Macrophage migration inhibitory factor (MIF) is a 12-kDa trimer protein consisting of 6 alpha helices and 3 beta sheets, which form a solvent channel through which negatively charged molecules may enter. MIF is expressed by T cells, macrophages, monocytes, and endothelial cells. MIF plays a prominent role in innate immunity and has been implicated in several inflammatory diseases including rheumatoid arthritis and pancreatitis as well as atherosclerosis. Strong MIF expression in macrophages has been shown to initiate and facilitate the atherogenesis process, and MIF also affects ischemia-reperfusion injury in the heart. However, the role of MIF in stroke has not been reported.

Stroke is a debilitating disease that can cause severe neurological and motor deficits, resulting in long-term care and contributing significantly to health care costs. Neuronal death after an ischemic insult has been attributed to a variety of molecular processes. Studies showed that mice deficient in T lymphocytes have reduced tissue damage after experimental stroke, and MIF is able to cause the arrest of circulating T cells and monocytes. MIF expression was shown to be hypoxia-inducible. Hypoxia is the major consequence of stroke. Although the effect of hypoxia on tissue viability has not been resolved, we showed that hypoxia facilitates Alzheimer disease pathogenesis.

In this report we investigated MIF gene expression in human stroke patients and stroke model rats. Our data demonstrate that MIF is upregulated under stroke, and hypoxia facilitates the transcription of MIF gene.

Materials and Methods

Human Subjects

The criteria for patient selection were followed according to the Fourth Chinese National Meeting for Cerebrovascular Disease (1996). Incidence of stroke was confirmed by head CT scan or MRI. The study included 102 patients (61 males and 41 females with an average age of 68.61±9.64) and 57 controls (32 males and 25 females with an average age of 68.12±10.84; P>0.05). There was also no significant difference in subjects having a history of hypertension, diabetes mellitus, drinking, and smoking between the two groups (P>0.05). The stroke patients had significantly higher levels of blood pressure, triglycerides, total cholesterol, and C-reactive protein than control subjects (P<0.05).

Animals, Western Blotting, ELISA, and qRT-PCR

All animal procedures were in accordance with The Hospital Animal Use and UBC Animal Care and Use Committee guidelines. Twelve-week-old male and female Wistar rats were subjected to the middle cerebral artery occlusion (MCAO). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus according to the manufacturer’s instructions. RNA was isolated using TRIzol reagent (Invitrogen), and qRT-PCR was performed as previously described. The human MIF gene specific primers were 5′-
MIF Is Significantly Increased in PBMCs After Acute Stroke

The MIF protein level was significantly higher in the patients (61.65±57.35 ng/mL) than in the controls (31.65±28.23 ng/mL, P<0.05; Figure 1A). The MIF mRNA level was also significantly higher in the patients than in the controls (169.57±11.03%; P<0.05; Figure 1B). To examine the MIF expression pattern after stroke, patients were divided into 3 groups based on stroke severity according to the NIHSS score: mild, <4; medium, 4 to 15; and severe, >15. Plasma MIF levels were highest in the severe group, 98.06±20.39 ng/mL; medium group, 45.87±17.34 ng/mL; and control (Figure 1C). MIF mRNA levels in PBMCs were also significantly increased in the early phase, 52.61±25.24 ng/mL, compared to 31.65±28.23 ng/mL in the control (Figure 1C). MIF mRNA levels in PBMCs were also increased in stroke patients, 126.09±9.56% (P<0.05), 178.26±11.42% (P<0.05), and 247.82±10.83% (P<0.05) in the mild, medium, and severe groups, respectively (Figure 1D).

We grouped the patients into the early phase (within 3 days) and the late phase (10 to 14 days) postischemic groups. The MIF protein (Figure 1E) and mRNA (Figure 1F) levels were significantly increased in the early phase, 52.61±25.24 ng/mL and 182.61±12.68%, respectively (P<0.001 relative to control). In contrast, there was no significant difference in the late phase, 39.18±17.34 ng/mL and 121.74±11.23%, respectively (P>0.05 relative to control). Our data demonstrate that MIF
gene expression was upregulated after stroke. MIF upregulation occurred during the acute phase, but decreased to normal levels during later stages of stroke, and the increase in blood MIF level correlates with the severity of ischemic damage to the brain.

MIF Expression Is Increased in the Brain of Rats After Focal Ischemia

To determine whether MIF is increased in the brain, we used an MCAO model of focal ischemia in rats. A modified neurological deficit score mNSS was used to assess stroke severity.15 The mNSS score was highest at 12 hours (14.30±1.22) after MCAO and gradually decreased over time (Figure 2A). MIF mRNA levels were significantly increased in the 4-, 12-, 24-hour, and 3-day ischemic groups with the highest increase at 12 hours (354.55±8.53%, P<0.01), and no significant increase in the 7- and 14-day groups (P>0.05) (Figure 2B). MIF protein levels were significantly increased in the 4-, 12-, 24-hour, and 3-day groups to 208.00±3.82%, 290.67±9.66%, 380.00±10.65%, and 308.00±9.80%, respectively, but not in the corresponding areas of control rat brains (Figure 2C and 2D, P<0.05). No significant increase was observed in the 7- and 14-day groups (P>0.05). These results suggest that MIF is increased in the brain after focal ischemia in rats, and the increase in MIF expression is associated with the mNSS score.

Hypoxia Facilitates MIF Gene Expression

The major consequence of stroke is hypoxia. To examine whether upregulation of MIF gene expression after stroke is...
attributable to hypoxia, we constructed a human MIF promoter plasmid pHIF-Luc containing 2672 bp of the 5′ flanking region of the human MIF gene (Figure 3A and 3B). pHIF-Luc displayed robust luciferase activity (14.5±0.76-fold higher than the control, P<0.001; Figure 3C). Sequence analysis revealed that the promoter contains 3 hypoxia response elements (HRE) at −600, −994, and −2564 bp (Figure 3D). To determine whether these HREs regulates MIF gene expression under stroke, pHIF-Luc was transfected into HEK293 cells and subjected to hypoxia treatment. Hypoxia markedly increased pHIF-Luc luciferase activity by 7.12±0.20-fold (P<0.0001; Figure 3E). Hypoxia also significantly increased the activity of the positive control pEpoE-Luc (P<0.0001), and had no effect on the vector control (P>0.05). Deletion of HRE sites in pHIFΔH-Luc abolished the effect of hypoxia on the MIF promoter activity (P>0.05; Figure 3E). These results indicate that the human MIF promoter contains functional hypoxia inducible enhancer elements. To determine whether the increase in MIF gene expression was attributable to hypoxia-induced HIF-1α overexpression, HIF-1α plasmid was cotransfected with promoter plasmids into cells. HIF-1α overexpression significantly increased pHIF-Luc luciferase activity by 8.07±0.20-fold (P<0.0001), and had no effect on HRE-deficient plasmid pHIFΔH-Luc or the vector control (P>0.05; Figure 3F). Furthermore, siRNA was used to knockdown the expression of HIF-1α, the mediator of hypoxia signaling pathway (Figure 3G). HIF-1α knockdown significantly reduced pHIF-Luc and pEpoE-Luc promoter activities to 19.54±0.37% and 29.59±0.55% of control under hypoxia (P<0.0001) but had no effect on control (P>0.05; Figure 3H). These results show that hypoxia facilitates the human MIF gene expression by transcriptional upregulation through HREs in its promoter.

**Discussion**

It is becoming increasingly clear that inflammation and innate immune responses play an important part in the extent of cell death after acute ischemic stroke. Our data suggest an interaction between MIF expression at or near the site of infarct and recruitment and infiltration of circulating T lymphocytes. Under stroke conditions, MIF may promote monocyte recruitment and infiltration as well as T lymphocyte infiltration. In summary, we have shown that MIF expression is increased at the transcriptional level in human stroke patients and in animal model of focal ischemia. The transcriptional upregulation of MIF results in an increase in MIF gene expression in the plasma, PBMCs, and cerebral cortex after stroke, and such upregulation is mediated by hypoxia signaling pathway through the effect of the HIF-1α transcription factor on HREs in the MIF gene promoter. MIF levels may serve as a biomarker for stroke. Future studies will determine the role of MIF in neuronal dysfunction after stroke and its potential use for diagnosis of and prognosis after stroke as well as in pharmaceutical development.

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**Disclosures**

None.

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