

In the family with familial HS, two of the five individuals recruited – the proband (IV-1) and her uncle (III-14) – met the inclusion criteria (Fig. 1a). The proband revealed widespread, marked scarring and fistulae, especially on the posterior neck and perineal region (Fig. 1b, c). The uncle demonstrated a milder HS phenotype (Fig. 1d). The proband's father (III-10) died of SCC on the upper back that arose in a region severely affected by HS.

A heterozygous single-nucleotide deletion in the *NCSTN* exon 5/intron 5 donor splice site (c.582+1delG) in the two affected individuals was identified (Fig. 2a). Notably, the unaffected individuals in the family were all wild-type for the mutation. The mutation was also absent in 100 ethnically matched control alleles.

To determine whether the splice site mutation in *NCSTN* identified in the proband causes the reduced γ-secretase activity, we extracted mRNA from her peripheral lymphocytes and measured the relative *NCSTN* mRNA level. This revealed a marked reduction in *NCSTN* mRNA expression in the proband compared with a healthy control (Fig. 2b), which suggests that the mutant mRNA was subjected to nonsense-mediated mRNA decay. Sequence analysis of the proband's cDNA revealed that this splice site mutation in *NCSTN* resulted in aberrant splicing variants that lack exon 6 or exons 6–8.

Nine Japanese patients with nonfamilial HS were recruited in this study. Mutation analysis of the γ-secretase genes was also performed. Remarkably, none of the patients carried any mutation in their γ-secretase genes, although only one of the patients was heterozygous for a missense mutation, p.Thr421Met, in *PSEN2*.

Discussion

Nicastrin, encoded by *NCSTN*, is a critical subunit of the γ-secretase complex,[11] which plays an important role in intramembranous cleavage of Notch and amyloid precursor protein.[3,12] In the skin, Notch is expressed in developing or differentiating epidermis and hair follicles, regulating the cell fate.[13] Notably,

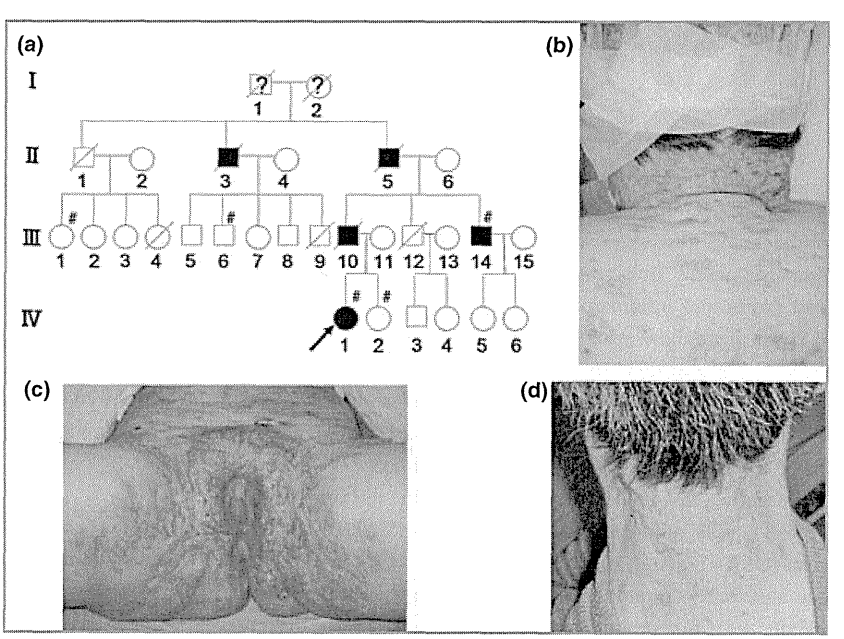

Fig 1. Clinical features of the Japanese pedigree with familial hidradenitis suppurativa (HS). (a) The family tree shows the autosomal dominant inheritance. Solid symbols denote affected individuals, open symbols denote unaffected individuals. # denotes individuals who were examined and sequenced in this study. The proband, IV-1 (b, c), demonstrated widespread sinuses, skin abscesses and disfiguring scars; her uncle, III-14 (d), showed a milder HS phenotype.

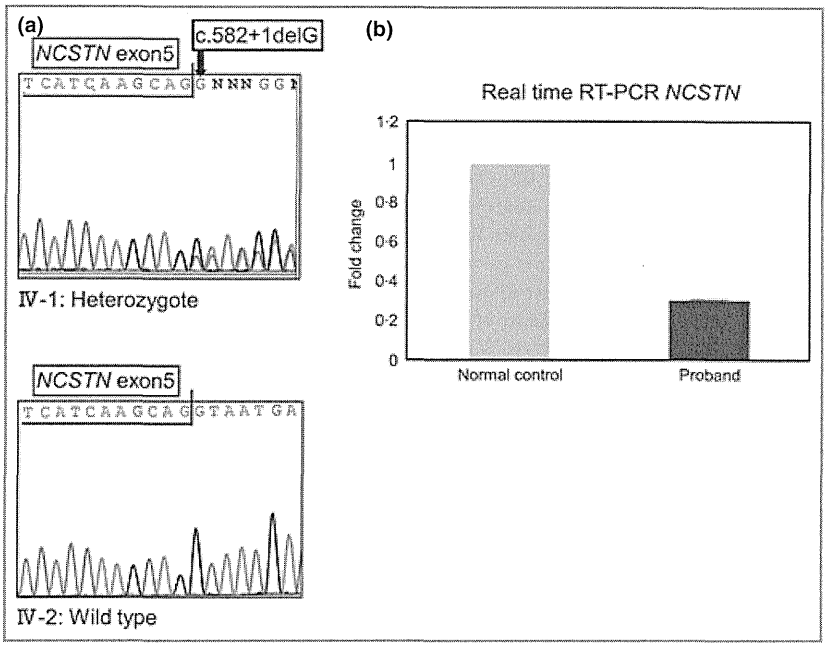

Fig 2. Mutation analysis of familial hidradenitis suppurativa. (a) A heterozygous single-nucleotide deletion in the nicastrin gene *NCSTN*, c.582+1delG, was identified in the proband, IV-1. Her unaffected sister, IV-2, was wild-type for this mutation. (b) Real-time reverse transcription–polymerase chain reaction analysis revealed marked reduction in *NCSTN* mRNA expression in the proband, IV-1, compared with an unaffected normal control.

disruption of a Notch signalling pathway causes epidermal and follicular hyperkeratosis and epidermal cyst formation.[13,14] Therefore, decreased Notch signalling due to a loss-of-function mutation in the γ-secretase genes is hypothesized to play a key role in the pathogenesis of HS via aberrant trichilemmal keratinization. This hypothesis is further supported by the fact that HS does not affect the palmoplantar regions, where no hair follicles exist.

In this study, we identified a novel splice site mutation, c.582+1delG, in *NCSTN*, which was confirmed to be a disease-causing mutation by real-time RT-PCR analysis. Our data provide further evidence that mutations resulting in haploinsufficiency of *NCSTN* are involved in the pathogenesis of familial HS.

Our data also indicate that mutations in the γ-secretase genes, including *NCSTN*, cause familial HS in the Japanese population. However, it was previously unknown whether the γ-secretase gene mutations were related to the development of nonfamilial HS. In the present study, eight of the nine patients carried no mutation in any of the γ-secretase genes, and only one of the patients was heterozygous for p.Thr421Met in *PSEN2*. We tried to analyse phenotype–genotype correlations in her family, but we were not able to obtain consent from her family for the DNA analysis. However, taking into account the fact that missense mutations in *PSEN1* and *PSEN2* have been identified in cases of familial Alzheimer's disease but not in cases of HS, and the fact that familial Alzheimer's disease and HS have been reported to be mutually exclusive, it is most likely that the missense mutation identified in the nonfamilial patient was not a cause of her HS phenotype.[3]

In conclusion, we demonstrated that a novel *NCSTN* mutation underlies familial HS in the Japanese population and revealed for the first time that a mutation in the γ-secretase genes is not linked to the development of nonfamilial HS. We believe that our findings further pave the way for understanding the contribution of γ-secretase to the pathogenesis of HS and might provide new insight into the pathogenesis of acne vulgaris, as this more common condition shares many clinical features with HS.

What's already known about this topic?

- Recently, loss-of-function mutations in the genes encoding γ-secretase were identified as a cause of familial hidradenitis suppurativa (HS) in the Chinese and British populations.
- To date, γ-secretase gene mutations have not been identified in other racial populations, including Japanese.
- It remains unclear whether *de novo* mutations within the same genes causing familial HS underlie nonfamilial HS.

What does this study add?

- We conducted mutation analysis of the γ-secretase genes in Japanese patients with familial HS. A novel splice site mutation, c.582+1delG, in the nicastrin gene *NCSTN*, which encodes a key component of γ-secretase, was identified and confirmed to be a disease-causing mutation using real-time reverse transcription polymerase chain reaction analysis.
- We also revealed for the first time that a γ-secretase gene mutation is not linked to the development of nonfamilial HS.

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Stevens-Johnson syndrome/toxic epidermal necrolysis mouse model generated by using PBMCs and the skin of patients

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Background: Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are life-threatening cutaneous reactions caused by drugs or infections and exhibiting widespread epidermal necrosis. Currently, there is no animal model that reproduces SJS/TEN symptoms.
Objective: We sought to develop a novel mouse model of SJS/TEN by using PBMCs and skin from patients who had recovered from SJS/TEN.
Methods: For our mouse model, patients' PBMCs were injected intravenously into immunocompromised NOD/Shi-*scid*, IL-2Rγ^{null} (NOG) mice, followed by oral administration of a causative drug. Subsequently, to replace human skin, unaffected skin specimens obtained from patients who had recovered from SJS/TEN were grafted onto NOG mice, after which patient-derived PBMCs and the causative drug were applied.
Results: Mice injected with PBMCs from patients with SJS/TEN and given the causative drug showed marked conjunctival congestion and numerous cell death of conjunctival epithelium, whereas there were no symptoms in mice injected with PBMCs from patients with ordinary drug skin reactions. $CD8^+$ T lymphocyte–depleted PBMCs from patients with SJS/TEN did not elicit these symptoms. In addition, skin-grafted mice showed darkening of the skin-grafted areas. Cleaved caspase-3 staining showed that dead keratinocytes were more numerous in the skin-grafted mice than in the healthy control animals.
Conclusion: We have established a novel human-oriented SJS/TEN mouse model and proved the importance of $CD8^+$ T lymphocytes in SJS/TEN pathogenesis. The mouse model promises to promote diagnostic and therapeutic approaches.
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Key words: *Stevens-Johnson syndrome, toxic epidermal necrolysis, animal models*

Abbreviations used
APC: Antigen-presenting cell
CTL: Cytolytic T lymphocyte
DC: Dendritic cell
FITC: Fluorescein isothiocyanate
GVHD: Graft-versus-host disease
GVHR: Graft-versus-host reaction
NK: Natural killer
NOG: NOD/Shi-*scid*, IL-2Rγ^{null}
ODSR: Ordinary drug skin reaction
PE: Phycoerythrin
sFasL: Soluble Fas ligand
SJS: Stevens-Johnson syndrome
TEN: Toxic epidermal necrolysis
TUNEL: Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are rare, life-threatening mucocutaneous reactions characterized by extensive detachment of the epidermis.[1] They are considered part of the same spectrum of diseases but with different severities. Patients with SJS have skin detachment on less than 10% of the body surface area, whereas patients with TEN have more extensive lesions.[2] The overall incidences of SJS and TEN have been estimated at 1 to 6 cases per million person-years and 0.4 to 1.2 cases per million person-years, respectively. The mortality associated with TEN is 25%. The eruptions are initially distributed on the face, trunk, and extremities but can rapidly extend to the whole body within just a few hours. Mucous membrane involvement is observed in approximately 90% of cases. Approximately 85% of patients have conjunctival lesions. Ocular complications include chronic conjunctivitis, conjunctival scarring, corneal vascularization, and corneal damage, which can lead to blindness. Ocular morbidity and visual loss can be caused by acute corneal complications, and progressive conjunctival scarring is also significantly associated with subsequent loss of vision.[3] Several treatments have been attempted, including high-dose corticosteroids, intravenous immunoglobulin, and plasmapheresis; however, some cases are resistant to these therapies.[4] In some cases only supportive therapy is applied out of concern over the immunosuppressive effect of these treatments.[5]

The pathologic mechanisms of SJS/TEN are not fully known.[6] Several mediators to induce SJS/TEN have been proposed, such as Fas ligand,[7] soluble Fas ligand (sFasL),[8] perforin, granzyme B,[9] and granulysin.[10,11] These mechanisms can induce massive epithelial cell death. Nevertheless, no one has been able to explain why these systemic "cell-death mediators" affect skin

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exclusively and result in widespread mucocutaneous erosions without dysfunction of other organs. Indeed, serum sFasL levels are increased not only in patients with SJS/TEN but also in those with viral infections[12] and graft-versus-host disease (GVHD).[13]

Because there is only 1 available animal model, basic research on SJS/TEN is still quite preliminary. Azukizawa et al[14,15] generated transgenic mice that express the foreign antigen ovalbumin only on keratinocytes. Injections of ovalbumin-specific cytotoxic T cells induced erosive skin manifestations and numerous apoptotic keratinocytes. Although this model showed widespread erosions and partially elucidated the pathomechanisms of skin lesions in patients with SJS/TEN, the model did not reproduce the drug-specific immune reactions that occur in the patients' blood cells and skin component cells. A more precise drug-triggered SJS/TEN mouse model is urgently required for a more complete understanding of SJS/TEN pathomechanisms and preclinical studies for novel therapeutic strategies.

In light of this, we aimed to develop a relevant animal model of SJS/TEN using patients' tissue samples to reproduce a reaction identical to that of SJS/TEN. Using immunocompromised mice, we successfully evoked the same reactions between the causative drug and human immune cells.

METHODS

Patients' samples

A total of 6 patients with SJS/TEN participated in this study (the patient information is detailed in the Table E1 in this article's Online Repository at www.jacionline.org). The causative drugs were acetaminophen in 4 patients, amoxicillin in 1 patient, and phenytoin in 1 patient. Blood samples were taken from patients with SJS/TEN at least 6 months to 3 years after complete remission of symptoms. Skin biopsy specimens were taken at least 1 year after complete remission of symptoms. The patients had received no systemic glucocorticoids before the study. Ordinary drug skin reactions (ODSRs) in our experiments included the maculopapular type and excluded other adverse drug reactions, such as drug-induced hypersensitivity syndrome/drug rash with eosinophilia and systemic symptoms and acute generalized exanthematous pustulosis. Samples were obtained from Hokkaido University Hospital. The collection of samples was approved by the local ethics committee and the institutional review board of Hokkaido University, and each patient provided written informed consent.

Mice

Immunocompromised NOD/Shi-*scid*, IL-2Rγ^{null} (NOG) mice at 6 to 7 weeks of age were purchased from the Central Institute for Experimental Animals (Tokyo, Japan). With human PBMCs, NOG mice have been used as models of human disease, such as HIV infection.[16] All the animal experiments were performed under the approval of the ethics committee for animal studies of Hokkaido University.

Analysis of graft-versus-host reactions

A graft-versus-host reaction (GVHR) was induced by means of intravenous injection of human PBMCs. Whole PBMCs (1×10^7) were obtained from healthy control subjects, suspended in 0.1 mL of PBS, and then injected intravenously into NOG mice. Skin, ocular, and mucous manifestations were observed. Body weight was monitored. Peripheral blood and splenocytes were analyzed by using flow cytometry to detect human cells. Skin and ocular lesions were investigated histopathologically.

ELISpot IFN-γ assay

PBMCs were prepared from patients' blood and isolated by using Ficoll-Isopaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation. Mouse peripheral cells and splenocytes were also isolated. The number of IFN-γ–producing cells was determined by using an ELISpot assay kit (Human IFN-γ ELISpot PVDF-Enzymatic; Diaclone, Besancon, France). Ninety-six-well nitrocellulose plates were washed 3 times with PBS before use, and PBMCs (2×10^5 in 100 μL) were incubated overnight with causative drugs in RPMI-1640 medium supplemented with 2 mmol/L L-glutamine, 25 mmol/L HEPES buffer, and 10% heat-inactivated autologous serum. Plates were washed 3 times with PBS, incubated for 2 hours with a biotinylated anti–IFN-γ antibody, and extensively washed. IFN-γ spot-forming cells were developed by using streptavidin–alkaline phosphatase, incubated for 2 hours, and washed before addition of the substrate (5-bromo-4-chloro-3-indolyl-phosphate). The number of spots was counted by using a dissecting microscope (SMZ1500; Nikon, Tokyo, Japan), and the frequency of IFN-γ lymphocytes was defined as the number of spots in 2×10^5 mononuclear cells. The drug-specific reactions between antigen-presenting cells (APCs) and antigen-specific T cells resulted in production of IFN-γ from drug-specific lymphocytes (ie, the IFN-γ–producing T cells are antigen-specific [causative drug–specific] T cells). Using the ELISpot assay, we detected causative drug–specific T cells.

SJS/TEN mouse model using patients' PBMCs

PBMCs were obtained from patients who had recovered from SJS/TEN. In some experiments isolated PBMCs were restimulated with causative drugs in completed RPMI media for 6 days. In other experiments $CD4^+$ or $CD8^+$ cells in PBMCs were depleted by using a magnet-activated cell sorter (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). PBMCs (2×10^6) were injected intravenously into the NOG mice, followed by oral administration of the causative drugs (acetaminophen, amoxicillin, or phenytoin, 100 μL). The dosage used in the model mice was based on milligrams per kilogram of body weight converted from the adult human normal dose. We administered the drug to the mice once daily. In addition, we confirmed that the dosage was under the median lethal dose in mice. Drug dosage was estimated by dose conversion by body weight. We checked for any changes of the skin, eyes, and mucosa, such as skin color or mucous hemorrhage. Peripheral blood and splenocytes were analyzed by using flow cytometry to detect human cells. Skin, ocular, and liver lesions were investigated by means of histopathologic examination and immunohistochemical staining.

Flow cytometric analysis

Cells were stained with the following antibodies: phycoerythrin (PE)–conjugated mouse CD45, fluorescein isothiocyanate (FITC)–conjugated human CD45, peridinin-chlorophyll-protein complex–conjugated human CD3, FITC-conjugated human CD4, PE-conjugated human CD8, or PE-Cy7–conjugated humanCD56 (BD Biosciences, San Jose, Calif). Analysis was performed by using a FACSAria with BD FACSDiva software (BD Biosciences).

Immunohistochemistry

Immunostaining of ocular, skin, and liver tissues was performed with antibodies to cleaved caspase-3 (Cell Signaling Technology, Beverly, Mass) and human CD4 and CD8 (BD Biosciences). FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, Pa) and TRITC-conjugated rabbit anti-rat IgG (Sigma-Aldrich, St Louis, Mo) were used as secondary antibodies. The nuclei were counterstained with propidium iodide. Fluorescence staining was detected with a confocal laser scanning fluorescence microscope (Fluoview FV1000; Olympus, Tokyo, Japan). We counted the number of stained cells from 5 separate fields, and the average was shown. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) is a method for detecting apoptotic cells with DNA fragmentation by labeling the terminal end of nucleic acids. The TUNEL assay was performed according to the manufacturer's protocol (Takara Bio, Shiga, Japan).