Evidence for a Role of Macrophage Migration Inhibitory Factor in Vascular Disease

Zhiping Chen, Masashi Sakuma, Alexandre C. Zago, Xiaobin Zhang, Can Shi, Lin Leng, Yuka Mizue, Richard Bucala, Daniel I. Simon

Objective—Inflammation plays an essential role in atherosclerosis and restenosis. Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that is widely expressed in vascular cells. However, there is no in vivo evidence that MIF participates directly in vascular injury and repair. Therefore, we investigated the effect of MIF blockade on the response to experimental angioplasty in atherosclerosis-susceptible mice.

Methods and Results—Carotid artery dilation (2.5 atm) and complete endothelial denudation were performed in male C57BL/6J LDL receptor-deficient mice treated with a neutralizing anti-MIF or isotype control monoclonal antibody. After 7 days and 28 days, intimal and medial sizes were measured and intima/media area ratio (I/M) was calculated. Intimal thickening and I/M were reduced significantly by anti-MIF compared with control antibody. Vascular injury was accompanied by progressive vessel enlargement or “positive remodeling” that was comparable in both treatment groups. MIF blockade was associated with reduced inflammation and cellular proliferation and increased apoptosis after injury.

Conclusion—Neutralizing MIF bioactivity after experimental angioplasty in atherosclerosis-susceptible mice reduces vascular inflammation, cellular proliferation, and neointimal thickening. Although the molecular mechanisms responsible for these effects are not yet established, these data prompt further research directed at understanding the role of MIF in vascular disease and suggest novel therapeutic interventions for preventing atherosclerosis and restenosis.

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Key Words: inflammation • cytokine • macrophage • atherosclerosis • restenosis

Atherosclerosis is increasingly regarded as a chronic inflammatory process that develops as a result of metabolic, physical, and environmental injury to the vasculature. The realization that atherosclerosis is an inflammatory “response to injury” involving various immune cells, particularly macrophages and T lymphocytes, was originally derived from studies of animal models. Early after the initiation of an atherogenic diet in animals, monocytes adhere to the vascular endothelium and accumulate in lesion-prone arterial sites. Adherent monocytes invade the arterial intima and differentiate into macrophages with the help of such modulators of inflammation as monocyte chemotactic protein-1 (MCP-1) and macrophage colony-stimulating factor, which are produced locally by smooth muscle cells (SMC). Resident macrophages then accumulate lipid to become foam cells and perpetuate the local inflammatory response. The adherence of monocytes to the endothelium and their entry into the intima are the earliest events of atherosclerotic lesion development. Selective absence of MCP-1 or its receptor, CCR2, decreases monocyte accumulation and lesion formation in murine models of atherosclerosis.

Vascular inflammation in atherosclerosis is mediated and propagated by cytokines, including IL-1β, macrophage colony-stimulating factor, and interferon-γ (INF-γ). A direct role for cytokines in athrogenesis has been defined in atherosclerotic-susceptible mice. For example, apolipoprotein E knockout mice that are deficient in either INF-γ receptors or cytokine had substantial reductions in atherosclerotic lesion size. Conversely, injection of INF-γ or the INF-γ-releasing factors, IL-12 and IL-18, enhances the extent of disease in apolipoprotein E-deficient mice.

Although macrophage migration inhibitory factor (MIF) was perhaps the first cytokine activity discovered, MIF has an undefined role in atherosclerosis. MIF is an important proinflammatory cytokine regulator of host inflammatory and immune responses. MIF modulates the activation of monocyte/macrophages and T lymphocytes, and experimental animal studies have established MIF to play a critical role in the host response to septic shock and in inflammatory pathologies responsible for arthritis, glomerulonephritis, and acute lung injury.

The expression and distribution of MIF have been investigated in rabbit and human atherosclerotic lesions and abdominal aortic aneurysms. MIF protein is detected in, and locally produced by, endothelial cells, vascular SMCs, T
lymphocytes, and macrophages, raising the possibility that MIF could play an important role during the progression of atherosclerosis. However, there is no in vivo evidence that MIF participates directly in vascular injury and repair. To begin to elucidate a role for MIF in atherosclerosis and restenosis, we investigated the effect of neutralizing MIF bioactivity on the response to experimental angioplasty in atherosclerosis-susceptible, LDL receptor-deficient (LDLR−/−) mice.

Methods

Carotid Injury

Male LDLR−/− C57BL/6j mice, maintained on a high-fat diet (20.1%) containing 1.25% cholesterol (Purina Mills, Richmond, Ind) for 12 weeks after weaning, underwent unilateral carotid artery dilation (2.5 atm) and complete endothelial denudation, as previously described. Briefly, animals were anesthetized on day 0 using ketamine (80 mg/kg intraperitoneally; Fort Dodge Laboratories) and xylazine (5 mg/kg intraperitoneally). All surgery was performed using sterile techniques with the aid of a dissecting microscope. To achieve complete endothelial denudation and controlled arterial bleeding or infection. Animal care and procedures were approved. All animals survived until the time of planned sacrifice for euthanasia.

Carotid arteries were embedded, and 2 cross-sections cut 1 mm apart were stained with hematoxylin and eosin and Verhoeff tissue elastin stain. A histologist blinded to the animal genotype measured the lumen, intimal, and medial areas of each cross-sectional plane using a microscope equipped with a CCD camera interfaced to a computer running NIH Image v1.60 software. Results for the 2 planes of each artery were averaged. For immunohistochemistry, standard avidin-biotin procedures for mouse CD45 (leukocyte common antigen; PharMingen, San Diego, Calif), mouse neutrophil-specific marker (mAb 7/4; Serotec, Indianapolis, Ind), mouse macrophage-specific marker Mac-3 (mAb M38/4; PharMingen), MIF (3.D.9), BrdU (DAKO, Carpinteria, Calif), and SMC α-actin (DAKO) were used. Apoptotic cells were detected by the TUNEL method using Apo Tag (Intergen, Purchase, NY). Immunostained sections were quantified as the number of immunostained positive cells divided by total number of nuclei.

Proliferation Studies

Low-passage rat vascular SMC (kindly provided by Dr Mark A. Perrella, Brigham and Women’s Hospital) were cultured until semi-confluent in 24-well plates containing DMEM and 10% heat-inactivated fetal bovine serum. The cells then were synchronized by overnight culture in 0.5% serum-containing media (DMEM). The medium was replaced with 10% serum-containing DMEM, PDGF (20 ng/mL; R&D Systems, Minneapolis, Minn), or 10% serum-containing DMEM together with a neutralizing anti-murine MIF or isotype control mAb. Reversal of the anti-MIF effect was examined by adding purified mouse recombinant MIF (rMIF) (250 ng/mL), as previously reported. The anti-MIF mAb 3.D.9 does not recognize mouse MIF that is present in trace quantities in fetal bovine serum. Cellular growth and proliferation were assessed using a colorimetric assay based on mitochondrial dehydrogenase cleavage of WST-1 reagent (Roche Molecular Biochemicals, Indianapolis, Ind) according to the manufacturer’s protocol. WST-1 solution was added to cells (1:10 vol:vol) and incubated in 5% CO2 at 37°C for 1 hour. An aliquot (100 μL) was removed to measure optical density (OD) at 450 nm.

MIF ELISA

Cell supernatants were assayed directly for MIF content by a sandwich ELISA using a rabbit polyclonal antiserum and a mouse monoclonal antibody raised to purified mouse recombinant MIF, as previously described.

Data Analysis

All data are presented as mean±SD. Comparisons between groups used a nonpaired t test; P<0.05 were considered significant.

Results

Neutralizing MIF Antibody Decreases Neointimal Thickening and Cellular Proliferation After Carotid Injury

To determine whether MIF bioactivity is causally related to neointimal formation, we performed carotid artery injury (ie,
Intimal and medial thickening were accompanied by progressive vessel enlargement (ie, “positive remodeling”), as determined by external elastic lamina area measurements over time, which was comparable in control antibody-treated and anti-MIF antibody-treated mice.

Because increasing evidence suggests that MIF plays an important role in regulating cellular proliferation,\textsuperscript{29,30} we assessed cellular proliferation by quantifying incorporation of BrdU. Substantial proliferation was observed 7 days after injury in control vessels (24.0% of medial cells), and proliferation was still evident at 28 days (5.9% of intimal cells). Neutralization of MIF bioactivity reduced medial proliferation at 7 days by 58% ($P=0.048$) and intimal proliferation at 28 days by 56% ($P=0.046$) (Table, Figure 2).

We went on to examine the role of MIF in the proliferation of vascular SMC in vitro. MIF protein exists preformed in several cell types, and previous studies have indicated that endogenously released MIF can act to stimulate cellular responses in an autocrine fashion.\textsuperscript{28,29,31} Serum supplementation is known to induce proliferation of quiescent cell populations\textsuperscript{32} and, in agreement with a previous study,\textsuperscript{28} we observed that the addition of serum or PDGF to SMC caused the release of immunoreactive MIF into culture supernatants in as little as 60 minutes (Figure 3a). The addition of neutralizing anti-MIF antibody inhibited the proliferative effect of serum to quiescent SMC by 83% ($P<0.01$) (Figure 3b), indicating that the release of endogenous MIF contributes significantly to the mitogenic effect of serum stimulation. The specificity of this effect was confirmed by the simultaneous addition of rMIF, which largely reversed the inhibitory effect of anti-MIF mAb.

**Neutralization of MIF Activity and Leukocyte Recruitment**

We have previously reported that mechanical carotid artery dilation and complete endothelial denudation is accompanied by prominent vascular inflammation.\textsuperscript{21,22} Altered leukocyte accumulation within vessels was observed in anti-MIF antibody-treated mice. Inflammatory cells (CD45-positive) invading the intima at 7 days were reduced by 27% from 49.7% ($\pm$1.6%) to 33.1% ($\pm$8.3%) ($P=0.01$) in anti-MIF–treated compared with control antibody-treated mice.

**Neutralization of MIF Activity and Apoptosis**

Because MIF provides a cell survival signal,\textsuperscript{33} we investigated the effects of neutralizing MIF bioactivity on apoptosis after injury. Anti-MIF antibody significantly increased the number of apoptotic (TUNEL-positive) cells in the intima (by 204\%, $P=0.021$) and media (179\%, $P=0.035$) at 7 days compared with control antibody (Table).

**Discussion**

Our study provides in vivo evidence that neutralizing MIF bioactivity reduces vascular inflammation, cellular proliferation, and neointimal thickening after experimental angioplasty in atherosclerosis-susceptible mice. These results establish a role for MIF in modulating the biological response to vascular injury.

Monocyte-mediated inflammation drives neointimal formation in atherosclerosis and restenosis. Monocytes are essential in atherogenesis as evidenced by the fact that selective absence of MCP-1\textsuperscript{3} or its receptor, CCR2,\textsuperscript{3} de-

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### Quantitative Morphometry and Immunohistochemistry

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Anti-MIF</th>
<th>$P$ Value</th>
</tr>
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<tr>
<td>Intimal area (mm$^2$)</td>
<td></td>
<td></td>
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<tr>
<td>7 d</td>
<td>$0.0057 \pm 0.0030$</td>
<td>$0.0055 \pm 0.004$</td>
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<td>$0.039 \pm 0.022$</td>
<td>$0.018 \pm 0.018$</td>
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<tr>
<td>Media area (mm$^2$)</td>
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<td></td>
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<tr>
<td>28 d</td>
<td>$0.065 \pm 0.020$</td>
<td>$0.058 \pm 0.021$</td>
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<td>Intima/Media, 28 d</td>
<td>$0.57 \pm 0.23$</td>
<td>$0.30 \pm 0.26$</td>
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<td>External elastic lamina</td>
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<td>7 d</td>
<td>$0.102 \pm 0.014$</td>
<td>$0.096 \pm 0.051$</td>
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<tr>
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<td>$0.200 \pm 0.086$</td>
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<td>BrdU-positive cells (%)</td>
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<td>$10.7 \pm 6.3$</td>
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<td>$2.6 \pm 1.1$</td>
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<tr>
<td>CD45-positive cells (%)</td>
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<td>$36.1 \pm 5.3$</td>
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<tr>
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<td>TUNEL-positive cells (%)</td>
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<td></td>
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<td>Intima, 7 d</td>
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<td>$5.7 \pm 0.9$</td>
<td>0.021</td>
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<tr>
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<td>$3.4 \pm 0.6$</td>
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creases monocyte accumulation and lesion formation in murine models of atherosclerosis. Emerging experimental and clinical data indicate that inflammatory cells may be central to neointimal growth after mechanical arterial injury. In animal models of balloon-induced and stent-induced injury, neutrophils and monocytes are recruited as a precursor to intimal thickening, and monocyte number within the vessel wall is associated with cellular proliferation and intimal thickening. Inflammation is a dominant histologic feature after endovascular stenting in human coronary arteries, with autopsy studies reporting a virtual carpet of luminal neutrophils and monocytes in the early hours and days after stenting, and the accumulation of large numbers of monocyte/macrophages in the neointima that comprise >60% of total cells at 1 month. It was previously uncertain whether the relationship between inflammation and experimental and clinical restenosis was causal or simply caused by covariation with other factors. Experimental observations by our laboratories and others now support a direct role for inflammation in the biological repair response to vascular injury. We found that antibody-mediated blockade or selective absence of Mac-1 (α5β2, CD11b/CD18) impaired diminished leukocyte accumulation and neointimal thickening after experimental angioplasty or endovascular stent implantation. Stenting in experimental models is associated with sustained elevation of MCP-1 after injury (≈14 days) compared with balloon-injured arteries (≈5 days), and antibody-mediated blockade of MCP-1 and CCR2 have reduced neointimal thickening after experimental angioplasty.

MIF is ideally positioned to play an important role during neointimal formation in atherosclerosis and restenosis. Carotid injury is associated with upregulation of medial MIF expression (Figure 1), and serial atherogenesis studies document that MIF protein is detected in, and locally produced by, endothelial cells, vascular SMCs, T lymphocytes, and macrophages. MIF regulates the activation of monocyte/macrophages and T lymphocytes and experimental animal studies have established MIF to play a critical role in the host response to septic shock and in the inflammatory pathologies responsible for arthritis, glomerulonephritis, and acute lung injury. Anti-MIF treatment was associated with suppression of monocyte recruitment, diminished production of tumor necrosis factor, IL-1, and IL-6, and reduced macrophage-mediated tissue injury in these models.

The precise mechanisms by which MIF promote neointimal formation are unknown. Immunoneutralization of MIF produces a significant inhibition of IL-1β, intercellular cell adhesion molecule-1, and vascular cell adhesion molecule-1 expression and macrophage accumulation in an experimental rat model of glomerulonephritis. We have observed that neutralizing MIF bioactivity is associated with reduced accumulation of inflammatory cells within the neointima. These antiinflammatory actions are likely to affect neointimal growth because targeting IL-1β, intercellular cell adhesion molecule-1, and vascular cell adhesion molecule-1 are all associated with reductions in neointimal thickening after vascular injury.

Increasing evidence also suggests that MIF plays an important role in regulating cellular proliferation. Both

Figure 1. MIF expression after carotid injury. Immunohistochemical staining of carotid artery before (0 days), 7 days, and 28 days after injury showing enhanced MIF staining within the media 7 days after injury (original magnification ×150). Arrow designates the internal elastic lamina.

Figure 2. Photomicrographs of mouse carotid arteries after injury. VerHoeff elastin stain 28 days after injury: (a) isotype control; (b) anti-MIF (original magnification ×38); (c) isotype control; (d) anti-MIF (×150). Neointima separates the internal elastic lamina (arrows) from the lumen. Proliferating (BrdU-positive) cells 7 days after injury: (e) isotype control; (f) anti-MIF (×150).
endogenously secreted and exogenously added MIF stimulate the proliferation of mouse fibroblasts via sustained activation of mitogen-activated protein kinase.\textsuperscript{28} Furthermore, cells deficient in MIF are defective in growth factor and integrin-induced cyclin D1 expression, Rb inactivation, and E2F-dependent transcription.\textsuperscript{44} We found that the addition of serum or PDGF to SMC caused the release of immunoreactive MIF into culture supernatants in as little as 30 minutes, and neutralizing anti-MIF antibody inhibited the proliferative effect of serum to quiescent SMC by 60%, indicating that the release of endogenous MIF contributes significantly to the mitogenic effect of serum stimulation. Indeed, neutralizing anti-MIF after carotid injury results in sustained reduction in cellular proliferation.

We also provide evidence that neutralizing MIF activity increases cellular apoptosis after vascular injury. Interestingly, MIF has been shown to protect cells from activation-induced, p53-dependent apoptosis, thereby enhancing inflammatory responses to infection,\textsuperscript{33} and stimulation of cells with MIF induces phosphorylation of Akt/protein kinase B. Because phospho-Akt functions as an anti-apoptotic protein, protecting against cell death induced by growth factor withdrawal or ischemia-reperfusion injury,\textsuperscript{45} the neutralization of MIF activity would be expected to be associated with diminished phosphorylation of Akt and increased apoptosis. We have reported previously using the same animal model that inhibition of Akt phosphorylation by simvastatin treatment is associated with increased apoptosis and reduced cellular proliferation, leukocyte accumulation, and neointimal formation after injury.\textsuperscript{22}

**Study Limitations**

To determine whether MIF bioactivity is causally related to neointimal formation, we performed carotid artery injury in mice treated with neutralizing anti-MIF monoclonal antibody. It is likely that neutralization of MIF bioactivity is incomplete, resulting in possible underestimation of the true role of MIF in the biological response to vascular injury. Accordingly, experiments are planned in mice genetically deficient in MIF. Also, we performed arterial injury in atherosclerosis-susceptible mice fed a high-fat diet. Our experimental design does not allow us to identify whether LDL receptor-deficiency, the high-fat diet itself, or the combination contributed to the enhanced leukocyte accumulation, cellular proliferation, and neointimal thickening observed in this study compared with our previous report in wild-type mice fed a normal chow diet.\textsuperscript{21} Finally, neointimal formation in this murine injury model may differ significantly from human atherosclerosis and restenosis.

**Conclusion**

Our results support the hypothesis that neutralization of MIF activity has antiinflammatory, antiproliferative, and pro-apoptotic actions relevant to attenuating neointimal formation. Although there is emerging evidence that mural thrombus formation plays an important role in neointimal thickening in atherogenesis\textsuperscript{46} and restenosis,\textsuperscript{47} the role of MIF in thrombosis is undefined. Further research may lead to new understanding of the actions of MIF and novel therapies for preventing atherosclerosis and restenosis.

**Acknowledgments**

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**References**


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