Overexpression of Macrophage Migration Inhibitory Factor Induces Angiogenesis and Deteriorates Prognosis after Radical Resection for Hepatocellular Carcinoma

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BACKGROUND. Macrophage migration inhibitory factor (MIF) is a pivotal cytokine that regulates inflammatory and immune responses. Recently, many investigators reported that MIF is expressed highly in several tumors, including hepatocellular carcinoma (HCC). However, the role of MIF in tumor angiogenesis and patient prognosis has not been examined in patients with HCC.

METHODS. The authors evaluated MIF expression in 56 samples of HCC by Western blot analysis, and the results were correlated with clinicopathologic factors and patient prognosis. MIF localization was determined by immunohistochemical methods, and the results were compared with tumor microvessel density (MVD), as assessed by anti-CD34 antibody. Furthermore, to validate the role of MIF in angiogenesis, both MIF expression during culture of HCC cells (using the Hep3B, HepG2, and Huh7 cell lines) under hypoxic condition and the angiogenic potential of recombinant MIF in an in vitro angiogenic model were examined.

RESULTS. Tumors with high MIF expression had high α-fetoprotein levels (P = 0.049) and frequent intrahepatic recurrence (P = 0.043). Immunohistochemical MIF scores had a significant correlation with MVD (P = 0.007). Patients who had tumors with high MIF expression levels had a significantly worse (P = 0.025) disease-free survival, and this finding remained significant as an independent prognostic factor in the multivariate analysis. Hep3B cells had high expression of MIF at 6 hours and 12 hours after hypoxic stress and exogenous MIF stimulated endothelial tube formation in in vitro angiogenesis.

CONCLUSIONS. The current findings suggest that MIF expression may play a pivotal role in the dismal prognosis of patients with HCC that may be attributable to the modulation of angiogenesis. Cancer 2005;103:588–98.

KEYWORDS: hypoxia, recurrence, endothelial cell, hepatoma cell line, microvessel density.

Macrophage migration inhibitory factor (MIF) was described originally as one of the first cytokines derived from activated T cells that inhibited the random migration of cultured macrophages.1,2 Later, it was found that MIF also is expressed by several types of immune-competent cells, including macrophages and eosinophils, and that it plays a pivotal role in the inflammatory process.3,4 In addition, it was reported that MIF could override the immunosuppressive effects of glucocorticoids, which accounts in part for the proinflammatory role of MIF in conditions such as septic shock, arthritis, and glomerulonephritis.5–13
Although MIF was discovered more than 40 years ago and has been well recognized as a pleiotropic cytokine, there has been renewed interest in MIF after the finding that MIF stimulates cell proliferation of quiescent, nontransformed, NIH/3T3 fibroblasts in an in vitro study.\(^\text{14}\) Many subsequent studies concluded that the action of MIF may extend beyond immune modulation and that MIF may function as a critical mediator of cell proliferation, tumorigenesis, and angiogenesis.\(^\text{15-21}\) With regard to hepatocellular carcinoma (HCC), most HCC lesions develop in a milieu of chronic inflammation produced by hepatitis viruses as well as dysregulated cellular proliferation due to cirrhosis formation. In this context, we were interested in determining the role of MIF in HCC. Indeed, Akbar et al.\(^\text{16}\) found that serum levels of MIF from patients with HCC and cirrhosis were higher than the levels in patients with chronic hepatitis. Kamimura et al.\(^\text{17}\) first showed that MIF expression in primary lung adenocarcinoma was related to patient prognosis. To the best of our knowledge, however, the relation between MIF expression and prognosis has yet to be addressed in patients with HCC.

Therefore, we set out to analyze the relationship between MIF expression and patient prognosis after radical hepatectomy for HCC as well as the angiogenic potential of MIF in HCC. Moreover, we evaluated hypoxia-induced changes in MIF expression in HCC cell lines, and we confirmed that recombinant MIF (rMIF) directly promotes vascularization in an in vitro model of coculture of human umbilical vein endothelial cells (HUVECs) and fibroblasts.

**MATERIALS AND METHODS**

**Patients**

Fifty-six patients (42 males and 14 females) were included in the current study. All patients underwent curative hepatic resection for HCC between July 1985 and April 2000. The age of patients ranged from 32 years to 77 years (mean standard ± deviation [SD], 62.0 years ± 9.5 years). Preoperative ultrasonography, computed tomography (CT) scans, and angiography were performed routinely in all patients. Our criteria for curability were published previously.\(^\text{22}\) To identify any residual tumor, all patients with uneventful recovery were followed 3–6 weeks postoperatively with ultrasonography, CT scans, and (if necessary) with angiography. Serum α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) were measured once per month at the outpatient clinic during the first 6 months. Imaging studies with ultrasonography and/or CT were repeated every 3 months. When tumor recurrence was suspected, the patient was hospitalized for angiography. None of the patients who were included in this study had received any preoperative chemotherapy or embolization therapy.

**Western Blot Analysis**

For the extraction of protein from cell lines, cells were washed three times with cold phosphate-buffered saline (PBS), and were harvested gently by scraping. Cell lysis was performed using prechilled buffer containing protease inhibitor cocktail (RIPA buffer; Roche, Mannheim, Germany). Tissue samples were homogenized in lysis buffer containing 50 mM Tris-HCL, pH 8.0; 400 mM NaCl; 1 mM ethylenediamine tetraacetic acid; and aprotinin at a final concentration of 2 μg/mL. Nuclei and cell debris were removed by centrifugation at 15000 revolutions per minute for 30 minutes at 4 °C. Protein Assay CBB Solution (Nacalai Tesque, Tokyo, Japan) was added to the supernatant fluids, and protein contents were measured with the Bradford method. Fifty micrograms of protein were used for each sample. Proteins were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to a nitrocellulose membrane (Millipore Corporation, Bedford, MA) overnight. The transblotted membrane was washed 3 times with Tris-buffered saline (TBS) containing 0.01% Tween 20 (TTBS). After blocking with TTBS containing 4% skim milk for 60 minutes, the membrane was incubated with primary antibody (anti-human MIF mouse immunoglobulin G [IgG]; 1:1000 dilution; Genzyme, Cambridge, MA) in TTBS containing 4% skim milk at room temperature for 60 minutes. The membrane was then probed with secondary antibody (i.e., antimouse goat IgG, horseradish peroxidase [HRP]; 1:500 dilution; Medical and Biological Laboratories Company Ltd., Osaka, Japan), for 60 minutes at room temperature and was washed 3 times with TTBS. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) using the ECL Western blot detection system (Amersham, Arlington Heights, IL). β-Actin (Sigma Chemical Company, St. Louis, MO) was used as an internal control. Digital images of immunoreactive bands of MIF were captured using a scanner (ES-2000; Seiko Epson, Tokyo, Japan) and Adobe Photoshop software (version 5.5; Adobe Systems, San Jose, CA). Immunoreactive bands were quantified using a public-domain National Institutes of Health (NIH) Image Program (developed at the U.S. NIH and available from URL: http://rsb.info.nih.gov/nih-image/ [accessed June 2003]). MIF-expressing tumors were divided into a high-expression group and low expression group according to the median MIF expression level.
Immunohistochemistry
Samples were fixed routinely in 10% formalin and subsequently embedded in paraffin. Serial, 4-μm-thick sections were prepared from paraffin blocks and stained for hematoxylin and eosin. Representative tumor samples that were free of any necrosis or hemorrhage were selected for immunohistochemical staining. Sections were deparaffinized and hydrated by sequential immersion in xylene and graded alcohol solutions. The sections were then incubated in 3% H₂O₂ for 30 minutes to block the endogenous peroxidase activity. Slides were treated with normal serum obtained from the same species in which the secondary antibody was developed for 30 minutes to block the nonspecific staining. Subsequently, slides were incubated with the primary antibodies 1) biotin-conjugated antihuman MIF goat IgG (Genzyme) at 1:100 dilution for 2 hours at room temperature and 2) antihuman CD34 mouse IgG (Dakopatts, Glostrup, Denmark) at 1:50 dilution for 90 minutes at room temperature. Slides also were incubated with the secondary antibodies 1) antibiotin rabbit IgG (HRP; Dakopatts) at 1:100 dilution for 30 minutes at room temperature and 2) antimouse rabbit antibody (Nichirei, Tokyo, Japan) for 30 minutes at room temperature. Then, they were incubated with peroxidase-conjugated streptavidin (Nichirei) for 30 minutes at room temperature. Washes in 0.075% Brij detergent/PBS followed all steps. Peroxidase activity was detected by incubating the samples with 3-amino-9 ethylcarbazol (Nichirei). Sections were counterstained with hematoxylin. For the negative controls, biotinylated normal goat IgG (Genzyme) was substituted for the anti-MIF antibody. For CD34 staining, counterstaining was not done.

Evaluation of MIF Immunostaining
Immunoreactivity for MIF was evaluated according to the intensity and extent of MIF staining. Intensity was scored on a scale from 0 to 3 (0 = negative staining, 1 = weakly positive staining, 2 = moderately positive staining, and 3 = strongly positive staining). Staining intensity was evaluated according to the maximum intensity among positive cells. The extent of positive staining was estimated and scored on a scale from 0 to 4 (0 = negative, 1 = 1–25% positive cells, 2 = 26–50%, positive cells, 3 = 51–75% positive cells, and 4 = 76–100% positive cells). The sum of the intensity score and the extent of staining score was used as the final staining score for MIF. Immunoreactivity scores for MIF were compared statistically with microvessel density (MVD).

Quantitation of MVD
Because of the technical difficulty in counting linearly immunostained sinusoids, we used an image-analysis system to assess the MVD as a percentage of the endothelial area. Because immunoreactivity for CD34 showed slight heterogeneity within the same tumor, the 5 most highly vascularized areas (hot spots; magnification, ×200) were selected for evaluation. The images were scanned with an Olympus charged-coupled device camera (Olympus, Tokyo, Japan) and stored on a computer for subsequent analysis. On the computer screen image, the background color was subtracted, and the 3-amino-9 ethylcarbazol-stained areas were evaluated as the number of pixels using the NIH image-analysis system.

Hypoxic Treatment of HCC Cell Lines
Three HCC cell lines were kindly provided by the Cell Resource Center for Biomedical Research (Tohoku University). Hep3B, HepG2, and Huh7 cells were cultured routinely in GIT, Dulbecco minimal Eagle medium, and RPMI with 10% fetal bovine serum, respectively. Fresh media was added 3 hours before hypoxic treatment. Cells were incubated in a hypoxic incubator (N₂/O₂/CO₂ incubator; SANYO, Tokyo, Japan), which maintained an environment of 1% O₂, 5% CO₂, and 94% N₂. The normoxic cells were maintained in a 37 °C incubator with 21% O₂. Cells were harvested at 70–80% cell confluence.

In Vitro Angiogenesis
A 24-multiwell tissue-culture plate format that was designed originally for the assessment of in vitro angiogenesis (KZ-1000; Kurabo, Osaka, Japan) was used for this study. After examining the cultures microscopically for cell morphology and signs of the growth of endothelial cells, rMIF at different concentrations (1.6 ng/mL, 3.1 ng/mL, 6.25 ng/mL, 12.5 ng/mL, 25.0 ng/mL, 50.0 ng/mL, 100.0 ng/mL, and 200.0 ng/mL) was added to the treatment wells. Duplicate wells were used for the control group (endothelial cell medium only), the positive control group (recombinant vascular endothelial growth factor A [rVEGF-A] 10 nM/mL), the negative control group (rVEGF-A 10 nM/mL plus suramin 50 μM/mL), and the rMIF group. The medium was changed every 3 days. After 11 days, cells were fixed in ice-cold 70% ethanol, and endothelial cell tubules were stained with a staining kit (ZHA-1225; Kurabo) according the manufacturer’s protocol. A primary antibody against human CD31 at a dilution of 1:4000 was used for detection of the endothelial tubules. Visualization of the staining was done with a
5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution.

Digital images were captured from four quadrants of each well using a charge-coupled device video camera (HC-300/OL; Olympus) and color image-freezer computer software (Photograb-300Z SH-3; Fujifilm, Tokyo, Japan), and they were stored on optical disks. Quantification of the CD31-stained area was performed as described above. The average of four measurements for each well was calculated, and the data were expressed as the number of pixels per unit area of field.

**Statistical Analysis**

Categorical comparisons of the data were evaluated with the Fisher exact test. Differences in the means of continuous measurements were tested with the Student t test or the Mann–Whitney U test, as appropriate. Survival rates were calculated with the Kaplan–Meier method, and the differences were compared using the log-rank or Wilcoxon test. Significant predictors of survival in the univariate analysis were then included in a multivariate Cox regression model to identify the independent risk factors. P values < 0.05 were considered statistically significant. All statistical analyses were performed on a personal computer with the StatView statistical software package (version 5.0 for Macintosh; Abacus Concepts, Inc., Berkeley, CA).

**RESULTS**

**MIF Expression by Immunohistochemistry**

MIF immunoreactivity was observed mainly in hepatocytes in the tumor tissues, showing a predominant cytoplasmic staining (Fig. 1A). MIF expression also was visualized in stromal cells, such as infiltrating mononuclear cells and vascular endothelial cells (Fig. 1B).

**MVD by Anti-CD 34 Expression**

The staining of sinusoid-like vessels inside and around the tumors was evident with anti-CD34 antibody (Fig. 1C). In the surrounding liver tissue, anti-CD34 antibody staining was confined to vessels of the portal triad, with weak staining in a few sinusoids at the perportal area. Tumors with MVD values equal to or greater than the median and tumors with MVD values less than the median were compared. We found no relation between tumor MVD and any of the clinicopathologic factors studied. However, a significant positive correlation was found between the MVD and the MIF immunostaining score (P = 0.007) (Fig. 2).

**MIF Expression by Western Blot Analysis and Clinicopathologic Findings**

Expression of MIF was detectable in all tumor samples. The immunoreactive bands of both tumor MIF and rMIF were detected as 12.5-kilodalton (kD) pro-
teins and were considered MIF (Fig. 1D). The MIF expression values ranged from 1010 to 6080 pixels per unit area (median value, 2953 pixels per unit area). The expression of MIF in tumor tissues was compared with the clinicopathologic factors and was correlated significantly with the serum AFP level ($P = 0.049$) and with intrahepatic recurrence ($P = 0.043$) (Table 1). However, no apparent correlation was observed between MIF expression and tumor size, portal invasion, histologic grade, or the number of tumors.

**MIF and Patient Survival**

Correlations between MIF expression and disease-free survival (DFS) and overall survival (OS) are shown in Table 2. Patients who had low expression of MIF had a significantly better DFS (log-rank test, $P = 0.025$; Wilcoxon test, $P = 0.037$) compared with patients who had high expression of MIF. However, MIF expression levels had no impact on overall patient survival (log-rank test, $P = 0.259$; Wilcoxon test, $P = 0.083$). The estimated 1-year, 3-year, and 5-year DFS rates in patients who had low expression of MIF were 74.2%, 47.1%, and 35.3%, respectively, compared with 59.7%, 26.1%, and 14.0%, respectively, in patients who had high expression of MIF (Fig. 3A). In the univariate Cox proportional hazard model, portal invasion and MIF expression (high vs. low) had significant effects on DFS (Table 2). Moreover, portal invasion (hazard ratio, 2.7; $P = 0.0028$) and high expression of MIF (hazard ratio, 2.0; $P = 0.033$) became independent predictors for DFS after curative hepatic resection in the multivariate Cox regression.

**TABLE 1**

| Variable                        | High ($n = 28$) | Low ($n = 28$) | $P$ value |
|---------------------------------|----------------|---------------|-----------|
| Microvessel density (x 10^7 pixels/unit area) | 16             | 11            | 0.999     |
| Capsule formation               |                |               | 0.648     |
| Present                         | 21             | 19            |           |
| Absent                          | 7              | 9             |           |
| Capsule invasion                |                |               | 0.789     |
| Present                         | 15             | 13            |           |
| Absent                          | 13             | 15            |           |
| Portal vein invasion            |                |               | 0.412     |
| Present                         | 13             | 9             |           |
| Absent                          | 15             | 19            |           |
| HBs-Ag                          |                |               | 0.043*    |
| Present                         | 7              | 12            |           |
| Absent                          | 21             | 16            |           |
| HCV-Ab                          |                |               | 0.546     |
| Present                         | 13             | 9             |           |
| Absent                          | 10             | 12            |           |
| Unknown                         | 5              | 7             |           |
| Child class                     |                |               | 0.079     |
| Class A                         | 16             | 23            |           |
| Class B or C                    | 12             | 5             |           |
| Histologic grade (differentiation) |            |               | 0.544     |
| Well                            | 6              | 8             |           |
| Moderate or poor                | 21             | 18            |           |
| Undifferentiated                | 1              | 2             |           |
| No. of tumors                   |                |               | 0.745     |
| Solitary                        | 23             | 21            |           |
| Multiple                        | 5              | 7             |           |
| $\alpha$-Fetoprotein (< 200 ng/mL) | 14             | 22            | 0.049*    |
| $\geq$ 200 ng/mL                | 14             | 6             |           |

MIF: macrophage migration inhibitory factor; IHR: intrahepatic recurrence; HBs-Ag: hepatitis B surface antigen; HCV-Ab: hepatitis C virus antibody.

*MIF Expression and Prognosis by Subgroups of Clinicopathologic Factors*

Next, a subgroup analysis was performed to reveal the impact of MIF expression on each of the clinicopathologic factors included in this study (Table 3). Patients with hepatitis B surface antigen (HBs-Ag)-positive tumors, solitary tumors, tumor size $\geq 3$ cm, portal vein invasion, and age $< 70$ years could be stratified into significant prognostic groups in terms of DFS and OS.
according to MIF expression. Among the patients with moderately or poorly differentiated tumors, MIF became a significant prognosticator only of DFS and had no impact on OS.

**Expression of MIF on Hypoxic Treatment**

Under normoxic conditions, MIF expression in HepG2 and Huh7 cells was higher compared with MIF expression in Hep3B cells. At the time of hypoxic treatment, significantly high expression of MIF was noticed only in Hep3B cells (normoxia vs. hypoxia [mean ± SD], 3668.8 ± 615.7 pixels vs. 4668.3 ± 724.8; \( P = 0.001 \)), and MIF expression was unaffected in HepG2 and Huh7 cells. In Hep3B cells, it was found that MIF was significantly high at 6 hours and 12 hours after hypoxia (6 hours, \( P = 0.008 \); 12 hours, \( P = 0.005 \)) (Fig. 4), but it was not significant at 18 hours or 24 hours after hypoxia (data not shown).

**Induction of In Vitro Angiogenesis by rMIF**

We administrated rMIF in a coculture with HUVECs and fibroblasts to examine whether rMIF could induce angiogenesis directly in an in vitro model. VEGF induced extensive endothelial tube formation (positive control), and the effect of VEGF was blocked by supplementation of suramin treatment (negative control). Tube formation scores were significantly higher in the MIF group compared with the control group (MIF group vs. control group [mean ± SD]: 66398.0 ± 11241.7 pixels per unit area vs. 22540.0 ± 7510.3 pixels per unit area; \( P = 0.005 \)) (Fig. 5A–E). However, angiogenesis induced by rMIF at the used doses in this
The study did not differ significantly from that induced by VEGF (MIF group vs. VEGF group [mean ± SD]: 66,398.0 ± 11,241.8 pixels per unit area vs. 83,467.6 ± 22,810.1 pixels per unit area; \( P = 0.310 \)). In addition, we could not find any dose-dependent effect of rMIF in this model of in vitro angiogenesis.

**DISCUSSION**

HCC remains a generally lethal disease that afflicts an estimated 1 million patients annually worldwide. When patients present with advanced-stage HCC, their survival may be calculated barely beyond 2 or 3 years, even after definitive therapy, due to frequent disease recurrences.\(^{24}\) To date, only a few genes have been identified that serve as useful prognostic markers for HCC. The results of the current study suggest that MIF may represent such a prognostic marker, in that it is expressed frequently in aggressive HCC tumors. Patients with high MIF-expressing tumors had a worse DFS; furthermore, among the patients with high-risk factors for tumor recurrence, including portal vein invasion, large tumor size, and HBs-Ag-positive tumors, MIF became a significant prognostic marker for both OS and DFS. Similarly, MIF has been reported to be a marker of biologically aggressive tumors and a worse prognostic marker in several other carcinoma types, including prostate, lung, breast, and colorectal carcinomas.\(^{15,17,19,25}\) An emerging body of evidence indicates that the tumor-progressive effect of MIF may be attributable to its action in inducing tumor-associated angiogenesis, immunomodulation, and alterations in the tumor suppressive pathway.\(^{19-21,26-28}\)

It has been well established that a vicious cycle of inflammation and healing may promote tumor formation. From this perspective, the role of MIF in HCC formation and progression deserves special attention, because most HCC tumors develop in a milieu of virus-induced hepatitis, resulting in healing in the form of cirrhosis. Originally, Akbar et al.\(^{18}\) reported a gradual increase in serum MIF levels in patients with hepatitis, cirrhosis, and HCC. We extended this finding by showing that MIF may play a critical role in inducing angiogenesis and metastasis, and MIF may be a marker of a worse prognostic in patients with HCC. The current findings are supported in a very recent report by Ren et al.,\(^{29}\) who showed that rMIF and supernatants of HCC cell lines enhanced the invasion and migration of HCC cells in an in vitro cell migration assay. In addition, in prostatic adenocarcinoma, expression of MIF mRNA in tumor cells reportedly increased during tumor progression and metastasis.\(^{15}\) Takahashi et al.\(^{16}\) showed that several growth factors induced MIF expression in colon cells, and transfection with an antisense MIF plasmid resulted in significant suppression of cell proliferation. Taken together, it appears that MIF acts as an autocrine downstream regulator of growth factor-dependent tumor cell proliferation, and this may contribute to tumor progression. However, Chesney et al.\(^{23}\) proposed a different hypothesis: They showed that the growth-inhibitory effect of MIF-specific antibody in a syngenic mouse lymphoma model was due to the inhibition of endothelial cell growth rather than tumor cell growth. Therefore, it is likely that, in addition to tumor cell proliferation, MIF enhances angiogenesis during tumorigenesis.

Angiogenesis is the most commonly recognized cause of tumor growth and metastases.\(^{30}\) In nearly all
solid tumors, it has been shown that hypervascular tumors grow rapidly, recur frequently, and indicate a worse prognosis. HCC is one of the highly vascular tumors that indicates a worse prognosis. In experimental settings, it has been shown that MIF in human melanoma cells stimulates incessant growth and invasion of melanoma concomitant with neovascularization. To the best of our knowledge, ours is the first study of its kind in HCC in which we showed a significant correlation between MIF expression and MVD. Similarly, Bando et al. demonstrated that cytoplasmic MIF staining was correlated significantly with MVD in human breast carcinoma.

Hypoxia in a rapidly growing tumor triggers transcription of an array of genes, including hypoxia-inducible factor, VEGF, and MIF. Including the current findings, several other reports have shown that tumor cells cultured under hypoxic or hypoglycemic conditions release large amounts of MIF into the culture media. Bacher et al. demonstrated that the up-regulation of MIF expression during hypoxic and hypoglycemic stress may play a critical role in neovascularization of glial tumors. It is possible that MIF secreted from tumor cells may work as a paracrine growth factor to initiate proliferation of the vascular endothelial cells and, thus, trigger tumor angiogenesis. In support of this concept, we observed that exogenous MIF at various concentrations stimulated HUVECs and promoted endothelial tube formation. Similar findings were reported in several other studies using other tumor cell lines, including melanoma and lymphoma. Although the exact mechanism of MIF action in inducing tumor angiogenesis remains undetermined, very recently, Ren et al. found that rMIF augments VEGF and interleukin 8 expression in HCC cell lines and, thus, promotes angiogenesis. We concur with those authors, in that we previously showed in our series of patients with HCC that VEGF played a significant role in early tumor angiogenesis. The phosphorylation and activation of the extracellular signal-regulating protein kinase subfamily of the mitogen-activated protein (MAP) kinases, which have been shown to be a prime step in tumor neovascularization.

### TABLE 3
Univariate Analysis by Subgroups According to Prognostic Variables in Patients with Hepatocellular Carcinoma as Evaluated by Disease-Free Survival and Overall Survival

| Variable                                | No. with high/low MIF expression | Disease-free survival | Overall survival |
|-----------------------------------------|----------------------------------|-----------------------|------------------|
|                                         |                                  | 5 yrs (%) | P value | 5 yrs (%) | P value |
| Age < 70 yrs                            | 24/22                            | 16.7/38.0 | 0.025*  | 36.1/56.9 | 0.049*  |
| HBs-Ag present                         | 7/12                             | 0.0/48.5  | 0.001*  | 0.0/72.9  | 0.001*  |
| Solitary tumors                         | 23/21                            | 6.9/38.6  | 0.005*  | 35.1/58.1 | 0.018*  |
| Tumor size ≥ 3 cm                      | 18/17                            | 9.0/28.2  | 0.048*  | 28.2/52.1 | 0.018*  |
| Moderate or poor histologic grade      |                                  |           |         |           |         |
| (differentiation)                      |                                  |           |         |           |         |
| PV invasion present                    | 13/9                             | 7.2/20.8  | 0.073   | 35.4/58.3 | 0.029*  |

MIF: macrophage migration inhibitory factor; HBs-Ag: hepatitis B surface antigen; PV: portal vein.

*P value < 0.05 (log-rank test).

**FIGURE 4.** Changes in macrophage migration inhibitory factor (MIF) expression levels are shown under hypoxic and normoxic conditions in hepatocellular carcinoma cell lines. MIF expression levels in Hep3B cells at 6 hours and 12 hours after hypoxic treatment were significantly higher compared with the levels in normoxic cells (6 hours, P = 0.008; 12 hours, P = 0.005).**
sor gene activity, and this may contribute further to enhanced angiogenesis by inducing VEGF expression.27

It is interesting to note that tumor-infiltrating macrophages and endothelial cells may serve as vital sources of MIF and may contribute to tumor angiogenesis. In our samples, large numbers of infiltrating mononuclear cells around the tumors and endothelial cells of tumor sinusoids expressed variable amounts of MIF. The interaction between host macrophages and tumor cells in nonsmall cell lung carcinoma increased angiogenic potential synergistically and was related to an increased release of angiogenic CXC chemokines.39 MIF expression in nonsmall cell lung carcinoma was associated closely with tumor CXC chemokines, vessel density, and recurrence after patients underwent resection for lung carcinoma.40,41

The tumorigenic effect of MIF may be attributable in part to its immune-suppressive effect. Tumors mimic immune-privileged areas largely due to the counterattack of immune-competent cells by Fas ligand-expressing tumor cells. Apte et al.28 reported that MIF secreted by corneal endothelial cells protects these cells from natural killer (NK) cells by inhibiting the release of perforin granules by NK cells. It also has been reported that MIF inhibits circulating T-lymphocyte activity by means of decreased interferon γ secretion and interleukin 2 receptor affinity.42 Others have reported that human uveal melanoma cells secrete MIF to protect themselves from NK cell-mediated kill-
ing.28,43 Taken together, these findings suggest that an additional mechanism of tumor evasion of the host-immune response may exist that is mediated by MIF and leads to a decrease in CD8-positive T-cell survival and lysis by NK cells. Further studies should be designed to address whether MIF protects HCC tumors from host-immune attack in a similar manner.

The results of the current study demonstrated that patients with HCC who had high expression levels of MIF had a poor prognosis, and MIF became an independent predictor of DFS. Moreover, the results showed that MIF expression in HCC cells was triggered by transient hypoxia, and rMIF stimulated vascular endothelial cells to form endothelial tubules in an in vitro experiment. MIF expression may play a role in HCC progression, invasion, and angiogenesis and, as such, may be a therapeutic target in patients with HCC.

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