nosorbent assay (ELISA) as previously described.[3] Briefly, the anti-human MIF antibody dissolved in 50 μl of phosphate-buffered saline was added to each well of a 96-well microtiter plate. After incubation for 1 h at room temperature, the plate was washed and 50 μl of biotin-conjugated anti-human MIF antibody was added. Following incubation, the plate was again washed. Then avidin-conjugated horseradish peroxidase was added to individual wells, which were further incubated for 1 h at room temperature. After washing again, 50 μl of substrate solution containing 200 μg of *o*-phenylenediamine and 10 ml of 30% hydrogen peroxide in 10 ml of citrate-phosphate buffer (pH 5.0) were added. Then the reaction was terminated with 50 μl of 1 N sulfuric acid. The absorbance at 492 nm was measured with an ELISA plate reader (Model 3550; Bio-Rad, Tokyo, Japan). 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. C-reactive protein (CRP) was measured using a Hitachi modular system 7350 (normal range, 0–0.39 mg/dl).

Table 1 summarizes the data. The mean serum MIF level before the TPT in the PPP patients was 11.32± 2.0 ng/ml ($n=29$), which was significantly higher than that of control subjects (4.78±0.32 ng/ml, $n=135$; $p=0.0027$). In contrast, the mean serum MIF level in the PPP patients was significantly decreased after the TPT (8.3±1.1 ng/ml)(before TPT versus after TPT, $p=0.016$). Elevation of CRP was detected after the TPT in each patient (data not shown).

To confirm these phenomena, two patients with acute tonsillitis had their changes in body temperature, CRP and serum MIF levels examined during the course of their illness. Six-year-old and 9-year-old male patients were admitted to Hokkaido University hospital with acute tonsillitis. In both cases, increased serum MIF levels preceded both fever and CRP elevation, MIF levels were also reduced at the onset of fever and elevation of CRP (Fig. 1).

**Table 1.** Serum MIF contents of PPP patients and MIF contents fell after the TPT

| Patients | Number of patients | Serum MIF (ng/ml)[a] |
|---|---|---|
| Control | 135 | 4.78±0.32 |
| PPP patients before the TPT | 29 | 11.32±2.0* |
| PPP patients after the TPT | 29 | 8.3±1.1** |

[a]The Mann Whitney *U*-test was used for analysis of the variance in serum MIF levels contents between several of the two groups. *Control versus patients before the TPT, $p=0.0027$, **PPP patients before the TPT versus after the TPT, $p=0.016$.

*In vitro* cytokine measurements of tonsil mononuclear cells stimulated with α-streptococcal antigens revealed significant tumor necrosis factor alpha (TNF-α) production in the culture supernatants from PPP patients but not controls.[4] These findings suggested a close immunologic link between the tonsillitis and proinflammatory cytokines. It has been shown that the TPT induces an increased level of some inflammatory cytokines such as TNF-α, interleukin-1 and interleukin-6 in peripheral blood monocytes of patients with certain focal infectious skin diseases. We have observed two cases with acute tonsillitis, which showed a transient increase in serum MIF preceding an elevation of CRP and fever. In addition, MIF levels were decreased at the onset of fever and the elevation of CRP. Furthermore, significantly higher MIF contents were observed in the sera of patients with PPP than in normal subjects. Unexpectedly, the MIF serum level fell after the TPT. The fact that the serum MIF level significantly decreased at the onset of fever and elevation of CRP after the TPT seems to be a surprising, but interesting, result.

Monocytes and macrophages are the main sources of MIF, which is released after exposure to bacterial endotoxins and exotoxins such as lipopolysaccharide and streptococcal pyrogenic exotoxin A.[2] Thus, these effects may be mediated by a number of proinflam-

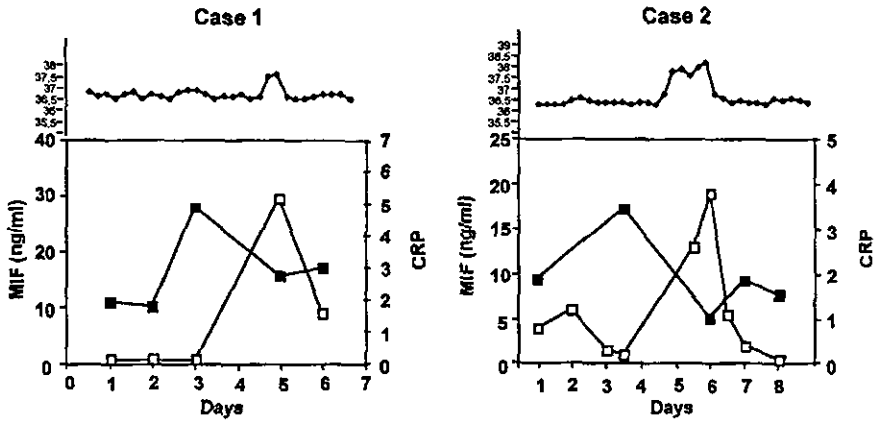


FIG. 1. Changes in body temperature, serum MIF, and CRP in patients with acute tonsillitis. The patients with acute tonsillitis (case 1, a 6-year-old male; and case 2, a 9-year-old male) were admitted to Hokkaido University hospital. Body temperature, CRP (□), and serum MIF (■) were examined during the course of admission.

matory cytokines, including TNF-α and IL-1.[2] Since MIF is an initiator of other proinflammatory cytokines such as TNF-α and IL-1, and regulates the induction of those cytokines,[1,5] our findings suggest that the induction of MIF may precede that of other inflammatory conditions.

ACKNOWLEDGEMENTS. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan (numbers 11670813, 13357008, 12671692, and 14571650).

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# Macrophage Migration Inhibitory Factor Is Induced by Thrombin and Factor Xa in Endothelial Cells*

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**Macrophage migration inhibitory factor (MIF), a proinflammatory cytokine, has been shown to play a role in wound-healing processes. In this study, we investigated whether protease-activated receptor (PAR)-1 and PAR-2 mediated MIF expression in human endothelial cells. Thrombin, factor Xa (FXa), and trypsin induced MIF expression in human dermal microvascular endothelial cells and human umbilical vein endothelial cells, but other proteases, including kallikrein and urokinase, failed to do so. Thrombin-induced MIF mRNA expression was significantly reduced by the thrombin-specific inhibitor hirudin. Thrombin receptor activation peptide-6, a synthetic PAR-1 peptide, induced MIF mRNA expression, suggesting that PAR-1 mediates MIF expression in response to thrombin. The effects of FXa were blocked by antithrombin III, but not by hirudin, indicating that FXa might enhance MIF production directly rather than via thrombin stimulation. The synthetic PAR-2 peptide SLIGRL-$NH_2$ induced MIF mRNA expression, showing that PAR-2 mediated MIF expression in response to FXa. Concerning the signal transduction, a mitogen-activated protein kinase kinase inhibitor (PD98089) and a nuclear factor (NF)-κB inhibitor (SN50) suppressed the up-regulation of MIF mRNA in response to thrombin, FXa, and PAR-2 agonist stimulation, whereas a p38 inhibitor (SB203580) had little effect. These facts indicate that up-regulation of MIF by thrombin or FXa is regulated by p44/p42 mitogen-activated protein kinase-dependent pathways and NF-κB-dependent pathways. Moreover, we found that PAR-1 and PAR-2 mRNA expression in endothelial cells was enhanced by MIF. Furthermore, we examined the inflammatory response induced by PAR-1 and PAR-2 agonists injected into the mouse footpad. As shown by footpad thickness, an indicator of inflammation, MIF-deficient mice (C57BL/6) were much less sensitive to either PAR-1 or PAR-2 agonists than wild-type mice. Taken together, these results suggest that MIF contributes to the inflammatory phase of the wound healing process in concert with thrombin and FXa via PAR-1 and PAR-2.**

The mechanism of wound healing is complex, consisting of inflammation, granulation, and remodeling of the tissue (1). Several growth factors and cytokines alone or in combination play important roles during tissue repair and enhance normal wound healing. Activation of endothelial cells is recognized as one of the early and important events in inflammation accompanied by production of proinflammatory cytokines and chemokines. During the inflammatory response, endothelial cells are exposed to bacterial products, proinflammatory cytokines, such as tumor necrosis factor (TNF)-α,[1] and extracellular proteases. These proteases, including thrombin and other serine proteases, are released from neutrophils, mononuclear cells, and mast cells (2).

Thrombin is a serine protease that plays a central role in hemostasis after tissue injuries by converting soluble plasma fibrinogen into an insoluble fibrin clot and by promoting platelet aggregation (3). In addition to these procoagulant effects, thrombin plays a major role in inflammation and repair of injured tissues. The proinflammatory effects of thrombin include stimulation of plasma extravasations and edema, increased expression of endothelial adhesion molecules that cause leukocyte adhesion and infiltration (4, 5), and release of proinflammatory cytokines by endothelial cells (6, 7). Thrombin also stimulates proliferation of endothelial cells, fibroblasts, and smooth muscle cells (8).

Tissue factor-activated factor VIIa complex cleaves and activates factors IX and X into factors IXa and factor Xa (FXa), respectively, which lead to thrombin generation (5). In addition to its role as a procoagulant activator, FXa has been shown to elicit inflammatory responses in endothelial cells such as proinflammatory cytokine production (9–11), and also acts as a mitogen for endothelial and smooth muscle cells (12, 13).

Macrophage migration inhibitory factor (MIF) was the first lymphokine shown to prevent the migration of macrophages out of capillary tubes (14). Although it was long thought that MIF was expressed exclusively in activated T cells, an array of recent reports has indicated that MIF is ubiquitously expressed in various cells and that macrophages in particular are a major source of this protein (15). It is of interest that MIF was reported as an anterior pituitary-derived hormone that could override the glucocorticoid-mediated suppression of inflammatory and immune responses (16, 17).

In this study, we examined the effects of thrombin and FXa on MIF expression using endothelial cells, human dermal microvascular endothelial cells (HDMEC), and human umbilical

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[1] The abbreviations used are: TNF-α, tumor necrosis factor-α; FXa, factor Xa; MIF, macrophage migration inhibitory factor; HDMEC, human dermal microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; ATIII, antithrombin III; NF-κB, nuclear factor-κB; PAR, protease-activated receptor; TRAP, thrombin receptor activation peptide; WT, wild type; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

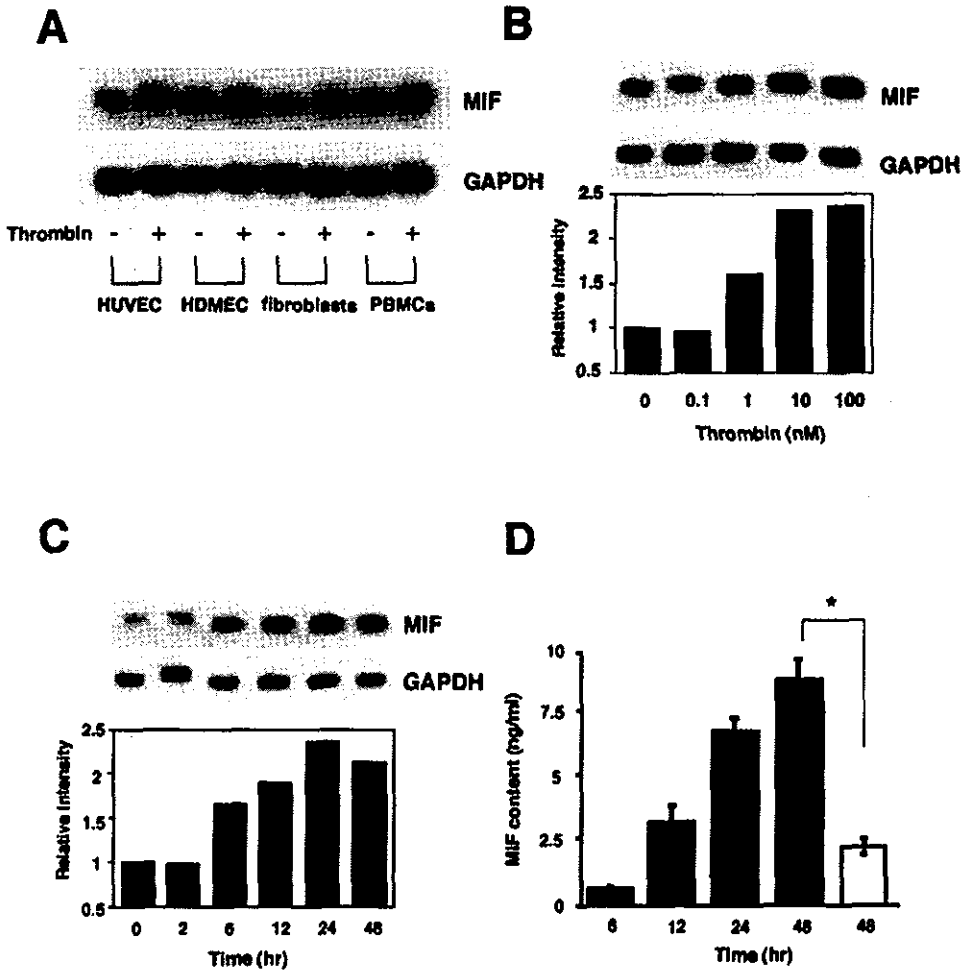


FIG. 1. **Thrombin-induced MIF mRNA expression in endothelial cells.** *A*, HDMEC, HUVEC, fibroblasts, and leukocytes were incubated for 24 h at 37 °C in the presence of thrombin (100 nM). The cells were harvested and subjected to Northern blot analysis as described under "Experimental Procedures." *B*, HDMEC ($1 \times 10^7$ cells/10 ml) were incubated for 24 h at 37 °C in the presence of various concentrations of thrombin (0.1 to 100 nM) and subjected to Northern blot analysis for MIF mRNA expression. *C*, time-course study of thrombin-induced MIF mRNA expression in HDMEC. HDMEC were stimulated with 100 nM thrombin and then Northern blot analysis was performed. *D*, MIF contents in culture media of HDMEC in response to thrombin. HDMEC were stimulated with thrombin (100 nM), and MIF contents in the culture media were measured by ELISA. The open column shows the MIF content in the absence of thrombin. Values are the means ± S.E. from three independent experiments. *, $p < 0.005$.

vein endothelial cells (HUVEC), and investigated their signal transduction. Based on the results, we discuss the possibility that the coagulation proteases in concert with MIF could promote wound healing at the sites of injury.

## EXPERIMENTAL PROCEDURES

*Materials*—The following materials were obtained from commercial sources: HDMEC (Cryo HMVEC-Neo) and HUVEC were from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD); human dermal fibroblasts were from Dainippon Seiyaku (Osaka, Japan); human thrombin, recombinant hirudin, trypsin, kallikrein, urokinase, concanavalin A and polymyxin B were from Sigma; antithrombin III (ATIII), purified human FXa, mitogen-activated protein kinase kinase inhibitor PD98089, p38 inhibitor SB203580, and NF-κB inhibitor SN50 were from Calbiochem; selective PAR-1 agonist peptide, thrombin receptor activation peptide (TRAP)-6 (SFLLRN) (18), TFLLR-$NH_2$, and selective PAR-2 agonist peptide SLIGRL-$NH_2$ were from Bachem AG (Bubendorf, Switzerland); the scrambled control peptides FSLLR-$NH_2$ (inactive on PAR-1) and LSIGRL-$NH_2$ (inactive on PAR-2) were synthesized by solid-phase methods, purified by high pressure liquid chromatography, and analyzed by mass spectrometry and amino acid analyses. The Isogen RNA extraction kit was from Nippon Gene (Tokyo, Japan); Moloney murine leukemia virus reverse transcriptase and Dulbecco's modified Eagle's medium were from Invitrogen; *Taq* DNA polymerase was from PerkinElmer Life and Analytical Sciences; horseradish peroxidase-conjugated goat anti-rabbit antibody was from Bio-Rad; the Micro BCA protein assay reagent kit was from Pierce; nylon membranes were from Schleicher & Schüll; and Ficoll-Paque PLUS and protein A-Sepharose were from Amersham Biosciences. All other chemicals were of analytical grade.

MIF-deficient mice were established by targeted disruption of the *MIF* gene as described previously (19), using a mouse strain bred onto a C57BL/6 background. Wild-type (WT) C57BL/6 mice were purchased from Japan Clea (Shizuoka, Japan) and maintained under specific-pathogen-free conditions.

*Cell Culture*—HDMEC and HUVEC were cultured according to the manufacturer's instructions in endothelial cell basal medium containing 3 mg/ml bovine brain extract, 10 μg/ml human epidermal growth factor, 1 mg/ml hydrocortisone, 0.5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 50 μg/ml sodium ascorbate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml Fungizone. The cells were grown in a 5% $CO_2$ incubator at 37 °C. For stimulation experiments, cells of passages 4 through 5 were used at a density of $1 \times 10^7$ cells/10 ml. Before stimulation, the cells were washed and placed in serum-free culture medium. Human PBMCs were prepared from heparinized blood by Ficoll-Paque PLUS density gradient centrifugation. In brief, we collected a cell layer at the density of 1.077 ± 0.001 g/ml. The PBMC was washed three times with sterile

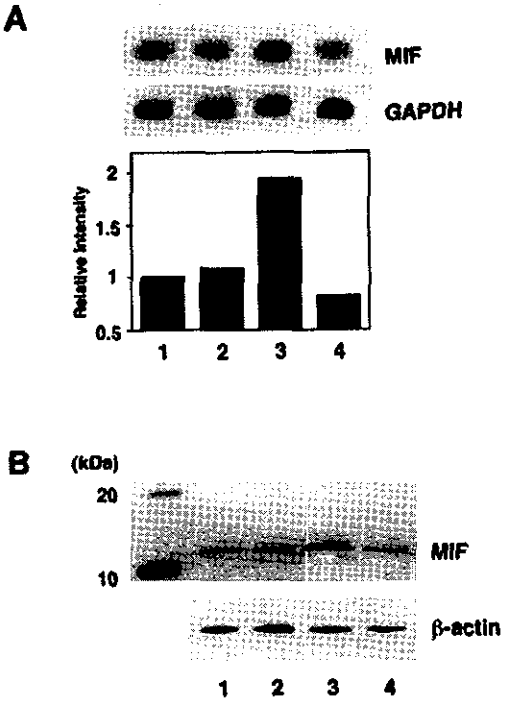


FIG. 2. **Effect of hirudin on thrombin-induced MIF expression.** *A*, HDMEC were treated with hirudin or medium alone for 30 min before the addition of thrombin and cultured for 24 h. The cells were harvested and subjected to Northern blot analysis as described under "Experimental Procedures." *Lane 1*, control (no stimulation); *lane 2*, hirudin (5 units/ml); *lane 3*, thrombin (100 nM); *lane 4*, thrombin (100 nM) + hirudin (5 units/ml). *B*, in a similar manner, HDMEC were treated with hirudin (5 units/ml), cultured for 24 h, and harvested. Western blot analysis was performed. *Lane 1*, control (no stimulation); *lane 2*, hirudin (5 units/ml); *lane 3*, thrombin (100 nM); *lane 4*, thrombin (100 nM) + hirudin (5 units/ml).

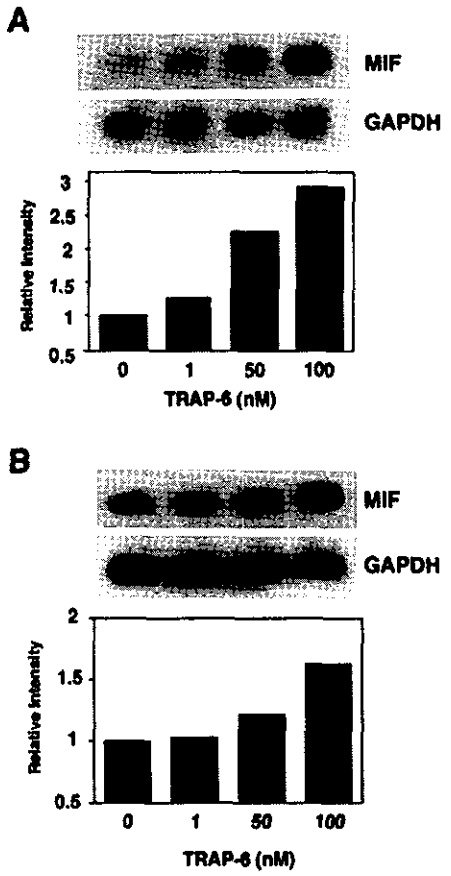


FIG. 3. **Enhancement of MIF mRNA expression by TRAP-6.** Northern blot analysis for MIF mRNA was performed using total RNA isolated from HDMEC and HUVEC after 24 h stimulation at 37 °C with various concentrations of TRAP-6 (0 to 100 nM). *A* and *B* show the results for HDMEC and HUVEC, respectively. The relative intensity was determined in comparison with that of the control (no stimulation).

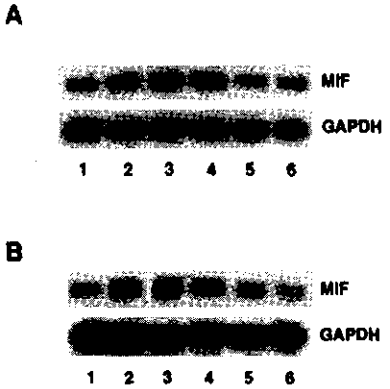


FIG. 4. **MIF mRNA expression by proteases in endothelial cells.** HDMEC and HUVEC were cultured for 24 h at 37 °C in the presence of various proteases and subjected to Northern blot analysis. *A* and *B* show the results for HDMEC and HUVEC, respectively. *Lane 1*, no stimulation; *lane 2*, thrombin (100 nM); *lane 3*, FXa (20 nM); *lane 4*, trypsin (100 nM); *lane 5*, kallikrein (200 nM); *lane 6*, urokinase (20 nM).

PBS. PBMCs ($1 \times 10^7$/10 ml) were cultured in RPMI 1640 medium containing 100 IU/ml ampicillin, 50 μg/ml streptomycin, and 30 μg/ml polymyxin B with use of 24-well plates at 37 °C in a 5% $CO_2$ incubator. The viability of these cells was >98%, as judged by the trypan blue dye exclusion method.

*Effects of Various Signal Inhibitors on MIF Expression*—To examine the signal transduction pathway of MIF, endothelial cells were stimulated with various inhibitors against molecules involved in the signal transduction pathway for 1 h at 37 °C before thrombin, FXa, or PAR-2 agonist peptide stimulation and incubated for 24 h.

*Assessment of Inflammation in Vivo*—The selective PAR-1 agonist peptide TFLLR-$NH_2$, the PAR-2 peptide SLIGRL-$NH_2$, and control peptides (FSLLR-$NH_2$, inactive on PAR-1, and LSIGRL-$NH_2$, inactive on PAR-2) dissolved in physiological saline (each 100 μg/50 μl) were injected in the footpads of MIF-deficient and WT mice. Inflammation was determined by the increases in footpad thickness of MIF-deficient and WT mice measured hourly for 6 h with an engineer's micrometer.

*Northern Blot Analysis*—Total cellular RNA was isolated from cells using an Isogen extraction kit according to the manufacturer's protocol. RNA (20 μg) was resuspended in Tris-EDTA (10 mM Tris-HCl and 1 mM EDTA, pH 7.4), denatured, and electrophoresed on 1% agarose formaldehyde gel. The RNA was then transferred to nylon membranes and cross-linked by UV irradiation. Prehybridization was carried out in 0.75 M NaCl, 0.02 M Tris-HCl, pH 7.5, 2.5 mM EDTA, 0.5× Denhardt's solution, 1% SDS, and 50% formamide at 42 °C for 4 h. Then hybridization was performed in the same buffer containing 10% dextran sulfate, 250 μg/ml salmon sperm DNA, and a radiolabeled probe at 42 °C for 20 h. The radiolabeled probe was prepared using human MIF cDNA as a template and labeled with a random primer labeling kit using [$\gamma$-$^{32}$P]dCTP. The primers used for PAR-1 were CAGTTTGGGTCTGAATTGTGTCG (sense) and TGCACGAGCTTATGCTGCTGAC (antisense) and those for PAR2 were TGGATGAGTTTTCTGCATCTGTCC (sense) and CGTGATGTTCAGGGCAGGAATG (antisense). The membrane was washed twice with 2× SSC (16.7 mM NaCl and 16.7 mM sodium citrate) at 22 °C for 5 min, twice with 0.2× SSC containing 0.1% SDS at 65 °C for 15 min, and twice with 2× SSC at 22 °C for 20 min before autoradiography. Quantitative densitometric analysis was performed using an MCID Image Analyzer (Fuji Film, Tokyo, Japan). The density of the MIF band was normalized by the intensity of GAPDH.

*Reverse Transcription-PCR*—Mouse ears from MIF-deficient and control (WT) C57BL/6 mice were surgically obtained, and total RNA was extracted with an Isogen RNA extraction kit. The reverse transcription of

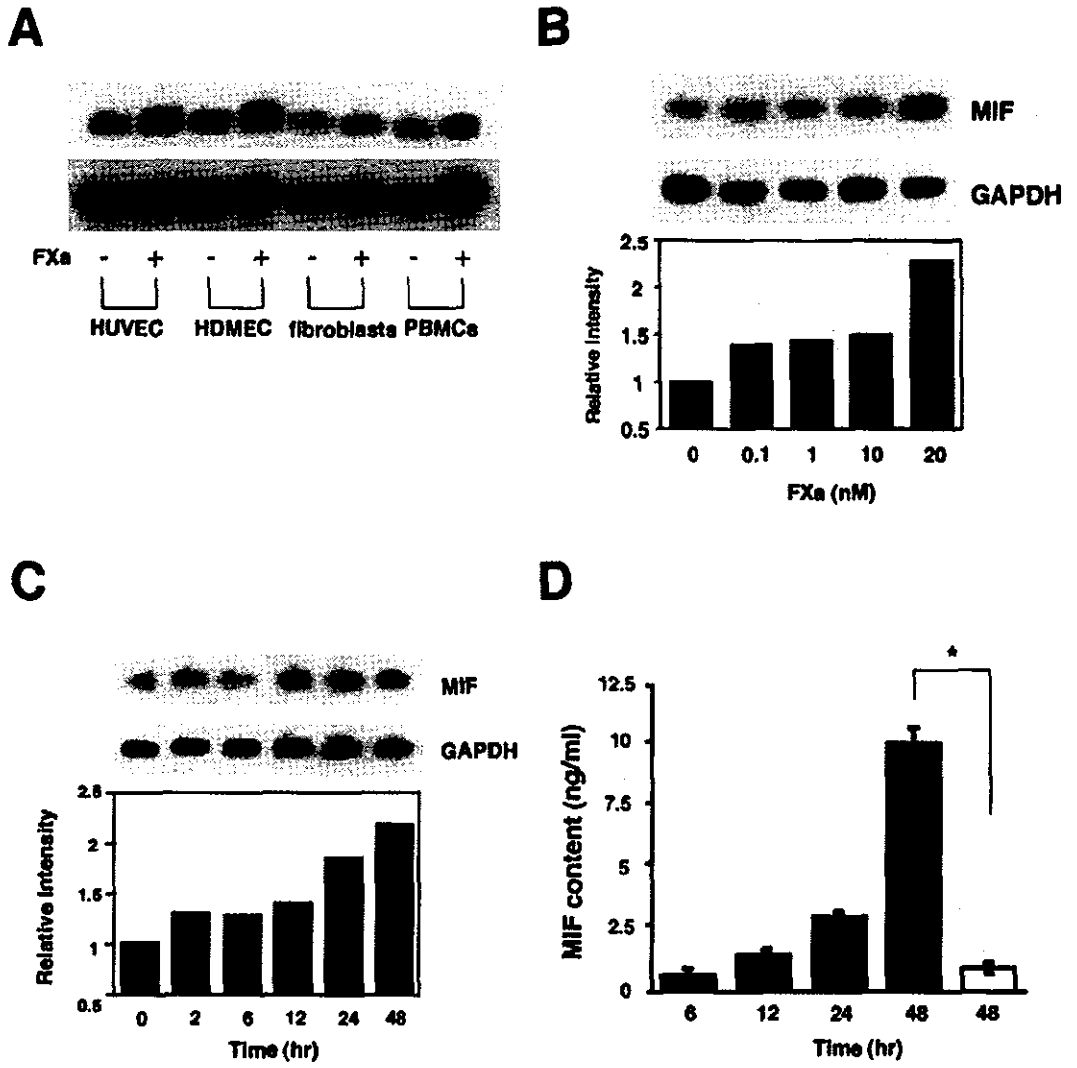


FIG. 5. **Enhancement of MIF mRNA expression by FXa in endothelial cells.** *A*, HDMEC, HUVEC, fibroblasts, and leukocytes were incubated for 24 h at 37 °C in the presence of FXa (20 nM). The cells were harvested and subjected to Northern blot analysis. *B*, HDMEC were treated with various concentrations of FXa at 37 °C for 24 h. The cells were harvested and subjected to Northern blot analysis for MIF mRNA expression. The relative intensity was determined in comparison with that of the control (without stimulation). *C*, time-course study of MIF mRNA expression in response to FXa (20 nM) in HDMEC. The density of MIF mRNA was normalized to the GAPDH signals. *D*, MIF contents in the culture media of HDMEC were measured after FXa (20 nM) stimulation by ELISA. The open column shows the MIF content in the absence of FXa. Values are the means ± S.E. from three independent experiments. *, $p < 0.001$.

the RNA was carried out with Moloney murine leukemia virus reverse transcriptase using oligo-dT primer and subsequent amplification using TaqDNA polymerase. PCR was carried out for 30 cycles with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min using a thermal cycler (Model 480, ABI). MIF primers used were 5′-GTTTCTGTCGGAGCTCAC-3′ (55–72) (forward) and 5′-AGCGAAG-GTGGAACCGTTCCA-3′ (215–236) (reverse). GAPDH was used as a positive control. Primers used were 5′-GAAGGTCGGTGTGAACGGATTT-G-3′ (6–28) (forward) and 5′-GTCCACCACCCTGTTGCTGTAGC-3′ (949–971) (reverse). After PCR, the amplified products were analyzed by 2% agarose gel electrophoresis.

*Western Blot Analysis*—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 bromphenol blue (0.04%), and the samples were heated at 100 °C for 5 min. The samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically onto nitrocellulose membranes. The membranes were blocked with 1% nonfat dry milk in phosphate-buffered saline (PBS), probed with anti-MIF antibodies and reacted 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.

*ELISA for MIF*—The ELISA was performed as described previously (20). In brief, the anti-human MIF antibody was added to each well of a 96-well microtiter plate and left for 1 h at room temperature. Before addition of the antibody, all wells had been filled with PBS containing bovine serum albumin (1%) for blocking and left for 1 h at room temperature. Samples were then added in duplicate to individual wells and incubated for 1 h at room temperature. After the plate was washed three times with PBS containing 0.05% Tween 20 (washing buffer), samples were again added in duplicate to individual wells and incubated for 1 h at room temperature. After the plate was washed three times, 50 μl of biotin-conjugated anti-human MIF antibody (IgG fraction) was added to each well. After incubation for 1 h at room temperature, the plate was again washed three times with washing buffer. Then, avidin-conjugated HRP was added to individual wells, followed by addition of the IgG antibody and incubation for 1 h at room temperature. Fifty microliters of substrate containing *o*-phenylenediamine and hydrogen peroxide in citrate-phosphate buffer, pH 5.0, was added to each well. After incubation for 20 min at room temperature, the reac-

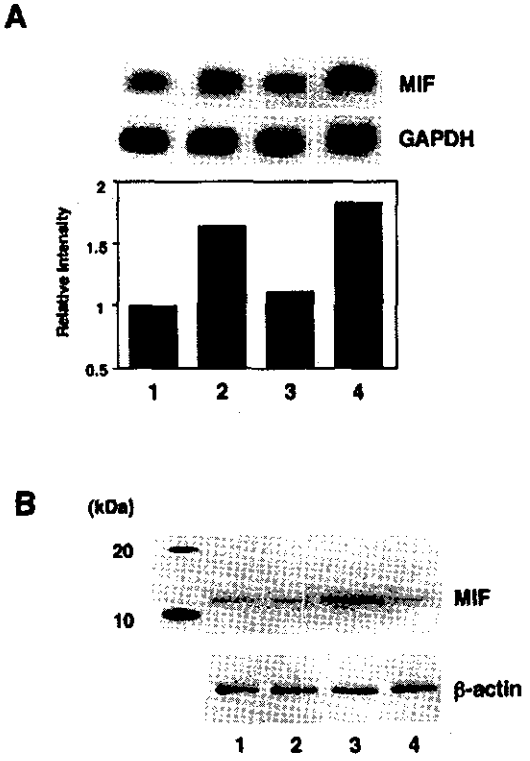


FIG. 6. **Effect of ATIII on MIF expression induced by FXa.** *A*, HDMEC were treated with ATIII or hirudin for 30 min before the addition of FXa. The cells were cultured for 24 h, harvested, and subjected to Northern blot analysis. *Lane 1*, no stimulation; *lane 2*, FXa (20 nM); *lane 3*, FXa (20 nM) + ATIII (160 nM); *lane 4*, FXa (20 nM) + hirudin (5 units/ml). *B*, HDMEC were stimulated with FXa with and without ATIII. MIF protein levels were examined by Western blot analysis. *Lane 1*, no stimulation; *lane 2*, ATIII (160 nM); *lane 3*, FXa (20 nM); *lane 4*, FXa (20 nM) + ATIII (160 nM).

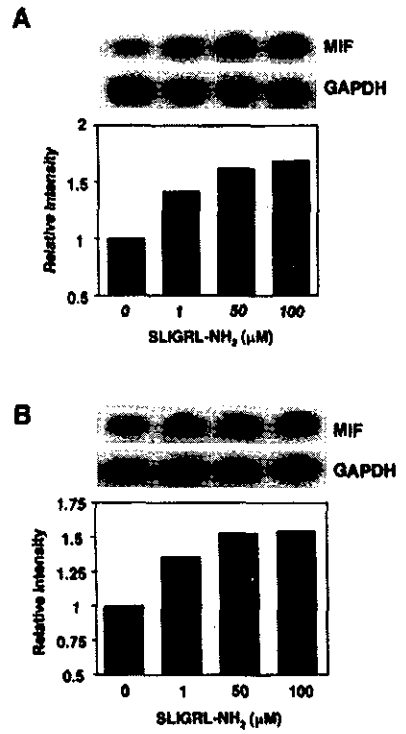


FIG. 7. **Enhancement of MIF mRNA expression by selective PAR-2 agonist peptide SLIGRL-$NH_2$ in endothelial cells.** Northern blot analysis was performed using total RNA isolated from HDMEC and HUVEC after 24 h stimulation at 37 °C with various concentrations (0 to 100 μM) of SLIGRL-$NH_2$. *A* and *B* show the results of HDMEC and HUVEC, respectively. The relative intensity was determined in comparison with that of the control (no stimulation).

tion was stopped with sulfuric acid, and the absorbance at 492 nm was measured using an ELISA plate reader (Model 3550; Bio-Rad).

*Statistical Analysis*—Data in the figures are presented as the mean ± S.E. of one representative experiment. Differences between the various treatments were tested by Student's *t* test and were considered statistically significant at $p < 0.05$.

## RESULTS

*Induction of MIF mRNA by Thrombin*—Thrombin induced increases in MIF mRNA levels in HDMEC, HUVEC, fibroblasts and PBMCs (Fig. 1*A*). HDMEC, HUVEC and PBMCs had strong MIF mRNA expression compared with fibroblasts. Incubation of HDMEC with thrombin (0 to 100 nM) induced a dose-dependent increase in the MIF mRNA level (Fig. 1*B*). Stimulation of the cells with 100 nM thrombin resulted in a 2.4-fold increase in MIF expression relative to the control (no stimulation). After stimulation with 100 nM thrombin, MIF mRNA levels were up-regulated at 6 h and reached a maximum at 24 h (Fig. 1*C*). Furthermore, MIF contents in the culture supernatants of HDMEC increased in response to thrombin (100 nM) in a time-dependent manner (Fig. 1*D*). We obtained similar results when we used HUVEC with regard to the dose-response curve and time-course. The specific thrombin inhibitor hirudin (5 units/ml) strongly inhibited the thrombin-induced MIF mRNA expression in HDMEC (Fig. 2*A*). By Western blot analysis, we confirmed that thrombin stimulated MIF production (3.5-fold increase compared with the control), and hirudin inhibited the thrombin-induced MIF production (Fig. 2*B*).

*Enhancement of MIF mRNA by TRAP-6*—Next we investigated whether the thrombin effect on MIF up-regulation was mediated via proteolytic cleavage of PAR-1 in endothelial cells. We evaluated the expression of MIF mRNA by HDMEC and HUVEC in response to various concentrations of TRAP-6, the PAR-1 agonist. TRAP-6 caused a dose-dependent increase in MIF mRNA expression in HDMEC (Fig. 3*A*). Similarly, the dose-dependent increase of MIF mRNA expression in response to TRAP-6 was seen in HUVEC, but was less significant (Fig. 3*B*). These results suggest that thrombin acts as an agonist for MIF, possibly via PAR-1, a specific thrombin receptor. We then examined other protease effects on MIF expression in endothelial cells. HDMEC or HUVEC were incubated for 24 h at 37 °C in the presence of various proteases. We found that thrombin, known as PAR-1 agonist, and FXa as well as trypsin, known as a PAR-2 agonist, induced MIF expression in endothelial cells, but other proteases, kallikrein and urokinase, failed to (Fig. 4).

*Enhancement of MIF mRNA and Production by FXa*—We examined the expression of MIF mRNA of HDMEC, HUVEC, fibroblasts and PBMCs by FXa stimulation. Enhanced MIF mRNA expression was observed in these cells, particularly in HDMEC, HUVEC and PBMCs (Fig. 5*A*). We performed Northern blot analysis to confirm that FXa was capable of inducing the expression of MIF mRNA in HDMEC. Treatment of HDMEC with 20 nM FXa resulted in a 2.3-fold increase in MIF mRNA expression compared with the unstimulated control (Fig. 5*B*). This induction of MIF mRNA expression by FXa (20 nM) occurred in a time-dependent manner (Fig. 5*C*). Furthermore, HDMEC secreted MIF into the culture media in a time-dependent manner in response to FXa (20 nM) (Fig. 5*D*). We obtained similar results regarding the dose-dependence and time-course in HUVEC (data not shown). The effect of FXa on MIF mRNA expression was inhibited by preincubation with

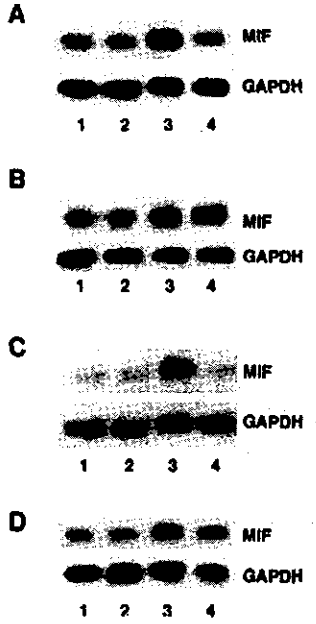


FIG. 8. **Effects of MAPK kinase inhibitors on thrombin or FXa-induced MIF mRNA expression.** HDMEC were incubated with the MEK inhibitor PD98089 or the p38 inhibitor SB203580 for 30 min at 37 °C before treatment with either thrombin or FXa for 24 h and subjected to Northern blot analysis. *A*, *lane 1*, no stimulation; *lane 2*, PD98089 (40 μM); *lane 3*, thrombin (100 nM); *lane 4*, thrombin (100 nM) + PD98089 (40 μM). *B*, *lane 1*, no stimulation; *lane 2*, SB203580 (25 μM); *lane 3*, thrombin (100 nM); *lane 4*, thrombin (100 nM)+ SB203580 (25 μM). *C*, *lane 1*, no stimulation; *lane 2*, PD98089 (40 μM); *lane 3*, FXa (20 nM); *lane 4*, FXa (20 nM) + PD98089 (40 μM). *D*, *lane 1*, no stimulation; *lane 2*, SB203580 (25 μM); *lane 3*, FXa (20 nM); *lane 4*, FXa (20 nM) + SB203580 (25 μM).

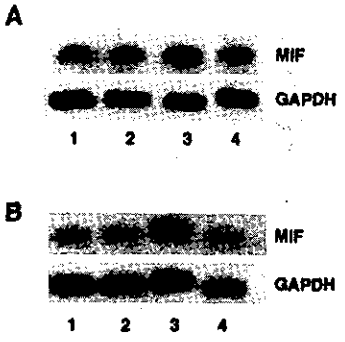


FIG. 9. **Effects of NF-κB inhibitor on thrombin- and FXa-induced MIF mRNA expression.** HDMEC were cultured in the presence of the NF-κB inhibitor SN50 for 30 min at 37 °C before treatment with either thrombin or FXa for 24 h and subjected to Northern blot analysis. *A*, *lane 1*, no stimulation; *lane 2*, SN50 (10 μM); *lane 3*, thrombin (100 nM); *lane 4*, thrombin (100 nM) + SN50 (10 μM). *B*, *lane 1*, no stimulation; *lane 2*, SN50 (10 μM); *lane 3*, FXa (20 nM); *lane 4*, FXa (20 nM) + SN50 (10 μM).

ATIII (160 nM), a physiological inhibitor of both FXa and thrombin (Fig. 6*A*). On the other hand, MIF mRNA induced by FXa was not inhibited by hirudin (5 units/ml), suggesting that FXa elicits these actions directly and not via thrombin. By Western blot analysis, we confirmed that ATIII strongly inhibited FXa-induced MIF production (Fig. 6*B*).

*Enhancement of MIF mRNA by PAR-2 Agonists*—Next we investigated whether the FXa effect on MIF up-regulation in endothelial cells was mediated via proteolytic cleavage of PAR-2. We evaluated the expression of MIF mRNA by HDMEC and HUVEC in response to various concentrations of SLIGRL-$NH_2$, a PAR-2 agonist. SLIGRL-$NH_2$ caused dose-dependent

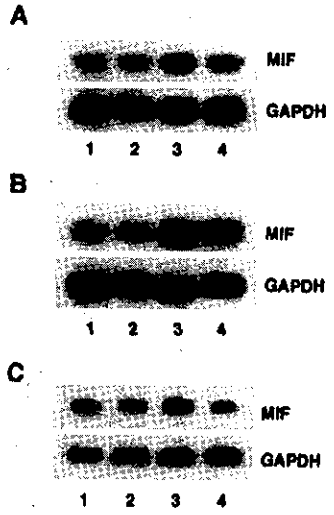


FIG. 10. **Effect of MAPK kinase inhibitors and NF-κB inhibitor on PAR-2-induced MIF mRNA expression.** HDMEC were cultured in the presence of the MEK inhibitor PD98089, the p38 inhibitor SB203580, and the NF-κB inhibitor SN50 for 30 min at 37 °C before treatment with the selective PAR-2 agonist peptide SLIGRL-$NH_2$ for 24 h. MIF mRNA levels were examined by Northern blot analysis. *A*, *lane 1*, no stimulation; *lane 2*, PD98089 (40 μM); *lane 3*, SLIGRL-$NH_2$ (100 μM); *lane 4*, SLIGRL-$NH_2$ (100 μM) + PD98089 (40 μM). *B*, *lane 1*, no stimulation; *lane 2*, SB203580 (25 μM); *lane 3*, SLIGRL-$NH_2$ (100 μM); *lane 4*, SLIGRL-$NH_2$ (100 μM) + SB203580 (25 μM). *C*, *lane 1*, no stimulation; *lane 2*, SN50 (10 μM); *lane 3*, SLIGRL-$NH_2$ (100 μM); *lane 4*, SLIGRL-$NH_2$ (100 μM) + SN50 (10 μM).

increases in MIF mRNA expression in HDMEC and HUVEC (Fig. 7, *A* and *B*).

*Signal Transduction of Thrombin, FXa, and PAR-2 Agonist for MIF Expression*—To examine the signal transduction of thrombin and FXa in endothelial cells with regard to MIF mRNA expression, we examined the effects of several inhibitors against molecules involved in the MAPK-dependent signal transduction pathway of HDMEC. We found that PD98089 (MAPK kinase inhibitor) significantly suppressed MIF mRNA induced by thrombin (Fig. 8*A*) or FXa (Fig. 8*C*). On the other hand, SB203580 (a p38 inhibitor) had little effect on the MIF mRNA expression induced by thrombin (Fig. 8*B*) or FXa (Fig. 8*D*), respectively. Next, we demonstrated that SN50 (an NF-κB inhibitor) significantly reduced MIF mRNA stimulated by thrombin (Fig. 9*A*) or FXa (Fig. 9*B*). We also examined the signal transduction of PAR-2 with regard to MIF mRNA expression in endothelial cells. The results showed that PD98089 significantly suppressed MIF mRNA expression induced by SLIGRL-$NH_2$ (Fig. 10*A*), whereas SB203580 had little effect (Fig. 10*B*). In addition, we demonstrated that SN50 significantly reduced MIF mRNA in response to SLIGRL-$NH_2$ (Fig. 10*C*). By using HUVEC, we obtained similar results regarding the effects of thrombin and FXa as well as these inhibitors (data not shown).

*Induction of PAR-1 and PAR-2 Expression by MIF*—We next assessed whether MIF directly up-regulated the expression of PAR-1 and PAR-2 in HDMEC. HDMEC were stimulated with various concentrations of MIF ranging from 0.1 ng/ml to 1000 ng/ml for 6 h, and then the levels of PAR-1 and PAR-2 mRNA were assessed. The results showed that the levels of both PAR-1 and PAR-2 mRNA were up-regulated, with PAR-2 mRNA being significantly elevated by 100 ng/ml MIF (3.4-fold elevation) (Fig. 11*A*). At the dose of 1000 ng/ml MIF, both PAR-1 and PAR-2 mRNA expression decreased (data not shown). Next, we performed a time-course study on PAR-1 and PAR-2 mRNA expression by MIF (100 ng/ml) in HDMEC.

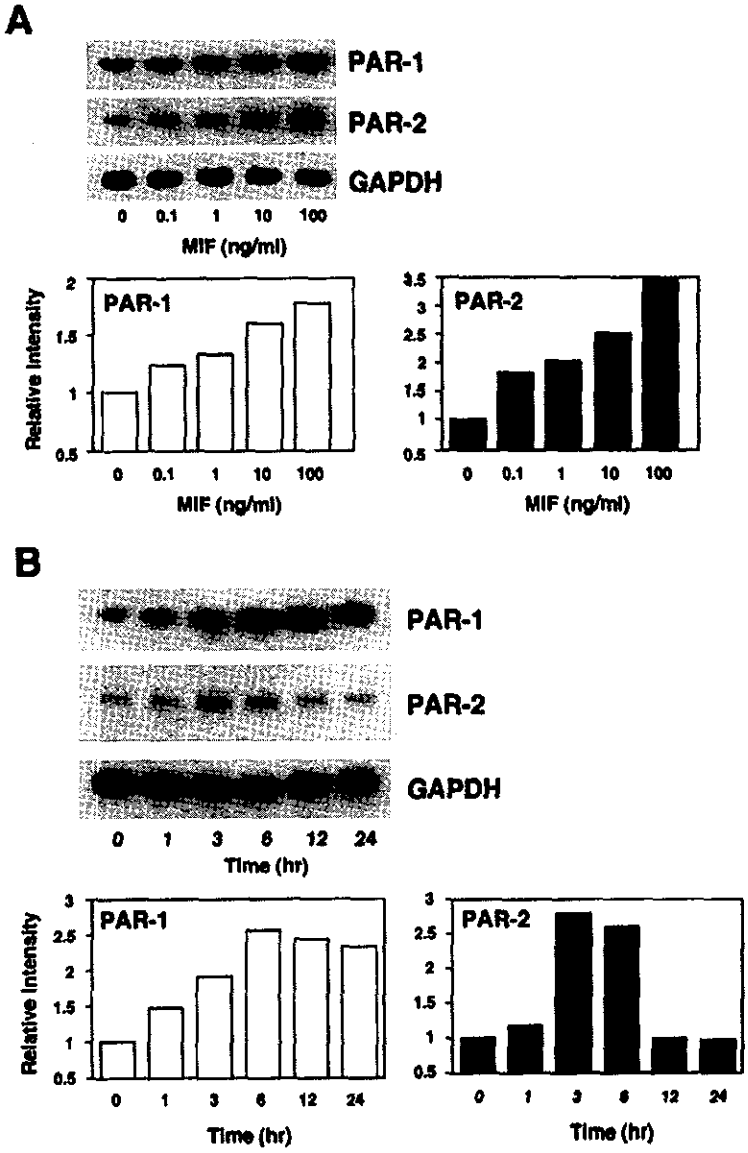


FIG. 11. **Effects of MIF on PAR-1 and PAR-2 mRNA levels in endothelial cells.** *A*, HDMEC were stimulated with the indicated concentrations of MIF for 6 h, and PAR-1 and PAR-2 mRNA levels were determined by Northern blot analysis. The PAR-1 and PAR-2 signals were normalized by the GAPDH signals, and the results are expressed as fold increases compared with the control (no stimulation). *B*, time-course study of PAR-1 and PAR-2 mRNA expression in response to 100 ng/ml MIF in HDMEC. The results are expressed as fold increases compared with the control (zero time).

PAR-1 mRNA expression in MIF-treated HDMEC was up-regulated at 1 h, peaked at 6 h, and then was sustained for up to 24 h (Fig. 11*B*). On the other hand, PAR-2 mRNA expression was up-regulated at between 3 and 6 h, but was markedly decreased at 12 h and 24 h. In a similar manner, MIF up-regulated PAR-1 and PAR-2 mRNA in UHVEC (data shown).

*Involvement of PAR-1 and PAR-2 in Vivo*—We first confirmed the deletion of the MIF gene in MIF-deficient mice by examining MIF mRNA of ears by reverse transcription-PCR (Fig. 12*A*). We assessed inflammation in the foot of the MIF-deficient mouse as for PAR-1 and PAR-2. The footpad swelling in WT mice caused by the selective PAR-1 agonist peptide TFLLR-$NH_2$ was maximal at 1 h and lasted for at least 6 h. Foodpad swelling of MIF-deficient mice was significantly decreased compared with WT mice (Fig. 12*B*). The selective PAR-2 agonist peptide SLIGRL-$NH_2$ also caused an increase of footpad swelling in WT mice that was maximal at 1 h, and footpad swelling of MIF-deficient mice was significantly decreased compared with WT mice, as with the PAR-1 agonist (Fig. 12*C*).

## DISCUSSION

MIF, the first reported lymphokine, was discovered in 1966 (14). It functions as an initiator of inflammation and the immune response via the positive regulation of a number of proinflammatory cytokines, including TNF-$\alpha$ and interleukin-1 (15). We demonstrated previously that MIF was present in human skin and characterized its tissue localization (21). Regarding cutaneous pathological features, high levels of MIF expression have been found in a variety of inflammatory conditions, such as atopic dermatitis in the epidermal layer of inflammatory skin lesions (20). In addition to its inflammatory properties, MIF is thought to be involved in cell proliferation and differentiation during wound repair (22) and tumor growth (23).

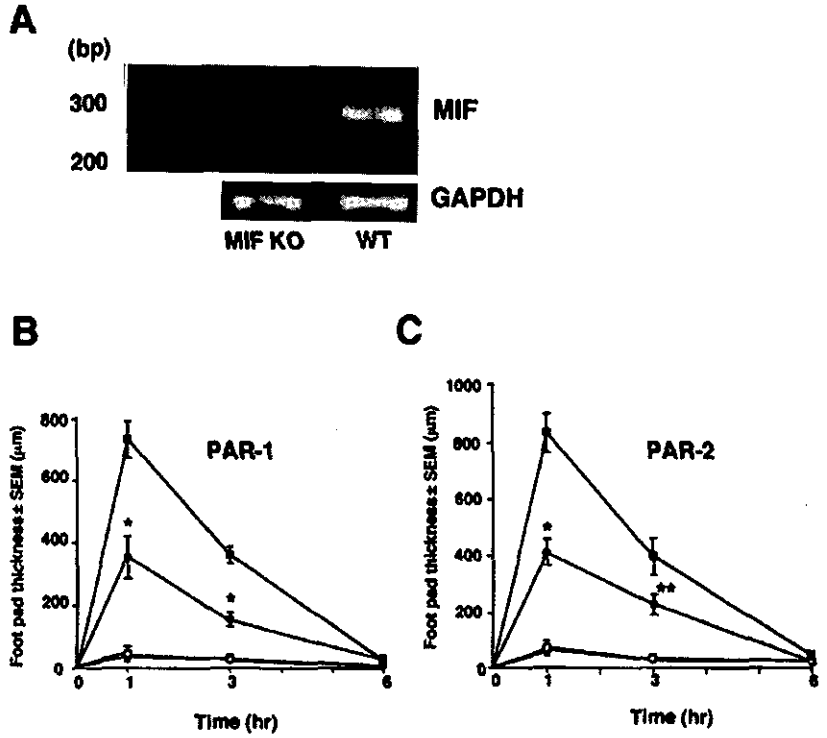


FIG. 12. **Assessment of inflammation *in vivo*.** *A*, MIF mRNA was detected by reverse transcription-PCR. Homogenates of ears of MIF-deficient and WT mice were prepared as described under "Experimental Procedures." GAPDH was used for controls. The selective PAR-1 agonist peptide, PAR-2 agonist peptide, and control peptides dissolved in physiological saline (each 100 μg/50 μl) were injected in the footpads of MIF-deficient and WT mice. The degree of inflammation was determined by footpad thickness as described under "Experimental Procedures." *KO*, knock-out. *B*, the effect of PAR-1 agonist peptide TFLLR-$NH_2$ and control peptide. PAR-1 agonist peptide TFLLR-$NH_2$-injected WT mice (■) and MIF-deficient mice (●); control peptide FSLLR-$NH_2$-injected WT mice (□) and MIF-deficient mice(○). *C*, the effect of PAR-2 agonist peptide and control peptide. PAR-2 agonist peptide SLIGRL-$NH_2$-injected WT mice (■) and MIF-deficient mice (●), control peptide LSIGRL-$NH_2$-injected WT mice (□) and MIF-deficient (○). Footpad thickness values are represented as mean ± S.E. ($n = 5$). *, $p < 0.001$; **, $p < 0.01$ for MIF-deficient *versus* WT mice.

Vascular endothelial cells are known to have the potential to produce MIF after inflammatory stimulation (24), and this cytokine acts as a potent mitogenic factor for human endothelial cells (25). In this context, MIF contributes to normal homeostasis and responses to stimuli such as wounds and infection (26, 27). As shown in this study, endothelial cells would be the major source of MIF in the events of inflammatory responses as well as in wound-healing processes. Thus, it is conceivable that MIF produced by endothelial cells may be of particular biological relevance to cutaneous inflammatory responses.

Thrombin is a multifunctional serine protease that plays an important role in the coagulation cascade, wound healing, and inflammatory response. In this study, we showed that thrombin induced MIF mRNA expression in endothelial cells and that this expression was specifically blocked by the thrombin inhibitor hirudin. Consistent with this finding, we showed that treatment with the PAR-1 receptor peptide TRAP-6 stimulated MIF expression in endothelial cells. Recent studies have provided evidence that endothelial cells express several PARs, including the thrombin receptors (PAR-1 and PAR-3) and thrombin-independent PAR-2 (2). PARs are G-protein-coupled receptors with seven transmembrane domains. Thrombin cleaves the receptors and exposes their new N-terminal sequences at the extracellular domain. The newly exposed domains then act as tethered ligands through which PAR can induce signal transduction. In this context, it is believed that synthetic hexapeptides carrying consensus sequences of the new amino-terminal region could mimic the effects of the respective proteases and that agonist peptides could be employed as tools to analyze the involvement of PAR in the regulation of cell functions.

We also showed that activation of endothelial cells through PAR-1 led to the production of MIF and, moreover, that endothelial cells would be a major source of MIF in the inflammatory response to tissue injuries. Thus, MIF facilitates the critical interaction between thrombin and PAR-1. Individual PARs mediate these events in response to thrombin, as exemplified by the fact that thrombin stimulates the secretion of IL-1 from activated macrophages (28). In association with this event, we previously reported that MIF expression was up-regulated in human fibroblasts by thrombin stimulation (29).

FXa induces thrombin production as a procoagulant activator and also has the potential to elicit inflammatory responses, such as cytokine production, in endothelial cells (9–11). FXa has also been shown to exert various cellular effects in a number of cell types and directly stimulates the proliferation of rat aortic smooth muscle cells and human mesangial cells (7, 11). There is evidence that FXa possesses the potential to activate PAR-2 or a PAR-2-related receptor, but the exact mechanism by which FXa exerts these cellular effects is not fully understood (30). As shown in this study, FXa potently enhances MIF. FXa-induced MIF expression was inhibited by ATIII but not by hirudin. This fact strongly indicates that the positive regulation of MIF mRNA expression by FXa is independent thrombin.

In addition, we revealed that the expression levels of both PAR-1 and PAR-2 mRNA were up-regulated by MIF in endothelial cells. It was not clear whether PAR-1 and PAR-2 were functionally important in the process of the MIF-induced inflammatory condition. PAR-1 mRNA expression started 1 h

after the stimulation, peaked at 6 h, and then was sustained up to 48 h. Similarly, MIF up-regulated PAR-2 mRNA expression at 3 h and after 6 h, but the expression decreased drastically thereafter. It has been reported that PAR-2 mRNA and protein levels are elevated by TNF-$\alpha$ in human umbilical vein endothelial cells but that the thrombin receptor gene (PAR-1) is not induced by TNF-$\alpha$ (31). Along with the present results, this finding indicates that MIF up-regulates both PAR-1 and PAR-2 mRNA levels in a manner distinct from the MIF-induced up-regulation of TNF-$\alpha$.

Both thrombin and FXa are known to exert their effects through a variety of downstream signaling mechanisms that may involve p44/p42 MAPK, p38 MAPK, PKC, and NF-$\kappa$B (32–36). In this study, we provided biochemical details of the signal transduction of thrombin and FXa within the pathways of MAP kinases and NF-$\kappa$B. In brief, PD98089, a specific inhibitor of MAPK kinase 1/2 that blocks the p44/p42 MAPK pathway and NF-$\kappa$B, prevented the effects of both thrombin and FXa on MIF mRNA expression. In contrast, SB203580, an inhibitor of p38 MAP kinase, had no effect. These facts proved that thrombin and FXa induced p44/p42 MAPK and NF-$\kappa$B activation, which led to MIF expression.

Finally, several growth factors/cytokines alone or in combination play important roles during tissue repair and enhance normal wound healing. Although it has long been speculated that proinflammatory cytokines and coagulation factors may play an important role in wound repair, the molecular-based mechanism has not been fully understood. In the present study, we demonstrated inflammation caused by injection of PAR-1 and PAR-2 agonists into mouse footpads, and the induced footpad thickness was much less severe in MIF-deficient mice than in WT mice. These facts indicate that MIF could play a critical role in functionally linking the cytokine network with the coagulation cascade during various wound-healing processes. Because endothelial cells are a potential source of MIF, MIF may play an important role as a positive regulator within the whole process of wound repair via up-regulation of PAR-1 and PAR-2.

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# Cetirizine, an H1-receptor antagonist, suppresses the expression of macrophage migration inhibitory factor: its potential anti-inflammatory action

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## Summary

*Background* H1-receptor antagonists are often effective in the treatment of allergic disorders such as atopic dermatitis. Cetirizine, a putative H1-receptor antagonist, has recently been shown to have anti-inflammatory properties through the inhibition of leucocyte recruitment and activation, and by the reduction of ICAM-1 expression on keratinocytes.

*Objective* To further elucidate the anti-inflammatory properties of cetirizine, we first examined its effects on antigen-induced eosinophilia and neutrophila *in vivo*. We then examined the anti-inflammatory effects of cetirizine on a human keratinocyte A431cell line.

*Methods* Mice were sensitized subcutaneously with ragweed pollen and were challenged intraperitoneally with the allergen. Cetirizine diluted in sterile water (0–20 mg/kg) or only sterile water was administered orally. Peritoneal cells were obtained at 8 and 24 h after challenge. The eosinophilia and neutrophilia induced by ragweed pollen extract were quantitated. Macrophage migration inhibitory factor (MIF), macrophage inflammatory protein 2 (MIP-2) and eotaxin contents of peritoneal fluid were also measured by mouse ELISA. The effects of cetirizine on MIF-induced IL-8 production in A431 cells were examined by ELISA. The effects of cetirizine on MIF expression and production in A431 cells were examined by human MIF ELISA and Northern blot analysis.

*Results* Eosinophilia and neutrophilia induced by ragweed pollen extract were found to be significantly reduced in cetirizine-treated mice (20 mg/kg). MIF, a pleuripotent cytokine, was significantly decreased at 8 and 24 h in the peritoneal fluid by cetirizine treatment. MIP-2 and eotaxin were also decreased 8 and 24 h, respectively, after challenge in the peritoneal fluid with cetirizine treatment. MIF stimulates IL-8 production in A431 cells. We found that MIF production in A431 cells was inhibited by 10 μM cetirizine. Consistent with this, cetirizine significantly inhibited MIF-induced IL-8 production.

*Conclusion* These results suggest that cetirizine exerts its anti-inflammatory effects by inhibiting MIF as well as IL-8 production, such as those involved in inflammatory allergic skin disease, suggesting a broad spectrum of action beyond its mere H1-receptor-antagonistic function.

**Keywords** cetirizine, chemokine, eosinophil, interleukin 8, macrophage migration inhibitory factor
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## Introduction

Cetirizine, a carboxylated metabolite of hydroxyzine, belongs to the new generation of H1-receptor antagonists, which inhibit the cutaneous early-phase reaction, and is effective as an oral treatment for patients with a number of allergic skin disorders, including atopic dermatitis (AD) [1, 2]. Cetirizine also exerts an array of anti-inflammatory activities, in addition to its anti-allergic activity. In particular, it has important effects on eosinophils that inhibit chemotaxis both *in vitro* and *in vivo* [3–5]. Expression of membrane ICAM-1 and soluble ICAM-1 in epithelial cells is significantly reduced in the presence of cetirizine [6]. High concentrations of cetirizine also markedly inhibit the IFN-γ-induced expression of membrane ICAM-1 in cultured human keratinocytes [6].

Macrophage migration inhibitory factor (MIF) was the first lymphokine reported to prevent the random migration of macrophages [7]. It plays an important role in delayed-type hypersensitivity [8]. We have recently reported the excessive expression of MIF mRNA and MIF protein in inflammatory skin lesions in AD [9, 10]. MIF originates from multiple cellular sources such as activated T lymphocytes, monocytes, eosinophils, and keratinocytes in AD [10–12]. The secretion of MIF is up-regulated *in vitro* by TNF-α and IFN-γ [13]. MIF in turn augments the secretion of TNF-α and IL-8 [14]. This inflammatory loop characterizes MIF as a pro-inflammatory cytokine. Keratinocytes are capable of producing a variety of cytokines, and are considered to be a principal

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source of cytokines in the epidermis. Cytokines are not only involved in the mediation of local inflammatory reactions within the epidermis, but also exert systemic effects by entering into the circulation.

It has recently been reported that cetirizine reduces the release of IL-8 from epithelial cells [15]. In this context, cetirizine might not only possess anti-inflammatory activity, but also reduce the chemotactic activity of effector cells [4, 5, 16]. We investigated here the effects of cetirizine on the production of IL-8 and MIF in human epidermal keratinocytes cell line A431. Furthermore, we examined the effects of cetirizine on antigen-induced eosinophilia and neutrophilia *in vivo* in response to oral administration of this drug.

## Materials and methods

### *Materials*

Male 8-week-old BALB/c mice (25–30 g) were obtained from SLC Inc. (Shizuoka, Japan). Human A431 cells were purchased from the Health Science Research Resources Bank (Osaka, Japan). Cetirizine was kindly provided by UCB Laboratories (Braine l'Alleud, Belgium). The following materials were obtained from commercial sources: ragweed pollen extract (1 : 100, wt/vol) in aqueous solution from Torii Pharmaceutical Co. (Tokyo, Japan); human IL-8-specific ELISA kit and mouse eotaxin-specific ELISA kit from genzyme TECHNE (Cambridge, MT, USA); mouse macrophage inflammatory protein 2 (MIP-2) ELISA kit from R&D Systems (Minneapolis, MN, USA); the Isogen RNA extraction kit from Nippon Gene (Tokyo, Japan); M-MLV reverse transcriptase from GIBCO (Grand Island, NY, USA); Taq DNA polymerase from Perkin–Elmer (Norwalk, CO, USA); horseradish peroxidase-conjugated goat anti-rabbit antibody from Pierce (Rockford, IL, USA); Vector ABC Kit from Vector Laboratories (Burlingame, CA, USA); nylon membranes from Schleicher & Schuell (Keene, NH); concanavalin A (Con A) and polymyxin B from Sigma (St Louis, MO, USA); Ficoll-Plaque Plus and Protein A Sepharose from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade.

### *Immunization and challenges*

The BALB/c mice were immunized and challenged with ragweed pollen according to the method of Kaneko et al. [17]. Briefly, mice were immunized by a series of five injections of a 1 : 1000 dilution of the ragweed pollen extract. On days 0 and 1, 0.1 mL was injected subcutaneously, and 0.2 mL was injected subcutaneously on days 6, 8, and 14. The mice were challenged on day 20 by an intraperitoneal injection of 0.2 mL of the dilution of the ragweed pollen extract. Experimental groups ($n = 5$) were treated with orally administered cetirizine diluted in sterile water (2 or 20 mg/kg) on days 18, 19, and 20. The control group was orally administered sterile water on days 18, 19, and 20. The naïve group was only challenged with no sensitization. Eight or 24 h after the challenge (day 21), mice were killed by incision of the carotid artery. Two millilitres of phosphate-buffered saline containing 1.0% fetal calf serum (FCS) and 5 U/mL heparin was injected intraperitoneally, and the abdomen was massaged. The peritoneal infusion was collected with a Pasteur pipette after the peritoneum had been opened. Differential leucocyte counts were made under a microscope after fixation and staining of the peritoneal cells with May–Grünwald–Giemsa stain. The peritoneal fluid contents were assayed for MIF, MIP-2, and eotaxin using mouse MIF, MIP-2, and eotaxin ELISA.

### *Cell culture*

A431 cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS and grown on plastic Petri dishes in a humidified atmosphere of 5% $CO_2$ and 95% air at 37 °C. Eighty percent confluent cells were used for the following experiments. Twenty-four hours prior to the experiments, the culture medium was changed with serum-free medium.

### *ELISA*

ELISA was used to measure the content of MIF secreted from cultured cells, as described in [10]. For this assay, we used recombinant human MIF to obtain the standard curve, with good linearity demonstrated between MIF contents (1–200 ng/mL) and absorbency. Human IL-8 release was determined with an IL-8-specific ELISA kit. Mouse eotaxin release was determined using an eotaxin-specific ELISA kit. MIF ELISA was also performed using mouse peritoneal fluid as described in [18]. MIP-2 and eotaxin contents in mouse peritoneal fluid were also measured using mouse MIP-2 and eotaxin ELISA kits, respectively.

### *Northern blot analysis*

Total cellular RNA was isolated from cultured A431 cells using an Isogen extraction kit. In brief, total RNA was extracted from cell pellets with an Isogen total RNA extraction kit. RNA was resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Total RNA (20 μg) was denatured and electrophoresed on 1% agarose formaldehyde gel. The RNA was then transferred to nylon membranes, and cross-linked by UV irradiation. Prehybridization was carried out in 0.75 M NaCl, 0.02 M Tris-HCl (pH 7.5), 2.5 mM EDTA, 0.5 × Denhardt solution, 1% sodium dodecyl sulphate (SDS), and 50% formamide at 42 °C for 4 h. Then hybridization was performed in the same buffer containing 10% dextran sulphate, salmon sperm DNA (250 mg/mL), and a radiolabelled probe at 42 °C for 20 h. The radiolabelled probe was prepared using human MIF cDNA as a template and labelled with a random primer labelling kit using [α-$^{32}$P]dCTP. The membrane was washed twice with 2 × SSC (16.7 mM NaCl, 16.7 mM sodium citrate) at 22 °C for 5 min, twice with 0.2 × SSC containing 0.1% SDS 65 °C for 15 min, and 2 × SSC at 22 °C for 20 min prior to autoradiography. Quantitative densitometric analysis was performed using an MCID Image Analyzer (Fuji Film, Tokyo, Japan). The density of each MIF band was normalized by the intensity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### *Statistics*

All statistical comparisons were performed with the Student's *t*-test. All *P*-values were two-sided, and summary data are expressed as means ± SEM.

## Results

### *Effects of cetirizine on antigen-induced neutrophilia and eosinophilia*

We first examined the effects of cetirizine on antigen-induced eosinophilia and neutrophilia. The neutrophilia at 8 h and eosinophilia at 24 h induced by ragweed pollen extract *per os* were significantly reduced in the mice treated with 20 mg/kg cetirizine (Fig. 1). We found that 2 mg/kg cetirizine had no effect on eosinophilia and neutrophilia. In this protocol, eosinophil accumulation reached a peak at 24 h, and the response gradually declined thereafter. In contrast, the accumulation of neutrophils reached a peak 8 h after challenge, and then their number declined [19]. This result indicated that cetirizine possessed not only potent H1-receptor-blocking activity, but also inhibitory action with regard to neutrophils and eosinophil infiltration into allergen-induced late-phase reaction sites. The MIF content of peritoneal fluid was significantly decreased in the mice treated with cetirizine compared with the control (Table 1).

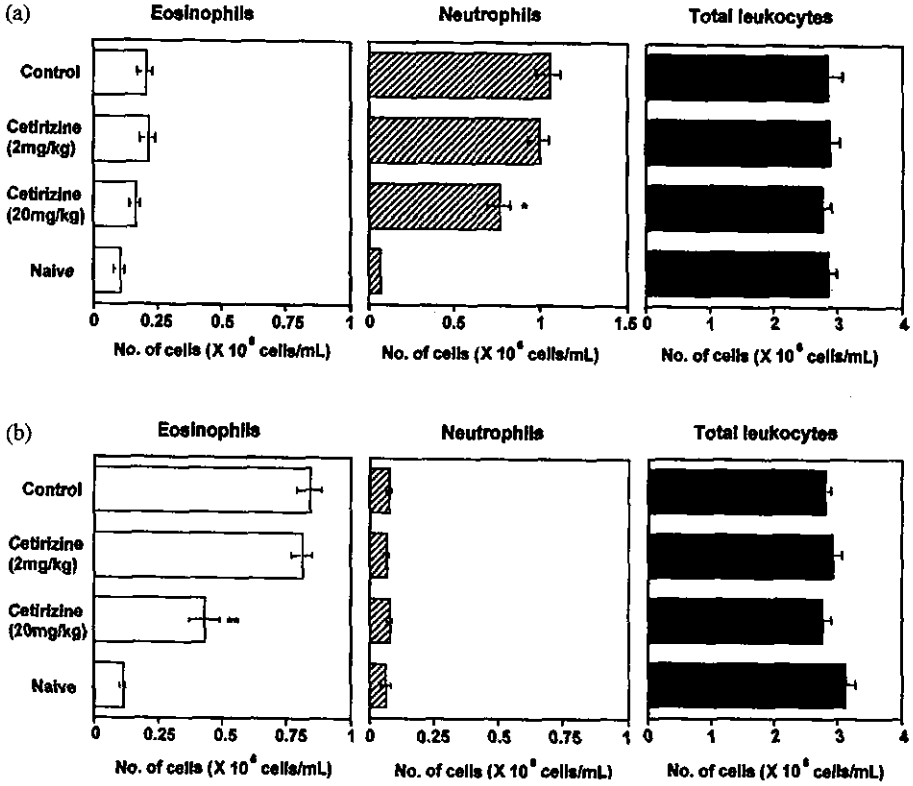


**Fig. 1.** Effects of cetirizine on antigen-induced neutrophilia and eosinophilia in mice. Mice were sensitized subcutaneously with an allergen on days 0, 1, 6, 8, and 14, and were challenged intraperitoneally with the same allergen on day 20. Peritoneal cells were obtained 8 h (a) or 24 h (b) after challenge. Cetirizine diluted in sterile water (2 or 20 mg/kg) or only sterile water (control group) was administered orally on days 18, 19, and 20. The naïve group was only challenged, with no sensitization. Each value represents the mean ± SEM of five animals. *$P<0.01$, **$P<0.005$ as compared with the control group.

**Table 1.** MIF, MIP-2 and eotaxin contents in peritoneal fluid

| | MIF(ng/mL)‡ | | MIP-2(pg/mL)‡ | | Eotaxin(pg/mL)‡ | |
|---|---|---|---|---|---|---|
| Treatment† | 8h | 24h | 8h | 24h | 8h | 24h |
| Control | 153.1 ± 15.7 | 169.5 ± 15.9 | 133.3 ± 9.4 | 11.0 ± 1.2 | 3.6 ± 1.4 | 44.8 ± 2.7 |
| Cetirizine(2mg/kg) | 147.6 ± 17.9 | 151.7 ± 16.7 | 125.2 ± 9.2 | 10.8 ± 2.3 | 8.2 ± 1.1 | 43.1 ± 3.5 |
| Cetirizine(20mg/kg) | 73.1 ± 9.9* | 75.7 ± 7.4* | 75.8 ± 9.1** | 8.7 ± 1.1 | 8.1 ± 1.4 | 24.1 ± 2.8** |
| Naïve | 38.4 ± 4.0 | 44.1 ± 6.9 | 13.3 ± 1.2 | 8.1 ± 0.5 | 5.2 ± 1.0 | 5.6 ± 0.9 |

†The mice were treated with orally administered cetirizine diluted in sterile water (2 or 20 mg/kg) on days 18, 19, and 20. The control group was orally administered with sterile water on days 18, 19, and 20. The naïve group was only challenged without sensitization ($n = 5$ in each group). ‡Eight or 24 hours after the challenge (day 21), MIF, MIP-2 and eotaxin contents of peritoneal fluid were assayed using mouse ELISA kits as described in Methods. MIF, migration inhibitory factory; MIP-2, macrophage inflammatory protein 2. *$P<0.005$; **$P<0.001$ vs. control.

Furthermore, the MIP-2 content at 8 h and eotaxin content at 24 h were significantly decreased by cetirizine treatments.

### *Stimulation of IL-8 release from A431 cells by MIF*

It has been reported that cetirizine also has anti-inflammatory properties [20]. In this context, we tested whether cetirizine exhibits any anti-inflammatory activity in relation to human keratinocytes. We carried out an experiment to determine whether MIF stimulates the production of IL-8, the multifunctional pro-inflammatory cytokine. The human keratinocyte cell line of A431 cells was incubated with various concentrations of MIF. The results indicate that MIF (10 ng/mL) enhanced IL-8 production within 24 h (Fig. 2).

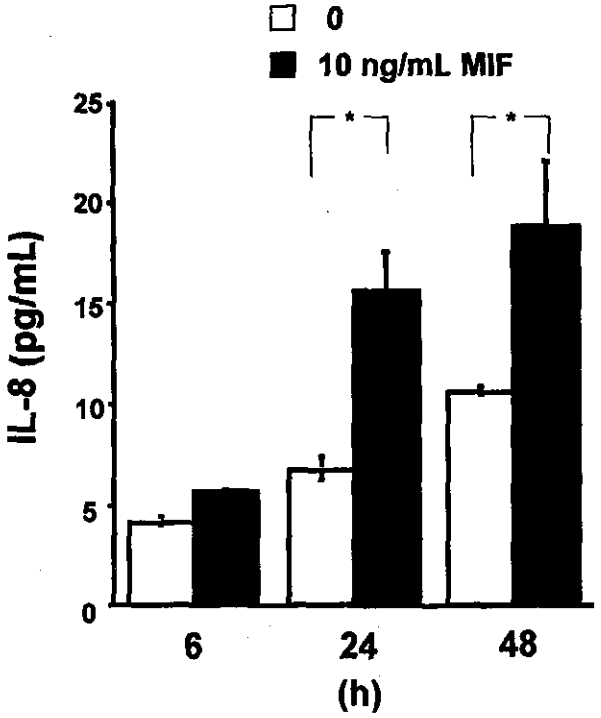


**Fig. 2.** IL-8 release from A431 cells stimulated by migration inhibitory factor (MIF). Human keratinocyte cell line A431 cells were incubated with various concentrations of MIF for 24 h. The IL-8 content was measured in the culture media of A431 cells with an IL-8-specific ELISA kit. Values are means ± SEM from three independent experiments. $^*P<0.01$.

### *Effects of cetirizine on MIF-induced IL-8 production in A431 cells*

To analyse whether cetirizine has inhibitory effects on the MIF-stimulated IL-8 release from A431 cells, the cells were pre-incubated with various concentrations of cetirizine, followed by stimulation with 10 ng/mL MIF. The addition of 0.1 μM cetirizine significantly inhibited the production of IL-8 in A431 cells at 24 h, as did that of 1 μM cetirizine at 6 h (Fig. 3).

### *Effects of cetirizine on MIF expression and production in A431 cells*

Next we examined whether cetirizine directly inhibits MIF production in A431 cells. Cells were pre-incubated with increasing concentrations of cetirizine, from 1 to 100 μM. After 6 and 24 h of treatment, the release of MIF was significantly inhibited at concentrations of 10 μM (Fig. 4a), indicating that cetirizine has the potential to inhibit not only IL-8 but also MIF production in A431 cells. To confirm the effects of cetirizine on MIF expression at the mRNA level, we carried out Northern blot analysis. The result showed that the MIF mRNA levels were significantly inhibited (0.46-fold decrease) by cetirizine after 6 h of treatment at 10 μM concentrations (Fig. 4b).

## Discussion

Cetirizine is a carboxylated analogue of hydroxyzine, that which potent and specific H1-receptor-blocking activity. Cetirizine has anti-inflammatory activities unrelated to its histamine antagonism. For example, it is also known to

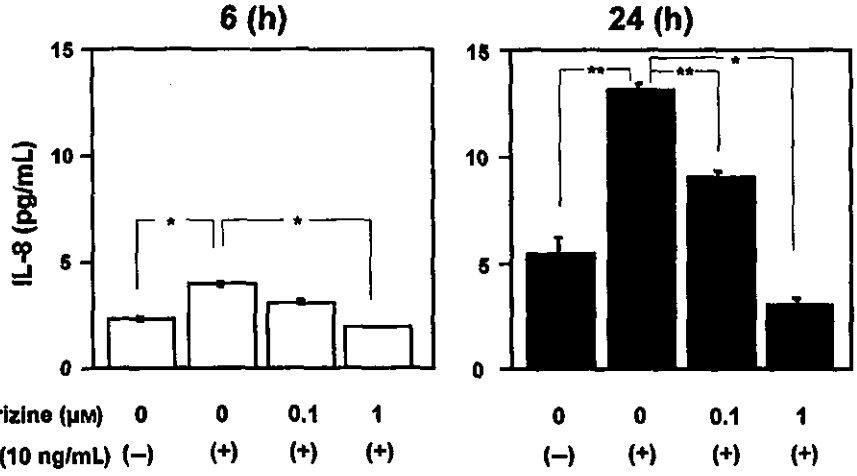


**Fig. 3.** Effects of cetirizine on IL-8 expression and release from A431 cells induced by migration inhibitory factor (MIF). A431 cells were pre-incubated with various concentrations of cetirizine, followed by stimulation with 10 ng/mL MIF. After incubation for 6–24 h, the IL-8 content was measured within the culture media of A431 cells with an IL-8-specific ELISA kit. Values are means ± SEM from three independent experiments. $^*P<0.05$, $^{**}P<0.01$.

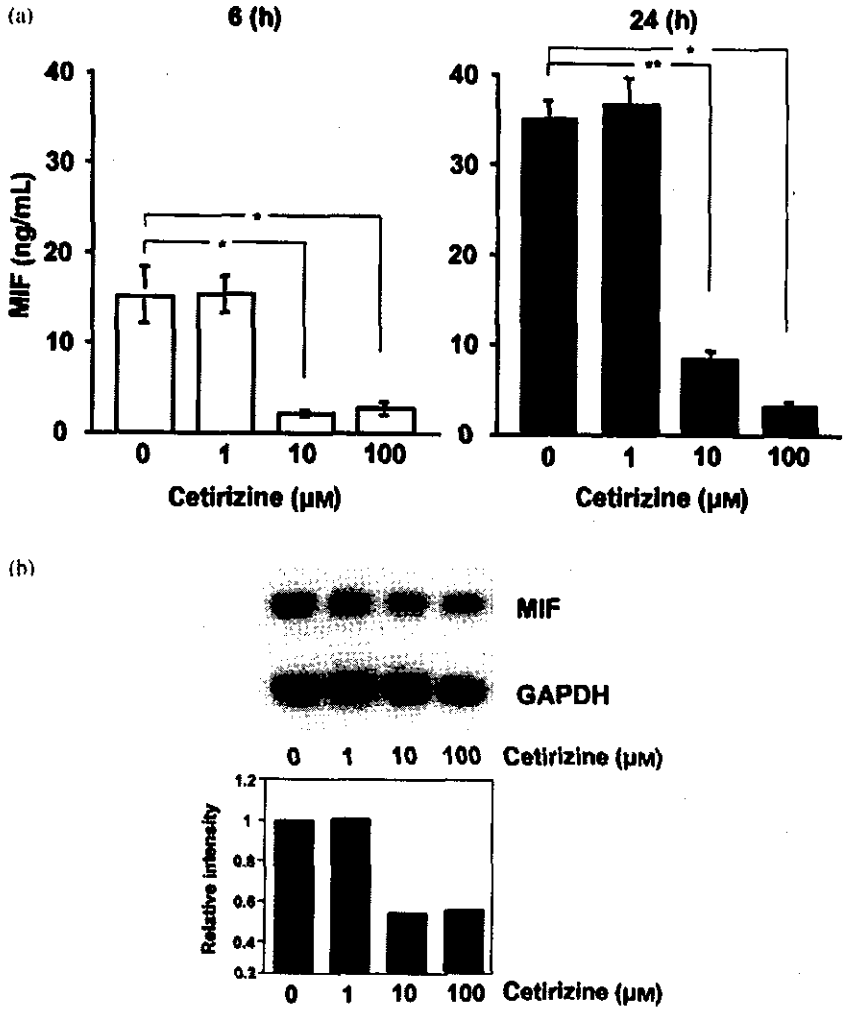


**Fig. 4.** Effects of cetirizine on migration inhibitory factor (MIF) mRNA expression and release from A431cells. (a) A431 cells were pre-incubated with increasing concentrations of cetirizine from 1 to 100 μM. After 6 and 24 h of treatment, an MIF ELISA measured the release of MIF into the culture media of A431 cells. Values are means ± SEM from three independent experiments. *$P<0.0001$, **$P<0.005$ vs. control (0 μg/mL cetirizine). (b) After 6 h of incubation with varying doses of cetirizine, total RNA was isolated from A431 cells. Northern blot analysis was performed as described in Materials and methods. The densities of MIF bands were normalized to the glyceraldehyde-3-phosphate dehydrogenase signals. The relative intensity was determined in comparison with that of the 0 μg/mL cetirizine.

inhibit eosinophil infiltration into allergen-induced late-phase reaction sites in humans [5, 21]. There is emerging evidence that eosinophils are important effector cells in allergic skin inflammation such as those occurring in AD. Previously it was reported that subcutaneous cetirizine treatment (15–30 mg/kg) markedly reduces ovalbumin-induced eosinophil accumulations [22]. The effects of high concentrations of cetirizine (10–30 mg/kg) have been reported [22, 23]. Our present results also demonstrated that oral cetirizine administration significantly reduced the eosinophilia induced by ragweed pollen extract in mice. However, a similar H1-blocker, terfenadine, is ineffective against eosinophil accumulations when administered subcutaneously (10 or 30 mg/kg) [22]. Thus, the effects of cetirizine appear not to be related to the H1-receptor-inhibition effects of histamines. Thomson et al. [24] reported that cetirizine exhibited potent *in vitro* inhibitory effects on eosinophil transmigration through monolayers of both lung and dermal microvascular endothelial cells. In a rat model, cetirizine has been found to inhibit the antigen-induced recruitment of eosinophils into the pleural cavity in animals sensitized with ovalbumin [25]. We also demonstrated that oral cetirizine administration significantly reduced neutrophilia. Cetirizine is also known to exert an anti-inflammatory effect on neutrophils [26]. Therefore, cetirizine not only exhibits potent H1-receptor-blocking activity but also inhibits eosinophil and neutrophil influx into allergen-induced late-phase reaction sites. In the present study, MIF, MIP-2, and eotaxin contents of peritoneal fluid were also significantly decreased by cetirizine treatment, indicating that the decrease in eosinophils and neutrophils caused by cetirizine is more profoundly involved in inhibiting these cytokines and chemokines. Indeed, MIF has the potential to cause eosinophils and neutrophils to accumulate [11, 12, 27]. In mice, a functional IL-8 homologue, MIP-2, induces migration of neutrophils, and eotaxin is a potent and specific eosinophil chemoattractant.

 

We have also found that cetirizine inhibited MIF release from A431 cells. In human allergic and inflammatory diseases, it has been reported that MIF has the potential to exacerbate asthma [12] and acute respiratory distress syndrome [14]. Therefore, it is hypothesized that cetirizine may alleviate acute inflammatory and allergic respiratory diseases through the suppression of MIF production. In the epidermis, we have demonstrated the presence of MIF in keratinocytes [28]. Following this report, we have demonstrated excessive expression of MIF mRNA as well as MIF protein in AD inflammatory skin lesions, with the serum MIF content changing in parallel with the clinical symptoms [10]. In this context, it is hypothesized that cetirizine exerts its anti-inflammatory action in allergic skin lesions by suppressing MIF production. Although a high cetirizine concentration was needed to inhibit MIF production and expression in A431 cells, this may be explained by the fact that there was no correlation between the concentration of H1-antihistamine which inhibited H1-receptor-independent anti-inflammatory effects and receptor-dependent histamine release [29]. Furthermore, MIF is known to stimulate the proliferation of fibroblasts and other cell lines [30, 31]. Recently, Cagnoni et al. [32] showed that cetirizine was able to inhibit fibroblast proliferation. These findings suggest that cetirizine may inhibit cell proliferation by the suppression of MIF bioactivity.

Furthermore, we have demonstrated in the present study that MIF stimulates the release of IL-8 in A431 cells. IL-8 may also play a role in inflammatory skin disorders such as AD, as it has been reported to be produced by keratinocytes [33]. IL-8 stimulates neutrophil chemotaxis and also affects other leucocyte cell types, including lymphocytes, basophils, and eosinophils. IL-8 is considered to play an important role in allergic responses at local sites of inflammatory skin disease. In this context, it is conceivable that cetirizine exerts its anti-inflammatory effects besides the anti-allergic potential to inhibit IL-8 and MIF production in epidermal keratinocytes, beyond its currently known H1-receptor-antagonistic activity.

In summary, cetirizine is a non-sedating selective H1 histamine antagonist, and is widely used for the treatment of allergic diseases, including perennial rhinitis and urticaria. In addition to these actions, cetirizine has been considered as having anti-inflammatory activity; however, the precise mechanism remains to be investigated. We here showed the potential of cetirizine to lower the levels of IL-8 and MIF. These findings strongly support the suggestion that MIF exerts anti-inflammatory actions in addition to its H1-receptor-antagonistic activity. Therefore, it is expected that cetirizine could be used more effectively to treat allergic diseases such as AD and other diseases involving severe inflammatory reactions.

## Acknowledgements

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# Characterization of Kdap, a Protein Secreted by Keratinocytes

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**Using a signal sequence-trap we identified a human gene encoding a polypeptide of 99 amino acids with a putative signal sequence. The gene was identical to keratinocyte differentiation-associated protein (Kdap), which was reported previously by Oomizu *et al* (Gene 256: 19–27, 2000) to be expressed in embryonal rat epidermis at the mRNA level. In humans, we found Kdap mRNA expression to be restricted to epithelial tissue at high levels. The 12.5 kDa protein was detected in culture supernatant of keratinocytes and those transfected adenovirally with the Kdap gene. In normal skin, Kdap protein was found exclusively within lamellar granules of granular keratinocytes and in the intercellular space of the stratum corneum. By contrast, in lesional skin of patients with psoriasis, Kdap was expressed more widely throughout suprabasal keratinocytes. When induced to differentiate *in vitro*, keratinocytes showed marked upregulation of Kdap mRNA expression similar to that of involucrin mRNA, but with differing kinetics. Finally, a spliced variant of Kdap mRNA was generated by alternative splicing mechanisms. Our studies indicate that human Kdap resembles rat Kdap with respect to tissue and cell expression at the mRNA level and that Kdap is a low-molecular-weight protein secreted by keratinocytes. Thus Kdap may serve as a soluble regulator of keratinocyte differentiation.**

Key words: alternative splicing/cDNA cloning/differentiation/keratinocyte/secreted protein
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As the outermost layer of skin, the epidermis serves as an interface between internal and external environments. It consists overwhelmingly of keratinocytes, which originate from stem cells and transiently amplifying cells in the basal layers, which in turn proliferate and differentiate first into spinous keratinocytes and then into granular keratinocytes (Watt, 2000). The latter cells' distal outer and inner cell membranes correspond, respectively, to lipid and protein envelopes that together form the cornified cell envelope (CCE). The protein envelope consists of an insoluble complex of proteins cross-linked by transglutaminase (TGase) to structural proteins such as involucrin and loricrin, whereas the lipid envelope consists of a monomolecular layer of ω-hydroxyceramides, covalently linked by ester bonds to the protein envelope (Kalinin *et al*, 2001).

The CCE a mechanical barrier that can forestall entry through the skin of infectious microbes, mutagenic ultraviolet radiation, noxious chemicals, and other injurious agents. Recent studies also suggest that it may play an active role in innate immunity. Molecular cloning and protein analyses have identified several secreted proteins of low molecular weight within the CCE (e.g., SKALP/elafin; cystatins) (Steinert and Marekov, 1995; Ishida-yamamoto and Iizuka, 1998; Schalkwijk *et al*, 1999), and functional studies have shown that many of these proteins exert proteinase inhibitor activity that may curtail microbial growth and/or dampen inflammation (Molhuizen and Schalkwijk, 1995).

Despite recent recognition of CCE components and elucidation of their functions, it is likely that several important components of the CCE remain undiscovered. The identification and elucidation of their function should improve our understanding of CCE. Hypothesizing that some of these novel CCE proteins are secreted and keratinocyte-specific proteins (e.g., involucrin, filagrin, and loricrin), we employed a previously established signal sequence (SS)-trap method (Tashiro *et al*, 1993) to isolate SS-coding genes from a human keratinocyte cDNA library because the N-terminal SS is unique to precursors of secreted and membrane-anchored proteins. SS-trapped cDNA clones were then screened for keratinocyte-specific genes using differential colony hybridization (keratinocytes *vs* dermal fibroblasts). Our efforts yielded several novel genes including one encoding a precursor of a secreted, low-molecular-weight protein expressed selectively in normal human epidermis by granular keratinocytes. Subsequent analyses have revealed the gene to be identical to keratinocyte differentiation-associated protein (Kdap), isolated by Oomizu *et al* (2000).

Oomizu *et al* identified Kdap using subtractive cDNA cloning of rat embryo skin at 14 *vs* 17 d of gestation. They

Abbreviations: CCE, cornified cell envelope; Kdap, keratinocyte differentiation-associated protein; PBS, phosphate-buffered saline

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also isolated cDNA clones for human and mouse homologs and identified spliced variants of mRNA for each species. Kdap mRNA was expressed specifically in the suprabasal layer of embryonal and neonatal rat skin. Because of Kdap's specific expression in skin and the possibility that it is secreted, we hypothesized Kdap to contribute to the regulation of human epidermal structure and/or function. Thus, we examined Kdap expression in human skin and other tissues, and characterized its biochemical properties and the regulation of gene expression during keratinocyte differentiation and of *in situ* protein expression in normal and psoriatic skin. Our findings expand our knowledge of Kdap, serving as the basis for further investigation into its function within epithelial tissue.

## Results

**Molecular cloning of novel SS-encoding genes expressed selectively by keratinocytes** To identify novel genes encoding secreted proteins expressed selectively by keratinocytes, we employed an SS trap (Tashiro *et al*, 1993) and differential colony hybridization. Because SS is encoded by 5'-end cDNA sequences, these sequences were enriched from cDNA synthesized with mRNA from primary-cultured human keratinocytes. We then inserted enriched cDNA into an expression vector to produce a fusion protein of cDNA-encoded polypeptide and SS-deficient hIL-2Rα. This cDNA library was transferred into COS-1 cells and examined for the ability of cDNA to escort hIL-2Rα-lacking SS to the cell surface, as judged by surface staining using anti-hIL-2Rα antibody (Ab) (Bonkobara *et al*, 2003). cDNA clones exhibiting surface expression were screened further using differential colony hybridization (Ariizumi *et al*, 1997) for genes expressed by keratinocytes, but not by dermal fibroblasts. These procedures yielded six novel genes (found in the EST database updated in the year of 2000) encoding SS that hybridized strongly with total cDNA probes prepared from keratinocyte mRNA, but only marginally or not at all with dermal fibroblast probes. Finally, full-length cDNA clones for each of the six EST genes were isolated and their entire nucleotide sequences were determined (Bonkobara *et al*, 2003).

**Amino acid structure of an EST clone** Among the six EST genes was one encoding a polypeptide (termed Kdap) consisting of 99 amino acids with a putative N-terminal SS of 22 amino acids (Fig 1). Comparison with known proteins in the human GenBank database (updated in 2000) failed to identify homologous proteins, but an EST database search revealed mouse and rat homologs (note that these sequences were not full-length cDNA clones). Indeed, we were able to clone the cDNA for these homologs using RT-PCR of mRNA from mouse and rat skin, respectively; nucleotide sequences were confirmed by comparison with EST clones. Analysis of amino acid sequences showed considerable conservation among the three species (54%–59% identity) (Fig 1). By contrast, the *IYP* sequence (located just after the SS) was found only in the mouse. A homology search conducted in 2003 revealed that our isolated gene is identical to Kdap, previously reported by Oomizu *et al* (2000).

**Keratinocyte-predominant expression of Kdap mRNA** As expected, northern blotting of the Kdap gene showed a transcript of approximately 1 kb expressed by keratinocytes, but not by dermal fibroblasts (Fig 2*A*). Human mRNA was detected only in the skin at a high level and in the thymus at a considerably lower level. Similar tissue specificity was noted for mouse mRNA (Fig 2*B*). We next examined RNA expression in different epithelia using RT-PCR (Fig 2*C*); there was high expression in oral mucosa, tongue, esophagus, and stomach, similar to that in the skin; much lower expression in the bladder and uterus; and no expression in the gastrointestinal mucosa. These results indicate that Kdap mRNA is expressed by various epithelia, especially those predominantly populated by keratinocytes.

**Kdap is a secreted protein** To determine whether the N-terminal amino acid sequence of Kdap acts as an SS to permit its secretion, we transfected an expression vector (pKdap-Fc) encoding a fusion protein of the entire Kdap sequence and the Fc portion of human IgG1 into COS-1 cells, and then harvested the supernatant (extracellular fraction) and whole-cell extracts (intracellular fraction) separately. Protein expression was determined by western blotting using anti-human IgG Ab (Fig 3*A*). Whereas Kdap-Fc protein was detected clearly as a single band of 49 kDa in the extracellular fraction, it was barely detectable in the intracellular fraction (control Ab did not produce specific bands), indicating that protein is secreted efficiently and also suggesting that the N-terminal sequence acts as an SS in transfected cells. To map the SS cleavage site, we used protein A-affinity chromatography to purify the Fc-fusion protein from the supernatant and automated Edman degradation to determine N-terminal amino acid residues. Our results indicated that the secreted Fc-fusion protein starts at alanine on amino acid 23, the cleavage site lying between this amino acid and glycine on amino acid 22 (Fig 1).

For biochemical characterization, we immunized rabbits with His-tagged recombinant Kdap protein (His-Kdap) to produce anti-human Kdap Ab. This Ab was used to examine Kdap secretion (without tags) in COS-1 cells that were gene transferred using an Adv with full-length Kdap cDNA (Fig 3*B*). By western blotting, we detected a single band of 12.5

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                                    ▽
Kdap_H MKIPVLPAVVLLSLLVLHSAQGATLGGPE---EESTIENYASRPEAFNTPF 48
Kdap_M MKIPILPVVALLSLLALHAVQGAALGHPTIYPEDSSYNNYPTATEGLNNEF 51
Kdap_R MKIPILPIVALLSLLALHAAQGAALGTPM---EDTTSSNYPSGTEGLS-EF 47

Kdap_H LNIDKLRSAFKADEFLNWHALFESIKRKLPFLNWDAFPKLKGLRSATPDAQ 99
Kdap_M LNFKRLQSAFQSENFLNWHVITDMFKNAFPFINWDFFPKVKGLRSAAPDSQ 102
Kdap_R LNFNKLQSAFKSDDFLNWHVLTDMFKKALPFINWEFFPKVKGLRSAVPDSQ 98
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**Figure 1**
**Amino acid sequences of human keratinocyte differentiation-associated protein (Kdap).** Amino acid sequence (deduced from nucleotide sequences of human Kdap cDNA, Kdap_H, GenBank accession number XM_097406) is aligned with those of mouse (Kdap_M, W30505) and rat (Kdap_R, AB011028) homologs. Human Kdap precursor is cleaved between amino acid 22 and 23 (shown by a reverse triangle).