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Spontaneous Remission of Solitary-Type Infantile Myofibromatosis

Kazuhiro Kikuchi^a Riichiro Abe^a Satoru Shinkuma^a
Erika Hamasaka^a Ken Natsuga^a Hiroo Hata^a
Yasuki Tateishi^a Masahiko Shibata^a Yuki Tomita^a
Yukiko Abe^a Satoru Aoyagi^a Makio Mukai^b
Hiroshi Shimizu^a

^aDepartment of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, and ^bDepartment of Pathology, Keio University School of Medicine, Tokyo, Japan

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].

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.

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.

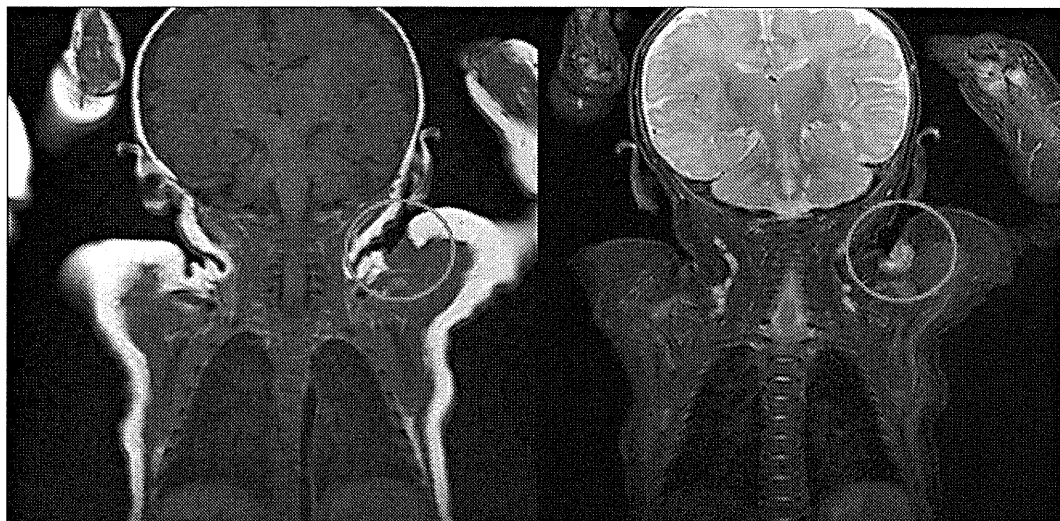


Fig. 2. MRI imaging showed the intensity of the nodule was similar to that of muscle. No additional lesions were found and infiltration of the tumor into the muscle was not observed.

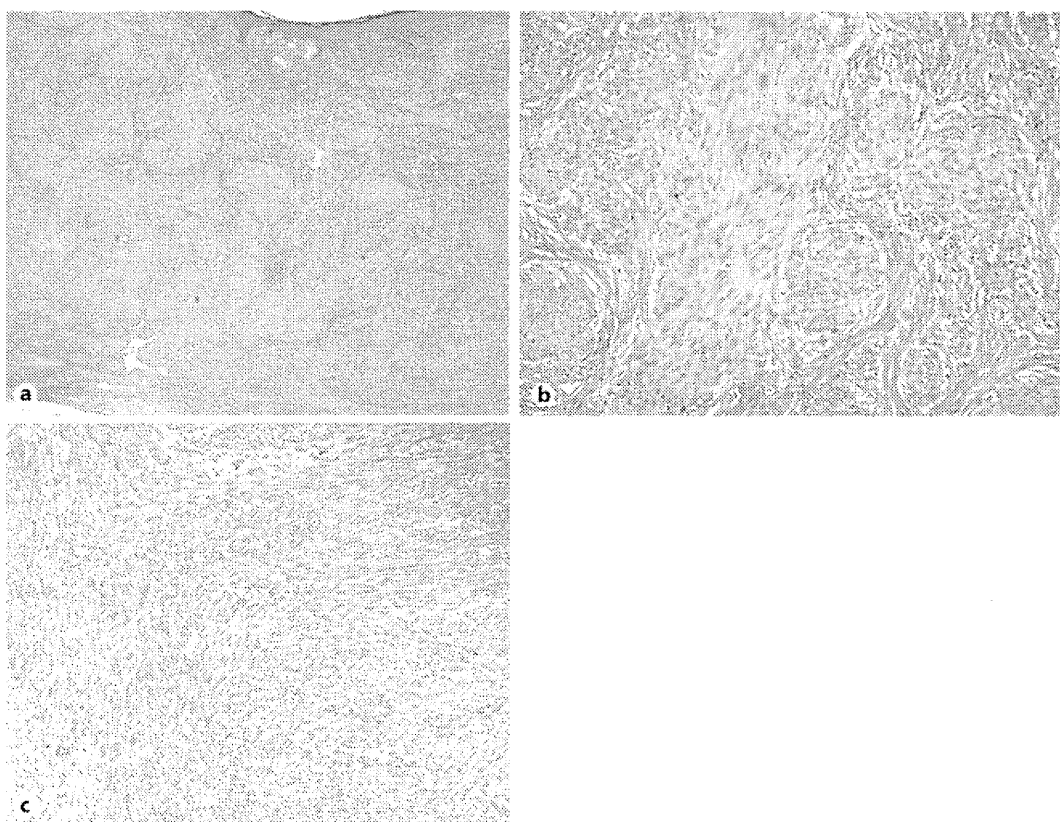


Fig. 3. Hematoxylin-eosin stain, original magnification $\times 20$ (**a**), and $\times 100$ (**b**). Specimen showed multifocal sclerotic dermal nodules composed of spindle cells and eosinophilic fibers. **c** Immunological staining of the tumor for α -smooth muscle actin ($\times 100$). Spindle cells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

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ORIGINAL ARTICLE

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

Yasuyuki Fujita, MD,^a Naoya Yoshioka, MS,^a Riichiro Abe, MD, PhD,^a Junko Murata, MD, PhD,^a
Daichi Hoshina, MD,^a Hirokatsu Mae, MS,^b and Hiroshi Shimizu, MD, PhD^a
Sapporo, Japan

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 10.1016/j.jaad.2010.04.042.)

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

Abbreviations used:

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

From the Department of Dermatology, Hokkaido University Graduate School of Medicine,^a and Sapporo Immuno Diagnostic Laboratory.^b

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Reprint requests: Riichiro Abe, MD, PhD, Department of Dermatology, Hokkaido University Graduate School of Medicine, N15 W7, Kita-ku, Sapporo 060-8638, Japan. E-mail: aberi@med.hokudai.ac.jp.

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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

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 al⁴ 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.

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).

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.

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 \pm 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).

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

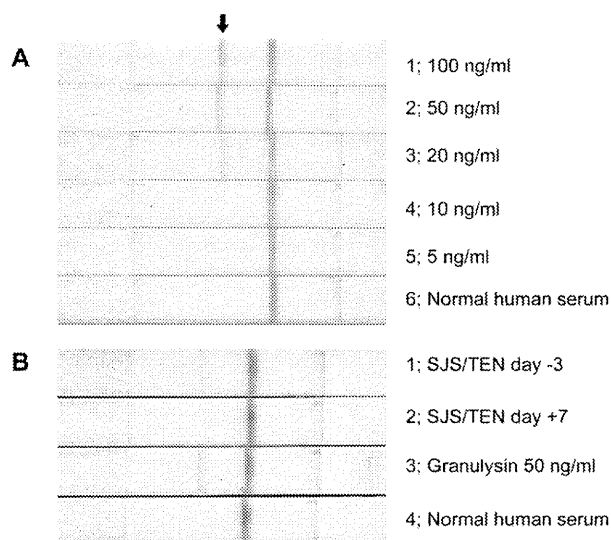


Fig 1. A, Immunochromatographic test strip detects elevated granulysin. 1 to 5, Diluted recombinant granulysin is applied. 6, Normal human serum as negative control (1.4 ng/mL). Positive results are shown as a band (indicated by the arrow). Approximately 10 ng/mL of granulysin is considered a positive 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).

apoptosis of target cells in a mechanism involving caspases and other pathways.¹¹ Chung et al⁴ 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

Yoko Yoshihisa^{1,3}, Teruhiko Makino^{1,3}, Kenji Matsunaga¹, Ayumi Honda², Osamu Norisugi¹, Riichiro Abe², Hiroshi Shimizu² and Tadamichi Shimizu¹

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

¹Department of Dermatology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Sugitani, Toyama, Japan and

²Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

³These two authors contributed equally to this work.

Correspondence: Teruhiko Makino or Tadamichi Shimizu, Department of Dermatology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Sugitani, Toyama 930-0194, Japan.
E-mail: tmakino@med.u-toyama.ac.jp or shimizut@med.u-toyama.ac.jp

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.

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

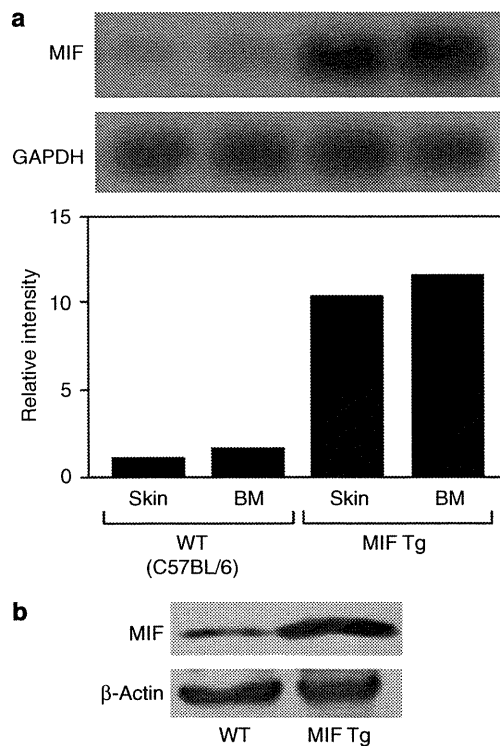


Figure 1. Expression of macrophage migration inhibitory factor (MIF) in tissues from MIF transgenic (Tg) mice. (a) Bone marrow (BM) and skin specimens were harvested from MIF Tg and wild-type (WT) mice, and the total RNA levels were determined by northern blot analysis as described in the Materials and 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.

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,

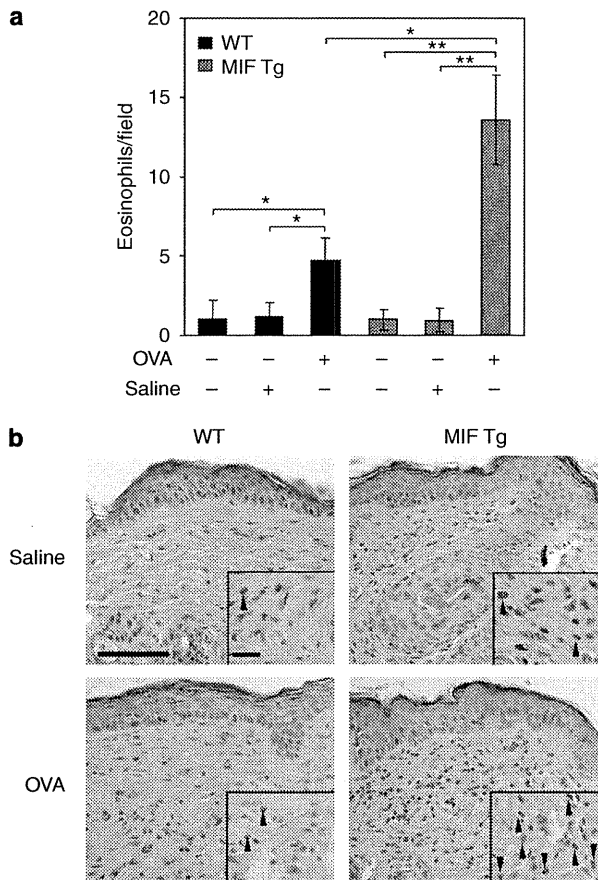


Figure 2. Eosinophil infiltration into ovalbumin (OVA)-sensitized skin sites of macrophage migration inhibitory factor (MIF) transgenic (Tg) mice. (a) The number of eosinophils in OVA-sensitized skin sites of MIF Tg mice was compared with the wild-type (WT) mice. Each value represents the mean \pm SD ($n=5$; * $P<0.001$, ** $P<0.0001$). (b) Histological features of OVA-sensitized skin sites in MIF Tg mice and WT mice. Scale bar for large panels = 50 μ m; scale bar for small panels = 10 μ m; hematoxylin and eosin section. Arrowheads point to eosinophils. The experiments were repeated three times and similar results were obtained.

IL-5, and IL-13, were low in the OVA-sensitized skin of MIF KO mice compared with WT mice (Figure 4).

The expression and production of eotaxin in cultured fibroblasts from MIF Tg mice and from MIF KO mice

To clarify the role of MIF in the expression of eotaxin, we performed *in vitro* experiments. A previous report described that IL-4 could dose-dependently induce the expression of eotaxin mRNA in dermal fibroblasts from humans and mice (Mochizuki *et al.*, 1998). Using this protocol, we analyzed the eotaxin expression in cultured fibroblasts from MIF Tg, MIF KO, and WT mice by stimulating them with IL-4. Unstimulated fibroblasts from these mice barely expressed eotaxin mRNA. However, fibroblasts from MIF Tg mice showed dramatically increased eotaxin mRNA after stimulation with 5 ng ml⁻¹ of IL-4 (Figure 5a). To evaluate whether there was an accompanying change in eotaxin protein production, the amount of eotaxin in fibroblast supernatants was also analyzed. Eotaxin proteins in

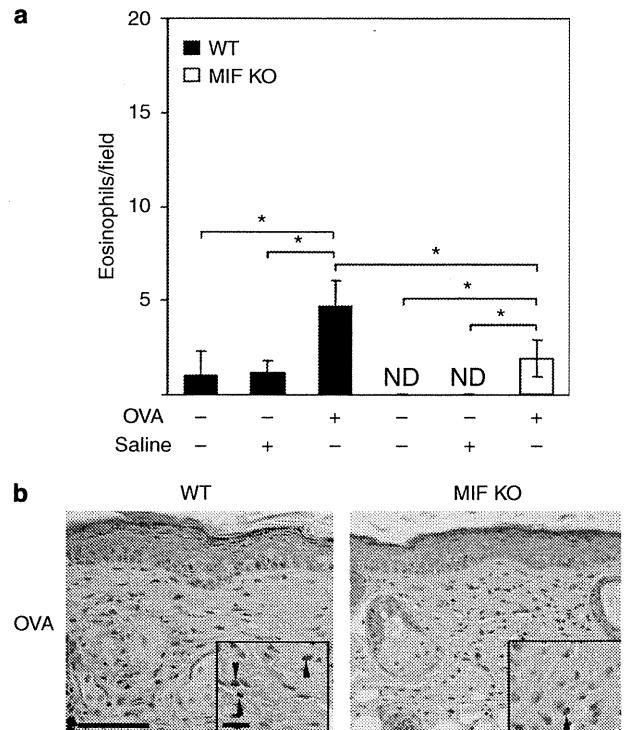


Figure 3. Eosinophil infiltration induced in ovalbumin (OVA)-sensitized skin sites of macrophage migration inhibitory factor (MIF) knockout (KO) mice. (a) The number of eosinophils in OVA-sensitized skin sites of MIF KO mice was compared with wild-type (WT) mice. Each value represents the mean \pm SD ($n=5$, * $P<0.05$). (b) Histological features of OVA-sensitized skin sites in MIF KO and WT mice. Scale bar for large panels = 50 μ m; scale bar for small panels = 10 μ m; hematoxylin and eosin section. Arrowheads point to eosinophils. The experiments were repeated three times and similar results were obtained each time.

the culture supernatant of fibroblasts from MIF Tg mice were also significantly increased compared with those from WT mice (* $P<0.005$). However, fibroblasts from MIF KO mice showed minimal expression of eotaxin mRNA even when stimulated with 10 ng ml⁻¹ of IL-4. Eotaxin production in the culture supernatant of fibroblasts from MIF KO mice was barely detectable (Figure 5b).

Recombinant MIF restored the expression and production of eotaxin in dermal fibroblasts from MIF KO mice

In dermal fibroblasts from WT mice, stimulation with IL-4 significantly induced the expression of eotaxin mRNA compared with unstimulated fibroblasts (Figure 6a). Addition of recombinant MIF significantly enhanced this increase in eotaxin expression. This suggests that the eotaxin expression in dermal fibroblasts from MIF Tg mice was markedly increased by IL-4 stimulation. A significant amount of eotaxin was also produced by combined stimulation with IL-4 (* $P<0.005$, ** $P<0.05$; Figure 6b). Although the fibroblasts from MIF KO mice showed minimal induction of eotaxin mRNA expression in response to stimulation with IL-4, both the expression of eotaxin mRNA and the production of eotaxin protein were restored by addition of recombinant MIF

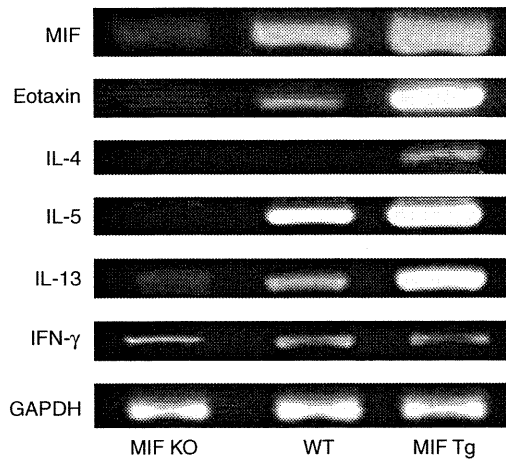


Figure 4. Expression levels of eotaxin and Th2-type cytokines in ovalbumin (OVA)-sensitized skin from macrophage migration inhibitory factor (MIF) transgenic (Tg) mice and MIF knockout (KO) mice. Reverse transcriptase-PCR analyses of eotaxin, IL-4, IL-5, IL-13, and IFN- γ levels in skin sites of MIF Tg and WT mice sensitized with OVA were performed. Eotaxin, IL-4, IL-5, and IL-13 mRNA expression levels were increased in OVA-sensitized MIF Tg; however, both eotaxin and Th2-type cytokines were markedly decreased in OVA-sensitized MIF KO mice, compared with WT mice. The experiments were repeated three times and similar results were obtained. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(Figure 6a and b). The levels of eotaxin production in MIF KO mouse fibroblasts exposed to MIF were similar to the levels in WT fibroblasts stimulated with IL-4 (Figure 6b).

DISCUSSION

There is growing evidence that the eosinophil is an important effector cell in allergic inflammatory diseases, such as asthma and AD. Accumulation of eosinophils in the skin is characteristic of inflammation associated with AD (Leiferman, 1989; Kapp, 1995). This study explored, for the first time, the significant increase in eosinophil infiltration in the skin of MIF Tg mice after OVA sensitization, compared with WT mice. However, in MIF KO mice, eosinophils failed to infiltrate the skin after repeated epicutaneous sensitization with OVA. Eosinophils accumulate at inflammatory sites and release numerous mediators capable of initiating and maintaining allergic inflammation. Yamaguchi *et al.* (2000) reported eosinophils to be an important source of MIF in allergic inflammatory diseases. The number of eosinophils was reported to be significantly decreased in lung tissue and in bronchoalveolar lavage fluid from MIF KO mice after stimulation with OVA, compared with those from WT mice (Mizue *et al.*, 2005; Magalhães *et al.*, 2007; Wang *et al.*, 2009). In an allergic rhinitis model, eosinophil recruitment into the nasal submucosa was also suppressed in MIF KO mice (Nakamaru *et al.*, 2005). Consistent with these findings, our current evidence indicates that MIF is essential for the infiltration of eosinophils into the OVA-sensitized skin.

This study also demonstrated that the expression of both eotaxin and IL-5 is markedly increased in the OVA-sensitized

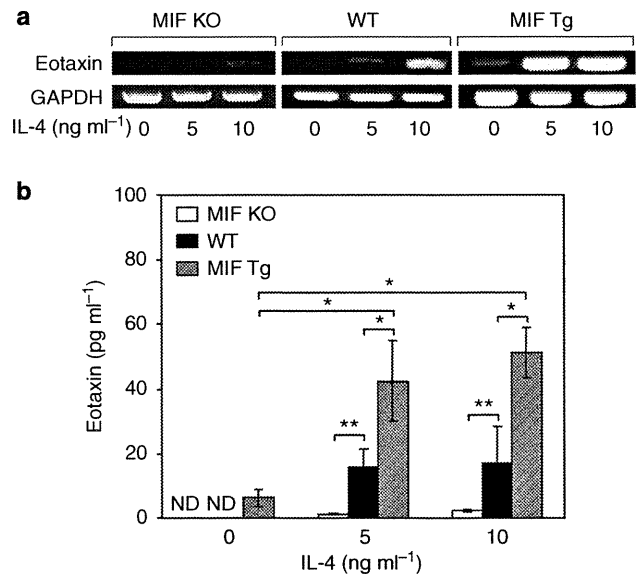


Figure 5. IL-4 induced eotaxin expression and production by fibroblasts from macrophage migration inhibitory factor (MIF) transgenic (Tg) and MIF knockout (KO) mice. Fibroblasts from MIF KO, MIF Tg, and wild-type (WT) mice were stimulated with IL-4 (5 or 10 ng ml⁻¹) for 24 hours. (a) RNA was extracted from the cells and the abundance of eotaxin mRNA was evaluated by reverse transcriptase-PCR. Data are from a representative experiment that was repeated three times and yielded similar results. (b) The eotaxin content of cultured supernatants was analyzed for eotaxin by ELISA. Each value represents the mean \pm SD of five specimens. * P <0.005, ** P <0.05. ND, not detected.

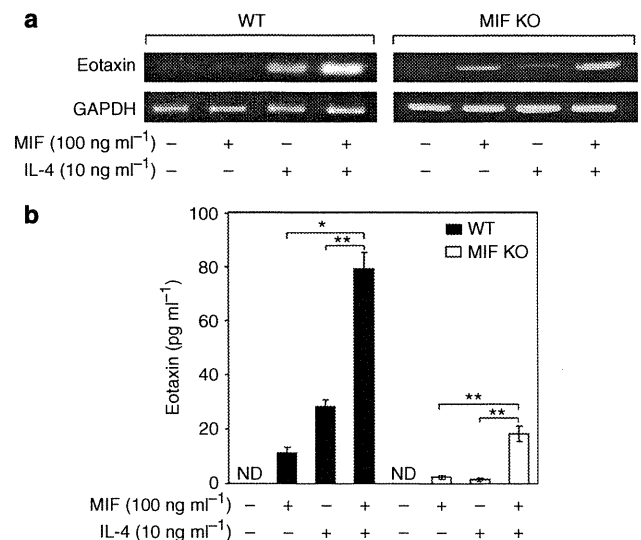


Figure 6. Recombinant macrophage migration inhibitory factor (MIF) restored eotaxin expression and production by IL-4 stimulation in dermal fibroblasts from MIF knockout (KO) mice. The fibroblasts were stimulated with IL-4 (10 ng ml⁻¹), MIF (100 ng ml⁻¹), or both IL-4 and MIF for 24 hours. (a) RNA was extracted from cells, and the abundance of eotaxin mRNA was evaluated by reverse transcriptase-PCR. Data are from a representative experiment that was repeated three times showing similar results. (b) The eotaxin contents of cultured supernatants were analyzed for eotaxin by ELISA. Each value represents the mean \pm SD of six specimens. * P <0.005, ** P <0.05. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ND, not detected.

skin sites of MIF Tg mice skin. The other Th2-type cytokines, IL-4 and IL-13, were also slightly increased in MIF Tg mice. On the other hand, the expression levels of eotaxin and Th2-type cytokines were markedly decreased in the OVA-sensitized skin sites of MIF KO mice. Acute AD involves a systemic Th2 response with eosinophilia, and marked infiltration of Th2 cells into skin lesions. These infiltrating T cells predominantly express IL-4, IL-5, and IL-13. Furthermore, the roles of cytokines in the induction of migration and the accumulation of eosinophils into an inflamed tissue have been extensively studied in recent years. Some of the important eosinophil chemoattractant cytokines include IL-5, IL-8, eotaxin, RANTES (regulated on activation, normal T cell expressed and secreted), and monocyte chemoattractant protein-3 (Lampinen *et al.*, 2004). Among these, eotaxin (CC chemokine ligand-11) is one of the most important eosinophil-selective chemoattractants (Jose *et al.*, 1994; Garcia-Zepeda *et al.*, 1996). Eotaxin is secreted by several cell types: epithelial cells, fibroblasts, and activated infiltrating leukocytes such as eosinophils (Garcia-Zepeda *et al.*, 1996; Ponath *et al.*, 1996; Ugucioni *et al.*, 1996). Eotaxin is reportedly related to the eosinophilia in allergic diseases, including AD and asthma (Ying *et al.*, 1997; Yawalkar *et al.*, 1999). IL-5 also has an important role in eosinophil development and differentiation (Sanderson, 1992). IL-5 KO mice had virtually no eosinophils in either saline-sensitized skin or in OVA-sensitized skin (Spergel *et al.*, 1999). Recently, Magalhães *et al.* (2009) reported that MIF was involved in IL-5-driven maturation of eosinophils and in tissue eosinophilia associated with *Schistosoma mansoni* infection. In addition, several earlier studies demonstrated that MIF KO mice failed to develop tissue eosinophilia, and that eotaxin, IL-4, and IL-5 were not induced in either allergic lung tissues or bronchoalveolar lavage fluid (Mizue *et al.*, 2005; Wang *et al.*, 2006). Accordingly, our results suggest that MIF is important in regulating both eotaxin and IL-5 in OVA-sensitized inflamed skin tissue.

In support of these *in vivo* observations, this study demonstrated that the expression of eotaxin was significantly increased after stimulation with IL-4 in fibroblasts from MIF Tg mice compared with WT fibroblasts, but not in fibroblasts from MIF KO mice. However, eotaxin expression in fibroblasts from MIF KO mice was restored by addition of recombinant MIF. These observations suggest that MIF is crucial to the expression of eotaxin, and antigen-induced eosinophil infiltration is suspected to be induced by eotaxin mainly by MIF, in addition with IL-5 production involved in MIF. Previous observations have shown that either IL-4 or IL-13 can increase eotaxin expression, and that they function synergistically with proinflammatory cytokines, such as tumor necrosis factor- α , to increase the production of eotaxin in epithelial cells and fibroblasts (Mochizuki *et al.*, 1998; Nakamura *et al.*, 1998; Li *et al.*, 1999; Stellato *et al.*, 1999; Fujisawa *et al.*, 2000; Terada *et al.*, 2000). Increases in both IL-4 and IL-13 in the inflamed skin of MIF Tg mice might involve enhancing the tissue eosinophilia. Furthermore, tumor necrosis factor- α secretion induced by MIF also has the ability to increase eotaxin expression in MIF Tg mice, on

the basis of the known capacity of MIF to trigger the secretion of several inflammatory cytokines, including tumor necrosis factor- α (Donnelly *et al.*, 1997). It was recently elucidated that MIF activates an extracellular signal-regulated kinase-1/2-mitogen-activated protein kinase signaling through its receptor CD74 (Leng *et al.*, 2003) and c-Jun N-terminus kinase-mitogen-activated protein kinase signaling through CD74/CXCR4 (Lue *et al.*, 2011), in addition to the endocytic pathway described previously (Kleemann *et al.*, 2000); however, the receptor-mediated mechanism involved in MIF-mediated IL-4-induced eotaxin release is unclear. This mechanism should therefore be an important focus of research in association with MIF-mediated skin allergy.

Finally, we suggest that the inhibition of MIF might be an effective treatment for AD, suppressing both eosinophil infiltration and eotaxin expression in the skin. We recently demonstrated that in murine models of AD, MIF-DNA vaccination elicited the production of endogenous anti-MIF antibodies, producing rapid improvement of AD skin manifestations (Hamasaka *et al.*, 2009). Our previous data and the current findings therefore hold promise for the development of MIF inhibitors as a therapeutic strategy for allergic diseases.

MATERIALS AND METHODS

Materials

The following materials were obtained from commercial sources: a mouse eotaxin-specific ELISA kit from Genzyme TECHNE (Cambridge, MA); Isogen RNA extraction kit from Nippon Gene (Tokyo, Japan); M-MLV reverse transcriptase from GIBCO (Grand Island, NY); Taq DNA polymerase from Perkin-Elmer (Norwalk, CT); nylon membranes from Schleicher & Schuell (Keene, NH); Ficoll-Plaque Plus and Protein A Sepharose from Pharmacia (Uppsala, Sweden); recombinant mouse IL-4 from R&D systems (Minneapolis, MN). Recombinant rat MIF (this recombinant MIF crossreacts with that of mice) was expressed in *Escherichia coli* BL21/DE3 (Novagen, Madison, WI) and was purified as described previously (Shimizu *et al.*, 2004). All other chemicals were of analytical grade.

Mice

The MIF-overexpressing Tg mice were established after complementary DNA microinjection. Physical and biochemical characteristics, including body weight, blood pressure, and serum cholesterol and blood sugar levels, were normal, as reported previously (Sasaki *et al.*, 2004). The transgene expression was regulated by a hybrid promoter composed of the cytomegalovirus enhancer and the β -actin/ β -globin promoter, as reported previously (Akagi *et al.*, 1997). The strain of the original MIF Tg mice was ICR, which were backcrossed with C57BL/6 for at least 10 generations. Tg mice were maintained by heterozygous sibling mating. Aged MIF Tg mice of 12 months or older developed neither skin allergies nor diseases. The MIF-deficient (KO) mice were established by targeted disruption of the *MIF* gene as described previously (Honma *et al.*, 2000), using a mouse strain bred onto a C57BL/6 background. MIF Tg, MIF KO, and WT mice were maintained under specific-pathogen-free conditions at the Institute for Animal Experiments of the Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama. All experiments were performed on 8-week-old female adult mice.

Epicutaneous sensitization

Epicutaneous sensitization of mice was performed as described previously (Spergel *et al.*, 1998). Briefly, each mouse was anesthetized with 10% nembutal (Hospira, Osaka, Japan), then shaved with a razor. One hundred mg of OVA (Sigma, St Louis, MO) in 100 μ l of normal saline were placed on a 1 \times 1 cm patch (Alcare, Tokyo, Japan), which was secured to the skin with a transparent bio-occlusive dressing (ALCARE). The patch was left in place for 1 week and then removed. At the end of the second week, an identical patch was reapplied to the same skin site. Each mouse had a total of three 1-week exposures to the patch, separated from each other by 2-week intervals. Inspection confirmed that the patch was still in place at the end of each sensitization period. Skin biopsies from treated areas were obtained for RNA isolation and histological evaluation. Six-micrometer thick skin sections were stained with hematoxylin and eosin (H&E). Eosinophils were counted under a microscope at a magnification of \times 400 and expressed as the mean number of the cells in five random fields (one section per mouse, five mice per group).

Northern blot analysis

Bone marrow cells were isolated from the femurs of MIF Tg or WT mice, and 1×10^6 cells ml^{-1} was collected. Total RNA was isolated from bone marrow cells and skin from mice using an Isogen RNA extraction kit according to the manufacturer's protocols. Twenty μ g of RNA from control and test samples were loaded onto a formaldehyde-agarose gel and the RNA was transferred onto a nylon membrane. RNA fragments obtained by restriction enzyme treatment for MIF and glyceraldehyde-3-phosphate dehydrogenase were labeled with [α - 32 P]deoxycytidine triphosphate using a DNA random primer labeling kit (Enzo Life Sciences International, Farmingdale, NY). Hybridization was carried out at 42 $^{\circ}$ C for 24–48 hours. Post-hybridization washing was performed in 0.1% SDS with $0.2 \times$ standard saline citrate ($1 \times$ standard saline citrate: 0.15 M NaCl, 0.015 M sodium citrate) at 65 $^{\circ}$ C for 15 minutes. The radioactive bands were visualized by autoradiography on Kodak X-AR5 film (Tokyo, Japan) and quantitatively analyzed using the NIH Image system (Bethesda, MD). The results were normalized by compensating for the glyceraldehyde-3-phosphate dehydrogenase mRNA levels.

Reverse transcription-PCR analysis

Total RNA was extracted from each mouse skin specimen. RNA reverse transcription was performed with M-MLV reverse transcriptase using random hexamer primers and subsequent amplification using Taq DNA polymerase. PCR was carried out for 35–40 cycles with denaturation at 94 $^{\circ}$ C for 30 seconds, annealing from 46 to 64 $^{\circ}$ C for 1 minute and extension at 72 $^{\circ}$ C for 45 seconds using a thermal cycler (PE Applied Biosystems Gene Amp PCR system 9700, Life Technologies Japan, Tokyo, Japan). The primers used in this study are described in Supplementary Table S1 online. After PCR, the amplified products were analyzed by 2% agarose gel electrophoresis.

Western blot analysis

The epidermis of each mouse was homogenized with a Polytron homogenizer (Kinematica, Lausanne, Switzerland). The protein concentrations of the cell homogenates were quantified using a Micro BCA protein assay reagent kit (Thermo Fisher Scientific,

Yokohama, Japan). Equal amounts of homogenates were dissolved in 20 μ l of Tris-HCL, 50 mM (pH 6.8), containing 2-mercaptoethanol (1%), SDS (2%), glycerol (20%) and bromophenol blue (0.04%), and then were heated to 100 $^{\circ}$ C for 5 minutes. The samples were then subjected to SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The membranes were blocked with 2.5% non-fat dry milk powder in phosphate-buffered saline, probed with antibodies against MIF (Shimizu *et al.*, 1996) and subsequently reacted with secondary IgG antibodies coupled with horseradish peroxidase. The resultant complexes were processed for the ECL detection system (Amersham Biosciences, Buckinghamshire, UK). The relative amounts of proteins associated with specific antibodies were normalized according to the intensities of β -actin (Sigma).

Cell culture

Skin specimens were obtained from the dorsal surfaces of newborn MIF Tg, MIF KO, and WT mice. The skin specimens were cut into 3–5 mm pieces and placed on a large Petri dish with the subcutaneous side down, followed by tissue incubation for 1 week in a humidified atmosphere of 5% CO_2 at 37 $^{\circ}$ C. Once sufficient numbers of fibroblasts had migrated out of the skin sections, pieces of the skin were removed and the cells were passaged by trypsin digestion in the same manner as wound-harvested fibroblasts. Fibroblasts were grown in DMEM containing 10% fetal calf serum and 1% penicillin/streptomycin. After 3 passages, the fibroblasts were used for the experiments. The fibroblasts from MIF KO and WT mice were stimulated with MIF (100 ng ml^{-1}), IL-4 (10 ng ml^{-1}), or MIF (100 ng ml^{-1}) in combination with IL-4 (10 ng ml^{-1}) for 24 hours. We also stimulated the fibroblasts from MIF Tg, MIF KO, and WT mice with IL-4 (5 or 10 ng ml^{-1}) alone for 24 hours. The cells were analyzed using reverse transcriptase-PCR. Culture supernatants were analyzed for eotaxin by ELISA.

Statistical analysis

Values are expressed as the means \pm SD of the respective test or control group. The statistical significance of differences between the control and test groups was evaluated by either Student's *t*-test or one-way analysis of variance.

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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Generalized Vitiligo and Associated Autoimmune Diseases in Japanese Patients and Their Families

Tomohiko Narita¹, Naoki Oiso¹, Kazuyoshi Fukai², Kenji Kabashima³, Akira Kawada¹ and Tamio Suzuki⁴

ABSTRACT

Background: Generalized vitiligo is an acquired disorder in which depigmented macules result from the autoimmune loss of melanocytes from the involved regions of skin. Generalized vitiligo is frequently associated with other autoimmune diseases, particularly autoimmune thyroid diseases (Hashimoto's thyroiditis and Graves' disease), rheumatoid arthritis, adult-onset type 1 diabetes mellitus, psoriasis, pernicious anemia, systemic lupus erythematosus, and Addison's disease.

Methods: One hundred and thirty-three Japanese patients with generalized vitiligo were enrolled in this study to investigate the occurrence of autoimmune diseases in Japanese patients with generalized vitiligo and their families.

Results: Twenty-seven of the patients with generalized vitiligo (20.3%) had autoimmune diseases, particularly autoimmune thyroid disease (sixteen patients, 12%) and alopecia areata (seven patients, 5.3%). Thirty-five patients (26.3%) had a family history of generalized vitiligo and/or other autoimmune diseases. Familial generalized vitiligo was present in fifteen (11.3%), including four families with members affected by autoimmune disorders. Twenty (15.0%) had one or more family members with only autoimmune disorders.

Conclusions: Among Japanese vitiligo patients, there is a subgroup with strong evidence of genetically determined susceptibility to not only vitiligo, but also to autoimmune thyroid disease and other autoimmune disorders.

KEY WORDS

alopecia areata, autoimmune disease, autoimmune thyroid disease, generalized vitiligo, rheumatoid arthritis

INTRODUCTION

Generalized vitiligo is an acquired disorder characterized by progressive, multifocal patches of depigmented skin, overlying hair, and mucous membranes resulting from the autoimmune loss of melanocytes in the involved areas. Generalized vitiligo is the most common hypopigmentation disorder, with a prevalence of approximately 0.4% in most populations,¹⁻³ though in the Chinese population, it is lower, approximately 0.093%.⁴ Studies of generalized vitiligo patients from a number of populations around the world have shown a strong epidemiological association with several other autoimmune diseases, particularly auto-

immune thyroid disease, rheumatoid arthritis, adult-onset type 1 diabetes mellitus, psoriasis, pernicious anemia, systemic lupus erythematosus (SLE), and Addison's disease.⁵⁻¹⁸ The most prevalent autoimmune disease in patients with generalized vitiligo is autoimmune thyroid disease (Hashimoto's thyroiditis and Graves' disease), with a reported overall frequency of 19.4% in Caucasian patients,³ 7.4% in Japanese patients,¹³ and 2.4-5.8% in Chinese patients.^{14,16} In the Caucasian population, family members are predisposed to vitiligo itself, an autoimmune thyroid disease, pernicious anemia, Addison's disease, SLE, and probably inflammatory bowel disease.¹⁹ In Japan, it is still uncertain whether Japanese family members

¹Department of Dermatology, Kinki University Faculty of Medicine, ²Department of Dermatology, Osaka City University Graduate School of Medicine, Osaka, ³Department of Dermatology, University of Environmental and Occupational Health, Fukuoka and ⁴Department of Dermatology, Yamagata University School of Medicine, Yamagata, Japan.
Correspondence: Naoki Oiso, Department of Dermatology, Kinki

University Faculty of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan.
Email: naoiso@med.kindai.ac.jp

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have a similar tendency, although affected persons have a predisposed relationship between generalized vitiligo and autoimmune disorders.¹³ The purpose of this investigation was to study the occurrence of concomitant autoimmune diseases in Japanese patients with generalized vitiligo and their families.

METHODS

The study protocol was approved by each institution's ethics committee, and signed informed consent was obtained from each subject. One hundred and thirty-three Japanese patients were enrolled in this study. They were diagnosed with generalized vitiligo at the Dermatology Clinic of Kinki University, Osaka City University, and Yamagata University.

The diagnosis of generalized vitiligo was determined by board-certified dermatologists (N.O., F.K., and T.S.) using standard diagnostic criteria; patients with segmental and localized types of vitiligo were excluded. Subjects were questioned about the age at onset, the duration of the disease, and the personal and familial medical history of generalized vitiligo, autoimmune thyroid diseases, rheumatoid arthritis, type 1 diabetes mellitus, psoriasis, pernicious anemia, SLE, Addison's disease, alopecia areata, and other autoimmune diseases. Multi-specialist medical evaluations were carried out to confirm the diagnoses in some patients and family members.

RESULTS

The characteristics of the one hundred and thirty-three patients are summarized in Table 1. Of these patients, twenty-seven (20.3%) had been diagnosed with other autoimmune disorders (Table 2). Sixteen (12%) had autoimmune thyroid disease, including Hashimoto's thyroiditis in ten (7.5%) and Graves' disease in six (4.5%). Three (two males and a female) had preceding Sutton's nevus. Fifteen (11.3%) reported a positive family history of generalized vitiligo (Table 3), including four families with members affected by autoimmune disorders. Seven (5.3%) had at least one first-degree relative (parent or child) with vitiligo, and four of these (3.0%) had at least one affected sibling. Twenty (15.0%) had a family history of only autoimmune disorders (Table 3).

DISCUSSION

We assessed the occurrence of autoimmune diseases in one hundred and thirty-three Japanese patients with generalized vitiligo. Generalized vitiligo is associated with higher prevalence of other autoimmune diseases in both the patients and their close relatives.^{8,11,13-18} In the present study, twenty-seven patients (20.3%) with generalized vitiligo had other autoimmune disorders. These results are similar to those for other populations,^{5-12,15} including the Chinese and Japanese populations.^{13,14,16} Seven patients (5.3%) also had alopecia areata, a ratio that is similar to that

Table 1 Demographics of 133 Japanese generalized vitiligo patients

	Patients
Mean age \pm SD (years)	49.3 \pm 19.8
Age range (years)	3-89
Gender (male : female)	57 : 76
Age (years)	
<20	20
20-59	57
\geq 60	56
Mean age at onset \pm SD (years)	41.2 \pm 20.9
Range of ages at onset (years)	3-88
Age at onset (years)	
<20	29
20-59	63
\geq 60	41
Mean duration \pm SD (years)	8.2 \pm 8.6
Duration range (years)	0-63

for generalized vitiligo patients in the African,²⁰ Indian,²¹ and Chinese populations,^{14,22} though not in the Caucasian population.⁸ Thirty-five patients (26.3%) had a positive family history of generalized vitiligo or other autoimmune diseases, which is again similar to the ratio reported in other populations,^{8,23} suggesting that these disorders involve shared susceptibility genes, although the specific genes and variants may differ among the populations.²⁴⁻²⁸

Recent genomewide association studies have identified at least seventeen confirmed vitiligo susceptibility genes.²⁴⁻²⁸ Many of these genes have also been implicated in other autoimmune diseases, particularly those that are epidemiologically associated with generalized vitiligo, such as autoimmune thyroid disease, rheumatoid arthritis, type 1 diabetes mellitus, and others. These shared immune related disease susceptibility genes may underlie our observed associations in patient with generalized vitiligo and their close relatives.

Hashimoto's thyroiditis is a fairly common disease in Japanese generalized vitiligo patients as shown in this study. This disease is characterized by the destruction of thyroid cells by various cell- and antibody-mediated immune processes.²⁹ However, the initiating process is not well understood to date, because it includes various environmental factors and inflammatory events. Recent epidermological findings⁵⁻¹⁸ including ours and genomewide association studies²⁴⁻²⁸ support the long-standing hypothesis that generalized vitiligo involves genetic susceptibility loci shared with other autoimmune diseases.³⁰ Similar to autoimmune thyroid disease, generalized vitiligo is now believed to be caused by the damage of melanocytes by various cell- and antibody-mediated immune mechanisms. Nevertheless, the initiating process has

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Table 2 Autoimmune diseases in patients with generalized vitiligo

	Number of patients			Number of patients		
	total	male	female	total	male	female
Autoimmune thyroid disease	16	5	11			
Hashimoto's thyroiditis				10	2	8
Graves' disease				6	3	3
Alopecia areata	7	3	4			
Psoriasis	2	2	0			
Pernicious anemia	1	1	0			
Adult-onset type 1 diabetes mellitus	(1†)	(1†)	0			
Acrodermatitis continua of Hallopeau	1	1	0			
Total	27	12	15			

†The patient also had Graves' disease.

Table 3 Occurrence of autoimmune diseases in families of generalized vitiligo

	Number of families	Number of families
Positive family history of generalized vitiligo	15	
Family member(s) with only generalized vitiligo	11	
Family member(s) with generalized vitiligo and other family member(s) with rheumatoid arthritis	3	
A family member with generalized vitiligo and another family member with alopecia areata	1	
Positive family history of only autoimmune disorders	20	
Autoimmune thyroid disease	13	
Hashimoto's thyroiditis		5
Graves' disease		5
Unknown		3
Alopecia areata	4	
Rheumatoid arthritis	1	
Psoriasis	1	
Adult-onset type 1 diabetes mellitus	1	
Total	35	

not well been elucidated. Further study is needed to identify the initiating factors inducing generalized vitiligo, autoimmune thyroid disease and other autoimmune disorders.

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CONFLICT OF INTEREST

No potential conflict of interest was disclosed.

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