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References

1 Stout AP: Juvenile fibromatosis. Cancer 1954;7:953–978.

2 Chung EB, Enzinger FM: Infantile myofibromatosis. Cancer 1981;48:1807–1818.

3 Larralde M, Hoffner MV, Boggio P, Abad ME, Luna PC, Correa N: Infantile myofibromatosis: report of nine patients. Pediatr Dermatol 2010;27:29–33.

4 Stanford D, Rogers M: Dermatological presentations of infantile myofibromatosis: a review of 27 cases. Australas J Dermatol 2000;41:156–161.

5 Goldberg NS, Bauer BS, Kraus H, Crussi FG, Esterly NB: Infantile myofibromatosis: a review of clinicopathology with perspectives on new treatment choices. Pediatr Dermatol 1988;5:37–46.

6 Hogan SF, Salassa JR: Recurrent adult myofibromatosis: a case report. Am J Clin Pathol 1992;97:810–814.

7 Wiswell TE, Sakas EL, Stephenson SR, Lesica JJ, Reddoch SR: Infantile myofibromatosis. Pediatrics 1985;76:981–984.

8 Ang P, Tay Y, Walford Y: Infantile myofibromatosis: a case report and review of the literature. Cutis 2004;73:229–231.

9 Dictor M, Elner A, Andersson T, Fernö M: Myofibromatosis-like hemangiopericytoma metastasizing as differentiated vascular smooth-muscle and myosarcoma. Am J Surg Pathol 1992;16:1239–1247.

10 Granter SR, Badizadegan K, Fletcher CD: Myofibromatosis in adults, glomangiopericytoma, and myopericytoma: a spectrum of tumors showing perivascular myoid differentiation. Am J Surg Pathol 1998;22:513–525.

11 Weedon D: Tumors and tumor-like proliferations; in Weedon D (ed): Skin Pathology, ed 2. Philadelphia, Churchill Livingstone, 2002, pp 767–768.

12 Variend S, Bax NMA, van Gorp J: Are infantile myofibromatosis, congenital fibrosarcoma and congenital hemangiopericytoma histogenetically related? Histopathology 1995;26:57–62.

13 Fields JP, Helwig EB: Leiomyosarcoma of the skin and subcutaneous tissue. Cancer 1981;47:156–169.

14 Staut AP, Hill WT: Leiomyosarcoma of the superficial soft tissues. Cancer 1958;11:844–854.

15 Toren A, Perlman M, Polak-Charcon S, Avigad I, Katz M, Kuint Y, Rechavi G: Congenital hemangiopericytoma/infantile myofibromatosis: radical surgery versus a conservative 'wait and see' approach. Pediatr Hematol Oncol 1997;14:387–393.

ORIGINAL ARTICLE

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 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 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).

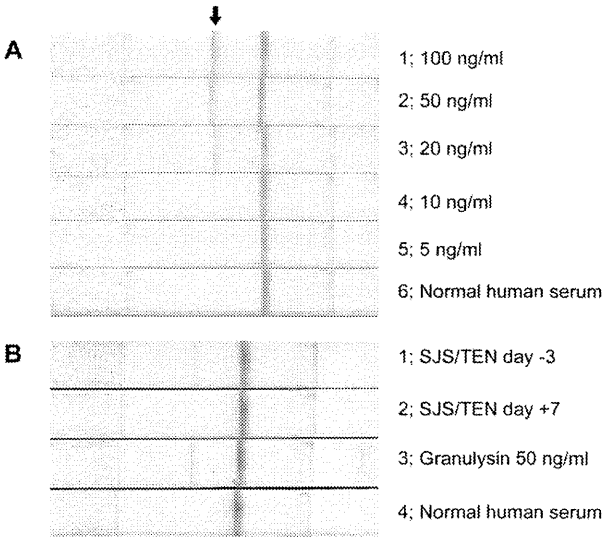

Fig 1. A, Immunochromatographic test strip detects elevated granulysin. *1 to 5*, Diluted recombinant granulysin is applied. *6*, Normal human serum as negative control (1.4 ng/mL). Positive results are shown as a band (indicated by the arrow). Approximately 10 ng/mL of granulysin is considered a positive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

REFERENCES

1. Roujeau JC, Kelly JP, Naldi L, Rzany B, Stern RS, Anderson T, et al. Medication use and the risk of Stevens-Johnson syndrome or toxic epidermal necrolysis. N Engl J Med 1995;333:1600-7.
2. Abe R, Shimizu T, Shibaki A, Nakamura H, Watanabe H, Shimizu H. Toxic epidermal necrolysis and Stevens-Johnson syndrome are induced by soluble Fas ligand. Am J Pathol 2003;162:1515-20.
3. Murata J, Abe R, Shimizu H. Increased soluble Fas ligand levels in patients with Stevens-Johnson syndrome and toxic epidermal necrolysis preceding skin detachment. J Allergy Clin Immunol 2008;122:992-1000.
4. Chung WH, Hung SI, Yang JY, Su SC, Huang SP, Wei CY, et al. Granulysin is a key mediator for disseminated keratinocyte death in Stevens-Johnson syndrome and toxic epidermal necrolysis. Nat Med 2008;14:1343-50.
5. Abe R, Yoshioka N, Murata J, Fujita Y, Shimizu H. Granulysin as a marker for early diagnosis of the Stevens-Johnson syndrome. Ann Intern Med 2009;151:514-5.
6. Ogawa K, Takamori Y, Suzuki K, Nagasawa M, Takano S, Kasahara Y, et al. Granulysin in human serum as a marker of cell-mediated immunity. Eur J Immunol 2003;33:1925-33.
7. Saigusa S, Ichikura T, Tsujimoto H, Sugasawa H, Majima T, Kawarabayashi N, et al. Serum granulysin level as a novel prognostic marker in patients with gastric carcinoma. J Gastroenterol Hepatol 2007;22:1322-7.
8. Pereira FA, Mudgil AV, Rosmarin DM. Toxic epidermal necrolysis. J Am Acad Dermatol 2007;56:181-200.
9. Bastuji-Garin S, Fouchard N, Bertocchi M, Roujeau JC, Revuz J, Wolkenstein P. SCORTEN: a severity-of-illness score for toxic epidermal necrolysis. J Invest Dermatol 2000;115:149-53.
10. Kaspar AA, Okada S, Kumar J, Poulain FR, Drouvalakis KA, Kelekar A, et al. A distinct pathway of cell-mediated apoptosis initiated by granulysin. J Immunol 2001;167:350-6.
11. Clayberger C, Krensky AM. Granulysin. Curr Opin Immunol 2003;15:560-5.

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.

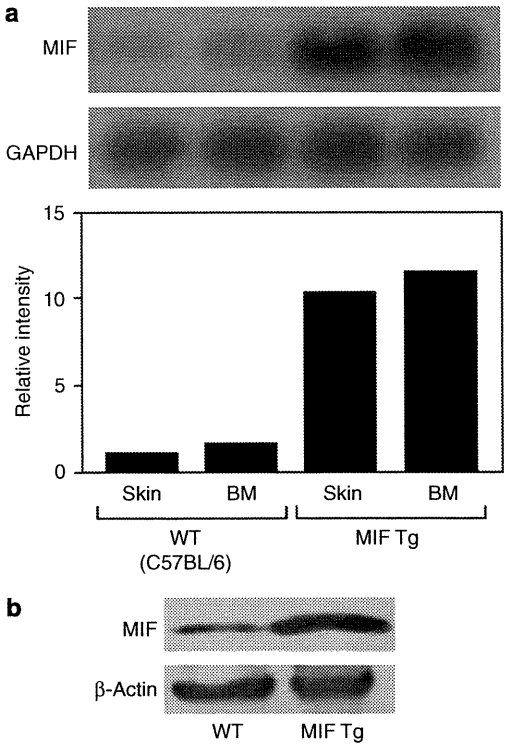

Figure 1. Expression of macrophage migration inhibitory factor (MIF) in tissues from MIF transgenic (Tg) mice. (**a**) Bone marrow (BM) and skin specimens were harvested from MIF Tg and wild-type (WT) mice, and the total RNA levels were determined by northern blot analysis as described in the Materials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

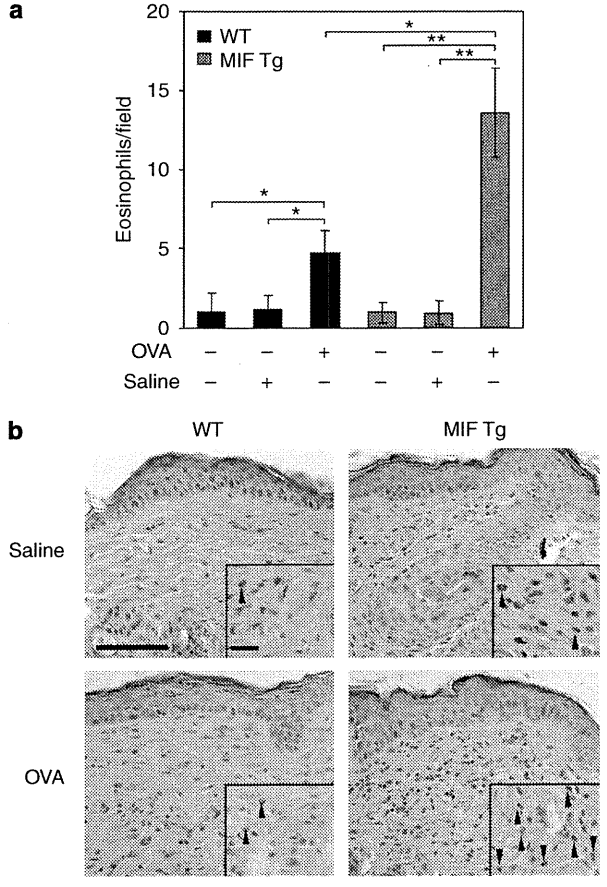

Figure 2. Eosinophil infiltration into ovalbumin (OVA)-sensitized skin sites of macrophage migration inhibitory factor (MIF) transgenic (Tg) mice. (**a**) The number of eosinophils in OVA-sensitized skin sites of MIF Tg mice was compared with the wild-type (WT) mice. Each value represents the mean ± 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 μm; scale bar for small panels = 10 μm; hematoxylin and eosin section. Arrowheads point to eosinophils. The experiments were repeated three times and similar results were obtained.

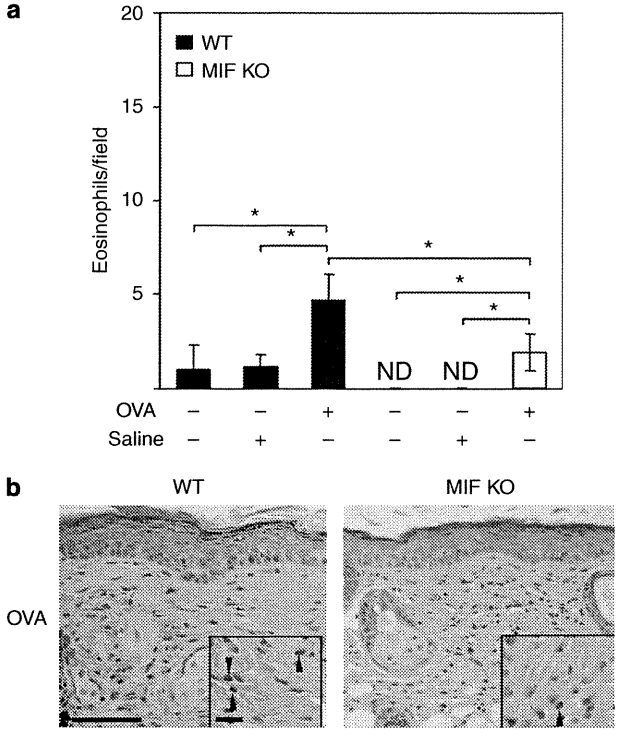

Figure 3. Eosinophil infiltration induced in ovalbumin (OVA)-sensitized skin sites of macrophage migration inhibitory factor (MIF) knockout (KO) mice. (**a**) The number of eosinophils in OVA-sensitized skin sites of MIF KO mice was compared with wild-type (WT) mice. Each value represents the mean ± 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 μm; scale bar for small panels = 10 μm; hematoxylin and eosin section. Arrowheads point to eosinophils. The experiments were repeated three times and similar results were obtained each time.

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 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

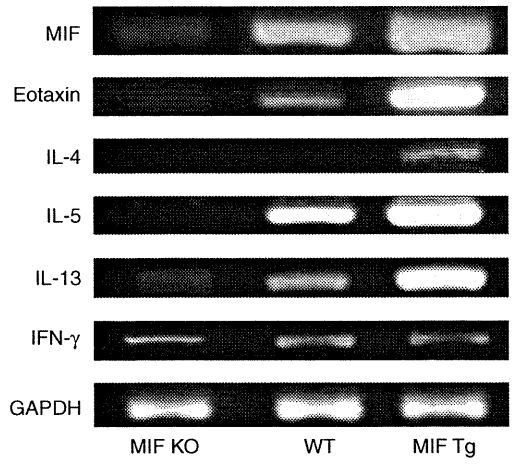

Figure 4. Expression levels of eotaxin and Th2-type cytokines in ovalbumin (OVA)-sensitized skin from macrophage migration inhibitory factor (MIF) transgenic (Tg) mice and MIF knockout (KO) mice. Reverse transcriptase-PCR analyses of eotaxin, IL-4, IL-5, IL-13, and IFN-γ levels in skin sites of MIF Tg 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.

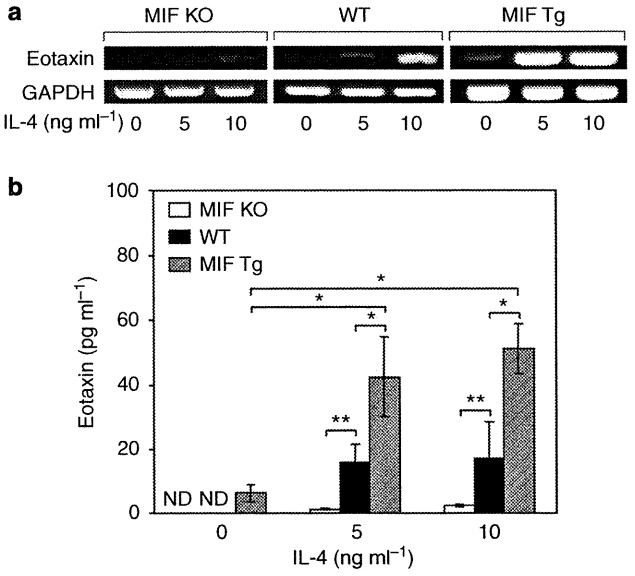

Figure 5. IL-4 induced eotaxin expression and production by fibroblasts from macrophage migration inhibitory factor (MIF) transgenic (Tg) and MIF knockout (KO) mice. Fibroblasts from MIF KO, MIF Tg, and wild-type (WT) mice were stimulated with IL-4 (5 or 10 ng ml^{-1}) for 24 hours. (**a**) RNA was extracted 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 ± SD of five specimens. *P<0.005, **P<0.05. ND, not detected.

(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 effecter 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

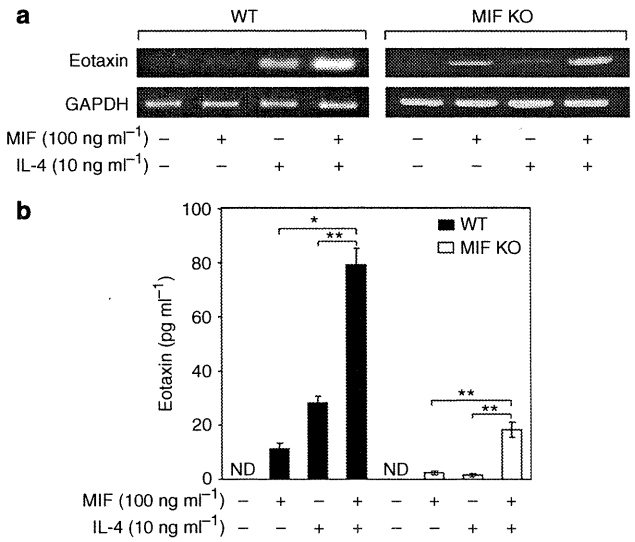

Figure 6. Recombinant macrophage migration inhibitory factor (MIF) restored eotaxin expression and production by IL-4 stimulation in dermal fibroblasts from MIF knockout (KO) mice. The fibroblasts were stimulated with IL-4 (10 ng ml^{-1}), MIF (100 ng ml^{-1}), or both IL-4 and MIF for 24 hours. (**a**) 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 ± 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; Uguccioni *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, MT); 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, CO); 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 μl of normal saline were placed on a 1 × 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 ×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 μg 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 °C for 24–48 hours. Post-hybridization washing was performed in 0.1% SDS with 0.2 × standard saline citrate (1 × standard saline citrate: 0.15 M NaCl, 0.015 M sodium citrate) at 65 °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 °C for 30 seconds, annealing from 46 to 64 °C for 1 minute and extension at 72 °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 μl 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 °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 °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 ± 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.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

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

REFERENCES

Akagi Y, Isaka Y, Akagi A *et al.* (1997) Transcriptional activation of a hybrid promoter composed of cytomegalovirus enhancer and beta-actin/beta-globin gene in glomerular epithelial cells *in vivo*. *Kidney Int* 51:1265–9

Bernhagen J, Calandra T, Bucala R (1998) Regulation of the immune response by macrophage migration inhibitory factor: biological and structural features. *J Mol Med* 76:151–61

Bernhagen J, Calandra T, Mitchell RA *et al.* (1993) MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. *Nature* 365:756–9

Bloom BR, Bennett B (1966) Mechanism of reaction *in vivo* associated with delayed-type hypersensitivity. *Science* 153:80–2

Bucala R (1996) MIF re-evaluated: pituitary hormone and glucocorticoid-induced regulator of cytokine production. *FASEB J* 7:19–24

Cheng Q, McKeown SJ, Santos L *et al.* (2010) Macrophage migration inhibitory factor increases leukocyte-endothelial interactions in human endothelial cells via promotion of expression of adhesion molecules. *J Immunol* 185:1238–47

Donnelly SC, Haslett C, Reid PT *et al.* (1997) Regulatory role for macrophage migration Inhibitory factor in acute respiratory distress syndrome. *Nat Med* 3:320–3

Fujisawa T, Kato Y, Atsuta J *et al.* (2000) Chemokine production by the BEAS-2B human bronchial epithelial cells: differential regulation of eotaxin, IL-8, and RANTES by TH2- and TH1-derived cytokines. *J Allergy Clin Immunol* 105:126–33

Garcia-Zepeda EA, Rothenberg ME, Ownbey RT *et al.* (1996) Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. *Nat Med* 2:449–56

Gregory JL, Leech MT, David JR *et al.* (2004) Reduced leukocyte-endothelial cell interactions in the inflamed microcirculation of macrophage migration inhibitory factor-deficient mice. *Arthritis Rheum* 50:3023–34

Gregory JL, Morand EF, McKeown SJ *et al.* (2006) Macrophage migration inhibitory factor induces macrophage recruitment via CC chemokine ligand 2. *J Immunol* 177:8072–9

Hamasaka A, Abe R, Koyama Y *et al.* (2009) DNA vaccination against macrophage migration inhibitory factor improves atopic dermatitis in murine models. *J Allergy Clin Immunol* 124:90–9

Honma N, Koseki H, Akasaka T *et al.* (2000) Deficiency of the macrophage migration inhibitory factor gene has no significant effect on endotoxaemia. *Immunology* 100:84–90

Jose PJ, Griffiths-Johnson DA, Collins PD *et al.* (1994) Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J Exp Med* 179:881–7

Kapp A (1995) Atopic dermatitis-the skin manifestation of atopy. *Clin Exp Allergy* 25:210–9

Kleemann R, Hausser A, Geiger R *et al.* (2000) Intracellular action of the cytokine MIF to modulate AP-1 activity and the cell cycle through Jab1. *Nature* 408:211–6

Lampinen M, Carlson M, Hakansson LD *et al.* (2004) Cytokine-regulated accumulation of eosinophils in inflammatory disease. *Allergy* 59:793–805

Leiferman KM (1989) Eosinophils in atopic dermatitis. *Allergy* 44:20–6

Leng L, Metz CN, Fang Y *et al.* (2003) MIF signal transduction initiated by binding to CD74. *J Exp Med* 197:1467–76

Leung DY, Nicklas RA, Li JT *et al.* (2004) Disease management of atopic dermatitis: an updated practice parameter. Joint Task Force on Practice Parameters. *Ann Allergy Asthma Immunol* 93:S1–21

Li L, Xia Y, Nguyen A *et al.* (1999) Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *J Immunol* 162:2477–87

Lue H, Dewor M, Leng L *et al.* (2011) Activation of the JNK signaling pathway by macrophage migration inhibitory factor (MIF) and dependence on CXCR4 and CD74. *Cell Signal* 23:135–44

Magalhães ES, Mourao-Sa DS, Vieira-de-Abreu A *et al.* (2007) Macrophage migration inhibitory factor is essential for allergic asthma but not for Th2 differentiation. *Eur J Immunol* 37:1097–106

Magalhães ES, Paiva CN, Souza HS *et al.* (2009) Macrophage migration inhibitory factor is critical to interleukin-5-driven eosinophilopoiesis and tissue eosinophilia triggered by Schistosoma mansoni infecton. *FASEB J* 23:1262–71

Mizue Y, Ghani S, Leng L *et al.* (2005) Role for macrophage migration inhibitory factor in asthma. *Proc Natl Acad Sci USA* 102:14410–5

Mochizuki M, Bartels J, Mallet AI *et al.* (1998) IL-4 induces eotaxin: a possible mechanism of selective eosinophil recruitment in helminth infection and atopy. *J Immunol* 160:60–8

Morar N, Willis-Owen SA, Moffatt MF *et al.* (2006) The genetics of atopic dermatitis. *J Allergy Clin Immunol* 118:24–34

Nakamaru Y, Oridate N, Nishihira J *et al.* (2005) Macrophage migration inhibitory factor (MIF) contributes to the development of allergic rhinitis. *Cytokine* 31:103–8

Nakamura H, Haley KJ, Nakamura T *et al.* (1998) Differential regulation of eotaxin expression by TNF-alpha and PMA in human monocytic U-937 cells. *Am J Physiol* 275:L601–10

Ponath PD, Qin S, Post TW *et al.* (1996) Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. *J Exp Med* 183:2437–48

Roger T, David J, Glauser MP *et al.* (2001) MIF regulates innate immune responses through modulation of Toll-like receptor 4. *Nature* 414:920–4

Rossi AG, Haslett C, Hirani N *et al.* (1998) Human circulating eosinophils secrete macrophage migration inhibitory factor (MIF): potential role in asthma. *J Clin Invest* 15:2869–74

Sanderson CJ (1992) Interleukin-5, eosinophils, and disease. *Blood* 79:3101–9

Sasaki S, Nishihira J, Ishibashi T *et al.* (2004) Transgene of MIF induces podocyte injury and progressive mesangial sclerosis in the mouse kidney. *Kidney Int* 65:469–81

Shimizu T (2005) Role of macrophage migration inhibitory factor (MIF) in the skin. *J Dermatol Sci* 37:65–73

Shimizu T, Abe R, Ohkawara A *et al.* (1997) Macrophage migration inhibitory factor is an essential immunoregulatory cytokine in atopic dermatitis. *Biochem Biophy Res Commun* 240:173–8

Shimizu T, Abe R, Ohkawara A *et al.* (1999) Increased production of macrophage migration inhibitory factor (MIF) by PBMCs of atopic dermatitis. *J Allergy Clin Immunol* 104:659–64

Shimizu T, Nishihira J, Watanabe H *et al.* (2004) Cetirizine, an H1-receptor antagonist, suppresses the expression of macrophage migration inhibitory factor (MIF): its potential anti-inflammatory action. *Clin Exp Allergy* 34:103–9

Shimizu T, Ohkawara A, Nishihira J *et al.* (1996) Identification of macrophage migration inhibitory factor (MIF) in human skin and its immunohistochemical localization. *FEBS Lett* 381:188–202

Spergel JM, Mizoguchi E, Brewer JP *et al.* (1998) Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. *J Clin Invest* 101:1614–22

Spergel JM, Mizoguchi E, Oettgen H *et al.* (1999) Roles of TH1 and TH2 cytokines in a murine model of allergic dermatitis. *J Clin Invest* 103:1103–11

Stellato C, Matsukura S, Fal A *et al.* (1999) Differential regulation of epithelial-derived C-C chemokine expression by IL-4 and glucocorticoid budesonide. *J Immunol* 163:5624–32

Terada N, Hamano N, Nomura T *et al.* (2000) Interleukin-13 and tumor necrosis factor-a synergistically induce eotaxin production in human nasal fibroblasts. *Clin Exp Allergy* 20:348–55

Uguccioni M, Loetscher P, Forssmann U *et al.* (1996) Monocyte chemotactic protein 4 (MCP-4), a novel structural and functional analogue of MCP-3 and eotaxin. *J Exp Med* 183:2379–84

Wang B, Huang X, Wolters PJ *et al.* (2006) Cutting edge: deficiency of macrophage migration inhibitory factor impairs murine airway allergic responses. *J Immunol* 177:5779–84

Wang B, Zhang Z, Wang Y *et al.* (2009) Molecular cloning and characterization of macrophage migration inhibitory factor from small abalone Haliotis diversicolor supertexta. *Fish Shellfish Immunol* 27:57–64

Weiser WY, Temple PA, Witek-Giannotti JS *et al.* (1989) Molecular cloning of a cDNA encoding a human macrophage migration inhibitory factor. *Proc Natl Acad Sci USA* 86:7522–6

Yamaguchi E, Nishihira J, Shimizu T *et al.* (2000) Macrophage migration inhibitory factor (MIF) in bronchial asthma. *Clin Exp Allergy* 30:1244–9

Yawalkar N, Uguccioni M, Schärer J *et al.* (1999) Enhanced expression of eotaxin and CCR3 in atopic dermatitis. *J invest Dermatol* 113:43–8

Ying S, Robinson DS, Meng Q *et al.* (1997) Enhanced expression of eotaxin and CCR3 mRNA and protein in atopic athma. Association with airway hyperresponsiveness and predominant co-localization of eotaxin mRNA to bronchial epithelial and endothelial cells. *Eur J Immunol* 27:3507–16

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ORIGINAL ARTICLE

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

Tomohiko Narita[1], Naoki Oiso[1], Kazuyoshi Fukai[2], Kenji Kabashima[3], Akira Kawada[1] and Tamio Suzuki[4]

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 autoimmune 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

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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).

Table 1 Demographics of 133 Japanese generalized vitiligo patients

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

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 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 findings[5-18] including ours and genomewide association studies[24-28] 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

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 aropecia 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 inducting generalized vitiligo, autoimmune thyroid disease and other autoimmune disorders.

ACKNOWLEDGEMENTS

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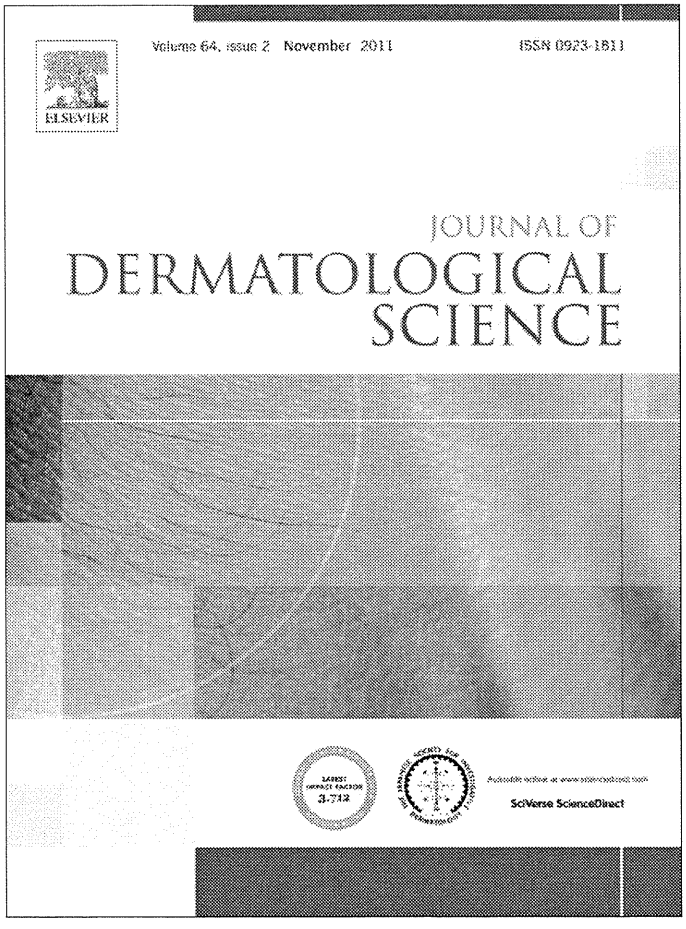
CONFLICT OF INTEREST

No potential conflict of interest was disclosed.

REFERENCES

1. Spritz RA. The genetics of generalized vitiligo and associated autoimmune diseases. *J Dermatol Sci* 2006;**41**:3-10.
2. Spritz RA. The genetics of generalized vitiligo and associated autoimmune diseases. *Pigment Cell Res* 2007;**20**:271-8.
3. Spritz RA. The genetics of generalized vitiligo. *Curr Dir Autoimmun* 2008;**10**:244-57.
4. Lu T, Gao T, Wang A, Jin Y, Li Q, Li C. Vitiligo prevalence study in Shaanxi Province, China. *Int J Dermatol* 2007;**46**: 47-51.
5. Betterle C, Caretto A, De Zio A *et al.* Incidence and significance of organ-specific autoimmune disorders (clinical, latent or only autoantibodies) in patients with vitiligo. *Dermatologica* 1985;**171**:419-23.
6. Hegedus L, Heidenheim M, Gervil M, Hjalgrim H, Hoier-Madsen M. High frequency of thyroid dysfunction in patients with vitiligo. *Acta Derm Venereol* 1994;**74**:120-3.
7. Schallreuter KU, Lemke R, Brandt O *et al.* Vitiligo and other diseases: coexistence or true association? Hamburg study on 321 patients. *Dermatology* 1994;**188**:269-75.
8. Alkhateeb A, Fain PR, Thody A, Bennett DC, Spritz RA. Epidemiology of vitiligo and associated autoimmune diseases in Caucasian probands and their families. *Pigment Cell Res* 2003;**16**:208-14.
9. Kurtev A, Dourmishev AL. Thyroid function and autoimmunity in children and adolescents with vitiligo. *J Eur Acad Dermatol Venereol* 2004;**18**:109-11.
10. Farrokhi S, Hojjat-Farsangi M, Noohpisheh MK, Tahmasbi R, Rezaei N. Assessment of the immune system in 55 Iranian patients with vitiligo. *J Eur Acad Dermatol Venereol* 2005;**19**:706-11.
11. Laberge G, Mailloux CM, Gowan K *et al.* Early disease onset and increased risk of other autoimmune diseases in familial generalized vitiligo. *Pigment Cell Res* 2005;**18**:300-5.
12. Birlea SA, Fain PR, Spritz RA. A Romanian population isolate with high frequency of vitiligo and associated autoimmune diseases. *Arch Dermatol* 2008;**144**:310-6.
13. Tanioka M, Yamamoto Y, Katoh M, Takahashi K, Miyachi Y. Vitiligo vulgaris and autoimmune disease in Japan. A

report from vitiligo clinic in Kyoto University Hospital. *Dermatoendocrinol* 2009;**1**:43-5.
14. Zhang Z, Xu SX, Zhang FY *et al.* The analysis of genetics and associated autoimmune diseases in Chinese vitiligo patients. *Arch Dermatol Res* 2009;**301**:167-73.
15. Amerio P, Di Rollo D, Carbone A *et al.* Polyglandular autoimmune diseases in a dermatological clinical setting: vitiligo-associated autoimmune diseases. *Eur J Dermatol* 2010;**20**:354-8.
16. Yang Y, Lin X, Fu W, Luo X, Kang K. An approach to the correlation between vitiligo and autoimmune thyroiditis in Chinese children. *Clin Exp Dermatol* 2010;**35**:706-10.
17. Akay BN, Bozkir M, Anadolu Y, Gullu S. Epidemiology of vitiligo, associated autoimmune diseases and audiological abnormalities: Ankara study of 80 patients in Turkey. *J Eur Acad Dermatol Venereol* 2010;**24**:1144-50.
18. Mosca M, Carli L, d'Ascanio A *et al.* Occurrence of organ-specific and systemic autoimmune diseases among the first- and second-degree relatives of Caucasian patients with connective tissue diseases: report of data obtained through direct patient interviews. *Clin Rheumatol* 2008; **27**:1045-8.
19. Alkhateeb A, Fain PR, Thody A *et al.* Epidemiology of vitiligo and associated autoimmune diseases in Caucasian probands and their families. *Pigment Cell Res* 2003;**16**: 208-14.
20. Grimes PE, Halder RM, Jones C *et al.* Autoantibodies and their clinical significance in a black vitiligo population. *Arch Dermatol* 1983;**119**:300-3.
21. Gopal KV, Rama Rao GR, Kumar YH, Appa Rao MV, Vasudev P; Srikant. Vitiligo: a part of a systemic autoimmune process. *Indian J Dermatol Venereol Leprol* 2007; **73**:162-5.
22. Liu JB, Li M, Yang S *et al.* Clinical profiles of vitiligo in China: an analysis of 3742 patients. *Clin Exp Dermatol* 2005;**30**:327-31.
23. Majumder PP, Nordlund JJ, Nath SK. Pattern of familial aggregation of vitiligo. *Arch Dermatol* 1993;**129**:994-8.
24. Jin Y, Mailloux CM, Gowan K *et al.* NALP1 in vitiligo-associated multiple autoimmune disease. *N Engl J Med* 2007;**356**:1216-25.
25. Jin Y, Birlea SA, Fain PR *et al.* Common variants in FOXP1 are associated with generalized vitiligo. *Nat Genet* 2010;**42**:576-8.
26. Jin Y, Birlea SA, Fain PR *et al.* Variant of TYR and autoimmunity susceptibility loci in generalized vitiligo. *N Engl J Med* 2010;**362**:1686-97.
27. Quan C, Ren YQ, Xiang LH *et al.* Genome-wide association study for vitiligo identifies susceptibility loci at 6q27 and the MHC. *Nat Genet* 2010;**42**:614-8.
28. Birlea SA, Jin Y, Bennett DC *et al.* Comprehensive Association Analysis of Candidate Genes for Generalized Vitiligo Supports XBP1, FOXP3, and TSLP. *J Invest Dermatol* 2011;**131**:371-81.
29. Hadj-Kacem H, Rebuffat S, Mnif-Féki M, Belguith-Maalej S, Ayadi H, Péraldi-Roux S. Autoimmune thyroid diseases: genetic susceptibility of thyroid-specific genes and thyroid autoantigens contributions. *Int J Immunogenet* 2009;**36**:85-96.
30. Spritz RA. Shared genetic relationships underlying generalized vitiligo and autoimmune thyroid disease. *Thyroid* 2010;**20**:745-54.



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Letter to the Editor

Repigmentation of leukoderma in a piebald patient associated with a novel *c-KIT* gene mutation, G592E, of the tyrosine kinase domain

Piebaldism (MIM 172800) is an autosomal dominant disorder showing localized poliosis and leukoderma of the frontal scalp, forehead, ventral trunk, and extremities. On the other hand, vitiligo is an acquired pigmentation disease, and the white patches distributed typically in an acral and periorificial regions. The depigmented regions have been believed to be stable with piebaldism because of the congenital absence of melanocytes in those regions involved, whereas, the size and the distribution of depigmented macules alters in vitiligo by a selective destruction of the melanocytes.

The *c-KIT* gene is located on chromosome 4q12 and encodes a type III tyrosine kinase receptor, c-KIT. The c-KIT protein functions as a receptor for stem cell factor (SCF), and mutations of the *c-KIT* gene are identified in 75% of subjects with piebaldism [1,2]. This c-KIT–SCF interaction is essential for melanocyte development, proliferation, survival, and migration. The binding of SCF to c-KIT initiates dimerization of c-KIT, which induces transactivation and auto-phosphorylation of tyrosine residues within the intracellular tyrosine kinase domains, followed by binding of signal transduction molecules [3]. Accordingly, mutations within the tyrosine kinase domain induce a severe phenotype of piebaldism because of their dominant negative effect [4]. A large number of subjects with piebaldism have intracellular mutations of *c-KIT*, and most of the patients with these mutations have severe phenotypes. On the other hand, there are several flame shift mutations and certain intracellular point mutations investigated with moderate phenotype including recent reports [5–7].

A 5-year-old Japanese female had a white section of hair in the front of her scalp, and leukoderma on her frontal scalp, forehead, abdomen, and knees. She also had hyperpigmented macules on her trunk and frontal thighs (Fig. 1). Her 35-year-old father had both leukoderma and hyperpigmentation with similar distributions (data not shown). We found progressive repigmentation within the leukoderma region on the patient's knees during inspections performed at 2 year intervals (Fig. 1). Her father had also inquired about progressive repigmentation within his leukoderma around his knees.

The parents provided written informed consent for their daughter's participation, and the study was approved by the Genetic Ethics Committee of Kinki University. We amplified the *c-KIT* gene by polymerase chain reaction (PCR) from genomic DNA of peripheral blood leukocytes obtained from the patient and her father, and the nucleic acid sequence of the *c-KIT* gene was analyzed by direct sequencing [5]. The identified mutation in *c-KIT* was confirmed by three independent sequencing reactions from the patient's DNA and one sequencing study of her father's DNA. The PCR products of exon 12 in the *c-KIT* from the patients (father and daughter) and 110 healthy volunteers were analyzed by a single strand conformational polymorphism analysis as described [8].

We found that there was a missense substitution at the 5′ first codon of exon 12 (Fig. 2). The G to A mutation at nucleotide position 1775 of the coding region of *c-KIT* resulted in an amino acid substitution from glycine to glutamic acid at position 592 within the intracellular tyrosine kinase domain. The substitution was not present in any of the 110 healthy controls, suggesting that the substitution is not a usual polymorphism but a novel mutation related to piebaldism.

Glycine 592 is conserved among fms family kinases (CSF-1, PDGFR), and is located 4 amino acids upstream from the ATP-binding motif (G-X-G-X-X-G), which is highly conserved among tyrosine kinases [3]. Hypopigmentation due to a L595P mutation located at one amino acid upstream of that motif was reported to be extensive, because L595 was included in ATP binding region [9]. However, the piebald phenotype of the present case was not severe, and G to E conversion of 4 amino acids upstream of G-X-G-X-X-G is also found in *C-SRC* kinase. Therefore 592G might affect activation of fms family kinase receptors regardless of ATP-binding capacity. Therefore G592E mutation may result in loss of function, which phenotype exhibits more mild than that by L595P mutation.

Alternatively, because G1775A is located at the first codon of exon 12 and the mutated allele is ctacagAGAAAA. In this situation, an aberrant splicing can occur as ctacagagAAAA according to the GT–AG splicing rule. This aberrant splicing resulted in a frame sift mutation, and produced nonsense mRNA. Consequently the presented mutation could be consequent to a loss of function.

Piebald patients with pigmental restoration of hypopigmented regions have been investigated in several previous studies, and a mild or modulate phenotype rather than a severe phenotype was observed. In those cases, frame shift mutations in the gene coding for the tyrosine kinase domain of *c*-KIT were identified [4]. It is conceivable that the intracellular frame shifts resulted in loss of function in only half of the c-KIT molecules, resulting in a mild or moderate phenotype.

Melanocytes were considered to exist within the repigmented regions in the present patients and the patients previously reported [4], and the possible existence of melanocytes was formerly investigated within the leukoderma of piebald patients [10]. Furthermore, it has been reported that the repigmented regions were either forehead or knees, which were easily exposed to sunlight [4]. A considerable number of intact c-KIT dimers on the melanocytes within these restricted regions of leukoderma may be required for repigmentation of the piebald patients in the presence

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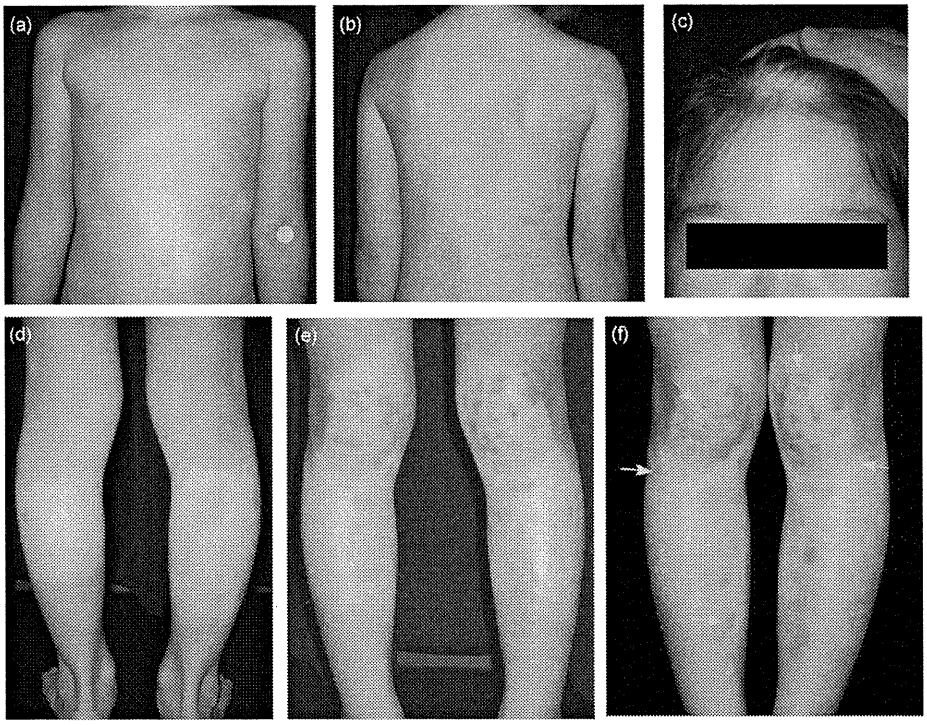

Fig. 1. (a) Leukoderma on the abdomen; (b) small pigmented patches on the back without leukoderma; (c) the white hair on front area of the patient's scalp (white forelock); (d) leukoderma around the popliteal fossas; (e) leukoderma with small pigmented patches around the knees at 5 years of age; (f) several new pigmented patches (indicated with blue arrows) emerged on the leukoderma around the knees at 7 years of age.

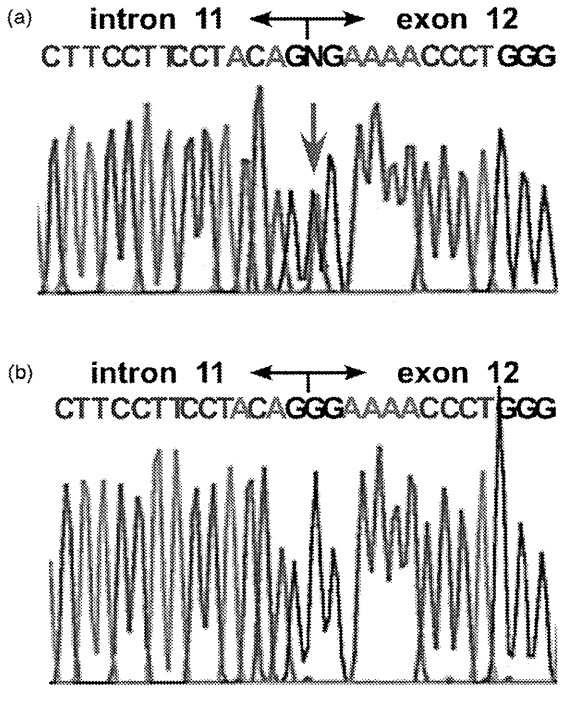

Fig. 2. (a) Genetic analysis of c-*KIT* from the patient revealed a single disease associated heterozygous nucleotide change of 1775 G > A indicated by a red arrow. (b) The single missense mutation was not seen in the analysis of wt DNA.

of sunlight long after birth. Further investigations will be expected to clarify the correlation between specific *c-KIT* mutations and pigment regeneration.

References

[1] Giebel LB, Spritz RA. Mutation of the KIT (mast/stem cell growth factor receptor) protooncogene in human piebaldism. Proc Natl Acad Sci U S A 1991;88:8696–9.
[2] Fleischman RA, Saltman DL, Stastny V, Zneimer S. Deletion of the c-kit protooncogene in the human developmental defect piebald trait. Proc Natl Acad Sci U S A 1991;88:10885–9.
[3] Mol CD, Lim KB, Sridhar V, Zou H, Chien EY, Sang BC, et al. Structure of a c-kit product complex reveals the basis for kinase transactivation. J Biol Chem 2003;278:31461–4.
[4] Murakami T, Fukai K, Oiso N, Hosomi N, Kato A, Garganta C, et al. New KIT mutations in patients with piebaldism. J Dermatol Sci 2004;35:29–33.
[5] Narita T, Oiso N, Fukai K, Motokawa T, Hayashi M, Yokoyama K, et al. Two children with a mild or moderate piebaldism phenotype and a father without leukoderma in a family with the same recurrent missense mutation in the kinase domain of KIT. Eur J Dermatol 2011;21:446–7.
[6] Duarte AF, Mota A, Baudrier T, Morais P, Santos A, Cerqueira R, et al. Piebaldism and neurofibromatosis type 1: family report. Dermatol Online J 2010;16:11.
[7] Chong KL, Common JE, Lane EB, Goh BK. A novel mutation in the kinase domain of KIT in an Indian family with a mild piebaldism phenotype. J Dermatol Sci 2010;59:206–9.
[8] Ito S, Suzuki T, Inagaki K, Suzuki N, Kono M, Tomita Y, et al. Two novel mutations detected in Japanese patients with oculocutaneous albinism. J Dermatol Sci 2006;44:116–8.
[9] Lin ZM, Xu Z, Bu DF, Yang Y. New mutations of KIT gene in two Chinese patients with piebaldism. Br J Dermatol 2006;155:1303–4.
[10] Hayashibe K, Mishima Y. Tyrosinase-positive melanocyte distribution and induction of pigmentation in human piebald skin. Arch Dermatol 1988;124:381–6.

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Letter to the Editor

Androgen receptor transactivity is potentiated by TGF-β1 through Smad3 but checked by its coactivator Hic-5/ARA55 in balding dermal papilla cells

We previously reported that TGF-β1 is a paracrine mediator from dermal papilla to hair follicle epithelium in the pathogenesis of androgenetic alopecia (AGA) [1,2]. Because TGF-β1 can induce catagen in hair cycling [3], it has been suggested that it functions as a paracrine pathogenic mediator from dermal papilla in AGA. On the other hand, TGF-β1 reportedly modulates androgen receptor (AR) transactivation in the monkey kidney cell line CV-1 as well as the human prostate cell lines PC-3 and DU145 cells [4,5]. However, it depends on the cell type or conditions whether TGF-β1 potentiates [5] or represses AR [4]. It is therefore of considerable interest to examine potential modulation by TGF-β1 and its downstream signaling for AR transcriptional activity in balding dermal papilla cells (bald DPCs). To address this issue, we used mouse mammary tumor virus long-terminal repeat (MMTV)-luciferase assays to examine whether TGF-β1 can alter AR transactivation in bald DPCs. The DPCs obtained at passages 4–6 from an AGA bald frontal scalp were cultured on a 12-well plate in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% charcoal-treated fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS, USA), penicillin (50 units/ml) and streptomycin (50 mg/ml) at 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2. At subconfluency, cells were transiently transfected by means of Fugene 6 (Roche Diagnostic Corp., Indianapolis, IN) with 0.1 μg pSG5-AR, 0.3 μg MMTV-luciferase reporter plasmid and 0.1 μg pRL-CMV vector as an internal control and 24 h later the medium was refreshed and 1 nM R1881 and 0.2 or 2.0 ng/ml of human recombinant TGF-β1 (R&D Systems Inc., Minneapolis, MN) or the corresponding mocks were added to the culture. After incubation for 24 h, the cells were harvested and subjected to luciferase assays using the Dual-Luciferase reporter assay system (Promega, Madison, WI). The results showed that 0.2 or 2.0 ng/ml of TGF-β1 can significantly enhance AR activity by a factor of 1.9 or 2.3 (Fig. 1), respectively, indicating that TGF-β1 signaling positively stimulates AR transactivation in bald DPCs. Next, to investigate the need for Smad3 to obtain this effect by TGF-β1, we examined the effect of Smad3 knockdown by siRNA on TGF-β1-induced AR transactivation. The bald DPCs were transiently transfected with 0.1 μg pSG5-AR, 0.3 μg MMTV-luciferase reporter plasmid, 0.1 μg pRL-CMV, and 100 pg/ml siRNA against Smad3 (siTrio, NM_005902; B-Bridge International, Inc., Cupertino, CA) or control RNA (siTrio negative control). Twenty hours later, the medium was refreshed and 1 nM R1881, 0.2 ng/ml human recombinant TGF-β1 or one of the corresponding mocks was added to the culture. After incubation for 24 h, the cells were harvested and subjected to luciferase assays. The results demonstrated that knockdown of Smad3 eliminated the effect of TGF-β1 on MMTV-luciferase activity (Fig. 2A, upper panel), indicating that Smad3 is necessary for TGF-β1 to exert its effect. The successful knockdown of Smad3 by siRNA was confirmed in this experiment (Fig. 2A, lower panel). In addition, because interaction of Smad3 and Hic-5/ARA55, which we previously reported is an androgen sensitivity regulator in DPCs [6], has been proven [7,8], we studied the effect of TGF-β1 on AR activity by using the MMTV-luciferase assays for bald DPCs

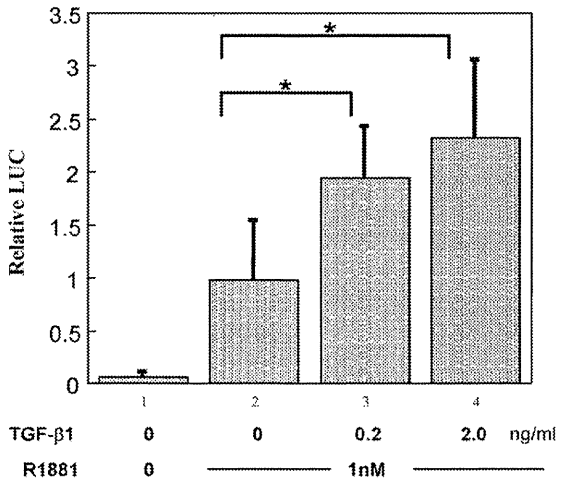

Fig. 1. Effect of TGF-β1 on transfected androgen receptor transactivity in balding dermal papilla cells (DPCs). The DPCs from AGA bald frontal scalp at subconfluency in a 12-well plate were transiently transfected at passages 4–6 with 0.1 μg pSG5-AR, 0.3 μg MMTV-luciferase reporter plasmid and 0.1 μg pRL-CMV vector using Fugene 6 as an internal control. At 24 h after transfection, 1 nM R1881 (lanes 2–4), synthetic androgen, or an ethanol mock solution (lane 1), and TGF-β1 at the indicated concentration (lanes 3 and 4) or a corresponding mock solution (4 mM HCl/0.1% BSA) (lanes 1 and 2) was added to the culture. After incubation for 24 h, the cells were harvested and subjected to luciferase assays. Each luciferase activity (relative LUC) is shown relative to the mean transactivation observed in the absence of TGF-β1 and the presence of R1881 (lane 2). Bars represent the mean ± standard deviations of three independent experiments. *$p < 0.05$; n.s., not significant ($p > 0.05$); Mann–Whitney's *U* test.