Macrophage Migration Inhibitory Factor Promotes Tumor Invasion and Metastasis via the Rho-Dependent Pathway

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ABSTRACT

Purpose: Macrophage migration inhibitory factor (MIF) plays an important role not only in the immune system but also in tumorigenesis. In this study, we investigated the potential role of MIF in association with tumor invasion and metastasis.

Methods: To assess the function of MIF, we knocked down the MIF mRNA using small interfering RNA (siRNA). Twenty-one base siRNA specific for the mRNA sequence of mouse MIF was introduced to a murine colon cancer cell line, colon 26. Tumor cell invasion was evaluated using a transwell method (8-μm pores) coated with Matrigel on the upperside membrane and with fibronectin on the underside membrane. Moreover, we investigated the signal transduction of lysophosphatidic acid (LPA) relevant to the Rho-dependent pathway and further examined the effect of MIF siRNA on this signal transduction system. In vivo, the tumor cells were pretreated with MIF siRNA and injected into the portal vein, and the effects on metastasis to the liver were evaluated.

Results: We found that MIF siRNA markedly reduced the invasion of the cells from the upperside to lowerside membranes. We revealed that the Rho-dependent pathway activated by LPA was suppressed by MIF siRNA. Next, we found that the tyrosine-phosphorylation of focal adhesion kinase and LPA-induced expressions of integrin β1 were significantly suppressed by MIF siRNA. In vivo, metastasis to the liver was significantly inhibited by pretreatment of the cells with MIF siRNA.

Conclusion: Taken together, these results suggest that MIF promotes tumor invasion and metastasis via the Rho-dependent pathway.
from Cytoskeleton (Denver, CO); Protein A-Sepharose from Pharmacia (Uppsala, Sweden); Effectene transfection reagent from Qiagen (Valencia, CA); and 1-oleoyl-2-LPA and type I collagen from Sigma (St. Louis, MO). All other chemicals used were of analytic grade.

Recombinant mouse MIF was expressed in E. coli BL21/DE3 and purified as described previously (17). A polyclonal anti-mouse MIF antibody was generated by immunizing New Zealand White rabbits with recombinant mouse MIF. The IgG fraction was prepared using Protein A-Sepharose according to the manufacturer’s protocol.

Mice. Four-week-old female BALB/c mice were purchased from Clea (Tokyo, Japan) and acclimatized for at least 1 week. They were used at 6 to 8 weeks of age. Mice were maintained under a 12-hour light/dark cycle (lights on from 6:00 a.m. to 6:00 p.m.) at a temperature of 20°C to 22°C. Food and water were available ad libitum. Animal studies conformed to the Regulations for Animal Experiments of the Institute for Animal Experimentation, Hokkaido University Graduate School of Medicine.

Cell Culture. The colon 26 cell line, established from BALB/c mice, was a generous gift from Dr. T. Kataoka (Cancer Chemotherapy Center, Tokyo, Japan). Colon 26 cells were cultured in RPMI 1640 containing 10% heat-inactivated FCS at 37°C under 5% CO₂ and subcultured every 3 days. For all experiments, logarithmically growing cells were used.

Transfection with siRNA. The RNAi technique is used for down-regulating the expression of a specific gene in living cells by introducing a homologous double-stranded RNA, and 21-base siRNAs are potent mediators of the RNAi effect in mammalian cells (16). The nucleotide sequences of dsRNA and complimentary dsRNA for mouse mRNA were 5'-CCGCAA-CUCACAGUAAGCUDdTdT-3' and 5'-CACGUUACUGUAGUGCCGdTdT-3', respectively. As a control RNA duplex (scramble RNA), 5'-GCCGCGCUUUGUAGAGAGGdTdT-3' and 5'-CGAUAUCUAACAGCGCUDdTdT-3' were used. Colon 26 cells (2 x 10⁵ cells) in culture dishes (60 mm in diameter) containing RPMI 1640 with FCS (10%) were transfected with either the MIF siRNA or the control RNA duplex using Effectene according to the manufacturer’s protocol. After 48 hours, the culture medium was removed and the cells were cultured under serum-free condition for 18 hours before stimulations.

RNA Extraction and Semiquantitative Reverse Transcription-PCR. For RNA extraction, the rats were killed after anesthesia with sodium pentobarbital at 4, 7, 14, 21, and 28 days postoperatively, and normal and fractured femora were harvested. The tissues were immediately frozen in liquid nitrogen and stored at −80°C until use in the RNA isolation. Total RNA was extracted using Trizol (Life Technologies, Rockville, MD) according to the manufacturer’s protocol. Total RNA (3.0 μg/mL) was incubated at 65°C for 10 minutes for denaturation. Denatured RNA (3.0 μg /mL), 5× RT buffer [1× RT buffer: 50 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 8.0 mmol/L MgCl₂, and 10 mmol/L DTT], 2.5 mmol/L deoxyoligonucleoside triphosphate, 100 pmol/L oligo-dT, and 0.5 μL of Monkey murine leukemia virus reverse transcriptase (Life Technologies), and 0.4 μL RNase inhibitor were incubated at room temperature for 10 minutes. After this process, 10 μL of this mixture were incubated at 42°C for 1 hour. Three microliters of the double-strand product were then mixed with 10× Taq/RT buffer [1× Taq/RT buffer: 10 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.01% gelatin, and 2.0 mmol/L DTT], 500 μmol/L deoxyoligonucleotide triphosphate mix, 25 mmol/L MgCl₂, 500 μmol/L of each sense and antisense oligonucleotide, and 0.25 μL Taq polymerase (Promega, Madison, WI). The PCR primers for amplification of rat MIF, matrix metalloproteinase (MMP)-13, and glycerolaldehydes-3-phosphate dehydrogenase (GAPDH) were designed as follows: MIF (360 bp), sense primer 5'-CACCATGCCTATTTCACTGTAACA-3' and antisense primer 5'-GCCCAGGTCTAAGCGAAGTT-GGAACCGT-3'; MMP-13 (424 bp), sense primer 5'-GCCGGAAATCCCTGAAGAGTCTAC-3' and antisense primer 5'-TTGGTCCAGGAGGAAAAGCG-3'; GAPDH (983 bp), sense primer 5'-TGAAGGTGGTGTACACAGGATTT-3' and antisense primer 5'-CATGATGGCCATGAGGTCC-3'. After a heating step at 94°C for 5 minutes, PCR was done for MIF, MMP-13, and GAPDH. Amplification was carried out under the following conditions: MIF, 94°C for 1 minute, 55°C for 1 minute, and 72°C for 30 seconds for 30 cycles; MMP-13, 94°C for 1 minute, 58°C for 2 minutes, and 72°C for 2 minutes for 22 cycles; GAPDH, 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute for 18 cycles. Following these steps, a final extension at 72°C for 7 minutes for these three samples using a thermal cycler (Perkin-Elmer, Norwalk, CT). The products were analyzed after separation by gel electrophoresis (2% agarose) and scanned densitometry to produce a standard curve that determined the linear range of quantifiable reaction products. GAPDH mRNA expression was used as a loading control.

Histology and Immunohistochemical Analysis. Liver tissues were fixed in 10% PBS-buffered formalin, and paraffin sections were stained with H&E and examined by light microscopy to assess the histologic changes. Immunohistochemical analysis was done using a Vectastain avidin-biotin complex kit according to the manufacturer’s protocol. Briefly, liver tissues obtained from mice were surgically excised at day 14 after inoculation. The livers were immersed in 10% PBS-buffered formalin. Sections were treated with methanol containing 0.3% hydrogen peroxide for 30 minutes to inactivate endogenous peroxidase. After washing with PBS, sections were incubated with a blocking solution for 30 minutes. An anti-mouse monoclonal antibody against CD31 was used as a marker for macrophages. The sections were incubated overnight at 4°C with the anti-CD31 antibody (1:100 dilution) and the reaction was visualized using 3,3’-diaminobenzidine tetrachloride containing 0.01% hydrogen peroxide. After counterstaining with hematoxylin, sections were microscopically examined and the numbers of positively stained cells were counted. Ten fields per section were counted using x100 objectives (n = 3).

Immunohot Analysis. Cells (1 x 10⁶ cells) were disrupted with a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The protein concentrations of the cell homogenates were quantitated using a Micro bicinchoninic acid protein assay reagent kit. Equal amounts of homogenates were dissolved in 20 μL of Tris-HCl, 50 mmol/L (pH 6.8), containing 2-mercaptoethanol (1%), SDS (2%), glycrol (20%), and bromphenol blue (0.04%) and heated at 100°C.
for 5 minutes. The samples were subjected to SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane (18, 19). The membranes were blocked with 5% nonfat dry milk and 0.1% Tween in PBS, probed with anti-focal adhesion kinase (FAK, pY397) phosphospecific antibody, and reacted with the anti-rabbit IgG antibody coupled with horseradish peroxidase. To assay for integrin β1, the membrane was probed with the anti-mouse CD29 (integrin β1 chain), and reacted with the rat anti-rat IgG antibody coupled with horseradish peroxidase. For the assay with MMP-13, anti-mouse MMP-13 antibody was used as a primary antibody, and anti-mouse IgG antibody was used as a secondary antibody. The resultant complexes were processed for detection by enhanced chemiluminescence using an enhanced chemiluminescence Western blotting detection system according to the manufacturer’s protocol.

For the assay with MIF, we did immunoblot analysis in a similar manner using the anti-rat MIF polyclonal antibody and the anti-rabbit IgG antibody coupled with horseradish peroxidase as primary and secondary antibodies, respectively. After the reaction, proteins were visualized with a Konica HRP-1000 immunostaining kit as recommended in the manufacturer’s protocol.

**Northern Blot Analysis.** Northern blot analysis was carried out as previously described (10). In brief, total RNA was extracted from colon 26 cells (5 × 10⁶ cells), separated by electrophoresis on agarose gels containing 0.6 mol/L formaldehyde and blotted onto nylon membrane filters. Hybridization was carried out with the mouse MIF cDNA probe, which was radiolabeled by [α-³²P]dCTP using the random primer labeling kit. The hybridization was done in a solution containing the radiolabeled cDNA probe, 50% formamide, 0.75 mol/L NaCl, 1% SDS, 20 mmol/L Tris–HCl (pH 7.5), 2.5 mmol/L EDTA, 0.5 × Denhardt’s solution (1 × Denhardt’s: 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficoll), and 10% dextran sulfate at 42°C for 16 hours. After hybridization, the filters were washed with 0.2 × SSC (1 × SSC: 0.15 mol/L NaCl, 0.015 mol/L sodium citrate, 0.1% SDS) at 65°C before autoradiography. Quantitative analysis was carried out using an MCID image analyzer (Imaging Research, Inc., Toronto, Ontario, Canada).

**Analysis of Invasion.** Invasion of colon 26 cells was measured by invasion of cells through Matrigel-coated transwell inserts (Becton Dickinson, Franklin Lakes, NJ). In brief, transwell inserts with 8-μm pores were coated on the upperside with Matrigel (40 μg per well) and the underside with fibronectin (10 μg per well) by passive adsorption. After treatment with MIF siRNA or control siRNA, the cells were cultured under serum-free RPMI 1640, and resuspended in PBS to a final concentration of 1 × 10⁴ cells per 100 μL. Cells were injected into the portal vein of BALB/c mice that had been anesthetized with ether and laparotomized. We divided mice into three groups: (i) a positive control group in which colon 26 cells were injected into the portal vein; (ii) a positive control group in which colon 26 cells treated with control siRNA were injected into the portal vein; and (iii) a test sample group in which colon 26 cells treated with MIF siRNA were injected into the portal vein. Fourteen days after inoculation with tumor cells, the mice were killed and the number of metastatic colonies in each liver was macroscopically counted. At the same time, the liver weight was recorded to evaluate tumor metastasis.

**Rho Pull-Down Assay.** The Rho pull-down assay was done according to the manufacturer’s protocol. Briefly, cells (5 × 10⁶ per well) were plated, allowed to attach for 12 hours, treated with MIF siRNA or control siRNA for 48 hours, and cultured under serum-free conditions for 24 hours. After incubation, cells were stimulated with 20 μmol/L LPA for 15, 30 and 60 minutes, washed twice with PBS, and lysed in immunoprecipitation assay buffer. Cell lysates were clarified by centrifugation, and equal volumes of lysates were incubated with Rhotekin RBD-agarose beads (30 μg) at 4°C for 45 minutes. The beads were washed thrice with washing buffer. Bound Rho proteins were detected by Western blot analysis using a monoclonal antibody against Rho. Western blot analysis of the total amount of Rho in cell lysates was done for the comparison of Rho activity (level of GTP-bound Rho) in different samples.

**Statistical Analysis.** All of the statistical analyses were carried out using Student’s t test. Ps < 0.05 were considered to indicate statistical significance.

**RESULTS**

First, we carried out a dose-dependent study to assess the effects of MIF siRNA on MIF protein expression by Western blot analysis using colon 26 cells. These cells (1 × 10⁶ cells per 5 mL), in FCS-free RPMI 1640 containing LPA (20 μmol/L), were treated with various doses of MIF siRNA, ranging from 0.1 to 1 μg/mL. After 48 hours, the cells were washed and subjected to Western blot analysis to measure levels of MIF protein. We found that MIF siRNA significantly suppressed MIF expression (Fig. 1A). Second, Northern blot analysis was carried out to assess the expression of MIF mRNA in response to LPA. LPA, ranging from 0.2 to 20 μmol/L, was added to colon 26 cells (1 × 10⁵ cells per 10 mL) in FCS-free RPMI 1640. After 24 hours of culture, MIF mRNA was found to have increased in a dose-dependent manner (Fig. 1B).

The expression levels of MIF mRNA and MIF protein of colon 26 cells were examined by reverse transcription-PCR and Western blot analysis, respectively. MIF mRNA was up-regulated by LPA (20 μmol/L) which was suppressed by the siRNA treatment (1 μg/mL) for 48 hours, whereas it was not significantly affected by control siRNA (Fig. 2A). As a control, the expression of GAPDH was measured. We next examined the effect of MIF siRNA on MIF protein production. The MIF content increased by LPA (20 μmol/L) was significantly reduced by ~50% by MIF siRNA as assessed by immunoblot analysis (Fig. 2B). The amount of β-actin was measured as a control. In the absence of LPA, MIF siRNA had no apparent effect on MIF mRNA expression (Fig. 2C). Consistent with this result, we...
found that MIF contents in the culture media were decreased by the MIF siRNA treatment (data not shown).

Invasion is the critical step in the metastatic cascade. To clarify the role of MIF siRNA in the tumor cell invasion in response to LPA, we did an in vitro invasion assay. The cells were treated with MIF siRNA (1 μg/mL) or control siRNA for 24 hours, and were cultured for another 24 hours in the absence of serum before LPA stimulation. Following this, we stimulated the cell with LPA (20 μmol/L). We found that LPA-induced migration and invasion was suppressed by MIF siRNA, whereas control siRNA did not affect the degree of tumor cell migration (Fig. 3). When we examined the effect of MIF siRNA on cell migration in the absence of LPA, we found siRNA to have no significant suppressive effect (data not shown). These findings suggest that MIF might play a key role in the migration and invasion of colon 26 cells.

To examine usefulness of siRNA for the treatment of the colorectal metastasis, we used the hepatic metastasis model. The metastatic abilities of colon 26 cells with MIF siRNA or control siRNA treatment (1 μg/mL) for 48 hours were determined by the number of metastatic nodules present 14 days after inoculation through the portal vein. Macroscopically, the number and size of metastatic foci were decreased by MIF siRNA treatment as compared with cells treated with the control siRNA (Fig. 4A). The number of metastatic nodules in the livers of mice injected with MIF siRNA-treated cells was much smaller than that by treatment with control siRNA (Fig. 4B). The weight of the livers containing the metastatic nodules produced by the MIF siRNA-treated cells was less than that by treatment with the control siRNA (Fig. 4C). MIF siRNA dose-dependently affected the number of metastatic modules and the liver weight (data not shown). We did Western blot analysis using whole livers to assess MIF protein levels in livers with or without metastatic tumors. Each whole liver injected with colon 26 cells treated with or without siRNA was homogenized and subjected to Western blot analysis. We found that MIF siRNA decreased the MIF protein level of the livers with metastatic tumors (Fig. 4D). These data indicate that MIF siRNA has the potential to suppress tumor growth and metastasis.

Histologic changes in the liver surrounding metastatic foci were examined to evaluate the effectiveness of MIF siRNA. In H&E staining, some mononuclear cells and vascularization were observed at the borders between normal tissue and tumor, when colon 26 cells without any pretreatment were injected through portal veins (positive controls; Fig. 5A and B). Similarly, significant vascularization was identified around the metastatic foci when colon 26 cells pretreated with the control siRNA were injected (controls; Fig. 5C and D). On one hand, vascularities were markedly reduced between normal and tumor tissues treated with MIF siRNA (Fig. 5E and F).

To confirm the neovascularization, we carried out immunohistochemical analysis using anti-CD31 antibody that reflects the vascular endothelial proliferation. Consistent with
the results of Fig. 4, we found the increased positive staining surrounding the metastatic foci of the cells nontreated (Fig. 6A) or treated with the control siRNA (Fig. 6B). In contrast, the number of CD31-positive-stained cells were markedly decreased (Fig. 6C).

To confirm the inhibitory effect of MIF siRNA on LPA-induced migration and invasion, we measured the intracellular levels of the GTP-bound, active form of Rho using the pull-down assay system. The level of the active form of Rho was elevated at 30 minutes and gradually decreased after the addition of LPA (20 μM) (Fig. 7). When we treated the cells with MIF siRNA (1 μg/mL) for 48 hours before LPA stimulation (20 μM), the elevation induced by LPA was markedly suppressed. The effect of MIF siRNA on Rho activation in the absence of LPA, but no significant change was found (Fig. 8B). The results show that MIF siRNA inhibited the Rho-mediated activation of actomyosin contractility and tyrosine phosphorylation of focal adhesion proteins by suppressing the prenylation of Rho.

We analyzed the effect of MIF siRNA on the phosphorylation of FAK by Western blot analysis. In preliminary experiments, we assessed the time course of the phosphorylation of FAK induced by LPA. By the pretreatment of MIF siRNA (1 μg/mL) for 48 hours, we found that tyrosine-phosphorylation of FAK was significantly elevated at 30 minutes after LPA treatment (20 μmol/L; Fig. 8A). Following this, we found that the tyrosine phosphorylation of FAK in response to LPA was markedly suppressed. Similar results were obtained at 15 and 60 minutes (data not shown). The total amounts of FAK proteins are shown at the bottom of each lane. The effect of MIF siRNA on FAK phosphorylation was assessed in the absence of LPA, but no significant change was found (Fig. 8A). The results show that MIF siRNA inhibited the Rho-mediated activation of actomyosin contractility and tyrosine phosphorylation of focal adhesion proteins by suppressing the prenylation of Rho.

We analyzed the effect of MIF siRNA on the LPA-induced integrin β1 expression by Western blot analysis. After colon 26 cells were treated with MIF siRNA (1 μg/mL) or control siRNA for 48 hours, the cells were cultured in the absence or
presence of LPA for 24 hours. Cells were lysed and the lysates were subjected to immunoblot analysis using anti-integrin β1, followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. We found that LPA induced the expression of integrin β1 (Fig. 9A). When the cells were treated with MIF siRNA, the expression of integrin β1 was markedly suppressed. In the absence of LPA, the effect of MIF siRNA on integrin β1 was not seen (Fig. 9B). These results suggest that MIF plays an important role for the integrin signaling in the event of LPA-induced colon 26 cells adhesion and invasion.

MMP is considered to be one of the important molecules involved in the induction of tumor invasion and metastasis. To examine the potential effect of MIF on MMP-13 expression in promoting tumor invasion and metastasis, we evaluated the effectiveness of MIF siRNA on MMP-13 production in response to LPA. We found that MMP-13 production was remarkably suppressed by MIF siRNA treatment (1 μg/mL) for 48 hours before LPA stimulation (20 μmol/L; Fig. 10A). MIF siRNA did not affect MMP-13 expression in the absence of LPA (Fig. 10B).

**Fig. 7** Effect of MIF siRNA on activation of Rho. The levels of the GTP-bound, active form of Rho were measured using the pull-down assay system as described in Materials and Methods. The cells were harvested at 0, 30, and 60 minutes after LPA (20 μmol/L) stimulation with treatment of MIF siRNA (1 μg/mL) or control siRNA.

**Fig. 8** Effect of MIF siRNA on LPA-stimulated tyrosine phosphorylation of FAK. A, colon 26 cells were treated with MIF siRNA (1 μg/mL) for 48 hours, and the phosphorylation of FAK in response to LPA (20 μmol/L) was analyzed. We harvested the cells at 30 minutes after LPA stimulation and assessed the amount of phosphorylated FAK by immunoblot analysis. Bottom of each lane, total amounts of FAK proteins. B, effect of MIF siRNA on FAK phosphorylation in the absence of LPA.
DISCUSSION

MIF was originally described as a T cell–derived lymphokine (1, 2), and this protein has been reevaluated as a pluripotent cytokine involved in broad-spectrum functions within and beyond the immune system (3, 4). Lanahan et al. first reported that the MIF gene was one of the early response genes in response to growth factors (20). With regard to tumorigenesis, the enhancement of MIF mRNA expression has been identified in lymph node metastasis of prostate cancer (9) and ductal breast carcinoma (21). Following these findings, it became evident that MIF was involved in the development of lymphoma and tumor-associated angiogenesis (22). Consistent with these findings, we showed that MIF plays a key role in tumor growth and angiogenesis in other types of tumors, such as melanomas (23) and colon cancers (24).

The molecular mechanism of tumor invasion on MIF remain to be investigated. In this study, we used siRNA to knockdown MIF mRNA for evaluation of its function in tumorigenesis, which showed that MIF siRNA markedly inhibited colon 26 invasion in vitro and liver metastasis in vivo. It is interesting that MIF siRNA had a significant effect without the complete knockdown of intracellular MIF by siRNA. In contrast to other cytokines, a large amount of MIF protein is stored in the cytosol of a variety of cells (4). Moreover, a significant amount of MIF protein remained in the cytosol, even a few days after MIF mRNA was significantly knocked down by siRNA. However, in vitro cellular functions, such as migration and proliferation, were markedly suppressed (data not shown). It is speculated that a certain amount of MIF protein is required for its biological functions to be exerted, or freshly biosynthesized MIF may have functionally distinct characteristics from stored MIF. Both of these speculations need further study.

The discovery of siRNA was first reported in plants by Hamilton et al. in 1999 (25). Their study showed that post-transcriptional gene silencing, a plant process similar to RNAi, was mediated by 25 nucleotide pieces of RNA capable of silencing homologous genes. Based on a large number of experiments using siRNA in mammals, it became evident that this gene knockdown system also worked effectively in mammals. Accordingly, the system has been widely used for the evaluation of targeted molecular functions.

Cell migration and invasion are critical stages in the metastatic cascade. Rho protein is one of the small GTPases, which exhibit both GDP/GTP binding and GTPase activities. Rho regulates signal transduction from receptors in the membrane to a variety of cellular events related to cell morphology, motility, cytoskeletal dynamics, and tumor progression (26, 27). More recently, it has been reported that LPA-induced migration is regulated by Rho-mediated activation in cancer cells (28).
On the other hand, integrin-mediated cell adhesion to the extracellular matrix plays an important role in a variety of biological processes, including tumor invasion and metastasis. The interaction among cellular signals is followed by enhanced tyrosine phosphorylation of various signals, such as FAK (29–31). The tyrosine phosphorylation of FAK is essential in LPA-induced cell migration (32) and is an important event downstream of Rho activation (33). In association with this event, Rho has been shown to induce sustained tyrosine phosphorylation of FAK, which plays an essential role in the movement of tumor cells (28).

In more detail, integrin β1 is a major cell adhesion molecule in tumor cells and plays a crucial role in supporting tumor cell attachment to extracellular matrix proteins (34). FAK and integrin contribute to various focal adhesions, including cell-extracellular matrix adhesions, and can alter the ability of the cell to attach and migrate through surrounding tissues (35). MMPs are proteolytic enzymes and considered to be one of the important molecules in the process of tumor invasion and metastasis. More recently, it has become clear that LPA contributes to metastatic dissemination of ovarian cancer cells via up-regulation of MMP activity and integrin β1 expression (36). We here showed that the activation of Rho, tyrosine phosphorylation of FAK, and expression of integrin β1 and MMP-13 were significantly down-regulated by MIF siRNA. Taken together, our findings strongly suggest that MIF plays a pivotal role in the invasion and metastasis of colon 26 cells via the Rho-dependent pathway. Because MIF siRNA suppressed most of the signaling steps involved in lipopolysaccharide (LPA) stimulation, it is conceivable that MIF might play a major role in LPA-mediated cell stimulation somewhere in the upper stream of the signaling pathway.

In clinical points of view, colorectal liver metastasis is associated with a very poor prognosis; most patients die within 2 years of diagnosis despite the availability of numerous therapies. Further improvement of the treatment modalities of liver metastasis is thus urgently needed. In a previous study, we found that MIF mRNA was up-regulated by LPA, suggesting that MIF plays an important role in intracellular signal transduction stimulated by LPA. We also revealed that the siRNA targeting MIF mRNA significantly inhibited the cell growth in response to LPA. In this study, we used this metastatic model using highly metastatic murine colon 26 carcinoma cells, and showed that siRNA markedly inhibited LPA-induced invasion of murine colon cancer cells. A hepatic metastatic murine model, produced by intraportal vein injection of colon cancer cells, is a useful tool for analyzing the molecular mechanism of liver metastasis (37). In addition, liver metastasis was strongly suppressed by pretreatment of the cells with siRNA. Based on these findings, it is hypothesized that MIF contributes not only to tumor growth, but also to tumor invasion and metastasis. According to several publications (13, 38, 39), the reference range of serum LPA concentration is considered to be 2 to 20 μmol/L. Thus, it is considered that an LPA study in vitro may be well correlated with that in vivo. In this context, these findings led us to hypothesize that MIF siRNA might be useful for the treatment of colorectal liver metastasis.

Finally, effective treatment of tumor invasion and metastasis constitutes the biggest challenge in clinical oncology. We here showed that tumor cell motility induced by LPA, a unique bioactive lysophospholipid, was significantly inhibited by down-regulation of MIF using RNAi, a novel gene-silencing technique. Moreover, MIF siRNA markedly inhibited LPA-induced invasion of murine colon cancer cells by attenuating the activation of Rho and thereby reducing the transmigration and focal adhesion assembly. Taken together, these results strongly indicate that MIF could be involved in the mechanism of tumor cell growth as well as in LPA-induced tumor invasion and metastasis. This may provide the basis for a new therapy that controls the metastasis of colon cancer.

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**REFERENCES**


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