keratinocyte from MSC easily. However, unfortunately there is no report about it. In our study, we showed BMP-4 induced keratinocyte differentiation in vitro, suggesting the receptor of BMP-4 or other related protein may be related to keratinocyte differentiation.

In this study, we showed that a specific chemokine may recruit circulating MSCs into the wound site, resulting in the stimulation of wound repair via the promotion of angiogenesis. Our findings indicate that MSCs together with tissue specific chemokines might be more effectively used for clinical applications.

Stem cells in bone marrow include hemopoietic stem cells, MSCs, and multipotent adult progenitor cells (12). It is still unknown whether hemopoietic stem cells are able to transdifferentiate into nonhemopoietic cells. Conversely, some specific environments, reported as niche microenvironments, are required to transdifferentiate into several organ-specific cells from bone marrow stem cells. In the skin, several reports showed there are a number of bone marrow cells that traffic through skin (34). Wounding stimulated the engraftment of bone marrow cells to the skin and induced bone marrow-derived cells to incorporate into and differentiate into nonhemopoietic skin structures. Although there are numerous reports of tissue-specific transdifferentiation from bone marrow, evidence has not suggested that direct transdifferentiation form bone marrow to specific tissue cells contributes to tissue regeneration. This also includes MSCs transdifferentiation. Other explanations of the effect of bone marrow application might be bone marrow cell-derived soluble factors, which regulate inflammation and angiogenesis. Recently, we reported that a specific chemokine, CTACK, is the major regulator involved in the migration of keratinocyte precursor cells from bone marrow into skin (31). Furthermore, increased bone marrow-derived keratinocyte migration by CTACK significantly accelerated the skin wound healing process. Because we demonstrated that MSCs migrate into wounded skin via SLC/CCL21-CCR7, it is interesting that MSCs ($CD34^-$ and bone marrow-derived keratinocyte precursor cells ($CD34^+$), which have a different phenotype, recruit and transdifferentiate into keratinocytes by different chemokine systems. In addition, chemokines induce wound repair via the accumulation of MSCs and bone marrow-derived keratinocyte precursors.

Finally, several clinical trials using MSCs have been attempted, including for spinal injury and myocardial infarction, which are difficult to heal by normal tissue regeneration. And it has been reported that MSCs application is very effective for these diseases. Therefore, we expect that MSCs therapy also accelerates skin wound healing especially refractory, common therapy-resistant skin ulcer.

Taken together, specific chemokine/chemokine receptor interactions involving stem cells are promising therapeutic candidates to regulate the regeneration phenomenon.

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Disclosures

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Interleukin-1β and macrophage migration inhibitory factor (MIF) in dermal fibroblasts mediate UVA-induced matrix metalloproteinase-1 expression

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Summary

Background: Exposure to solar UV radiation is the main environmental factor that causes premature aging of the skin. Matrix metalloproteinases (MMP)-1 is a member of the MMP family and degrades types I and III collagens, which are the major structural components of the dermis.
Objective: We evaluated the involvement IL-1β and macrophage migration inhibitory factor (MIF) in MMP-1 expression under ultraviolet A (UVA) irradiation.
Methods: IL-1β and MIF in MMP-1 expression in cultured human dermal fibroblasts and the UVA effects on MMPs production using IL-1α/β-deficient mice were analyzed. Furthermore, fibroblasts derived from MIF-deficient mice were used to analyze the effect of IL-1β-induced MMPs production.

Abbreviations: IL-1, interleukin-1; MIF, macrophage migration inhibitory factor; MMP, matrix metalloproteinase; UV, ultraviolet; TNF-α, tumor necrosis factor-α; TIMP, tissue inhibitor of matrix metalloproteinase; JNK, c-Jun N-terminal kinase.

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Results: IL-1β-enhanced MIF expression and induced MMP-1 in cultured human dermal fibroblasts. IL-1β-induced MMP-1 expression is inhibited by neutralizing anti-MIF antibody. Dermal fibroblasts of IL-1α/β-deficient mice produced significantly decreased levels of MMPs compared to wild-type mice after UVA irradiation. Furthermore, fibroblasts of MIF-deficient mice were much less sensitive to IL-1β-induced MMPs production. On the contrary, IL-1β produced significantly decreased levels of MMPs in MIF-deficient mice fibroblasts. The up-regulation of MMP-1 mRNA by IL-1β stimulation was found to be inhibited by a p38 inhibitor and a JNK inhibitor. In contrast, the MEK inhibitor and inhibitor were found to have little effect on expression of MMP-1 mRNA.

Conclusions: IL-1β is involved in the up-regulation of UVA-induced MMP-1 in dermal fibroblasts, and IL-1β and MIF cytokine network induce MMP-1 and contribute to the loss of interstitial collagen in skin photoaging.

1. Introduction

Exposure to solar UV radiation is the main environmental factor that causes premature aging of the skin (photoaging). Human skin aging resulting from UV irradiation is a cumulative process that occurs based on the degree of sun exposure. Quantitative and qualitative changes in the dermal extracellular matrix proteins such as elastin, glycosaminoglycans, and interstitial collagens are also associated in dermal photodamage. There are several morphological and biochemical indications that collagen type I is reduced in UV actinically damaged skin [1]. Various types of UV-induced matrix-degenerating metalloproteinases present in dermal fibroblasts contribute to the breakdown of dermal interstitial collagen and other connective tissue components.

As for the underlying biological mechanisms of action involved in skin damage, the skin is known to secrete a number of cytokines, including interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α [2–4]. UV irradiation up-regulates the production of these cytokines, and UV-induced collagenases such as matrix metalloproteinase (MMP)-1 from dermal fibroblasts are mediated in part by IL-1α and IL-1β [3]. Furthermore, collagenase activity has been shown to be inhibited by a tissue inhibitor of metalloproteinases (TIMP) [5].

Macrophage migration inhibitory factor (MIF) is originally identified, as a lymphokine that concentrates macrophages at inflammatory loci, is a potent activator of macrophages *in vivo* and is considered to play an important role in cell-mediated immunity [6,7]. It has been reported that MIF is expressed primarily by T cells and macrophages; recent studies have however revealed, that this protein is ubiquitously expressed by various cells, thus indicating its involvement beyond the immune system in a variety of pathologic states [8,9]. It is of interest that MIF functions as a cytokine, an anterior pituitary-derived hormone, and a glucocorticoid-induced immunomodulator [10].

In the skin, MIF expression is expressed in the epidermis, particularly in the basal layer [11]. We recently demonstrated that MIF mediates the up-regulation of MMP-1 expression in response to the stimulation of UVA irradiation [12]. However, the precise role of MIF and IL-1β interaction in the effects of UVA on MMP-1 expression of dermal fibroblasts remain unknown. In the present study, we evaluated the involvement IL-1β and MIF in MMP-1 expression in cultured human dermal fibroblasts. We then examined the UVA effects on MMPs production using IL-1α/β-deficient mice. Furthermore, fibroblasts derived from MIF-deficient mice were used to analyze the effect of IL-1β-induced MMPs production.

2. Materials and methods

2.1. Materials

The following materials were obtained from commercial sources. PD98089, SB203580, SP600125 and GF109203X were purchased from Calbiochem (San Diego, CA); Dulbecco's modified eagle medium (DMEM) from Invitrogen (Groningen, Netherland); Isogen RNA extraction kit was from Nippon Gene (Toyama, Japan); Biotrack MMP-1 assay kit, [α-^{32}P]dCTP, and Hybond N nylon membrane were from Amersham Bioscience (Piscataway, NJ); DNA random primer labeling kit was from Takara (Kyoto, Japan); Centriprep YM-30 was from Millipore (Bedford, MA); Anti-IL-1β antibody were from Genzyme TECHNE (Cambridge, MT); recombinant human IL-1β and recombinant mouse IL-1β were from R&D systems (Minneapolis, MN). Anti-MMP-1 antibody, anti-β-actin antibody and gelatin were purchased from Sigma–Aldrich (St. Louis, MO); anti-MMP-13

(Collagenase-3) antibody was from Chemicon (Temecula, CA); anti-MIF polyclonal antibody was prepared as described previously [11]. Recombinant human MIF was expressed in *Escherichia coli* BL21/DE3 (Novagen, Madison, WI) and purified as described previously [13]. This MIF contained less than 1 pg of endotoxin/μg of protein, as determined by chromogenic Lumulus amoebocyte assay (Bio-Whittaker, Walkerville, MD).

2.2. Cells and skin tissues

Human dermal fibroblasts were purchased from Dainippon Seiyaku (Osaka, Japan). Cells of passages 3–4 were used for the experiments. In brief, cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, glutamine (2 mM), sodium ascorbate (50 μg/ml), penicillin (100 U/ml penicillin), streptomycin (100 μg/ml), and amphotericin B (100 μg/ml). The cells were grown in a moist atmosphere in a 5% CO_2 incubator at 37 °C. IL-1α/β-deficient mice were established by targeted disruption of the IL-1α/β gene, using a mouse strain bred onto a BALB/c background [14]. Wild-type (WT) BALB/c mice were purchased from Japan Clea (Shizuoka, Japan). By targeted disruption of the MIF gene, a mouse strain (bred onto a C57BL/6 background) deficient in MIF was established previously [15]. Mice were maintained under specific-pathogen-free conditions. Newborn mouse skin was carefully shaven, a segment of skin excised, and fibroblasts were obtained using the standard explant technique. Briefly, the skin was cut into 3 × 5-mm pieces and placed onto large petri dishes with the subcutaneous side down. Once a sufficient number of fibroblasts had migrated out from the skin sections, pieces of the skin were removed and the cells were passaged by trypsin digestion in the same manner as for the fibroblasts. Fibroblasts were grown in DMEM containing 10% FCS and penicillin/streptomycin. The cells from passages 3 were used for the experiment.

2.3. IL-1β or MIF stimulation in fibroblasts

Human dermal fibroblasts after reaching 70% confluence were stimulated with various concentration of IL-1β for 24 h, and cells or supernatants were subjected to Northern blot, Western blot, zymography or ELISA analysis. Cells were also stimulated with various concentration of MIF, and subjected to Northern blot or Western blot analysis. To examine the effects of anti-MIF antibody on the IL-1β-induced MMP-1 mRNA, fibroblasts were stimulated by IL-1β in the presence of an anti-MIF antibody (1 or 10 μg/ml) in DMEM supplemented with 10% FCS and then further incubated for 24 h. Expression of MMP-1 mRNA and protein levels were assessed by Northern blot and MMP-1 ELISA. To examine the signal-transduction pathway of IL-1β, human dermal fibroblasts were stimulated with or without IL-1β and various inhibitors of molecules involved in the signal-transduction pathway for 24 h and subjected to Northern blot analysis. Dermal fibroblasts from MIF-deficient mice or WT mice were harvested after reaching 70% confluence. Then, the cells were stimulated with IL-1β for 24 h, and MMP-13 production was evaluated by Western blot analysis.

2.4. UVA irradiation

The UVA irradiation source was a FL20S/BLB fluorescent lamp (Clinical Supply, Tokyo, Japan) that emitted an energy spectrum with high fluency in the UVA region (300–430 nm), with a peak at 352 nm. A 6-mm thick glass plate was used to block UVB emissions. The emitted dose was calculated using a UVA radiometer photodetector (Torex, Tokyo, Japan). The human dermal fibroblasts, or dermal fibroblasts from IL-1α/β-deficient mice or WT-mice were washed with phosphate-buffered saline (PBS), suspended in Hank's buffer, and subjected to UVA irradiation. The duration of UV irradiation delivered to cells was altered by sliding a plastic lid covered with aluminum foil onto a flat-bottomed six-well plate. After irradiation, the cells were cultured in DMEM with 10% FCS at 37 °C. Control samples were mock-irradiated and maintained under the same culture conditions as those used for the UVA-irradiated specimens. *In vivo* analysis, the abdomens of IL-1α/β-deficient mice or WT mice were carefully shaved, and irradiated with UVA (0–30 J/cm^2). After UVA irradiation for 24 h, skin was surgically obtained. Cultured fibroblasts or skin was then assessed by Northern blot or Western blot analysis.

2.5. Northern blot analysis

Total RNA was isolated from monolayer cultures using an Isogen RNA extraction kit according to the manufacturer's protocols. To examine the signal-transduction pathway of IL-1β, human dermal fibroblasts were stimulated with or without IL-1β and various inhibitors of molecules involved in the signal-transduction pathway for 24 h. RNA was quantified by spectrophotometry, and equal amounts of RNA (10–15 μg) from samples were loaded on a formaldehyde-agarose gel. The gel was stained with ethidium bromide to visualize the RNA standards, and the RNA was transferred onto a nylon membrane. Fragments obtained by restriction enzyme

treatments for MMP-1, MIF, IL-1β and GAPDH were labeled with [α-^{32}P]dCTP using a DNA random primer labeling kit. Hybridization was carried out using the human MIF cDNA probe as previously described [15]. Complete coding cDNA for human MMP-1 in a pSP64 vector was obtained from the American Type Culture Collection. The templates of human MIF, IL-1β, and GAPDH cDNA for Northern blot analysis were obtained by RT-PCR from a human cDNA library of human fibroblasts. The preparation of each template was as follows: for IL-1β primers used were (870 bp), sense 5′-ATTCTCTTCAGCCAATCTTCATT-3′ (34–56) and antisense 5′-CTGGGTACAGCTCTCTTTAGGAA-3′ (881–903) (GenBank accession no. X02532); and GAPDH (1024 bp), sense 5′-CGGGATCCATGGGGAAGGTGAAGGTC-3′ (59–78) and antisense 5′-CGGGATCCTTACTCCTTGGTGGCCAT-3′ (1051–1070) (GenBank accession no. M33197) [16]. Hybridization was carried out at 42 °C for 24 h. Post-hybridization washes were performed twice in 0.1% SDS, 0.2 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate) at 65 °C for 15 min. The radioactive bands were visualized by autoradiography on Kodak X-AR5 film and quantitatively analyzed using the NIH image system. Multiple autoradiographic data were examined to ensure that the results reflected those produced in the linear range of the film. The results were normalized by GAPDH mRNA levels. Comparison of ethidium bromide-stained gels with the corresponding GAPDH mRNA levels showed that GAPDH mRNA levels reflected the total RNA loaded onto the gels.

2.6. ELISA for MIF

To examine the concentration of MIF from cultured fibroblasts, supernatants from cultured fibroblasts by IL-1β stimulation were examined using an MIF ELISA system as described previously [17]. For this assay we used recombinant human MIF to obtain the standard curve, in which good linearity was demonstrated between MIF concentrations (1–200 ng/ml) and absorbency.

2.7. ELISA for MMP-1

After reaching confluence, the cells were trypsinized and then plated on a 24-well culture dish at 4×10^4 cells per well in 0.5 ml of DMEM containing 10% FCS. After 48 h, the medium was replaced with 0.5 ml of serum-free DMEM containing various doses of IL-1β. After 24 h, the supernatants were collected and subjected to ELISA for MMP-1. MMP-1 was assayed by an ELISA using a Biotrack MMP-1 assay kit according to the manufacturer's protocol. The minimal sensitivity of the assay system was 6.25 ng/ml, and good linearity was observed at amounts up to 100 ng/ml. Using this ELISA system, all forms of MMP, including pro-MMP-1, MMP-1, and MMP-1 complexes with TIMP-1, could be measured.

2.8. Determination of MMP-1 activity in culture media of fibroblasts

Culture media of dermal fibroblasts were collected in the presence of IL-1β at 24 h, and concentrated (10-fold) using Centriprep YM-30 (Millipore, Bedford, MA) for further analyses. Then, MMP-1 activities were determined by heparin-enhanced zymography as previously described [18]. In brief, gelatin (0.5 mg/ml) was embedded in 7.5% SDS-PAGE gel. Samples (15 μg protein for each sample) were treated with sample buffer without dithiothreitol at room temperature and electrophoresed until the dye-front was near the bottom of the gel. To produce the enhancing effects, 10 μl heparin (0.3 mg/ml in 1× sample buffer without SDS) was added to the lanes 20–30 min after electrophoresis began. Each gel was washed two times with 2.5% Triton X-100, 50 mM Tris, pH 7.5, 4 °C, 20 min each, to remove SDS and then two times with buffer plus 5 mM $CaCl_2$. The gel was washed three times with incubation buffer (50 mM Tris, pH 7.5, 5 mM $CaCl_2$) and then incubated in this buffer with added protease inhibitors (50 μM each of z-phe-chloromethylketone and tosyl-phe-chloromethylketone and aminoethyl benzenesulfonyl fluoride) for 18 h at 37 °C with gentle shaking. Gels were stained with 0.1% Coomassie blue in 40% MeOH, 10% acetic acid, for 45 min, and destained with 7% acetic acid. A positive control for recombinant human MMP-1 was used to detect the molecules of MMP-1.

2.9. Western blot analysis

Fibroblasts (1×10^6 cells) were disrupted with a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The protein concentrations of the cell homogenates were quantified using a Micro BCA protein assay reagent kit. Equal amounts of homogenates were dissolved in 20 μl of Tris–HCl, 50 mM (pH 6.8), containing 2-mercaptoethanol (1%), sodium dodecyl sulfate (SDS) (2%), glycerol (20%), and bromophenol blue (0.04%), and the samples were heated to 100 °C for 5 min. The samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically onto a nitrocellulose membrane. The membranes were blocked with 1% non-fat dry milk in PBS, probed with anti-MIF, anti-IL-1β, anti-MMP-1 or anti-MMP-13 antibody, then allowed to react with goat anti-rabbit IgG Ab coupled with horseradish

peroxidase. The resultant complexes were processed for the detection system according to the manufacturer's protocol. For loading controls, we carried out Western blot analysis on β-actin using an anti-β-actin antibody.

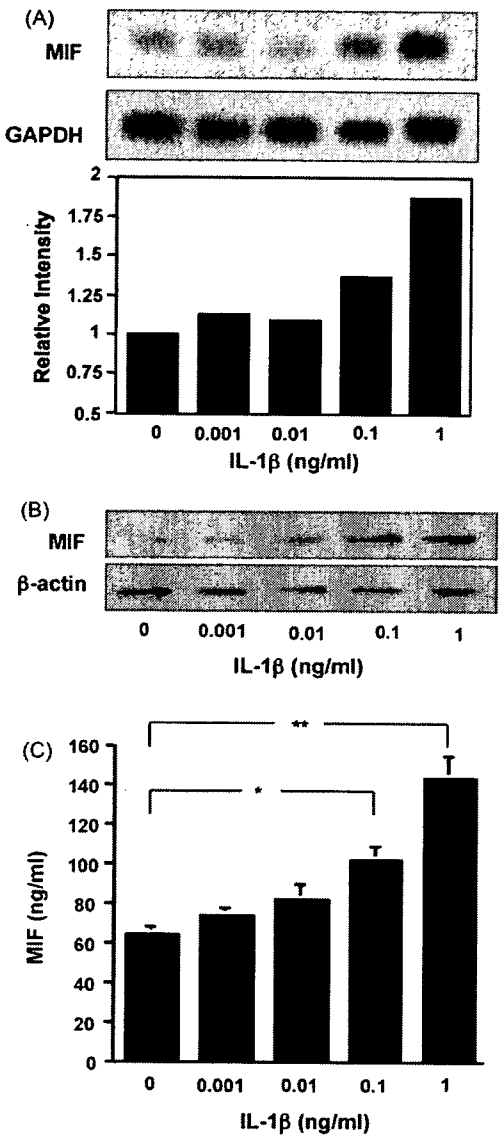

Fig. 1 IL-1β-induced MIF expression and production in human fibroblasts. Fibroblasts were treated with various concentrations of IL-1β (0–1 ng/ml) and cultured for 24 h. (a) Total RNAs extracted from IL-1β-stimulated fibroblasts and Northern blot analysis was carried. The membranes were hybridized with radiolabeled cDNA probes of MIF and GAPDH and then visualized by autoradiography. (The experiments were repeated three times with similar results.) (b) Cell lysates (40 μg) of IL-1β-stimulated fibroblasts cultured were subjected to Western blot analysis with anti-MIF antibody. (The experiments were repeated three times with similar results.) (c) MIF in the culture media was measured by ELISA, as described under Section 2. The values are the mean ± S.E. of three different experiments. $^{*}p < 0.05$ and $^{**}p < 0.005$ vs. 0 ng/ml IL-1β.

2.10. Statistics

Differences between the various treatments were statistically tested using the Student's *t*-tests. For comparisons of multiple groups, one-way ANOVA was applied to the data. *p* values of <0.05 considered statistically significant. Data in the figures are shown as the mean ± S.E.M. of several experiments.

3. Results

3.1. IL-1β-induced MIF expression in human dermal fibroblasts

We first examined whether IL-1β is able to stimulate expression and production of MIF in dermal fibroblasts. Fibroblasts were stimulated by IL-1β, and it was found that IL-1β up-regulates MIF mRNA in a dose-dependent manner after 24 h (Fig. 1a). MIF content in cell lysate is up-regulated in a dose-dependent manner by Western blot analysis (Fig. 1b). After 24-h IL-1β stimulation at a dosage of 1 ng/ml, the MIF released from dermal fibroblasts was remarkably elevated by MIF ELISA (2.3-fold, $p < 0.005$) (Fig. 1c). On the other hand, MIF is unable to stimulate IL-1β mRNA expression in dermal fibroblasts (Fig. 2a). In addition, UVA (10 J/cm^2)

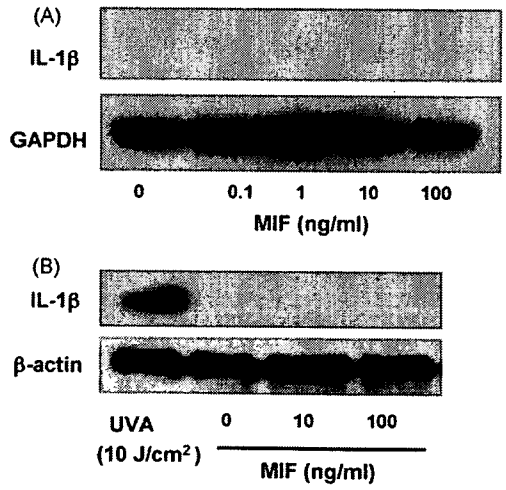

Fig. 2 MIF-induced IL-1β expression and production in human fibroblasts. Fibroblasts were treated with various concentrations of MIF (0–100 ng/ml) and cultured for 24 h. (a) Total RNAs extracted from MIF-stimulated fibroblasts. Northern blot analysis was carried out. The membranes were hybridized with radiolabeled cDNA probes of IL-1β and GAPDH and then visualized by autoradiography. (b) Cell lysates (40 μg) of 10 J/cm^2 UVA or MIF (0–100 ng/ml) stimulated fibroblasts cultured for 24 h were subjected to Western blot analysis with anti-IL-1β antibody. (The experiments were repeated three times with similar results.)

up-regulated stimulate IL-1β production, whereas MIF (up to 100 ng/ml) failed to stimulate IL-1β production by Western blot analysis (Fig. 2b).

3.2. Effects of IL-1β on MMP-1 mRNA expression, production and activation

MMP-1 mRNA was up-regulated by 1 ng/ml IL-1β stimulation in human dermal fibroblasts (Fig. 3a). MMP-1 protein was also up-regulated in the culture supernatant of human dermal fibroblasts at doses of 1 ng/ml IL-1β stimulation at 24 h ($p < 0.01$) (Fig. 3b). To investigate the effect of IL-1β-induced MMP-1 activity, we performed zymography. We used heparin to enhance the signal, because MMP-1 is difficult to detect at low levels in conventional gelatin zymography [18]. MMP-1 in the active form (45 kDa) in fibroblasts was enhanced by 1 ng/ml IL-1β stimulation at 24 h (Fig. 3c).

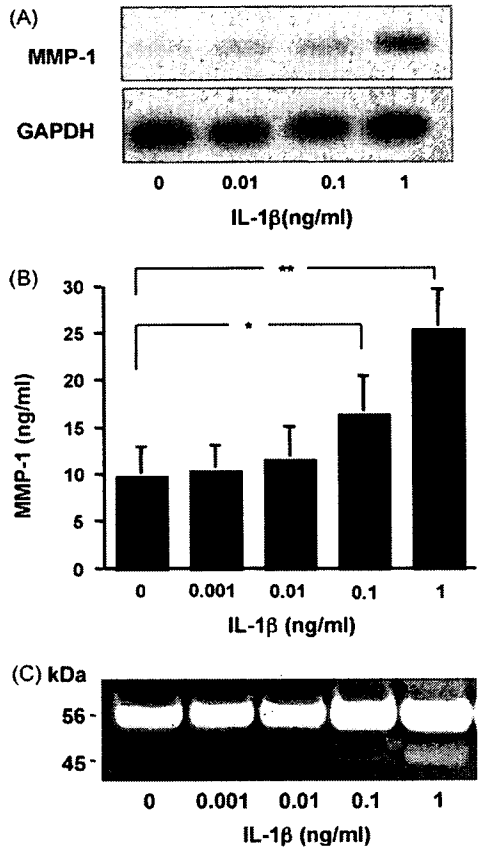

Fig. 3 Effects of IL-1β on MMP-1 mRNA expression and production in dermal fibroblasts. (a) Total RNAs extracted from dermal fibroblasts were treated with IL-1β (0–10 ng/ml) at 24 h. Northern blot analysis was carried out for MMP-1 and GAPDH. (The experiments were repeated three times with similar results.) (b) Aliquots of the culture supernatants of dermal fibroblasts in serum-free medium were collected after treatment with various concentrations of IL-1β for 24 h, then subjected to ELISA for MMP-1 ($n = 5$). $^*p < 0.05$ and $^{**}p < 0.01$ vs. control (0 ng/ml). (c) Culture supernatants of dermal fibroblasts were collected at the indicated doses of IL-1β for 24 h. The supernatants were concentrated 10-fold and subjected to heparin-enhanced zymography. Molecular weight markers at 56 and 45 kDa show latent and active forms of MMP-1, respectively.

3.3. Inhibition of IL-1β-induced MMP-1 expression and production of dermal fibroblasts by a neutralizing anti-MIF antibody

We attempted to determine whether neutralizing anti-MIF antibody influences IL-1β-induced MMP-1 expression in human dermal fibroblasts. By Northern blot analysis, we found that the anti-MIF antibody (10 μg/ml) significantly down-regulated (37%) the expression of MMP-1 mRNA-induced by IL-1β stimulation (1 ng/ml) (Fig. 4a). On the other hand, non-

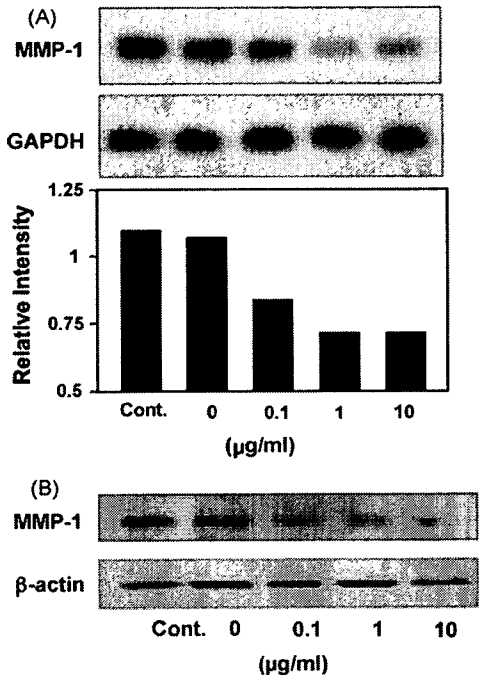

Fig. 4 Effects of neutralizing anti-MIF antibody on the IL-1β-induced MMP-1. Fibroblasts were stimulated by IL-1β (1 ng/ml) in the presence of neutralizing anti-MIF antibody (0–10 μg/ml) in DMEM supplemented with 10% FCS and then further incubated for 24 h. (a) Expression of MMP-1 mRNA was assessed by Northern blot analysis. Cont. non-immunized control antibody. (The experiments were repeated three times with similar results.) (b) Cell lysates (40 μg) of IL-1β (1 ng/ml)-stimulated fibroblasts with anti-MIF antibody (0–10 μg/ml) cultured for 24 h were subjected to Western blot analysis using an anti-MMP-1 antibody. The results with anti-β-actin antibody are shown as a control. Cont. non-immunized control antibody. (The experiments were repeated three times with similar results.)

immunized control antibody had no effect on the expression of MMP-1 mRNA-induced by IL-1β stimulation. Based on the results of the Western blot analysis, we confirmed that IL-1β-induced MMP-1 production is inhibited by neutralizing anti-MIF antibody (Fig. 4b).

3.4. Effects of MAP kinase and PKC inhibitors on IL-1β-induced MMP-1 mRNA expression

To examine the signal-transduction pathway of IL-1β, we examined the effects of several inhibitors of molecules involved in the signal-transduction pathway when dermal fibroblasts were stimulated with IL-1β relevant to MMP-1 up-regulation.

Several MAP kinase inhibitors were tested, including MEK inhibitor PD98089, p38 inhibitor SB203580 and JNK inhibitor SP600125. We found that these p38 inhibitor and JNK inhibitor significantly reduced MMP-1 mRNA stimulated by IL-1β (61% and 41%, respectively) (Fig. 5b and c). In contrast, the MEK inhibitor failed to inhibit the up-regulation of MMP-1 mRNA (Fig. 5a). Furthermore, we found that PKC inhibitor GF109203X also significantly suppressed MMP-1 mRNA stimulated by IL-1β (Fig. 6).

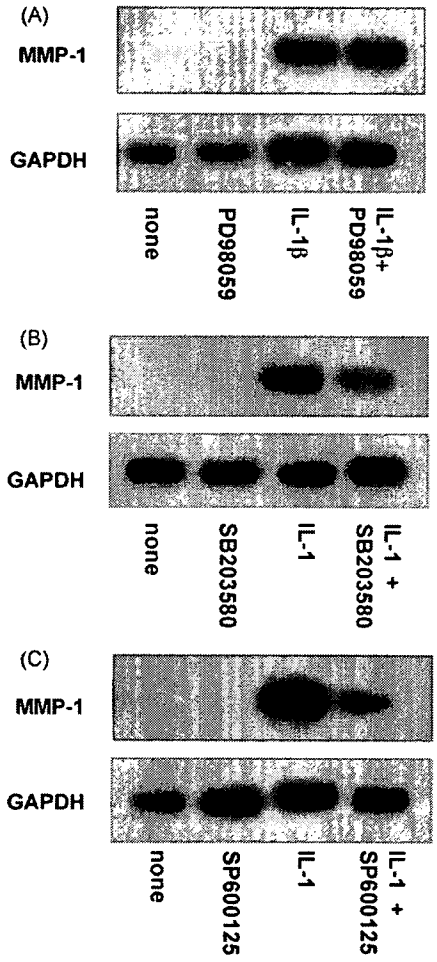

Fig. 5 Effects of MAP kinase inhibitors on IL-1β-induced MMP-1 mRNA expression. Dermal fibroblasts were preincubated for 30 min with various concentrations of MAP kinase inhibitors prior to challenge with IL-1β. The cells were then incubated for 24 h and assessed for MMP-1 mRNA. (a) MEK inhibitor PD98089; lane 1, no stimulation; lane 2, PD98059 (40 μM); lane 3, IL-1β (1 ng/ml); lane 4, IL-1β (1 ng/ml) + PD98059 (40 μM). (b) p38 inhibitor SB203580; lane 1, no stimulation; lane 2, SB203580 (10 μM); lane 3, IL-1β (1 ng/ml); lane 4, IL-1β (1 ng/ml) + SB203580 (10 μM). (c) JNK inhibitor SP600125; lane 1, no stimulation; lane 2, SP600125 (30 μM); lane 3, IL-1β (1 ng/ml); lane 4, IL-1β (1 ng/ml) + SP600125 (30 μM).

3.5. UVA-induced MMP-13 mRNA expression and production in cultured dermal fibroblasts and skin tissue *in vivo* from IL-1α/β-deficient mice

To clarify whether synthesis of MMP is required for the UVA-induced collagenase, we used dermal fibroblasts from IL-1α/β-deficient mice in the production of mouse collagenase MMP-13. Although MMP-13 plays a restricted role in human tissues, it is the predominant tissue collagenase in rodents. Twenty-four hours after UVA irradiation, a significant decrease (50%) in viability was observed only after more than 20 J/cm^2 UVA irradiation in fibroblasts from IL-1α/β-deficient mouse (data not shown); we therefore used up to 10 J/cm^2 UVA irradiation for experiment. After 24 h of 10 J/cm^2 UVA irradiation, MMP-13 production was observed in cell lysates of dermal fibroblasts in control WT mice in a dose-dependent

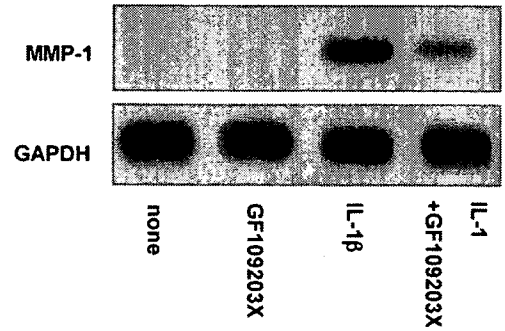

Fig. 6 Effects of PKC inhibitor on IL-1β-induced MMP-1 mRNA expression. Dermal fibroblasts were preincubated for 30 min with various concentrations of PKC inhibitor prior to challenge with IL-1β, then assessed for MMP-1 mRNA. The cells were then incubated for 24 h in the presence or absence of PKC inhibitor GF109203X. Lane 1, no stimulation; lane 2, GF109203X (10 μM); lane 3, IL-1β (1 ng/ml); lane 4, IL-1β (1 ng/ml) + GF109203X (10 μM). (The experiments were repeated three times with similar results.)

manner (Fig. 7a). On the other hand, UVA irradiation appeared to have little effect on MMP-13 production in dermal fibroblasts of IL-1α/β-deficient mice. Since IL-1 is involved in mechanism of MMP-13 production, MMP-13 expression is low level in IL-1α/β-deficient mice. IL-1α/β-deficient mouse is low level of MMP-13 expression and less sensitive to UVA irradiation. Consistent with these results *in vitro*, elevated MMP-13 production was also observed in the skin of control WT mice in a dose-dependent manner after UV irradiation *in vivo*, whereas UVA irradiation had little effect on MMP-13 production in the skin of IL-1α/β-deficient mice (Fig. 7b).

3.6. MMP-13 production in MIF-deficient mice after IL-1β stimulation

To examine the involvement of IL-1β and MIF in MMPs production, MMP-13 production from dermal fibroblasts stimulated with IL-1β in MIF-deficient mice were analyzed. Western blotting revealed dermal fibroblasts of MIF-deficient mice were much less sensitive to IL-1β-induced MMP-13 production compared to WT mice (Fig. 8).

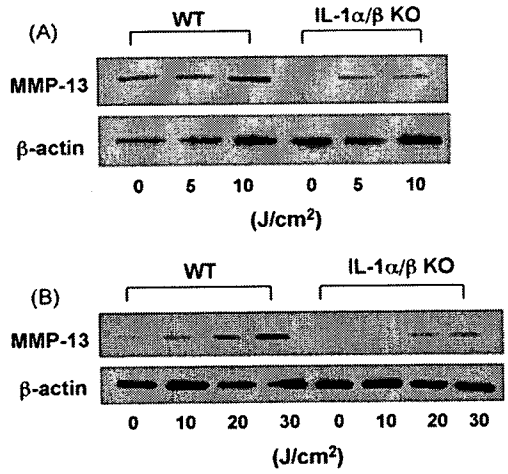

Fig. 7 UVA-induced MMP-13 production from cultured dermal fibroblasts and skin tissues of IL-1α/β-deficient mice. (a) Cultured third passage dermal fibroblasts from IL-1α/β-deficient mice or WT mice were irradiated with UVA at 0, 5, or 10 J/cm^2. After 24 h UVA irradiation, cell lysates (40 μg) were subjected to Western blot using anti-MMP-13 antibody. The results with anti-β-actin antibody are shown as a control. (The experiments were repeated three times with similar results.) (b) Shaved abdomens of IL-1α/β-deficient mice or WT mice were irradiated with UVA at 0, 10, 20, or 30 J/cm^2. After 24 h, skin homogenates were subjected to Western blotting using an anti-MMP-13 antibody. (The experiments were repeated three times with similar results.)

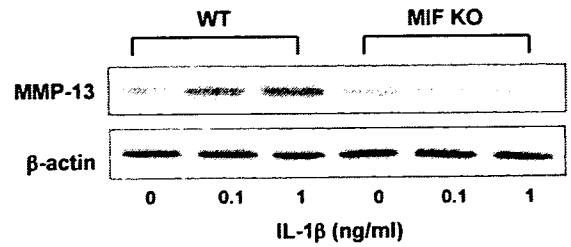

Fig. 8 Effects of IL-1β on MMP-13 production from cultured dermal fibroblasts of MIF-deficient mice. Cultured third passage dermal fibroblasts from MIF-deficient mice or WT mice were stimulated with various concentration of IL-1β. After 24 h, cell lysates (40 μg) were subjected to Western blot using anti-MMP-13 antibody. The results with anti-β-actin antibody are shown as a control. (The experiments were repeated three times with similar results.)

4. Discussion

Regarding the environmental damage to skin, the most common physical injury is that caused by UV irradiation. UV irradiation substantially increases the risk of actinic damage to the skin. Photoaged skin is biochemical characterized by a predominance of abnormal elastic fibers in the dermis and by a dramatic decrease in distinct collagen types. Interstitial collagens, the major structural components of the dermis, have been found to be particularly diminished in skin actinically damaged by UV irradiation [19–21]. It is of note that UVA irradiation reaches the reticular dermis, rendering fibroblasts accessible targets [22]. UV irradiation induces MMPs responsible for alterations in the collagenous extracellular matrix of connective tissue, resulting in impaired integrity.

The molecular mechanisms of UV-induced MMPs have yet to be defined. UV-induced expression of pro-inflammatory cytokines such as IL-1β and TNF-α may also in part account for the expression of MMPs. Constitutive collagenase synthesis has been reported to be regulated by an IL-1β autocrine mechanism [23]. In the present study, IL-1β up-regulates MMP-1 mRNA as well as protein levels and MMP-1 activity by zymography in dermal fibroblasts. We also found that inhibitors for p38 inhibitor and JNK inhibitor significantly reduced MMP-1 mRNA stimulated by IL-1β. In contrast, the MEK inhibitor failed to inhibit the up-regulation of MMP-1 mRNA. Furthermore, we found that PKC inhibitor GF109203X also significantly suppressed MMP-1 mRNA stimulated by IL-1β. These results suggest that IL-1β is involved in the up-regulation of UVA-induced MMP-1 in dermal fibroblasts through p38, JNK and PKC-dependent pathways. We previously demonstrated that MIF is involved in the up-regulation of UVA-induced MMP-1 in dermal fibro-

blasts through PKC-, PKA-, src-family tyrosine kinase-, MAPK-, c-jun-, and AP-1-dependent pathways [12]. In particularly, the up-regulation of MMP-1 by MIF stimulation was inhibited by a MEK inhibitor, and a JNK inhibitor, but not a p38 inhibitor [12]. These observations indicate that signal-transduction pathway by IL-1β or MIF-induced MMP-1 in dermal fibroblasts were different in MAP kinas cascade.

Moreover, we observed that dermal fibroblasts of IL-1α/β-deficient mice produced significantly decreased levels of MMP-13, and UVA irradiation had little effect on MMP-13 production in the skin of IL-1α/β-deficient mice. These observations strongly support the previous report that UVA-induced IL-1β play a central role in the synthesis of collagenase/MMP-1 [3].

MIF functions as a pleiotropic cytokine by participating in inflammation and immune responses. MIF was originally discovered as a lymphokine involved in delayed hypersensitivity and various macrophage functions, including phagocytosis, spreading, and cell growth activity [24–26]. MIF was recently reevaluated as a proinflammatory cytokine and pituitary-derived hormone that potentiates endotoxemia [9]. This protein is ubiquitously expressed in various organs, including the brain, and kidney [27–33]. It is currently known that MIF is a proinflammatory cytokine that plays an essential role in the activation of T cells after antigenic stimulation. Furthermore, this protein plays a key role in cell proliferation and angiogenesis [34]. In the skin, MIF is expressed in the epidermis, particularly in the basal layer [11]. MIF is known to play an important role in the skin in immune responses, inflammation and cell proliferation [35]. In relation with MIF and photoaging, we recently demonstrated that after UVA irradiation, MIF up-regulates MMP-1 mRNA as well as protein levels and MMP-1 activity by zymography in dermal fibroblasts [12]. However, the precise role of MIF and IL-1β interaction in the effects of MMP-1 expression of dermal fibroblasts are not well understood.

In this study, we have here demonstrated that IL-1β stimulation leads to a significant increase in specific MIF mRNA and protein levels in human dermal fibroblasts. We also demonstrated that neutralizing anti-MIF antibody suppresses the expression of MMP-1 induced by IL-1β. On the other hand, MIF is unable to stimulate IL-1β expression and production in dermal fibroblasts. It is therefore possible that after UVA irradiation, IL-1β induce MMP-1 expression. In addition, MIF induced by IL-1β also enhance the MMP-1 expression in dermal fibroblasts. In this context, we also analyzed MIF-deficient mice, and revealed that dermal fibroblasts from MIF-deficient mice were much less sensitive to IL-1β-induced MMP-13 production. It is therefore possible that IL-1β may stimulate MMP-13 production via MIF in dermal fibroblasts. In fibroblasts, we can suggest that UVA irradiation may stimulate IL-1β production by an autocrine loop of both IL-1β and MIF. Then, both IL-1β and MIF play an important role in the synthesis of MMP-1 (MMP-13).

In conclusion, we suggest the UV-induced cytokine network consisting of IL-1β and MIF interrelated loops induce MMP-1 (MMP-13) and thus may contribute to the loss of interstitial collagen in cutaneous photoaging. This newly identified mechanism may contribute to our understanding of photo-induced dermal connective tissue damage, which results in photoaging.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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LETTER TO THE EDITOR

Macrophage migration inhibitory factor (MIF) in bullous pemphigoid

Bullous pemphigoid (BP) is an inflammatory subepidermal blistering disease associated with an autoimmune response to two hemidesmosomal antigens, BP230 and BP180. In the upper dermis of lesion, various inflammatory cells, including eosinophils and lymphocytes, have been identified. The blister cavity typically contains fibrin and eosinophils. Furthermore, various cytokines are also thought to play crucial roles in the inflammatory responses.

Macrophage migration inhibitory factor (MIF) is one of the immunoregulatory cytokines involved in T cell activation and delayed-type hypersensitivity. T cells and macrophages are the primary source of MIF [1]. Keratinocytes or eosinophils have also been reported to be an important source of MIF in allergic inflammatory diseases [2,3]. MIF is considered to act, by both paracrine and autocrine stimulatory pathways, to augment the activation of these cells [3]. Increased MIF expression has been reported in lesions from many immune/inflammatory diseases, including psoriasis, atopic dermatitis, asthma and so on [3–6]. However little is known about the contribution of MIF in BP. To elucidate the involvement of MIF in the pathogenesis of BP, serum and blister fluid MIF levels of patients with BP were examined. Immunofluorescent staining was also performed in order to determine the distribution of MIF in the skin lesions.

The study groups included 21 patients with BP (mean age was 71.6 years old, range 46–87) and 45 healthy volunteers (mean age was 45.1 years old, range 21–64). All of the BP patients were diagnosed based on clinical, histological and immunofluorescent criteria. Serum samples were obtained from all of the BP patients at the active clinical stage before treatments and from the healthy volunteers. At the same stage, bullous fluid samples were also collected from six BP patients. Three bullous fluid samples from mechanical bulla were collected as controls. At the inactive stage, which was defined as the period when skin lesions disappeared as a result

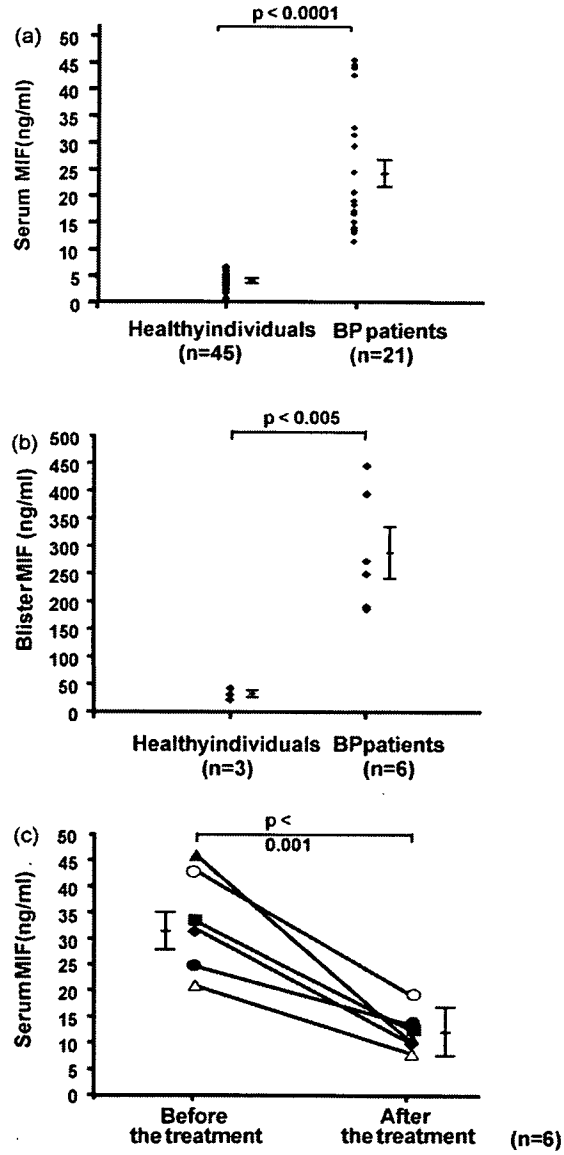

Fig. 1 (a) The serum MIF concentrations of the BP patients in comparison to healthy individuals. (b) The blister MIF concentrations of the BP patients in comparison to those of mechanical bulla. (c) The serum concentrations of MIF before and after treatment in patients with BP.

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of treatment with oral corticosteroid, serum samples were obtained from six BP patients. At this stage, all the patients were controlled with low dose of medicine. Skin biopsies were taken from the skin lesions during the active stage. Aliquots of serum and bullous fluid, and biopsies specimens were stored at −80 °C until used. MIF levels were determined using a human MIF enzyme linked immunosorbent assay (ELISA; Sapporo Immuno Diagnostic Laboratory, Sapporo, Japan) as described previously [6]. Double immunofluorescent staining for the BP skin was performed using seven biopsy specimens. A rabbit polyclonal anti-human MIF antibody and an anti-eosinophil major basic protein (MBP) antibody (CHEMICON International, Temecula, CA) were used as the primary antibodies. All data were presented as the mean ± S.E.M. Statistical analysis was performed using the Mann–Whitney *U*-test and paired *t*-test. A *p*-value of less than 0.05 was considered to be statistically significant.

The average MIF level in the serum collected from BP patients at the active stage was 24.0 ± 1.94 ng/ml. On the other hand, the mean serum MIF levels were 4.1 ± 0.26 ng/ml in the healthy control subjects. BP patients during the active clinical stage showed higher levels of MIF ($p < 0.0001$) in comparison to the healthy control subjects (Fig. 1(a)). The average MIF level in blister fluid collected from BP was 289.7 ± 43.9 ng/ml. The mean MIF levels were 31.9 ± 6.23 ng/ml in mechanical bulla (Fig. 1(b)). Blister fluid from the BP showed higher levels of MIF ($p < 0.005$) in comparison to the controls. The elevated serum MIF levels in BP patients decreased significantly after treatment (Fig. 1(c)). MIF was expressed on the entire epidermal layer and the infiltrating cells in the upper dermis of skin lesions. Most of the cells in the upper dermis also expressed eosinophil MBP (Fig. 2).

This study demonstrated that the MIF levels in the serum and blister fluid were significantly elevated in BP patients and the serum MIF levels decreased in accordance with the improvement of BP. The immunofluorescent analysis demonstrated that the keratinocytes and the infiltrating eosinophils in the skin lesions were identified as the dominant source of MIF in BP. These results in BP were similar to those in atopic dermatitis or psoriasis except for the fact that MIF expression on the infiltrating cells was not detected in these diseases [3,6]. The source of MIF in the sera was not precisely demonstrated at this time. It is possible that the circulating eosinophils release MIF as reported previously [4].

Recent studies have revealed the levels of various cytokines, including IL-1 beta, IL-4, IL-5, IL-6, IL-8, IL-10, TNF-alpha and IFN-gamma to increase in the serum or the blister fluid of BP [7]. The secretion of

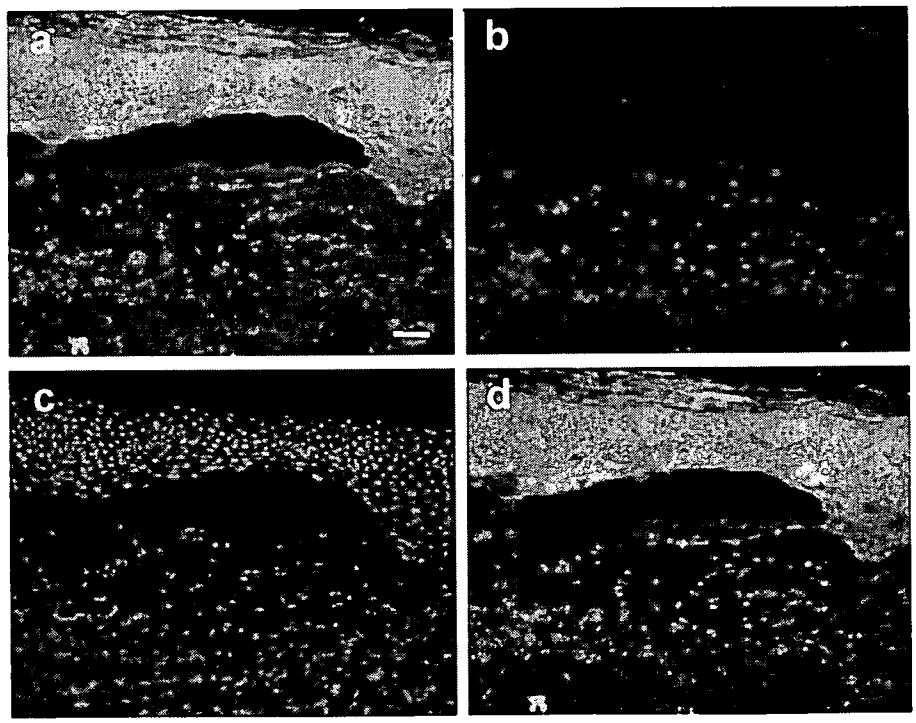

Fig. 2 The immunofluorescent observation of the skin in a BP lesion. MIF was expressed throughout the entire epidermal layer and it was co-expressed with eosinophil MBP in the upper dermis. (a) Immunostaining for MIF, (b) immunostaining for eosinophil MBP, (c) staining for DAPI, 4′,6-diamidino-2-phenylindole and (d) merged image. The scale bar, 20 μm, is the same for all panels.

MIF is up-regulated *in vitro* by TNF-alpha and IFN-gamma [1]. MIF in turn augments the secretion of TNF-alpha and IL-8 [8]. This inflammatory loop characterizes MIF as a proinflammatry cytokine. Therefore MIF might induce the local inflammatory and immunological responses associated with BP in cooperation with other cytokines. Furthermore, chemokines such as eotaxin and TARC also increased in the sera of patients with BP [9,10]. However, little is known about the relationship between MIF and chemokines. As a result, more investigations are called for to clarify this relationship in the future.

These results suggest that MIF might be involved in the pathogenesis of BP via both the inflammation of the epidermis and the infiltration of various cells, such as eosinophils, and it may therefore be a promising target as a potential antibody therapy of BP.

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(Fig. 1c, black arrows). There were numerous blood vessels in the peripheral region of the tumour (Fig. 1d), especially where matrix deposition was absent. Many of these blood vessels were dilated and showed a large sinusoidal configuration. The major histological features of PMTMCT described by Folpe *et al.*[8] are: (i) spindle- to stellate- or round-shaped tumour cells with normochromatic, small nuclei and indistinct nucleoli, (ii) myxoid to myxochondroid matrix, (iii) calcification with osteoclast-like giant cells, and (iv) prominent blood vessel formation with a pattern similar to that of a haemangiopericytoma. Our patient showed all of these features and thus we diagnosed him as having PMTMCT associated with OOM. Immunohistochemically, the tumour cells showed reactivity for vimentin (data not shown). The tumour cells were not reactive with S100, CD34 or epithelial membrane antigen (data not shown). The tumour matrix contained variable amounts of mucus, which stained with alcian blue (data not shown). Blood vessels were not reactive with α-smooth muscle actin. To determine whether the tumour expressed FGF23 protein, immunohistochemical analysis using antihuman FGF23 antibody was performed as described previously.[10] The majority of tumour cells stained with FGF23 (Fig. 1e). No FGF23 expression was observed in the smudgy matrix.

Our case supports the involvement of FGF23 produced from PMTMCT in the pathogenesis of OOM. Identification of the origin of the tumour cells and elucidation of the mechanism of high expression of FGF23 in the tumour cells are necessary for understanding the pathophysiology of PMTMCT.

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Unilateral periorbital oedema due to sarcoid infiltration of the eyelid: an unusual presentation of sarcoidosis with facial nerve palsy and parotid gland enlargement

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SIR, Causes of unilateral eyelid swelling include cellulitis, angioedema, granulomatous blepharitis and malignant lymphoma. However, cases of sarcoidosis that present with unilateral eyelid swelling are rare. We report a patient with sarcoidosis who had a remarkable, unilateral eyelid swelling. Interestingly, the patient also had facial nerve palsy in the first branches of the right trigeminal nerve resulting facial weakness, in just the same area as the skin involvement.

A 54-year-old man was initially seen with a 5-month history of persistent and asymptomatic eruptions on his right eyelid with low-grade fever and malaise. Physical examination revealed remarkable swelling on the right eyelid with a brown-coloured erythema but without heat, tenderness or pruritus (Fig. 1a). Wrinkling around the right side of the forehead was indistinct. In addition, he had a right parotid gland swelling. The initial clinical diagnosis was granulomatous blepharitis. Histopathological examination of his upper right eyelid showed numerous noncaseating granulomas in the dermis, composed of epithelioid cells with a few scattered lymphocytes and histiocytes surrounding the granulomas (Fig. 2a, b).

Laboratory data showed elevated levels of angiotensin-converting enzyme (ACE) at 29·7 U L^{-1} (normal 8·3–21·5),

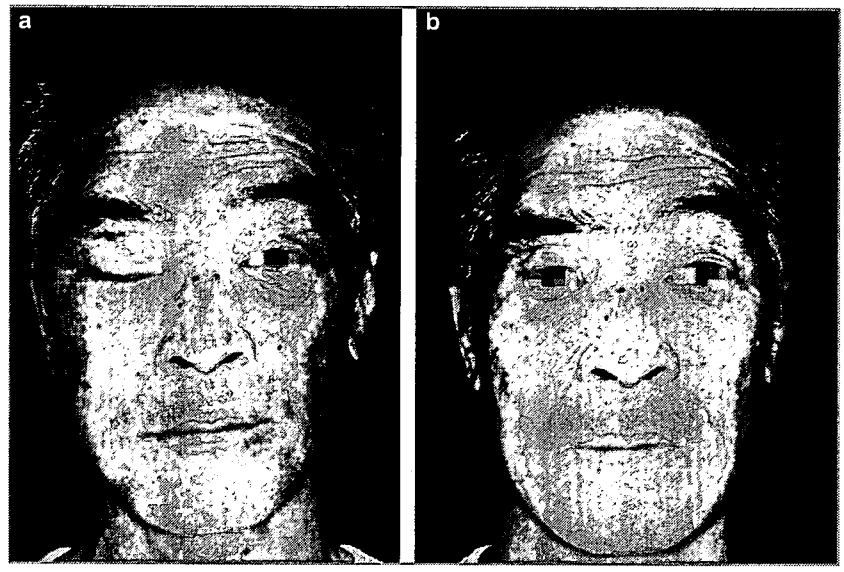

Fig 1. (a) Upon admission, the right upper and lower eyelid and right cheek were swollen. There was slight contraction of the right frontalis muscle on attempting to wrinkle the forehead. (b) After systemic prednisolone therapy. The eyelid and cheek swelling had disappeared, enabling the right eye to open.

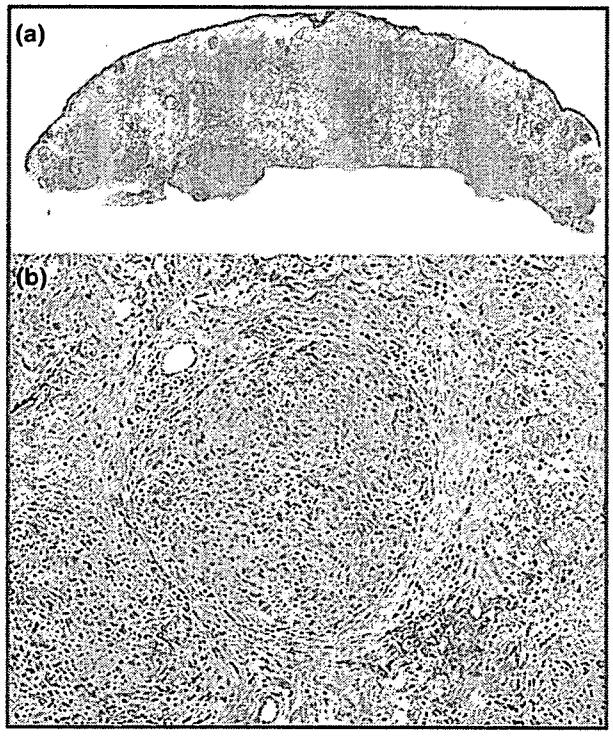

Fig 2. (a) Histopathological features of the right upper eyelid showed multiple nodules throughout the dermis. (b) Multiple noncaseating granulomas composed of epithelioid cells were present in the dermis. Haematoxylin and eosin; (a) low power magnification; (b) high power magnification.

and an erythrocyte sedimentation rate of 20 mm in the first hour. Intradermal injection of purified protein derivative for *Mycobacterium tuberculosis* gave negative results. Ophthalmological examination was unremarkable. An electrocardiogram revealed a ventricular extrasystole of 2000 beats per day. Computed tomography and magnetic resonance imaging showed swelling of his right parotid gland, and lymph node enlargement at the upper mediastinum, both sides of the aorta, and bilateral inguinal lymph nodes, whereas no bilateral hilar lymphadenopathy was detected.

Based on the clinical and histological findings, a diagnosis of sarcoidosis was made. In addition, the patient's symptoms, including fever, facial nerve palsy and swelling of the right parotid gland, were compatible with an incomplete type of Heerfordt syndrome. Because of arrhythmia, facial nerve palsy and remarkable eyelid swelling, we started the patient on oral prednisolone 40 mg daily. Within several days, rapid subsidence of the periorbital swelling was obtained (Fig. 1b). Furthermore, ACE levels normalized and the extrasystole improved slightly.

Sarcoidosis is a multisystemic granulomatous disorder of unknown aetiology, most frequently manifest in the lung, skin, lymph node and eye. Cases of sarcoidosis-related eyelid swelling have previously been reported,[1–8] but in only one case were sarcoidosis lesions confirmed by skin biopsy.[7] In the other cases, eyelid swelling might have occurred as a result of an inflammatory reaction occurring in cutaneous sarcoidosis. In addition, eyelids were involved bilaterally in these reports, whereas our patient showed only unilateral eyelid involvement.

Furthermore, our patient was also diagnosed as having Heerfordt syndrome, which is a rare association of sarcoidosis features characterized by fever, uveitis, swelling of the parotid gland and facial nerve palsy.[9] This syndrome can be further divided into complete and incomplete types. Our case is classified as an incomplete type, as uveitis was absent. The facial nerve palsy in this syndrome might occur as a result of compression of the facial nerve by the parotid gland swelling or direct infiltration of sarcoidal granulomas to the nerve. In our patient, the eyelid swelling, swelling of the parotid gland and facial nerve palsy were observed on the same (right) side of the face, with the first branches of the right trigeminal nerve. To our knowledge, this is the first case of sarcoidosis with Heerfordt syndrome that was simultaneously limited to just one side of the face.

As our patient had shown arrhythmia, facial nerve palsy and marked swelling of the eyelid, we decided to treat him with a systemic corticosteroid at 40 mg daily. Rapid subsidence of the eyelid swelling and facial nerve palsy was obtained within several days, and no recurrence was seen at 7 months.

Here we have reported an interesting case of sarcoidosis with remarkable unilateral eyelid swelling associated with Heerfordt syndrome. In conclusion, we believe that eyelid swelling should be added to the list of clinical signs associated with sarcoidosis.

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Pityriasis rubra pilaris in a mother and two daughters

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SIR, Pityriasis rubra pilaris (PRP) is a disorder of keratinization of unknown cause. Most cases of PRP are sporadic but familial clustering has been reported.[1–4] We report three members of one family with clinical and histological features of PRP (Fig. 1). To our knowledge, this is the first report of congenital PRP affecting two daughters.

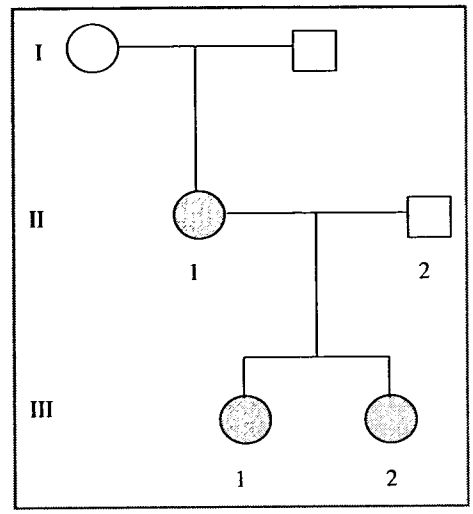

Fig 1. Family pedigree showing hereditary pityriasis rubra pilaris.

Patient 1 (generation II, offspring 1). The mother, now aged 33 years, developed PRP when she was 12 years old. There was no prior family history of PRP, psoriasis or atopy. She has the classical type of adult-onset PRP typified by a cephalocaudal eruption of follicular hyperkeratotic papules progressing to generalized erythroderma with islands of sparing (Fig. 2a,b). She also has a scaly scalp, yellow thickening of the palms and soles and subungual hyperkeratosis. The clinical diagnosis of PRP was supported by histological findings of hyperkeratosis, patchy parakeratosis varying in both horizontal and vertical planes and follicular plugging.

She has been treated with potent topical steroids, psoralen and ultraviolet A phototherapy, narrowband ultraviolet B phototherapy, isotretinoin, methotrexate and ciclosporin with variable response. She used emollients only during pregnancy when she noticed some improvement followed by a postnatal relapse.

Patient 2 (generation III, offspring 1). The first daughter was born with thick scales on the scalp (Fig. 2c) and areas of superficial peeling on the face (Fig. 2d), genital region, palms and soles. Within weeks she developed follicular erythematous papules on the face, trunk and limbs (Fig. 2e,f) which enlarged into pink plaques with a scaly edge. Some of the plaques coalesced on the trunk. There was no palmoplantar thickening. A skin biopsy showed alternating orthokeratosis and parakeratosis in both the vertical and horizontal directions with lipping of the follicular ostia and associated follicular plugging. These appearances were consistent with PRP. She is now 3 years old and her skin is mostly clear, requiring emollients and intermittent use of mild topical steroids for redness affecting the creases.

Patient 3 (generation III, offspring 2). The second daughter was born with similar findings to her sister, although less severely affected. She had greasy scales on the front of her scalp and forehead extending to the eyelids (Fig. 2g). She also had

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See related article on pg 802

Soluble Fas Ligand: Is It a Critical Mediator of Toxic Epidermal Necrolysis and Stevens–Johnson Syndrome?

Junko Murata[1] and Riichiro Abe[1]

Although soluble Fas ligand (sFasL) is an important candidate in toxic epidermal necrolysis (TEN) and Stevens–Johnson syndrome (SJS), Stur and colleagues report that elevated sFasL has been detected in maculopapular rashes. In addition to sFasL, other factors, including predisposing genetic factors, should also be investigated to determine their precise pathogenesis in TEN and SJS.

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Toxic epidermal necrolysis (TEN) and Stevens–Johnson syndrome (SJS) are among the most severe cutaneous adverse reactions seen in humans. TEN and SJS are life-threatening diseases with a mortality rate approaching 25%. The main causes of these diseases are drug intake and, to a lesser extent, viral infection. In TEN and SJS patients' epidermis, marked keratinocyte apoptotic events are frequently observed. This keratinocyte apoptosis results in blister formation and widespread skin detachment. In contrast to TEN and SJS, maculopapular rashes (MPRs) are milder variants of cutaneous reactions also due to drug allergy or viral infection. Occasional apoptotic keratinocytes are sometimes observed histologically in MPR epidermis.

So far, numerous possible mediators of keratinocyte apoptosis have been suggested, such as peripheral cytotoxic T cells, inflammatory cytokines, nitric oxide, granzyme B, and perforin. For example, Nassif *et al.* (2002) showed that T lymphocytes present within TEN lesions may exhibit drug-specific cytotoxicity against autologous cells without restimulation. However, although TEN and SJS lesional skin contained few inflammatory cells, there were no other convincing ways to explain how keratinocyte apoptosis might occur. In addition, tumor necrosis factor-α, a potent apoptotic mediator, has been suggested as a critical factor in TEN and SJS (Paquet *et al.*, 1994). As increases in serum levels of tumor necrosis factor-α have been observed in various inflammatory diseases, it is unlikely that tumor necrosis factor-α alone is a specific mediator in TEN and SJS.

In 1998, Viard *et al.* (1998) reported that the activation of Fas through Fas ligand (FasL) is an important primary step leading to keratinocyte apoptosis in TEN. The generally held concept is that Fas and FasL are derived from keratinocytes, and that FasL expressed by keratinocytes causes keratinocyte apoptosis in TEN in either an autocrine or a paracrine fashion (Viard *et al.*, 1998).

Conversely, we demonstrated that the levels of soluble FasL (sFasL) in patients' sera were elevated in an initial phase and significantly declined between 3 and 6 days after the start of the disease course. We also showed that sFasL secretion from peripheral blood mononuclear cells could be induced after the causative drug stimulation *in vitro* (Abe *et al.*, 2003).

Clinically, there are some difficulties in diagnosing TEN and SJS. Because patients sometimes show only maculopapular eruptions without any mucosal involvement in the early stage of disease, it is quite difficult to distinguish TEN and SJS from MPR. It is also difficult to determine from a patient's clinical appearance and laboratory data alone whether the disease is due to drug intake or viral infection.

Stur and colleagues (2007, this issue) show that sFasL levels were elevated in sera from SJS patients but were low in TEN patients. In addition to this result, they detected high sFasL levels in sera from MPR patients. In particular, sFasL serum levels were elevated in drug-induced MPR; however, no significant increase was

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observed in the patients with viral infection. The authors therefore suggest that determination of sFasL serum concentration might be useful to distinguish MPRs caused by drugs versus viral infection.

So far it has been assumed that high sFasL levels mediated marked keratinocyte apoptosis and resulted in TEN and SJS. Previous reports demonstrated that sFasL serum levels in MPR patients were not elevated (Abe *et al.*, 2003). MPR, SJS, and TEN are considered to be part of the same spectrum of diseases, and it was expected that sFasL levels might be correlated to the severity of these diseases.

> Genetic backgrounds may influence organ-specific sensitivity of sFasL.

Now, important scientific issues arise from the data in this paper: If serum sFasL levels are elevated in MPR patients, what other unknown factors might affect disease progressing to TEN and SJS? Furthermore, what are the indicators that differentiate MPR from TEN and SJS? Finally, are there other factors that can push the disease process resulting in TEN and SJS associated with viral infection?

The authors explained the differences between their results and the results of other investigators by (1) the larger number of MPR patient sera (n = 42) that was examined; (2) a rapid decline of sFasL levels during the first few days of disease onset, as we reported (Abe *et al.*, 2003); and (3) that fact that the sFasL concentration in MPR patients seems to be dependent on the type of drug.

To further clarify whether sFasL levels are elevated in TEN and SJS, we have collected a number of TEN and SJS serum samples from a range of medical institutions in Japan. In fact, our data from more than 20 TEN patients indicate that high sFasL levels were detected only in the early stage before mucosal lesions or bullae appeared (J.M. *et al.*, manuscript in preparation).

What is the true mediator of apoptosis in TEN and SJS? Several papers have reported genetic factors related to drug hypersensitivity and the influencing of drug metabolism and the immune response, including HLA genotypes. Chung *et al.* (2004) found that the alleles within the HLA region occurred at an increased frequency in patients with carbamazepine-induced SJS; particularly, HLA-B*1502 was present in 100% of carbamazepine-SJS patients but in only 3% of carbamazepine-tolerant patients and in 8.6% of the general population. These findings could explain the rarity of TEN and SJS (approximately 100 cases per million per year). On the other hand, Fas and FasL polymorphisms might be associated with an increased risk of particular diseases. A single-nucleotide polymorphism, identified at nucleotide position –844C in the 5′ promoter of the FasL gene, and basal expression of FasL were also significantly higher in –844C than in –844T homozygous donors (Wu *et al.*, 2003). The –844C homozygous genotype may lead to an increased expression of sFasL and alter FasL-mediated signaling in lymphocytes, enhancing the risk of autoimmunity, such as in systemic lupus erythematosus (Wu *et al.*, 2003). Furthermore, this single-nucleotide polymorphism confers host susceptibility to cancers via enhanced activation-induced death of antigen-specific T cells (Sun *et al.*, 2005). It is therefore possible that some genetic factors inducing susceptibility to Fas and FasL might influence the occurrence of TEN and SJS.

Another possibility is that susceptibility to Fas/FasL may affect organ-specific differences in apoptotic sensitivity. Previous reports have shown that acute-hepatitis patients have high sFasL concentrations in their sera (Shiota *et al.*, 1998). We also have data indicating that high serum sFasL levels are detected in patients with drug-induced acute hepatitis (Murata, J. *et al.*, unpublished data). We speculate that genetic backgrounds may influence organ-specific sensitivity of sFasL. Increases in sFasL expression may lead to development of TEN and SJS in patients with highly sFasL-sensitive keratinocytes, whereas they may develop liver dysfunction in patients with high hepatocyte sFasL sensitivity.

An urgent review of pathophysiology in TEN and SJS is needed to resolve this issue and to determine an effective treatment. Determining a single mediator in the pathogenesis of TEN and SJS may be difficult, because no single factor may explain the whole pathogenesis. The complicated relationship among soluble factors, cells, and their controlling genetic factors is likely to play a vital role in the pathogenesis of TEN and SJS.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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