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 SLLGRL-NH2 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), 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 IXα and factor Xα (FXα), respectively, which lead to thrombin generation (5). In addition to its role as a procoagulant activator, FXα 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 vein endothelial cells (HUVEC), human umbilical

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.
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-NH₂, and selective PAR-2 agonist peptide SLIGRL-NH₂ were from Bachem AG (Bubendorf, Switzerland); the scrambled control peptides FSLLR-NH₂ (inactive on PAR-1) and LSIGRL-NH₂ (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 ng/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% CO₂ incubator at 37 °C. For stimulation experiments, cells of passages 4 through 5 were used at a density of 1 × 10⁶ 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. 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 × 10⁶ 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.](https://www.jbc.org/)

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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). 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).

PBS. PBMCs (1 × 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% CO2 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 TFFLR-NH2, the PAR-2 peptide SLIGRL-NH2, and control peptides (FSLLR-NH2, inactive on PAR-1, and LSIGRL-NH2, 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-Cl 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-Cl, 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 [γ-32P]dCTP. The primers used for PAR-1 were CAGTCTGGCTCTGCACTTTGCTG (sense) and TCCAGGGCTTTAGTCTGCTGAC (antisense) and those for PAR2 were TGGATGAGTTTTCTGCATCTGCC (sense) and CGTGTGTTAGGGAGAATG (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
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'-AGCGAAGGTGGAGACCGTTCCA-3' (215–236) (reverse). GAPDH was used as a positive control. Primers used were 5'-GAAGGTCGGTGTGAACGGATTTG-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 subjected to Northern blot analysis using 2% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrochemically 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 with PBS containing 0.05% Tween 20, samples were added to individual wells and incubated for 1 h at room temperature. 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 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-
Thrombin and FXa Induce MIF in Endothelial Cells

RESULTS

Induction of MIF mRNA by Thrombin—Thrombin induced increases in MIF mRNA levels in HDMEC, HUVEC, fibroblasts and PBMCs (Fig. 1A). 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. 1B). 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. 1C). Furthermore, MIF contents in the culture supernatants of HDMEC increased in response to thrombin (100 nM) in a time-dependent manner (Fig. 1D). 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. 2A). 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. 2B).

Enhancement of MIF mRNA by TRAP-6—Next we investi-
Thrombin and FXa Induce MIF in Endothelial Cells

FIG. 7. Induction of PAR-1 and PAR-2 expression by MIF.

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 investigated 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. 8A) or FXa (Fig. 8C). On the other hand, SB203580 (a p38 inhibitor) had little effect on the MIF mRNA expression induced by thrombin (Fig. 8B) or FXa (Fig. 8D), respectively. Next, we demonstrated that SN50 (an NF-κB inhibitor) significantly reduced MIF mRNA stimulated by thrombin (Fig. 9A) or FXa (Fig. 9B). 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-NH2 (Fig. 10A), whereas SB203580 had little effect (Fig. 10B). In addition, we demonstrated that SN50 significantly reduced MIF mRNA in response to SLIGRL-NH2 (Fig. 10C). 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. 11A). 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.

ATIII (160 nM), a physiological inhibitor of both FXa and thrombin (Fig. 6A). 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. 6B).

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-NH2, a PAR-2 agonist. SLIGRL-NH2 caused dose-dependent increases in MIF mRNA expression in HDMEC and HUVEC (Fig. 7, A and B).

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).
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. 11B). 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. 12A). 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-NH₂ was maximal at 1 h and lasted for at least 6 h. Footpad swelling of MIF-deficient mice was significantly decreased compared with WT mice (Fig. 12B). The selective PAR-2 agonist peptide SLIGRL-NH₂ 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. 12C).

**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-α 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).
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

![Assessment of inflammation in vivo](image-url)
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-α in human umbilical vein endothelial cells but that the thrombin receptor gene (PAR-1) is not induced by TNF-α (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-α.

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-κB (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-κB. In brief, PD98059, a specific inhibitor of MAPK kinase 1/2 that blocks the p44/p42 MAPK pathway and NF-κB, 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-κB 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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Macrophage Migration Inhibitory Factor Is Induced by Thrombin and Factor Xa in Endothelial Cells

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