MACROPHAGE MIGRATION INHIBITORY FACTOR: ROLES IN REGULATING TUMOR CELL MIGRATION AND EXPRESSION OF ANGIOGENIC FACTORS IN HEPATOCELLULAR CARCINOMA

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Macrophage migration inhibitory factor (MIF) may contribute to multiple aspects of tumor progression, including control of cell proliferation, differentiation, cell survival and angiogenesis. However, the potential roles of MIF in regulating hepatocellular carcinoma (HCC) tumor cell migration and the expression of angiogenic factors by HCC tumor cells have not been studied yet. In our study, we reported that intracellular MIF mRNA and protein were overexpressed in HCC tissues compared to nontumor tissues by using in situ hybridization and immunohistochemical staining. HCC tumor cell lines also secreted large amounts of MIF into the supernatants of tumor cell culture. To assess the role of MIF in HCC, we employed the transwell invasion chamber to study the effect of MIF on tumor cell migration. Our results showed that recombinant MIF and the supernatants of tumor cell line culture could enhance the invasion and migration of HCC cells. This effect can be inhibited by the addition of a neutralizing anti-MIF antibody. We observed that increased MIF serum levels correlated with higher levels of interleukin-8 (IL-8) and IL-8 promoter activity of p53, and recently, overexpression of MIF was found in human melanoma,25 breast carcinoma,26 metastatic prostate cancer27 and adenocarcinoma of the lung.28 In a recent report, Akbar et al.29 showed that the levels of MIF were significantly higher in the sera from patients with HCC and liver cirrhosis than from patients with normal controls. However, the sources of MIF in HCC and the function of MIF in tumor biology remain unclear. There are no other data in the literature on the role of MIF in HCC.

Key words: macrophage migration inhibitory factor; hepatocellular carcinoma; angiogenesis; metastasis

Hepatocellular carcinoma (HCC) is one of the most common human cancers in the world, and its incidence is particularly high among the Chinese. HCC has been the second largest cause of cancer death in China since the 1990s.1 Although morbidity and mortality rates have decreased in patients with surgically treated HCC in recent years, the long-term prognosis remains unsatisfactory because of a high recurrence rate.2 The high recurrence rate is related to a propensity of HCC for vascular invasion and metastasis. Although the risk factors of tumor recurrence in HCC are well known, the precise molecular mechanisms contributing to the invasiveness of HCC still remain unclear. Understanding of the molecular mechanisms of the complex multistep process of tumor invasion and metastasis could facilitate the development of better treatment modalities and preventive measures.

Tumor growth and metastasis depend upon the ability of the tumor to induce its own blood supply. This process is dependent on the induction of angiogenesis mediated by angiogenic factors secreted by the tumor cells. Tumor cells secrete a wide variety of angiogenic factors that participate in the development of microvasculature in the tumor.3,4 Among the identified angiogenic factors, vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) are the most potent and representative angiogenic factors.5–8 Other angiogenic factors, such as basic fibroblast growth factor (bFGF), angiopoietin-1 (Ang-1) and Ang-2 and transforming growth factor-α (TGF-α) have also been shown to induce angiogenesis in vitro and in vivo.9,10 VEGF is present in various human tumors, including lung,11 breast,12 gastrointestinal13 and neuroblastoma.14 VEGF expression is higher in HCC cells than in noncancerous tissue.15,16 IL-8 is expressed by a number of human malignancies, and its expression correlates with the metastatic potential of the tumors.17,18 It has been recently reported that IL-8 is an angiogenic factor of HCC.19 However, the mechanisms that regulate expression of angiogenic factors in HCC are still not well understood.

Macrophage migration inhibitory factor (MIF) has emerged to play a central role in the control of the host inflammatory and immune response. MIF normally circulates at basal levels in serum, and additional MIF is secreted as a product both of the anterior pituitary gland and of activated monocytes/macrophages in response to various invasive stimuli.20–22 In addition to its potent effects on the immune system, MIF has been linked by several reports to fundamental processes that control cell proliferation, differentiation, angiogenesis and tumor progression.23 Hudson et al.24 have shown that MIF can inactivate the tumor suppressor activity of p53, and recently, overexpression of MIF was found in human melanoma,25 breast carcinoma,26 metastatic prostate cancer27 and adenocarcinoma of the lung.28 In a recent report, Akbar et al.29 showed that the levels of MIF of patients with HCC were significantly higher in the sera from patients with HCC and liver cirrhosis than from patients with normal controls. However, the sources of MIF in HCC and the function of MIF in tumor biology remain unclear.

There are no other data in the literature on the role of MIF in HCC.

Abbreviations: Ang, angiopoietin; APAAP, alkaline phosphatase anti-alkaline phosphatase complexes; bFGF, basic fibroblast growth factor; DIG, digoxigenin; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; Ig, immunoglobulin; IL-8, interleukin-8; MIF, macrophage migration inhibitory factor; mAb, monoclonal antibody; PAP, peroxidase anti-peroxidase complexes; SSC, standard saline citrate; TCM, tumor-conditioned media; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

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In our study, we showed that HCC cells produced MIF and demonstrated the possible biologic function of MIF with regard to the enhancement of tumor cell migration and the production of angiogenic factors.

MATERIAL AND METHODS

Patients and tissue samples
Between January 1998 and June 1999, 55 Chinese patients who underwent resection of HCC in the Department of Surgery of the University of Hong Kong in Queen Mary Hospital were studied. There were 40 males and 15 females. The median age of the patients at diagnosis was 51 years (range, 16–73 years). Tissues from the resected specimens of patients were prospectively collected in a fresh state and then fixed in 10% formalin. Blood samples were collected, before surgical resection, from the 55 patients with HCC and 15 healthy subjects. The study was approved by the Human Research Ethics Committee, Queen Mary Hospital, University of Hong Kong, Hong Kong. Informed consent was obtained according to the regulations of the Committee.

Reagents and antibodies

All regents were obtained from Sigma (St. Louis, MO) unless otherwise stated. Recombinant human MIF was cloned, expressed in Escherichia coli, and purified by anion exchange and reverse phase chromatography as described previously. The following mouse anti-human monoclonal antibodies (mAbs) were used: anti-MIF mAb, anti-human CD3 specific for T cells (R&D Systems, Minneapolis, MN); anti-CD68, which recognizes most monocytes and macrophages (R&D); and isotype controls (R&D). Peroxidase and alkaline phosphatase-conjugated goat-anti-mouse immunoglobulin G (IgG), mouse peroxidase anti-alkaline phosphatase complexes (APAAP), and mouse alkaline phosphatase-anti-peroxidase complexes (PAP) were purchased from Dako (Glostrup, Denmark).

A 520-bp fragment of human MIF cDNA cloned into pCRII plasmid was used to prepare digoxigenin (DIG)-labeled antisense and sense cRNA probes for in situ hybridization according to the manufacturer’s protocol (Boehringer Mannheim GmbH, Mannheim, Germany).

In situ hybridization

In situ hybridization was performed on paraffin sections (4 μm) of formalin-fixed tissue as previously described. Sections were treated by being heated in a microwave oven, incubated with 0.2 M HCl for 15 min, followed by 1% Triton X-100 for 5 min, and finally digested with 10 μg/ml proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) at 37°C for 20 min. After being washed in 2× standard saline citrate (SSC), the sections were prehybridized, and then hybridized with DIG-labeled sense or antisense MIF cRNA probe overnight at 42°C in a hybridization buffer containing 50% deionized formamide, 4× SSC, salmon sperm DNA (1 mg/ml) and yeast tRNA (1 mg/ml). Sections were then washed in 0.1× SSC at 42°C, and the hybridized probe was detected using alkaline phosphatase-conjugated sheep anti-DIG F(ab) fragments and color development with nitro blue tetrazolium/X-phosphate.

Immunohistochemical staining

One-color immunohistochemical staining was performed as described previously. Paraffin sections were incubated with anti-MIF mAb at 1:1,000 dilution overnight at 4°C. The sections were then washed with PBS (pH 7.4). Endogenous peroxidase was inactivated with 3% H₂O₂ in methanol, and then incubated with peroxidase-conjugated goat anti-mouse IgG. After being washed with PBS, sections were incubated with PAP and developed with 3,3-diaminobezidine to produce a brown color. Double immunohistochemical staining was performed as described previously. Briefly, sections were placed in 10 mM sodium citrate (pH 6.0) and heated using a microwave oven. The sections were then incubated with mAbs using the 3-layer PAP. After a second round of microwave oven heating, sections were labeled with the mAbs overnight at 4°C. After being washed, the sections were incubated with APAAP, and then developed with Fast Blue BB Salt (4-benzoylazino-2,5-diethoxybenzenediazonium chloride hemi[zinc chloride] salt).

Isolation of human hepatocytes from normal and HCC tissues

The tumor tissues were from patients with HCC who underwent resection in our hospital, and the human normal livers were from healthy organ donors. The isolation of human hepatocytes was performed as described previously, with minor modification. Liver specimens were cut into small tissue fragments (1–2 mm³) in DMEM (Gibco, Grand Island, NY) and then incubated with 0.2% pronase (Boehringer, Mannheim, Germany) and 0.8 μg/ml DNase ( Worthington, UK) at 37°C with continuous pH registration and correction with 1 N NaOH. pH was kept between 7.3 and 7.5. After 30 min, the suspension and rest fragments were filtered through a gauze pore (60 μm) for further fragments of remaining liver tissue, and reincubated for another 15 min. The suspension was centrifuged at 300g for 10 min and washed twice with PBS containing 0.8 μg/ml DNase. The erythrocytes were removed by using 16% Nycodenz (Nycosaid, Oslo, Norway) gradient for 20 min, 600g at 4°C. The low-density fraction was collected and washed with PBS. The final pellet was resuspended in DMEM for use.

Cell culture and stimulation assay

Three human HCC cell lines (PLC, Huh 7 and Hep 3B) and normal control cell line (CL-48, human fetal liver) were purchased from the American Type Culture Collection (Rockville, MD). All cell lines were maintained in DMEM, supplemented with 10% FBS (Gibco) and antibiotic agents (100 U/ml penicillin G and 80 μg/ml streptomycin, Gibco). HCC cells line PLC was cultured in the presence of 10% FBS for 72 hr, and the supernatants were collected as tumor-conditioned media (TCM). For stimulation experiments, cells were seeded at 5 × 10⁶/ml in 24-well plates and then cultured in DMEM with 10% FBS for 24 hr. Cells were washed with PBS and treated with DMEM with 1% BSA and different concentrations of recombinant human MIF (ranging from 0.1–200 ng/ml).

Flow cytometric analysis

Intracellular MIF expression was evaluated by immunofluorescent staining with anti-MIF mAb (R&D Systems, Minneapolis, MN). Cells were maintained in 4% paraformaldehyde/PBS for 10 min and followed by treatment with 1% saponin for 20 min at room temperature. The treated cells were incubated with anti-MIF mAb and secondary mAb conjugated with fluorescein isothiocyanate (FITC), both for 45 min at 20°C. After a final wash, cells were resuspended in 1% paraformaldehyde/PBS. Labeled cells were assayed by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) that automatically and simultaneously measured the fluorescence of individual cells identified by their size-dependent light-scattering properties.

Western blot

Cell lysates were prepared from the HCC cultured cells lines. Lysed cells were treated with SDS-reducing buffer (4-fold volume) and subjected to SDS-PAGE. Proteins on the gel were transferred onto nitrocellulose membranes, which were then immersed in a blocking solution containing 5% skimmed milk and 0.1% Tween for 20 min. Afterwards, the membranes were incubated with biotin-labeled anti-human MIF goat polyclonal antibody (R&D Systems) for 2 hr at room temperature. After being washed with PBS, the membranes were incubated with streptavidin-biotin complex for 1 hr at room temperature. The antigen was detected using enhanced chemiluminescence Western blotting detection reagents (Amersham, Arlington Heights, IL).
FIGURE 1 – The analysis of MIF expression by in situ hybridization and immunohistochemic staining in the tumor and nontumor tissues from HCC patient. (a) Tumor cells showing MIF mRNA expression (blue) with strong cytoplasmic staining in HCC. (b) Positive MIF mRNA expression (blue) in cytoplasm of hepatocytes in liver cirrhosis tissue. (c) Weakly positive expression of MIF mRNA in healthy liver. (d) Positive cytoplasmic expression of MIF protein (brown in tumor cells of HCC. (e) Positive cytoplasmic expression of MIF protein (brown) in liver cirrhosis tissue. (f) Negative or weakly positive expression of MIF protein in healthy liver.
Measurement of the levels of MIF, VEGF and IL-8 in the sera of patients with HCC and in the supernatants of tumor cell culture

MIF, VEGF and IL-8 in the sera of patients with HCC and in culture supernatants of cell lines were measured by using ELISA kits (R&D Systems). Cells at $5 \times 10^4$ were cultured in 24-well plates in DMEM with 10% FBS for 24 hr and then washed twice with PBS, and further cultured in medium with different concentrations of MIF for 24 hr. The culture supernatants of cells were collected after each experiment, centrifuged at 300g for 5 min to remove cells and debris. The ELISA was performed according to the manufacturer's instructions.

Migration assay

Tumor cell invasion and migration were assayed in an invasion chamber (Becton Dickinson Labware, Franklin Lakes, NJ) with 8 μm-porosity polycarbonate filter membrane.33,34 The upper sides of the membranes were precoated with Matrigel matrix (Becton Dickinson Labware, 30 μg/insert), which is a material that mimics the basement membrane. The coated insert was placed in each well of a 24-well plate filled with 500 μl of medium (DMEM with 1% FBS) in the presence of various amounts of MIF or TCM. The upper well contained a suspension of HCC cell lines or control cell line CL-48 (200 μl, $1 \times 10^5$ cell/ml medium). The cells in chambers were incubated for 24 hr at 37°C, and migration to the underside of the precoated filter was measured. After incubation at 37°C for 24 hr, the cells on the upper surface of the filters were removed by swabbing with a cotton swab and the cells that had migrated to the lower surface were counted under a microscope. For each chamber, the number of migrated cells in 5 randomly chosen high-power fields was counted. All assays were performed in triplicate, and at least 3 independent experiments were performed.

Statistical analysis

Continuous variables were expressed as mean ± SD and compared using the unpaired t-test. Correlation between continuous variables was performed using the Pearson correlation coefficient.
Expression of MIF in HCC

By using in situ hybridization, we analyzed human HCC specimens that contained adjacent cirrhotic liver tissue as well as healthy human liver specimens. We found that MIF mRNA expression was strongly positive in HCC tissue. MIF mRNA was located in the cytoplasm of tumor cells and hepatocytes of cirrhotic liver (Fig. 1a and b). In contrast, the hepatocytes from healthy liver were negative or weakly positive for MIF mRNA staining (Fig. 1c). Controls employed a sense MIF cRNA probe labeled to the same specific activity as the antisense probe. No staining was observed in specimens from healthy or HCC subjects using the sense probe or with no probe at all (data not shown). Furthermore, by using immunohistochemical staining, we observed that MIF protein was positive in tumor cells (Fig. 1d) and hepatocytes of cirrhotic livers (Fig. 1e). MIF protein expression in healthy liver was considerably less intense than HCC specimens (Fig. 1f). In addition, 2-color immunostaining showed that macrophages, infiltrating T cells and endothelial cells in the HCC samples had strong expression of MIF (data not shown).

We further quantified the MIF expression in freshly isolated tumor cells from HCC and hepatocytes from healthy livers by using flow cytometry. Figure 2a showed that MIF expression in the cytoplasm of HCC cells was much higher than that of the hepatocytes from healthy liver (92.10% vs. 20.59%).

Expression of MIF in human HCC cell lines

Expression of MIF in 3 HCC cell lines (Hep 3B, PLC and Huh 7) was assessed by flow cytometry. MIF expression was predominantly identified in the cytoplasm of tumor cells (Fig. 2b). The expression of MIF in cell lines was confirmed by Western blot, which had an MW of 12.5 kd (Fig. 2c). The amount of secreted MIF protein in the supernatants of cultured HCC cell lines was measured by ELISA after 24 hr incubation. The MIF concentrations obtained from 3 independent experiments were as follows: 29.8 ± 5.6 ng/ml in PLC; 15.7 ± 3.9 ng/ml in Hep 3B; and 20.6 ± 7.8 ng/ml in Huh 7 (Fig. 3). MIF concentration in TCM was 212.2 ± 25.9 ng/ml.

Statistical significance was taken as p < 0.05. All statistical analyses were performed using statistical software SPSS for Windows 9.0 (SPSS, Inc., Chicago, IL).

RESULTS

Expression of MIF in HCC

Serum levels of MIF, IL-8 and VEGF in patients with HCC

The levels of MIF, IL-8 and VEGF in the sera of patients with HCC were significantly increased compared to healthy subjects (Table I). Furthermore, the levels of serum MIF correlated positively with the serum levels of IL-8 (r = 0.327, p = 0.028), suggesting that MIF and IL-8 expression in HCC patients may be related. The correlation between serum levels of MIF and VEGF was not statistically significant (r = 0.232, p = 0.126), but there was a significant correlation between serum IL-8 and serum VEGF levels (r = 0.491, p = 0.001).

Effect of MIF on stimulation of VEGF and IL-8 in HCC cells

To detect whether MIF contributes to the production of VEGF and IL-8 by HCC cell lines, Hep 3B, PLC and Huh 7 were stimulated with MIF for 24 hr. The cytokines in the supernatants of cell lines were measured by ELISA. Figure 4 showed that the levels of both VEGF and IL-8 were increased in the supernatants of MIF-stimulated Hep 3B, PLC and Huh 7 cell cultures, in a dose-dependent manner. However, MIF had no effect on stimulating the production of IL-8 and VEGF in control cell line CL-48 (Fig. 4). Furthermore, the production of IL-8 and VEGF by HCC cell lines can be inhibited by neutralizing MIF mAb, but not by control antibody (Fig. 5).

Effect of MIF on HCC cell migration

To assess the role of MIF on HCC cell migration, migration of HCC cells in response to MIF was examined by the invasion chamber method. Various doses of MIF from 1–200 ng/ml or the TCM were added in the lower chambers. Migration of PLC cells was enhanced by exogenously added recombinant MIF in a dose-dependent manner (Fig. 6a). In contrast, MIF was not able to enhance the migration of control cell line CL-48 (Fig. 6a). In addition, migration of other cell lines (Hep 3B and Huh 7) exhibited the same pattern in response to MIF (data not shown). Furthermore, TCM can also increase PLC cell migration. This enhanced migration of PLC by the TCM can be inhibited by a neutralizing antibody against MIF, but not by the addition of a control antibody (Fig. 6b).

DISCUSSION

Recent studies suggest that MIF may contribute to multiple aspects of tumor progression and neoplasia. In this investigation, expression of MIF mRNA and protein has been shown in human HCC specimens and cell lines. In comparison, MIF expression in freshly isolated hepatocytes from healthy liver was much less than that of tumor cells isolated from HCC. These results conform well with the observation by Akbar et al.29 Epidemiologic and clinical studies clearly indicate that most of the patients suffer from liver cirrhosis caused by chronic hepatitis viral infection or alcohol for a period of time before developing HCC, and liver cirrhosis is regarded as a precancerous state of HCC.35,36 Our results demonstrated that the expression of MIF mRNA and protein was also high in the hepatocytes from patients with liver cirrhosis, suggesting that MIF may have a role in both induction and progression of HCC.
One of the important activities of MIF-promoting tumorigenesis is to stimulate angiogenesis. In vitro, MIF is also an angiogenic factor by its ability to stimulate endothelial cell growth by a direct effect. IL-8 and VEGF are potent angiogenic factors reported to promote tumor growth and metastasis in numerous studies. Patients with high IL-8 levels in cancerous tissue of HCC had a significantly higher frequency of portal vein invasion, venous invasion and bile duct invasion. Increased expression of VEGF has also been shown to correlate with tumor progression and intrahepatic metastasis. Furthermore, there is evidence that a high serum VEGF level is a predictor of intrahepatic metastasis and microscopic
venous invasion in HCC. The relationship, if any, between MIF and expression of IL-8 and VEGF by HCC cells remains to be elucidated. In our study, we showed that MIF, VEGF and IL-8 levels are elevated in serum of patients with HCC, and the levels of MIF correlated with the levels of IL-8, suggesting that MIF and IL-8 expression in HCC may be related. There was, however, no significant correlation between serum MIF and VEGF, suggesting that other factors may play a role in regulating serum VEGF production in patients with HCC. Thus, we investigated whether MIF was able to induce the production of IL-8 and VEGF by tumor cells. We demonstrated that recombinant MIF could induce a panel of 3 HCC tumor cell lines to express IL-8 and VEGF in vitro in a dose-dependent manner. These results provided evidence for an additional novel mechanism by which MIF promotes angiogenesis, which is its ability to induce expression of angiogenic factors by tumor cells.

It is of great interest that enormous MIF expression was detected in HCC tissues and HCC cell lines. This finding indicated that MIF may also participate in the regulation of tumor cell behavior. We focused on the effect of MIF on the invasiveness and metastatic potential of HCC cell lines in our experiments. Shimizu et al. reported that MIF stimulates migration of human melanoma cells.

We observed that on monolayer culture, exogenous recombinant human MIF did not stimulate the growth of the HCC cells (data not shown), while it markedly enhanced the migration of HCC cells but not control cells.

Furthermore, in our study, we clearly demonstrated that HCC cell lines secreted abundant amounts of MIF in the medium, and the concentration of MIF in the medium was sufficiently high to enhance the migration of HCC cells and production of IL-8 and VEGF. The activity of MIF was partially inhibited by anti-MIF neutralizing antibody. However, other secreted factors such as hepatocyte growth factor in the supernatants of HCC cell culture can also increase the migration of HCC cells and production of IL-8 and VEGF in HCC cells (unpublished data).

Crucial to the interpretation of these data is the finding that MIF has no effect on stimulating IL-8 and VEGF production and enhancing migration in control cell line CL-48. One of the possible explanations is the lack of membrane receptor on the CL-48 cell surface, leading to no response to MIF. There is very little available data to explain how MIF interacts with cells, as no membrane-bound receptor has been reported for MIF to date. It is very interesting and important to study cell-based models and pathways so as to reveal mechanisms of how MIF exerts its effects from the cell surface.

On the basis of previous studies and the current study, we hypothesized a potential autocrine/paracrine regulatory mechanism of MIF in tumor environment. In our study, tumor, stroma and endothelial cells produced MIF, which may promote tumor invasion by stimulating HCC cell migration, and may promote angiogenesis indirectly by stimulating tumor cells to produce angiogenic factors IL-8 and VEGF. These angiogenic factors are able to promote angiogenesis in vivo and are correlated with poor
prognosis of HCC. It will be worthwhile to study whether blocking the MIF expression would benefit the tumor control in HCC via inhibiting angiogenesis in addition to inhibiting tumor cell invasion and migration.

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