Involvement of Macrophage Migration Inhibitory Factor (MIF) in the Mechanism of Tumor Cell Growth

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Abstract

Background: Macrophage migration inhibitory factor (MIF) was recently rediscovered as a cytokine, pituitary hormone, and glucocorticoid-induced immunomodulator. MIF is constitutively expressed in various cells and enhances production of inflammatory cytokines such as tumor necrosis factor-α, interleukin-1, and interferon γ. Recently, it was reported that MIF mRNA was overexpressed in prostatic tumors, which suggests that MIF is a protein involved in tumor cell growth beyond inflammatory and immune responses.
Materials and Methods: We examined the expression of MIF in the murine colon carcinoma cell line colon 26 by Western and Northern blot analyses and immunohistochemistry. Next, we investigated the effects of transforming growth factor (TGF) β, basic fibroblast growth factor (b-FGF), and platelet-derived growth factor (PDGF) on the expression of MIF mRNA. Furthermore, we examined whether MIF is involved in tumor cell proliferation, using an MIF anti-sense plasmid transfection technique.

Results: We demonstrated that MIF protein and its mRNA were highly expressed in colon 26 cells, using Western and Northern blot analyses, respectively. By immunohistochemical analysis, we found that MIF was localized largely in the cytoplasm of the tumor cells. In response to TGF-β, b-FGF, and PDGF, MIF mRNA expression was significantly up-regulated. Following this, we transfected the cells with an anti-sense MIF plasmid, which revealed that this treatment induced significant suppression of cell proliferation.
Conclusion: Although MIF plays multifunctional roles in a broad spectrum of pathophysiological states, little has been done to investigate the role of this protein in association with tumor growth. The current results suggest the possibility that MIF induces tumor cell growth in concert with other growth factors, which encouraged us to investigate a novel approach for tumor therapy using an anti-MIF antibody and an MIF anti-sense plasmid transfection technique.

Introduction

Little progress has been made in the treatment of patients with advanced or metastatic colorectal

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cancers. Cytological examination of tumor cells to determine the potential for proliferation and metastasis is important for the prognosis and therapy of the patient. Colon cancer cells secrete and/or respond to several growth factors, including transforming growth factor (TGF) β, platelet-derived growth factor (PDGF) and basic fibroblast growth factor (b-FGF), and express specific

receptors for these proteins (1). These growth factors and their receptors play a pivotal role in regulating cellular proliferation and transformation. With regard to the histological characteristics of colorectal tumors, macrophages are frequently localized at the periphery of stromal tissues adjacent to the zones of metastatic tumor invasion, where they secrete a variety of growth factors and cytokines.

In the last few years, several reports, including ours, demonstrated that constitutive expression of macrophage migration inhibitory factor (MIF) was not limited to T cells. It is also expressed by macrophages and various organs, including brain, liver, and kidney (2–4). As for the biological functions, MIF was discovered to be a pituitary hormone, inflammatory cytokine, and glucocorticoid-induced immunoregulator (5). Other than these biological properties, it has been considered that MIF might also be involved in cell proliferation and differentiation, based on the following reports. MIF was predominantly localized in differentiating cells of the embryonic chicken lens (6), and specifically detected at the proliferative basal cells of the human epidermis (7) as well as corneal endothelium (8). Moreover, MIF was identified as one of the growth factor–induced, delayed early-response genes (9). In support of this finding, we found that MIF mRNA was up-regulated at 3–6 hr after penetrating injury of the cornea, a finding similar to that of other delayed early-response genes (10).

Recently, it was reported that MIF was markedly expressed in primary and metastatic prostate cancer tissues, compared to normal prostatic cells (11). This finding suggests that MIF is involved in the proliferation and differentiation of tumor cells. On the basis of this finding, we employed Western and Northern blot analyses and immunohistochemistry, as well as an MIF anti-sense plasmid transfection technique, to investigate whether MIF is involved in tumor cell growth.

Materials and Methods

Materials

All of the following materials were obtained from commercial sources. Nitrocellulose membrane filters were purchased from Millipore (Bedford, MA); Isogen RNA extraction kit was from Nippon Gene (Toyama, Japan); SspI and random primer DNA labeling kit was from Takara (Kyoto, Japan); horseradish peroxidase (HRP)-conju-

gated goat anti-rabbit IgG antibody and Micro BCA protein assay kit were from Pierce (Rockford, IL); Konica HRP-1000 immunostaining kit was from Konica (Tokyo, Japan); 3,3'-diaminobenzidine tetrahydrochloride was from Wako (Osaka, Japan); Histofine SAB-PO kit was from Nichirei (Tokyo, Japan); pBK-CMV vector was from Invitrogen (San Diego, CA); G418 (geneticin) was from Sigma (St. Louis, MO); fetal bovine serum (FBS) and RPMI-1640 were from Gibco BRL (Grand Island, NY); human TGF-β and rat PDGF (BB) were from R & D Systems (Minneapolis, MN); human b-FGF was from Progen (Heidelberg, Germany); CellTiter 96 AQ (MTS) assay kit was from Promega (Madison, WI), and Protein A-Sepharose was from Pharmacia (Uppsala, Sweden). All other chemicals used were of analytical grade.

Anti-MIF Antibody

A polyclonal anti-rat MIF antibody was generated by immunizing New Zealand White rabbits with recombinant rat MIF as previously described (12). The IgG fraction was prepared using Protein A-Sepharose according to the manufacturer's protocol. Since only a single amino acid residue is different between rat and mouse MIFs, the anti-rat MIF immunologically cross-reacted with mouse MIF.

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 medium containing 10% heat-inactivated FBS at 37°C under 5% CO_2, and subcultured twice a week. For all experiments, logarithmically growing cells were used.

Western Blot Analysis

Western blot analysis was carried out by the method of Towbin et al. (13). In brief, colon 26 cells (5 × 10^6 cells) were suspended in 200 μl of Tris-HCl 50 mM, pH 6.8, containing β-mercaptoethanol (1%), sodium dodecyl sulfate (SDS) (2%), glycerol (20%), and bromophenol blue (0.04%), and boiled at 100°C for 5 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (14). The proteins on the gel were electrophoretically transferred onto a nitrocellu-

lose membrane at 50 mA for 1 hr using a semi-dry blot transfer apparatus (Bio-Rad). The nitrocellulose membrane was intensively washed with phosphate-buffered saline (PBS), and incubated with the anti-rat MIF polyclonal antibody (4 μg/ml IgG) at room temperature for 1 hr. After washing with PBS containing 0.02% Tween 20, the membrane was further incubated with HRP-conjugated anti-rabbit IgG (1 μg/ml) for 1 hr. After the reaction, proteins were visualized with a Konica HRP-1000 immunostaining kit as recommended in the manufacturer's protocol. Protein concentration was determined with a Micro BCA protein assay reagent kit.

Immunohistochemistry

Immunohistochemistry for colon 26 cells was performed using a Histofine SAB-PO kit according to the manufacturer's instructions. In brief, the harvested cells were mounted on gelatinized glass slides. They were immersed in methanol containing 0.3% H_2O_2 for 30 min to block endogenous peroxide activity. Following three washing steps in cold PBS containing 10% sucrose, nonspecific staining was blocked by 10% normal goat serum in PBS for 10 min, and the tissue specimen was incubated with the anti-rat MIF antibody (10 μg/ml PBS) for 30 min at room temperature. Following three washes in cold PBS containing 10% sucrose, the specimen was further incubated with biotinylated goat anti-rabbit IgG and avidin-biotin-peroxidase complex for 30 min. After additional washes, the positive staining was visualized by 3,3′-diaminobenzidine tetrahydrochloride containing 0.01% H_2O_2. The cells were counterstained with 1% methyl green solution.

ELISA

The MIF concentration was measured by enzyme-linked immunosorbent assay (ELISA) as previously described (15). In brief, the anti-rat MIF antibody (4 μg/ml) was added to each well of a 96-well microtiter plate and left for 1 hr at room temperature. All wells were filled with PBS containing BSA (1%) for blocking and left for 1 hr at room temperature. Samples were added in duplicate to individual wells and incubated for 1 hr at room temperature. After the plate was washed three times, 50 μl of biotin-conjugated anti-rat MIF antibody (4 μg/ml IgG) was added to each well. After incubation for 1 hr at room temperature, streptavidin-HRP-conjugated goat

anti-rabbit IgG antibody was added to individual wells and they were incubated for 1 hr 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 reaction was stopped with 4N sulfuric acid (25 μl). The absorbance at 492 nm was measured using an ELISA plate reader (Biorad, Model 3550).

Northern Blot Analysis

Northern blot analysis was carried out as previously described (3). In brief, total RNA was extracted from colon 26 cells (5 × 10^6 cells) and separated by electrophoresis on agarose gels containing 0.6 M formaldehyde, and blotted onto nylon membrane filters. Hybridization was carried out with the rat MIF cDNA probe, radiolabeled by [α-^{32}P]dCTP using a random primer labeling kit. The hybridization was performed in a solution containing the radiolabeled cDNA probe, 50% formamide, 0.75 M NaCl, 1% SDS, 20 mM Tris-HCl (pH 7.5), 2.5 mM EDTA, 0.5× Denhardt's solution (1× Denhardt's; 0.2% BSA, 0.2% polyvinylpyrolidone, 0.2% Ficoll), and 10% dextran sulfate at 42°C for 16 hr. After hybridization, the filters were washed with 0.2× standard saline citrate (SSC) (1× SSC; 0.15 M NaCl, 0.015 M sodium citrate, 0.1% SDS) at 65°C before autoradiography. After washing, the same filters were also reacted with a radiolabeled murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe as an internal control. Quantitative analysis was carried out using an MCID Image analyzer (Fuji Film, Tokyo, Japan).

Construction of Anti-sense MIF Plasmid

The coding region of mouse MIF cDNA was prepared by polymerase chain reaction according to a previous report (16), which was subcloned into the *Bam*HI site of pBK-CMV vector, a eukaryotic expression vector driven by the human cytomegalovirus promoter. Subcloning into the *Bam*HI restriction site yielded an insert in the anti-sense orientation (pBK-MIFAS) (Fig. 1). The orientation and proof of completeness of the insert were determined by means of restriction enzyme analysis and DNA sequencing using a DNA sequencer (ABI, Model 377A).
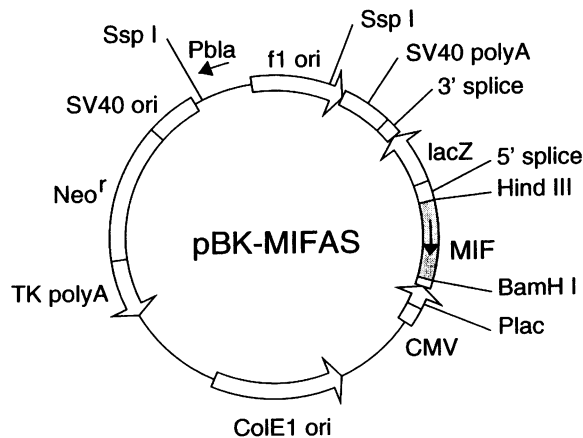
Fig. 1. Construction of the anti-sense MIF plasmid (pBK-MIFAS). The coding region of mouse MIF cDNA was subcloned into the *Bam*HI restriction site of pBK-CMV vector, a eukaryotic expression vector driven by the human cytomegalovirus promoter.
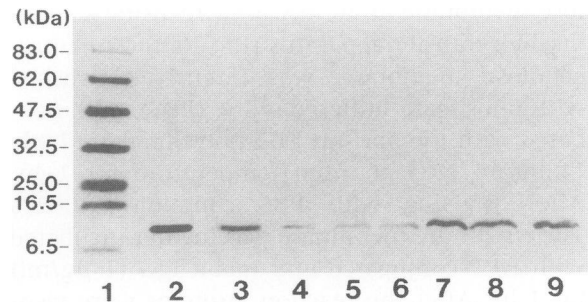

Fig. 2. Expression of MIF protein in colon 26 cells and transfected cells. Western blot analysis was carried out to examine the expression of MIF protein by each cell type. The transfected bands were visualized by a Konica HRP-1000 immunostaining kit. Lane 1, molecular weight markers; lane 2, recombinant rat MIF (100 ng/ml); lane 3, colon 26 cells (2 × 10^5 cells); lanes 4–6, colon 26 cells (2 × 10^5 cells) transfected with pBK-MIFAS; lanes 7–9, colon 26 cells (2 × 10^5 cells) transfected with pBK vector alone.

Transfection with Anti-sense MIF Plasmid

Colon 26 cells (2 × 10^5 cells) suspended in RPMI-1640 without FBS were transfected with either the pBK-MIFAS or the pBK-CMV vector alone linearized with SspI digestion by electroporation using a T820 optimizer (BTX, San Diego, CA) at 200 V with a single pulse of 10 msec. Drug selection was started 24 hr after the transfection by the addition of G418 (400 μg/ml) to the culture medium containing 10% FBS. The cells were fed every 3 days with fresh medium for 3 weeks. Then G418-resistant colonies were separately transferred to individual wells of a 48-well plate and colonies were expanded.

Cell Proliferation Assay

Colon 26 cells with and without anti-sense MIF plasmid transfection were seeded on 96-well culture plates at the initial density of 5 × 10^3 cells/well. The MTS assay was performed after incubation for 96 hr according to the manufacturer's protocol. Twenty microliters of 2 mg/ml MTS solution was added to wells and incubated for 2 hr at 37°C. The plates were placed on a shaker for 1 min, and the absorbance was measured with a plate reader read at 490 nm. Each assay was repeated at least three times.

Statistical Analyses

All data were expressed as mean values and standard deviations. For assessment of effects on cell growth rate, one-way factorial analysis of variance (ANOVA) was performed, followed by Fisher's protected least-significant difference (PLSD) analysis for multiple comparison. For all statistical analyses, $p > 0.05$ was taken as the criterion of significance.

Results

Identification of MIF Protein

To confirm the expression of MIF protein in the colon 26 cells, Western blot analysis was carried out. The MIF protein band migrated to the corresponding molecular mass of MIF, about 12.5 kDa, which was visualized with a Konica HRP-1000 immunostaining kit (Fig. 2, lanes 1–3). In addition, we examined secretion of MIF from colon 26 cells into their culture medium by ELISA. MIF was secreted into the culture medium of colon 26 cells without any treatment. The MIF concentration of culture medium collected at 24 hr culture was 6.1 ± 1.8 ng/10^4 cells. This result showed that MIF protein was expressed constitutively in colon 26 cells.

We also examined the intracellular MIF content of three different pBK-MIFAS transfected cell types by Western blot analysis, which showed significant decreases (~0.4-fold) compared to that of pBK vector alone (Fig. 2, lanes 4–9).
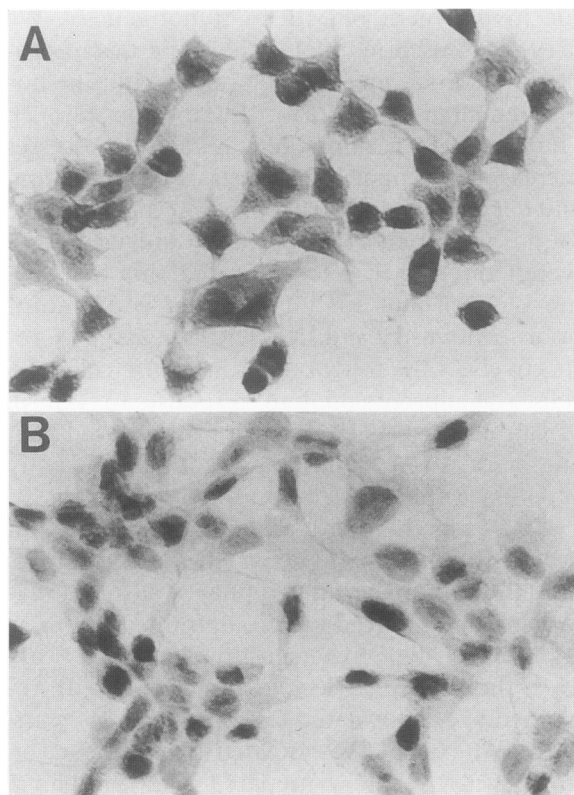
Fig. 3. Immunohistochemistry of colon 26 cells for MIF. (A) Immunohistochemical analysis was carried out using an anti-rat MIF antibody to detect its localization in colon 26 cells. (B) The control cells reacted with the anti-MIF antibody preincubated with an excess amount of rat MIF (10 μg/ml).

Fig. 4. Northern blot analysis of MIF mRNA expression in colon 26 cells in response to TGF-β, PDGF, and b-FGF. Total RNA was extracted from cultured cells after 24 hr in RPMI-1640 containing 1% FBS in the presence of various doses of each growth factor. (A) TGF-β; lane 1, 0 ng/ml; lane 2, 0.1 ng/ml; lane 3, 1 ng/ml; lane 4, 10 ng/ml; lane 5, 100 ng/ml. (B) PDGF: lane 1, 0 ng/ml; lane 2, 0.1 ng/ml; lane 3, 1 ng/ml; lane 4, 10 ng/ml; lane 5, 100 ng/ml. (C) b-FGF: lane 1, 0 ng/ml; lane 2, 0.1 ng/ml; lane 3, 1 ng/ml; lane 4, 10 ng/ml; lane 5, 100 ng/ml. The profile of GAPDH is shown at the bottom of each lane.

Immunohistochemical Analysis

We performed immunohistochemical analysis using the anti-rat MIF antibody to examine its localization in colon 26 cells. Positive MIF staining was observed largely in the cytosol, and weak positive staining was also observed in the nuclei (Fig. 3A). The control cell sample reacted with the primary antibody premixed with an excess amount of rat MIF (10 μg/ml) did not show any specific positive staining (Fig. 3B).

Influence of Growth Factors on MIF mRNA Expression

We found that MIF mRNA was increased by the stimulus with TGF-β (0.1–100 ng/ml), PDGF (0.1–100 ng/ml), or b-FGF (0.1–100 ng/ml) (Fig. 4). MIF mRNA expression reached the maximum level at 12 hr after stimulation by these growth factors, and remained at a plateau until at least 24 hr. Therefore, we examined all dose
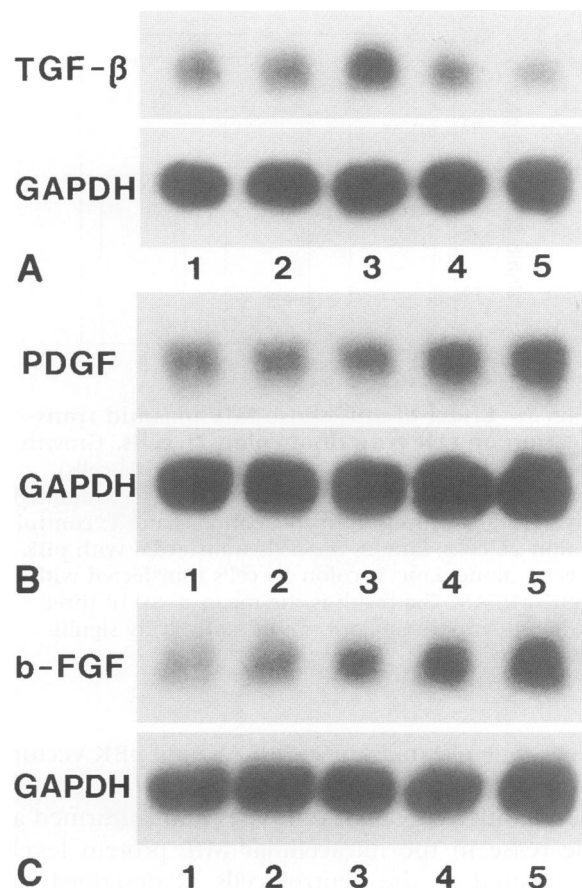
dependency at 24 hr after stimulation. TGF-β up-regulated the expression to the maximum level with the dose of 1 ng/ml (1.4-fold), but the expression level was gradually decreased by more than 10 ng/ml. PDGF and b-FGF induced the up-regulation of MIF mRNA in a dose-dependent manner, and increased the level 1.7-fold and 1.6-fold with a dose of 100 ng/ml concentration, respectively.

Effect of Anti-sense MIF Plasmid Transfection on Cell Proliferation

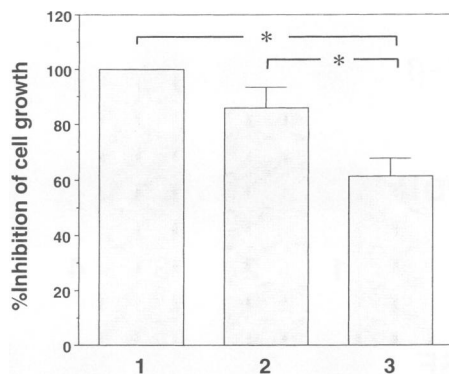We examined the effect of anti-sense MIF plasmid transfection on cell proliferation of colon 26

Fig. 5. Effect of anti-sense MIF plasmid transfection on cell growth of colon 26 cells. Growth of colon 26 cells (initial cell count, 5×10^3 cells) transfected with anti-sense MIF cDNA was examined by MTS assay after 96 hr incubation. Lane 1, control colon 26 cells; lane 2, the cells transfected with pBK vector alone; lane 3, colon 26 cells transfected with pBK-MIFAS. The result is the mean ± SD of three different experiments ($n = 9$); *statistically significant ($p < 0.01$).

cells. The plasmids, pBK-MIFAS, and pBK vector were linearized with SspI before use. We isolated three different clonal cell types and confirmed a decrease in the intracellular MIF protein level compared to the control cells as described in Figure 2. The control cells were prepared by electroporation without any plasmid vector. The cell growth rate was examined by MTS assay for colon 26 cells transfected with either the pBK-MIFAS or pBK vector alone. The proliferation rate of the transfected colon 26 cells with pBK-MIFAS was markedly suppressed (61.1 ± 10.5%) compared to the control cells (Fig. 5). In the cells transfected with the vector alone, it was slightly decreased (85.8 ± 12.3%). From these results, it was concluded that the intracellular content of MIF might be a critical component of promoting cell growth.

Discussion

Meyer-Siegler et al. identified enhanced MIF mRNA levels in prostatic adenocarcinoma and its metastatic tumors in comparison with normal prostatic tissues by means of the differential display-polymerase chain reaction technique (11). Consistent with this finding, we identified higher expression of MIF in human primary and metastatic colon cancer tissues than in normal colonic mucosal tissues (unpublished observation). These observations prompted us to investigate

the involvement of MIF in tumorigenesis. We previously reported biological properties of proliferation, invasion, and metastasis of murine undifferentiated carcinoma cell line colon 26 (17). The metastatic variant of colon 26 cells possesses increased potential for metastasis and invasion, and has been well characterized. Accordingly, we found at first that MIF protein and its mRNA were expressed in colon 26 carcinoma cells, using Western blot analysis together with immunohistochemistry and Northern blot analysis, respectively. MIF mRNA expression of colon 26 cells was up-regulated in response to TGF-β, PDGF, and b-FGF. Moreover, we showed that anti-sense MIF plasmid transfection significantly suppressed the proliferation of colon 26 cells.

MIF was the first lymphokine discovered in the culture medium of activated T cells, and this protein accumulated macrophages at inflammatory loci and induced various immune responses, e.g., delayed-type hypersensitivity (18,19). However, the precise molecular function of MIF remained to be elucidated for more than 25 years. Since MIF cDNA was isolated by Weiser et al. from a cDNA library prepared using human T cells (20), previously unrecognized properties of this protein have been discovered. Bernhagen et al. demonstrated that MIF is the major secretory protein released by anterior pituitary cells in response to lipopolysaccharide, and an anti-MIF antibody fully prevented lethality from endotoxin shock (21). Moreover, MIF was determined to be essential for T cell activation (22). It should also be noted that MIF secreted from anterior pituitary cells can counterregulate the effect of glucocorticoids (23). MIF is also expressed in a variety of organs. Macrophages, in particular, are the major source of this protein (2).

There are several reports (6–9) suggesting the possible involvement of MIF in cell growth; however, there has been no clear evidence demonstrating a direct link of MIF with cell growth. In this study, we found that tumor proliferation was significantly suppressed by anti-sense MIF plasmid transfection as described above. This fact strongly indicated that intracellular MIF might be profoundly involved in the mechanism of tumor cell growth. Although cloned anti-sense transfected cells showed a decreased proliferation rate until 4–6 weeks after isolation, the proliferation rate was gradually restored to the control level or higher in accordance with the duration of culture, in spite of the fact that the cells were still resistant to G418. To further in-

vestigate this phenomenon, we carried out Northern blot analysis of these long-term cultured clonal cells. The result revealed that the level of MIF mRNA of these cells was higher than that of the control (data not shown). In addition, during the process of cloning transfected cells, we observed microscopically that highly and effectively transfected cells appeared to be remarkably transformed, with vacuoles in the cytosol, and never divided. On the basis of these observations, it is conceivable that MIF is essential for cell growth.

Growth factors and cytokines released from macrophages appear to be important for the response of the host with cancer. For example, TGF-β is associated with progression of tumorigenicity and invasion, and increase of TGF-β is found in human colon cancer correlated with disease progression to metastasis (24). PDGF is elevated in good correlation with tumor size, and the protein released from colon carcinoma cells stimulates the growth of NIH3T3 cells in a paracrine manner (25). Moreover, b-FGF contributes to cancer cell proliferation, as the mRNA is over-expressed in correlation with progression of cancer cells (26). As demonstrated in this study, MIF mRNA expression of colon 26 cells was significantly induced by these growth factors. These data suggest that MIF is involved in the mechanism of tumor cell growth in concert with these growth factors. We found that the growth rate of colon 26 cells was minimally affected by the addition of recombinant MIF to the culture medium. Therefore, it is speculated that an increase in intracellular MIF content rather than extracellular MIF would be critical for cell growth. This finding is consistent with the results of MIF antisense plasmid transfection. Thus, we hypothesized that growth factors released from the tumor cells as well as tumor-associated macrophages could induce tumor cell growth via elevation of the intracellular MIF content.

Finally, we presented evidence that MIF could be an important protein for promotion of tumor growth, possibly in concert with other growth factors. The current results indicate that it may be possible to establish a new therapeutic approach using an anti-MIF antibody and antisense MIF plasmid transfection. To verify this possibility, we are currently investigating the effect of anti-MIF antibody on tumor cell proliferation and tumor metastasis in vivo.

Acknowledgments

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