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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

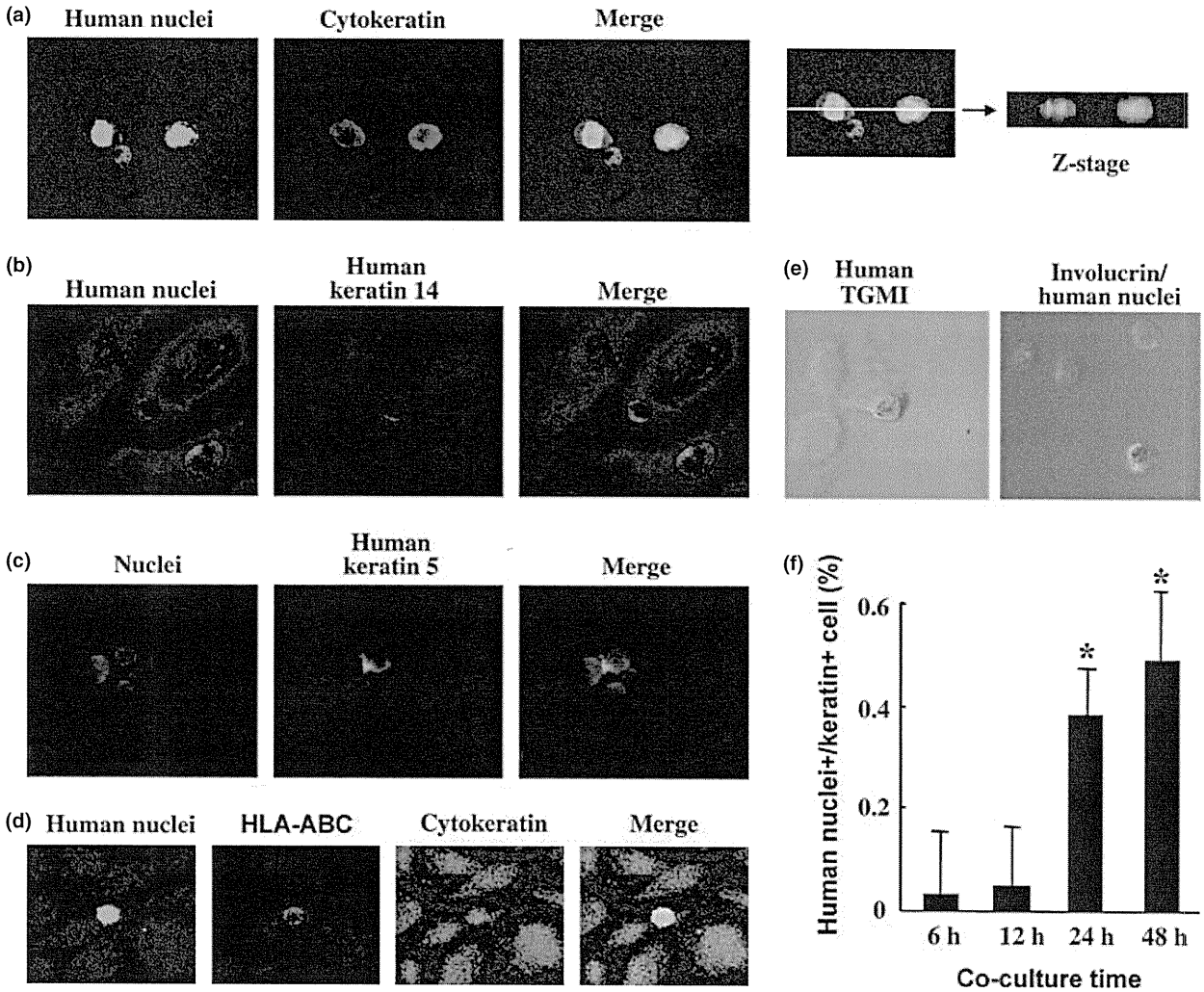

Figure 1 Coculture of human haematopoietic stem cells (hHSCs) and a mouse keratinocyte cell line, Pam212. (a) Expression of human nuclei (green) and cytokeratin (red) after 2 days in coculture, and cross-sectional analysis of the same cells (Z-axis). (b) Expression of human nuclei (green) and human cytokeratin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

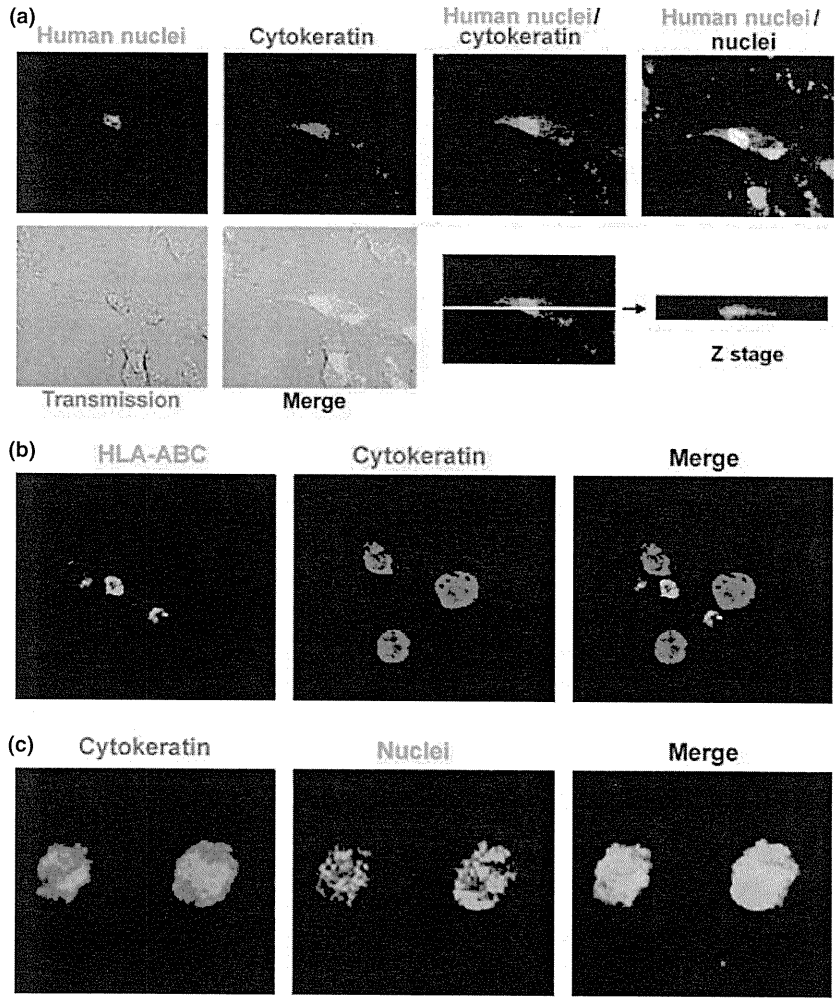

Figure 2 Coculture of human haematopoietic stem cells (hHSCs) and Pam212 cells fixed with 0.5% paraformaldehyde, and culture of hHSCs in conditioned medium of Pam 212 cells. (a) Expression of human nuclei (green), mouse nuclei (blue) and cytokeratin (red) after 2 days in coculture of hHSCs and fixed 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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observations. First, cutaneous mosaicism has been demonstrated only in dermal fibroblasts or adnexal keratinocytes,[9] both cell types following different embryologic paths from that of melanocytes and both giving rise to nevi which always follow Blaschko's lines (exception: Becker's nevus). Second, melanocytic nevi, in which nevus cells most likely carry the genetic defect, never follow Blaschko's lines, the only exception seemingly being the recently framed "nevus lentiginosus linearis".[10] Thus, I suggest that the relative non-specificity of the syndromic associations of mosaic hypomelanosis and hypermelanosis (excluding McCune–Albright syndrome) might rely on the fact that melanocytes are just "innocent bystanders" of mosaic states affecting other cells and tissues.

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Yellow nail syndrome: Nail change reflects disease severity

Dear Editor,

The triad of yellow nail syndrome (YNS) includes nail yellowing and thickening, lymphedema, and respiratory manifestations.[1] Although the pathogenesis of YNS is unknown, acquired lymphatic dysfunction and microvasculopathy with protein leakage have been thought to be the predominant mechanisms underlying the clinical manifestations.[1,2] YNS has been described in association with malignancies,[3,4] immunodeficiencies[4] and connective tissue diseases.[5] Herein, we report a case of YNS, in which the first manifestation was nail changes alone, then lymphedema and pleural effusion became prominent.

A 71-year-old man was referred to our department with a 5-year history of yellow discoloration of the fingernails and toenails. For 10 years, he had suffered recurrent episodes of chronic sinusitis and pneumonitis. From 3.5 years before his visit, general malaise, dry cough, exertional dyspnea and edema of both legs had presented. Edema of both legs had been improving due to the use of a diuretic. However, the nail changes remained.

When he visited our department, the fingernails and toenails all showed yellowish discoloration, slow growth, absent lunulae, increased curvature and thickening (Fig. 1). Fungal infection was ruled out by KOH examination of the nails. Neither fungus nor bacterium was cultured in the samples taken from the nails.

X-ray and computed tomography of the chest showed bilateral pleural effusions, predominantly in the right lung (Fig. 2).

Thoracentesis revealed light yellow fluid and exudative pleural effusion. No malignant cells were found. Culture of pleural fluid did not identify any bacterial infection. The common causes of transudates (cardiac failure, hepatic cirrhosis, nephropathy) and exudates (lymphoma, metastatic disease, connective tissue disease,

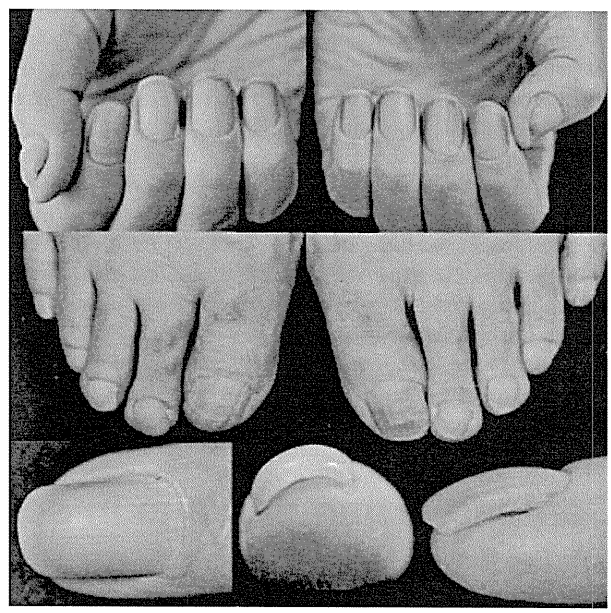

Figure 1. Clinical findings. The fingernails and toenails show yellowish discoloration, thickening, increased curvature and absent lunulae.

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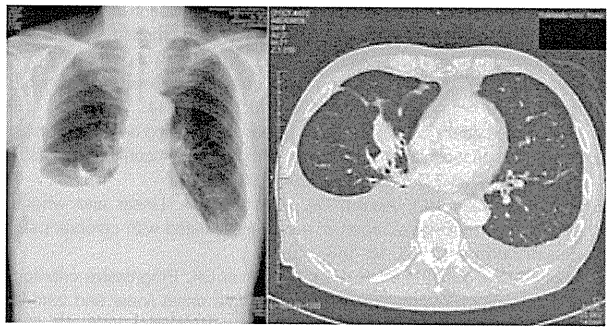

Figure 2. Bilateral pleural effusion. X-ray and computed tomography scans of the chest show bilateral pleural effusion.

infection) were excluded based on analysis of the pleural fluid. Diagnosis of YNS was made.

Yellow nail syndrome is characterized by the triad of yellow nails, lymphedema and respiratory manifestations. The presence at any given time of one of these three manifestations is sufficient to establish the diagnosis.[1] Characteristic nail features in YNS seem to be the most variable finding and the first to be recognized.[6] We questioned whether there are other manifestations suggesting YNS when we found yellow discoloration of the nails. The complete triad, which was seen in our case, is observed in only approximately 23.4% of YNS patients.[7]

Many conditions have been associated with YNS, particularly respiratory manifestations, such as bronchiectasis and recurrent lower respiratory tract infection, which are present in approximately half of the patients.[8] Other conditions with YNS include immunodeficiency states,[6] connective tissue diseases, and several malignancies, such as breast cancer[3] and lymphoproliferative disorders.[4] Rheumatoid arthritis is the autoimmune disease that is most commonly associated with YNS.[5]

The pathogenesis of the YNS manifestations is unknown. Recent studies have suggested that microvasculopathy with protein leakage may be more likely than functional lymphatic insufficiency as an explanation for the etiology of YNS.[8] The characteristic discoloration of the nails may be due to accumulation of lipofuscin, which is the product of fatty acid oxidation in the nail plate.[9] Another suggestion is that there are melanin particles in the nails, which become apparent when the nail matrix becomes inflamed.

Although there are no established effective treatments for the nail manifestations, partial or complete improvement occurs spontaneously in up to one-third of patients.[5] Some cases were reported to improve with better control of the respiratory manifestations.[6] Another paper reported that the nails returned to normal when a complicated tumor regressed.[3] Therefore, yellow nails may be an indicator of other coexistent manifestations of YNS or complications. In our case, worsening of the nail manifestations might be associated with the severity of lymphedema or pleural effusion.

The manifestations seen in YNS are not necessarily coincidental. When a patient clinically shows yellow nails, we should carefully consider YNS and conduct follow ups for any complications.

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Can end organ damage in scleroderma be predicted based on nail fold dermatoscopy findings?

Dear Editor,

Systemic scleroderma (SSc) is a connective tissue disease characterized by fibrosis and thickening of the skin. Decreased number of capillaries, dilated capillary loops and giant capillaries are frequently observed on nail fold examination.[1] Basillar pulmonary fibrosis, pulmonary arterial hypertension (PAH) and esophageal dysmotility are the most common comorbidities, and frequently cause SSc-related mortality.[2]

A total of 35 Turkish patients; 15 with diffuse cutaneous SSc (dcSSc) and 20 with limited cutaneous SSc (lcSSc), were

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Spontaneous Remission of Solitary-Type Infantile Myofibromatosis

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Key Words
Infantile myofibromatosis · Leiomyosarcoma · Solitary type

Abstract
Infantile myofibromatosis is a rare fibrous tumor of infancy. The cutaneous solitary type has typically an excellent prognosis. However, histologically, it is important to rule out leiomyosarcoma, which has a poor prognosis. The low frequency of mitosis was definitive for a diagnosis of infantile myofibromatosis. We present a cutaneous solitary-type case of infantile myofibromatosis. Following incisional biopsy, the tumor remitted spontaneously.

Introduction

Infantile myofibromatosis is a benign fibrous tumor of infancy and was first described by Stout in 1954 [1]. In most cases, it is present at birth, and in 90% of cases, the tumor appears within the first 2 years of life [2, 3]. The prognosis is excellent in the solitary type, which is limited in the skin, muscle, and subcutaneous lesions [2–4]. In contrast, the multicentric form of infantile myofibromatosis, which has visceral involvement, can be life-threatening [4, 5]. The solitary type is usually benign and the recurrence rate is low at 10%. Therefore, surgical excision is recommended [2, 6].

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We present a case of a 3-week-old girl showing features of infantile myofibromatosis (solitary type). Excision was performed and no recurrence was detected in 24 months' follow-up.

Case Report

A 3-week-old, otherwise healthy Japanese girl had a solid, red-colored, cutaneous nodule on left shoulder. The nodule had a central concavity with a crust on the surface and measured 20 × 21 mm in diameter (fig. 1).

Physical examination and CT imaging of the head, chest, abdomen and pelvis revealed no additional lesions. No infiltration of the tumor into the muscle was identified by MRI imaging (fig. 2). Incisional biopsy was performed when the patient was 4 months old. The specimen showed multifocal sclerotic dermal nodules. The nodules were composed of spindle cells with round or oval nuclei and eosinophilic cytoplasm. Delicate bundles of eosinophilic fibers separated the cellular aggregates (fig. 3a, b). A diagnosis of infantile myofibromatosis, leiomyoma, leiomyosarcoma, histiocytoma, or other sarcoma was suggested. Spindle cells expressed smooth muscle actin (fig. 3c), but not caldesmon, desmin or S100 protein (not shown). The mitotic figures were very infrequent [6 mitoses per 10 low-power images (40×)]. These results were confirmed to be consistent with infantile myofibromatosis. The tumor gradually regressed until it completely disappeared 24 months after biopsy.

Discussion

Infantile myofibromatosis usually develops at birth or during the first years of life. Chung and Enzinger found the median age at presentation to be 3 months [2]. A slight male predominance among patients with both the solitary and multicentric variants was noted by Wiswell et al. [7]. Most cases of infantile myofibromatosis are solitary nodules, accounting for up to 70% of cases in one study [2], and up to 80% in another series [4]. The prognosis is excellent in the solitary type [2–4]. In the case of solitary-type infantile myofibromatosis, spontaneous regression can be expected [3, 4]. In contrast, a quarter of the cases with the multicentric form may have visceral involvement and can be life-threatening [2, 4, 5]. The solitary type of infantile myofibromatosis is usually benign and is typically found in the dermis, subcutis, or deep soft tissues. The distribution is predominantly on the head, neck, and trunk like our case. Involvement of the extremities is reported to be rare [2]. Solitary infantile myofibromatosis on an upper extremity accounted for only 13.3% in one study of 45 cases [8].

The histologic hallmark of infantile myofibromatosis is an un-encapsulated, well-circumscribed lobule of peripheral spindle cells, which bear a close resemblance to smooth muscle [9, 10]. Often there is a central area of hemangiopericytoma-like small rounded cells surrounding blood vessels [11, 12]. This combination of features gives infantile myofibromatosis its recognizable biphasic appearance, though the hemangiopericytoma-like appearance was not detected in this case. The presence of smooth muscle actin in the spindle cells indicates the diagnosis of infantile myofibromatosis or leiomyosarcoma. Considering the difference in prognosis, it was necessary to rule out leiomyosarcoma [13] in this case. While at least 1 mitotic cell per field in high-power (×200 or ×400) fields is detected in leiomyosarcoma [14], very infrequent mitotic figures [6 mitotic cells per 10 low-power (×40) fields] were observed, which definitively indicated infantile myofibromatosis in this case.

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Previously, radical excision had been advocated as the treatment of choice, because it had been believed that the solitary form gave rise to multiple nodules with potential visceral involvement by metastases [15]. However, it is now more probable that the solitary and multicentric forms are distinct entities and that the solitary form remains localized and can regress [15]. Therefore, a wait-and-see approach has been suggested more recently as a treatment option [15]. However, in our patient, the decision was ultimately made to treat with surgical removal to exclude a diagnosis of leiomyosarcoma, which would have had a poor prognosis. The nodule disappeared completely after excision. The course was consistent with previous reports of solitary-type infantile myofibromatosis [2, 4, 15], and supports our histological diagnosis.

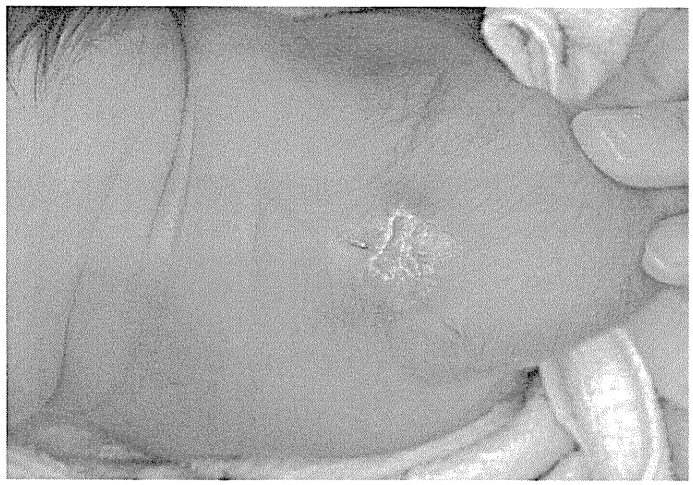

Fig. 1. Solid, red-colored subcutaneous nodule with a central concavity on the left shoulder.

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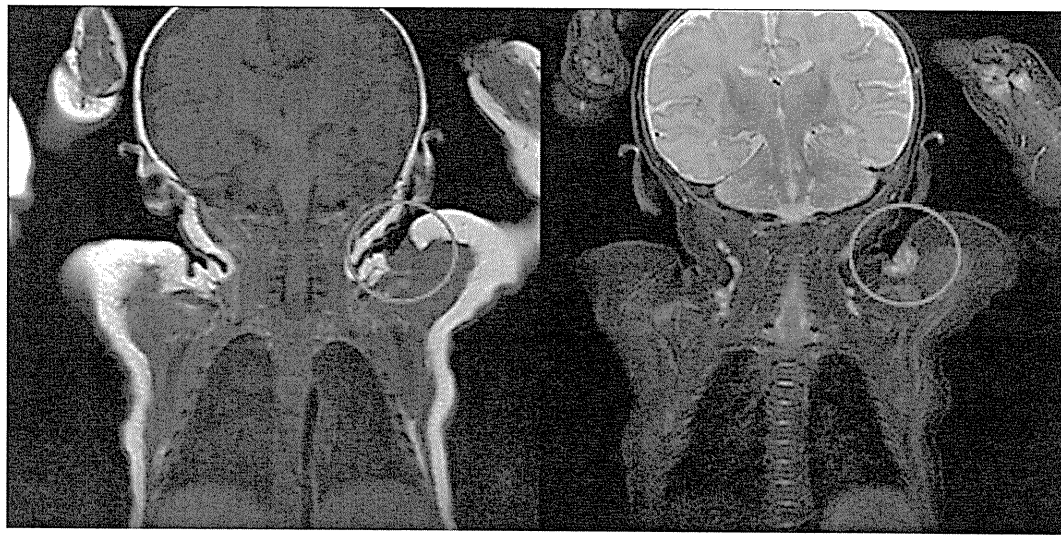

Fig. 2. MRI imaging showed the intensity of the nodule was similar to that of muscle. No additional lesions were found and infiltration of the tumor into the muscle was not observed.

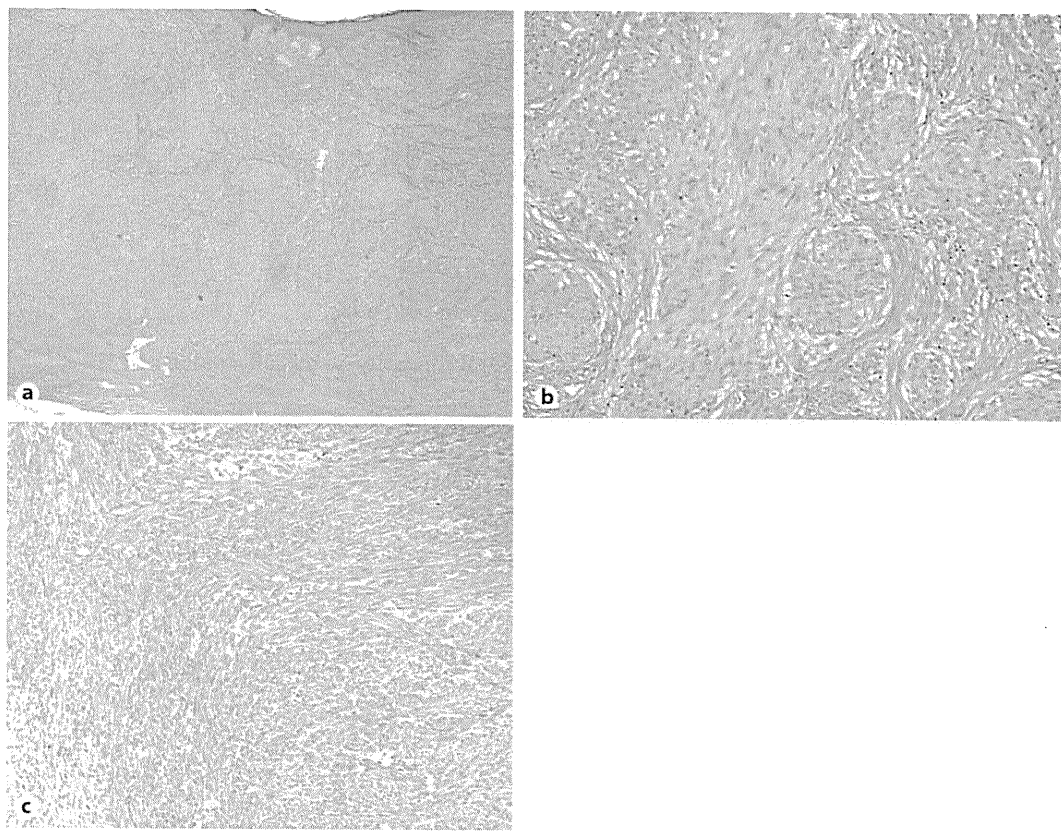

Fig. 3. Hematoxylin-eosin stain, original magnification ×20 (**a**), and ×100 (**b**). Specimen showed multifocal sclerotic dermal nodules composed of spindle cells and eosinophilic fibers. **c** Immunological staining of the tumor for α-smooth muscle actin (×100). Spindle cells express smooth muscle actin.

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Rapid immunochromatographic test for serum granulysin is useful for the prediction of Stevens-Johnson syndrome and toxic epidermal necrolysis

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Background: Life-threatening adverse drug reactions such as Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) sometimes start with clinical features of ordinary drug-induced skin reactions (ODSRs) and it may be difficult to make a correct diagnosis before severe mucocutaneous erosions occur. We have reported that serum granulysin levels are elevated (cut off: 10 ng/mL) in patients with SJS/TEN before generalized blisters form.

Objective: We sought to develop a rapid detection system for elevated serum granulysin to predict the progression from ODSRs.

Methods: Serum samples from 5 patients with SJS/TEN at 2 to 4 days before mucocutaneous erosions formed were analyzed. Sera from 24 patients with ODSRs and 31 healthy volunteers were also investigated as control subjects. We developed a rapid immunochromatographic assay for the detection of high levels of serum granulysin using two different antigranulysin monoclonal antibodies.

Results: The immunochromatographic test showed positive results for 4 of 5 patients with SJS/TEN but only one patient of 24 with ODSRs. The results correlated closely with those of enzyme-linked immunosorbent assays.

Limitations: The validation of the long-time stability in this test strip has not been investigated.

Conclusion: This novel test enables the prediction of SJS/TEN occurrence in patients even when only features of ODSRs are noted clinically. (J Am Acad Dermatol 2011;65:65-8.)

Key words: adverse drug eruption; diagnostic test; granulysin; Stevens-Johnson syndrome; toxic epidermal necrolysis.

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are life-threatening adverse drug reactions characterized by blister formation and widespread skin detachment.[1] In the early stage, SJS/TEN presents clinically as edematous papules or erythema multiforme–like target rashes, which are very similar to those of ordinary drug-induced skin reactions (ODSRs). Such a clinical course makes it difficult to reach a diagnosis of SJS/TEN in the early stage, and this results in high mortality. There is an urgent need for a method to distinguish between early-stage SJS/TEN and ODSRs.

The method should be as fast as possible, because SJS/TEN usually occurs within a few days. Furthermore, the technique should be as clinically

Abbreviations used:

ODSRs: ordinary drug-induced skin reactions
sFasL: soluble Fas ligand
SJS: Stevens-Johnson syndrome
TEN: toxic epidermal necrolysis

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simple as possible, such as using immunochromatographic test strips that are available for the detection of influenza infections. Among several candidates for diagnostic markers, we examined soluble Fas ligand (sFasL) and found that it is elevated in the sera of patients with SJS/TEN in the early stage, before mucocutaneous erosions appear.[2,3] It would be very useful to be able to predict the occurrence of SJS/TEN, but sFasL serum levels are too low (cut off: 100 pg/mL) for use in a rapid diagnostic device.

Chung et al[4] recently reported that granulysin is highly expressed in blisters of patients with SJS/TEN. We found that both serum granulysin and sFasL are higher in patients with early-stage SJS/TEN than in patients with ODSRs.[5] Serum levels of granulysin are 100 times higher (cut off: 10 ng/mL) than those of sFasL. Based on these observations, we developed a rapid immunochromatographic assay for the detection of high-level serum granulysin to diagnose and predict the early stage of SJS/TEN.

CAPSULE SUMMARY

- Drug reactions sometimes start with edematous papules, and it may be difficult to distinguish life-threatening drug reactions from ordinary drug reactions early in their course.
- We recently found that serum granulysin levels are increased in patients who later develop Stevens-Johnson syndrome or toxic epidermal necrolysis.
- We report a novel immunochromatographic assay to detect high levels of serum granulysin. With this test, we can predict whether patients with nonspecific edematous papules will develop severe drug eruptions.

METHODS

Patients

SJS refers to cases with mucosal erosions and epidermal detachment of less than 10% of the body surface area, and TEN refers to those with more than 30% involvement. Disease onset in patients with SJS/TEN was defined as the day when the mucocutaneous or ocular lesion first eroded or ulcerated (day 1).[3] From multiple Japanese institutions, we obtained serum samples from 35 patients with SJS/TEN.[3] Of these, we investigated 5 patients whose sera had been collected before the diagnosis of SJS/TEN (day −2 to −4). The patient information is listed in Table I. Serum samples from patients with ODSRs (n = 24) and healthy volunteers (n = 31) were also analyzed. Informed consent was obtained from all patients, and the procedures were approved by the Ethical Committee of the Hokkaido University Graduate School of Medicine, Sapporo, Japan.

Immunochromatographic assay

In the immunochromatographic test, a murine monoclonal antibody specific to human granulysin (RB1, MBL, Nagoya, Japan) was conjugated with microparticles and then placed on the glass membrane area of the test device in a dry state. Another granulysin monoclonal antibody (RC8, MBL) was immobilized on a nitrocellulose membrane to form a result line. Likewise, a control line was created by the immobilization of antimouse IgG. The granulysin in the serum sample specifically bound to the microparticles via RB1 and comigrated upward until the granulysin was sandwiched with the immobilized RC8, revealing a visible result line. The entire test procedure was completed within 15 minutes.

Enzyme-linked immunosorbent assay

The granulysin concentrations of the serum samples were measured with a sandwich-enzyme-linked immunosorbent assay as previously described.[6,7] In brief, 96-well flat-bottomed plates were coated with 5 mg/mL of RB1 antibody and stored overnight at 4°C. The plates were then washed and blocked with phosphate-buffered saline containing 0.1% Tween-20 (washing buffer) and blocked with 10% fetal bovine serum in washing buffer at room temperature for 2 hours. The samples and standards (recombinant granulysin, R&D Systems, Minneapolis, MN) were incubated for 2 hours at room temperature. Then they were reacted with 0.1 mg/mL of biotinylated RC8 antibody for 1 hour. The plates were then treated with 0.2 mg/mL of horseradish-peroxidase-conjugated streptavidin (Roche Diagnostics, Basel, Switzerland) for 30 minutes at room temperature. The plates were incubated with tetramethylbenzidine substrate (Sigma, St Louis, MO) for 30 minutes at room temperature, and then 1 mol/L sulfuric acid was added. The optical density was measured at 450 nm using a microplate reader (Mithras LB940, Berthold Technologies, Thoiry, France).

RESULTS

We first applied diluted recombinant human granulysin protein to the immunochromatographic test strips, to confirm the threshold and reliability of the assay. Approximately 10 ng/mL of sample yielded a result line, and 3 repeated investigations brought the same results (Fig 1, *A*).

Table I. Patient information

Patient No.	Age, y	Sex	Diagnosis	Affected skin area	Causative drug	Serum granulysin (d)
1	17	M	SJS	20%	Carbamazepine	52.1 (−3)
2	66	F	TEN	70%	Imatinib	14.2 (−3)
3	27	F	SJS	<10%	Unknown	42.2 (−4)
4	80	M	SJS	5%	Phenytoin	12.9 (−2)
5	25	F	SJS	Only mucosal lesions	Unknown	2.7 (−2)

F, Female; *M*, male; *SJS*, Stevens-Johnson syndrome; *TEN*, toxic epidermal necrolysis.

Based on this observation, we then applied serum samples to detect the elevated granulysin levels. Four of 5 SJS/TEN samples showed positive results (Fig 1, *B*). All the positive samples had elevated granulysin as detected by enzyme-linked immunosorbent assay analysis (30.35 ± 9.91 ng/mL, average ± SEM). The only sample with a negative result had granulysin at the normal level of 2.7 ng/mL. Conversely, one in 24 ODSRs samples and none of 31 healthy volunteers showed positive bands in this immunochromatographic assay. The test showed a sensitivity of 80% and a specificity of 95.8% for SJS/TEN versus ODSRs. The results of the immunochromatographic test correlated closely with early diagnosis for SJS/TEN ($P = 1.02 \times 10^{-3}$, analyzed by Fisher exact probability test).

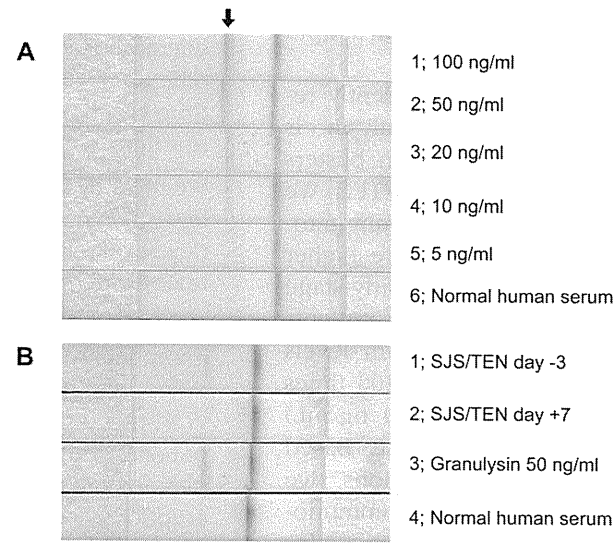

Fig 1. A, Immunochromatographic test strip detects elevated granulysin. *1 to 5*, Diluted recombinant granulysin is applied. *6*, Normal human serum as negative control (1.4 ng/mL). Positive results are shown as a band (indicated by the arrow). Approximately 10 ng/mL of granulysin is considered a positive result. **B**, Detection of serum granulysin by immunochromatographic assay. *1*, Serum taken from patient 1 with early Stevens-Johnson syndrome (*SJS*)/toxic epidermal necrolysis (*TEN*) 3 days before blister formation. Although patient showed only edematous erythema and papules without mucosal manifestations, serum granulysin was 52.1 ng/mL. *2*, Seven days after blister formation in same patient with SJS/TEN. No bands are observed, and serum granulysin has decreased to 5.7 ng/mL. *3*, Recombinant human granulysin as positive control. *4*, Normal human serum as negative control (3.5 ng/mL).

DISCUSSION

We succeeded in developing a rapid immunochromatographic test for the detection of high-level serum granulysin that puts our previous findings to practical use. Although 20% of the cases could be missed, it would be a useful adjunct in diagnosing SJS/TEN. It would not be necessary for every morbilliform drug eruption. We suggest that the test be applied when clinical findings hinting at SJS/TEN, such as target lesions, are seen. However, two biopsies should be done as soon as SJS/TEN are suspected, for hematoxylin-eosin processing and immediate frozen sections, in order to look for necrotic keratinocytes, which is another sensitive test.[8] If the results of either method are negative, careful daily and hourly monitoring of the patient for a few days should take place. Furthermore, to assess the severity of illness and to predict mortality, we should use the mathematical tool called SCORTEN that has been developed.[9]

Granulysin, a member of the saposin-like protein family of lipid-binding proteins, exhibits potent cytotoxicity against a broad panel of microbial targets, including tumor cells, transplanted cells, bacteria, fungi, and parasites, damaging negatively charged cell membranes.[10] Granulysin plays important roles in host defense against pathogens, and it induces apoptosis of target cells in a mechanism involving caspases and other pathways.[11] Chung et al[4] reported that granulysin was identified as the most highly expressed cytotoxic molecule in blisters of patients with SJS/TEN. Very recently, we showed that granulysin levels of sera from patients with SJS/TEN are significantly elevated before the development of skin detachment or mucosal lesions.[5] The elevated serum granulysin levels decrease rapidly within 5 days after disease onset. This pattern is similar to that

observed with sFasL.[3] When granulysin levels for patients with SJS/TEN in the early stage were compared with those levels for patients with ODSRs and healthy control subjects, the differences were statistically significant.[5]

This novel test enables the early diagnosis of SJS/TEN in patients with cutaneous adverse drug reactions that are otherwise indistinguishable from ODSRs.

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Macrophage Migration Inhibitory Factor Is Essential for Eosinophil Recruitment in Allergen-Induced Skin Inflammation

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Macrophage migration inhibitory factor (MIF) is a pluripotent cytokine that has an essential role in the pathophysiology of experimental allergic inflammation. Recent findings suggest that MIF is involved in several allergic disorders, including atopic dermatitis (AD). In this study, the role of MIF in allergic skin inflammation was examined using a murine model of AD elicited by epicutaneous sensitization with ovalbumin (OVA). We observed the number of skin-infiltrating eosinophils to significantly increase in OVA-sensitized MIF transgenic (Tg) mice compared with their wild-type (WT) littermates. On the other hand, eosinophils were virtually absent from the skin of MIF knockout (KO) mice and failed to infiltrate their skin after repeated epicutaneous sensitization with OVA. The mRNA expression levels of eotaxin and IL-5 were significantly increased in OVA-sensitized skin sites of MIF Tg mice, but were significantly decreased in MIF KO mice in comparison with the levels in WT littermates. Eotaxin expression was induced by IL-4 stimulation in fibroblasts in MIF Tg mice, but not in MIF KO mice. These findings indicate that MIF can induce eosinophil accumulation in the skin. Therefore, the targeted inhibition of MIF might be a promising new therapeutic strategy for allergic skin diseases.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic, relapsing inflammatory disease of the skin with significant morbidity and an adverse impact on patient well-being (Morar *et al.*, 2006). AD is considered to result from a dysregulation of the normal interactions between the environment and genes, defects in skin barrier function, and systemic and local immunological responses (Leung *et al.*, 2004). The contribution of the immune response to the pathogenesis of AD has been attributed largely to abnormalities in adaptive immunity, with key roles being played by T-helper 1(Th1)/Th2 cell dysregulation, IgE production, dendritic cell signaling, and mast-cell hyperactivity, leading to the pruritic, inflammatory dermatosis that characterizes AD (Leung *et al.*, 2004). In addition, accumulation of eosinophils is characteristic of the inflammation associated with AD (Honma *et al.*, 2000).

Macrophage migration inhibitory factor (MIF) was the first lymphokine reported to prevent random migration of macrophages (Bloom and Bennett, 1966). As the molecular cloning of MIF complementary DNA (Weiser *et al.*, 1989), MIF has been re-evaluated as a proinflammatory cytokine and pituitary-derived hormone that potentiates endotoxemia (Bernhagen *et al.*, 1993; Bucala, 1996). MIF has an important role in delayed-type hypersensitivity (Bernhagen *et al.*, 1998). Recently, it has been demonstrated that MIF also upregulates the expression of Toll-like receptor-4, which mediates lipopolysaccharide binding and activation of macrophages (Roger *et al.*, 2001). MIF is now recognized as a cytokine that exhibits a broad range of immune and inflammatory activities, including induction of inflammatory cytokines, and regulation of macrophage and lymphocyte proliferation. Furthermore, MIF induces the endothelial expression of E-selectin, ICAM-1, vascular cell adhesion molecule-1, IL-8, and monocyte chemoattractant protein-1, thus resulting in leukocyte recruitment (Gregory *et al.*, 2004, 2006; Cheng *et al.*, 2010). MIF originates from multiple cellular sources such as activated T lymphocytes, monocytes, eosinophils, and keratinocytes (Rossi *et al.*, 1998; Shimizu *et al.*, 1999; Yamaguchi *et al.*, 2000). MIF has also been shown to

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Abbreviations: AD, atopic dermatitis; KO, knockout; MIF, macrophage migration inhibitory factor; Tg, transgenic; WT, wild type

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exacerbate human allergic and inflammatory diseases, such as asthma (Rossi *et al.*, 1998) and acute respiratory distress syndrome (Donnelly *et al.*, 1997).

We recently reported excessive expression of MIF mRNA and protein in inflammatory skin lesions and in sera from AD patients (Shimizu *et al.*, 1999; Shimizu, 2005). We also showed that the serum MIF levels decrease as the clinical features of this disease improve, thus suggesting that MIF has a pivotal role in the inflammatory response in AD (Shimizu *et al.*, 1997). These studies raise the possibility that MIF is an important component of Th2-mediated immunopathology in general, and might therefore be relevant to chronic inflammatory allergic conditions.

Eosinophils may aggravate the inflammatory response in the skin of AD patients. Spergel *et al.* (1998, 1999) reported a murine model of allergic skin inflammation elicited by epicutaneous sensitization with ovalbumin (OVA). This model displays many of the features of human AD, including elevated total and specific IgE, dermatitis characterized by infiltration of the dermis by $CD4^+$ T cells and eosinophils, and increased local expression of mRNAs for the cytokines IL-4, IL-5, and IFN-γ. In our present study, MIF transgenic (Tg) mice and MIF knockout (KO) mice were used to assess the potential role of MIF in the pathogenesis of AD in this murine model of allergic skin inflammation. We also investigated the effects of MIF on eotaxin expression of dermal fibroblasts.

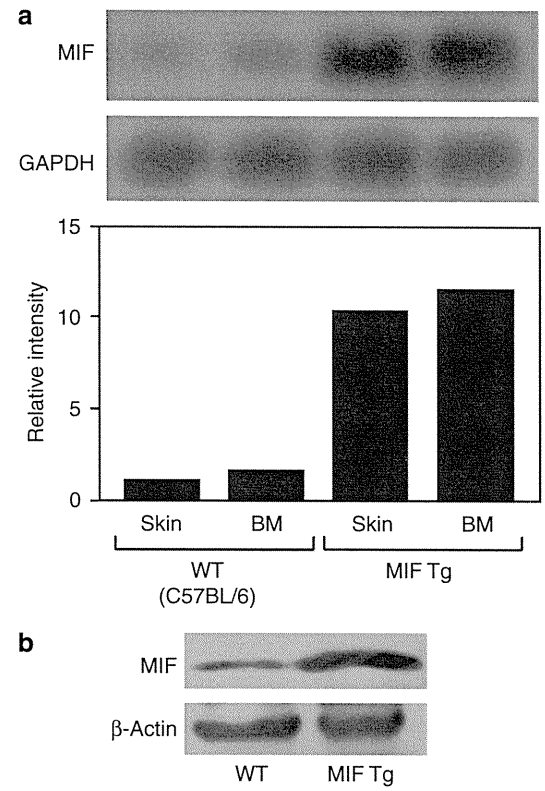

Figure 1. Expression of macrophage migration inhibitory factor (MIF) in tissues from MIF transgenic (Tg) mice. (**a**) Bone marrow (BM) and skin specimens were harvested from MIF Tg and wild-type (WT) mice, and the total RNA levels were determined by northern blot analysis as described in the Materials and Methods. The density of MIF bands was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signals. BM and skin from MIF Tg mice showed an ~10-fold higher level of MIF mRNA expression than those from WT mice. (**b**) Western blot analysis of skin from MIF Tg mice showed that the MIF protein level was also higher in MIF Tg mice than in WT mice.

RESULTS

The expression of MIF was increased in bone marrow and skin from MIF Tg mice

MIF Tg mice exhibited no lethal or prominent pathological lesions in the organs examined. A northern blot analysis revealed the MIF mRNA expression in bone marrow and skin from MIF Tg mice to be ~10 times higher than that in wild-type (WT) mice (Figure 1a). MIF protein was also increased in the skin from MIF Tg mice compared with that from WT mice, as demonstrated by western blotting (Figure 1b).

OVA-sensitized skin sites of MIF Tg mice showed marked eosinophil infiltration

To examine the role of MIF in eosinophilic infiltration, MIF Tg and WT mice were subjected to epicutaneous OVA sensitization. Only a few eosinophils were present in saline-sensitized skin from MIF Tg and WT mice, while eosinophilic infiltration of the dermis was significantly increased following epicutaneous sensitization with OVA. The mean number of eosinophils after OVA sensitization was 13.6 ± 2.84 in MIF Tg mice, but only 4.8 ± 1.37 in WT mice ($P<0.001$; Figure 2a). Figure 2b shows the histological features of OVA-sensitized skin sites in MIF Tg and WT mice. The epidermis was slightly thickened, and numerous eosinophils and mononuclear cells infiltrated the upper dermis around the vessels, in the OVA-sensitized skin of MIF Tg mice.

Eosinophil numbers were not increased in the OVA-sensitized skin of MIF KO mice

To further clarify the roles of MIF in eosinophilic infiltration, MIF KO mice were subjected to epicutaneous OVA sensitization. The mean number of eosinophils after OVA sensitization was 2.0 ± 0.94 in MIF KO mice, and did not differ from that after saline sensitization. Furthermore, this value was significantly lower than that of WT mice (4.8 ± 1.37, $P<0.05$; Figure 3a). Histological features also confirmed only a few eosinophils to be present in the dermis after OVA sensitization in MIF KO mice (Figure 3b).

The expression of eotaxin and Th2-type cytokines increased in the OVA-sensitized skin of MIF Tg mice, but decreased in the OVA-sensitized skin in MIF KO mice

We next examined the expression of mRNAs for eotaxin and cytokines in OVA-sensitized skin specimens from MIF Tg, MIF KO, and WT mice. The expression levels of eotaxin and Th2-type cytokines, especially IL-5, were increased in the OVA-sensitized skin of MIF Tg mice compared with WT mice. However, IFN-γ, a Th1-type cytokine, did not differ between MIF Tg and WT mice. Conversely, low eotaxin mRNA expression was observed in the OVA-sensitized skin of MIF KO mice compared with WT mice. Similarly, the mRNA expression of the Th2-type cytokines, including IL-4,