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

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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. **(*Arterioscler Thromb Vasc Biol*. 2004;24:709-714.)**

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.[1] 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.[1] 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 chemoattractant 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[2] or its receptor, CCR2,[3] decreases monocyte accumulation and lesion formation in murine models of atherosclerosis.

Vascular inflammation in atherosclerosis is mediated and propagated by cytokines, including IL-1β,[4] macrophage colony-stimulating factor,[5] and interferon-γ (INF-γ).[6] A direct role for cytokines in atherogenesis has been defined in atherosclerotic-susceptible mice. For example, apolipoprotein E knockout mice that are deficient in either INF-γ receptors[7] or cytokine[8] had substantial reductions in atherosclerotic lesion size. Conversely, injection of INF-γ[9] or the INF-γ–releasing factors, IL-12[10] and IL-18,[11] enhances the extent of disease in apolipoprotein E-deficient mice.

Although macrophage migration inhibitory factor (MIF) was perhaps the first cytokine activity discovered,[12] 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[13] and in inflammatory pathologies responsible for arthritis,[14] glomerulonephritis,[15] and acute lung injury.[16]

The expression and distribution of MIF have been investigated in rabbit and human atherosclerotic lesions[17–19] and abdominal aortic aneurysms.[19] MIF protein is detected in, and locally produced by, endothelial cells, vascular SMCs, T

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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 stretching, we modified the air-drying model of Fishman et al.[20] Application of this model to wild-type and atherosclerotic-susceptible mice has been previously reported by our group.[21,22] Briefly, the right carotid artery was surgically exposed and isolated from the surrounding tissues. The proximal common and internal carotid arteries were occluded with microvascular clamps, and the external carotid artery identified and ligated with a 6-0 silk suture. An incision was made in the external carotid proximal to the ligature, and a 30-gauge needle connected with polyethylene tubing (PE-10; Becton Dickinson & Co) to a saline-filled syringe was introduced. After gently irrigating the isolated common carotid segment with saline to remove blood, the 30-gauge needle was secured within the external carotid by placing a ligature around the external carotid artery proximal to the needle insertion site. The syringe was replaced with an angioplasty inflation device (Advanced Cardiovascular Systems/Guidant, Inc) and the saline-filled isolated common carotid segment dilated with 2.5 atm of pressure for 30 seconds. The inflation device was then replaced with an air-filled 60-mL syringe, and a 30-gauge air exit hole was made at the proximal end of the common carotid. Endothelial denudation was performed by air-drying the carotid for 3 minutes (20 mL/min). After air-drying, the artery was refilled with saline, the needle removed, the external carotid ligated proximal to the needle insertion site, and the clamps removed to re-establish normal anterograde flow. A cotton-tip applicator was applied to the air exit hole in the common carotid to tamponade bleeding, the wound irrigated with saline, the incision closed with running 4-0 silk suture, and topical antibiotic ointment applied. All animals survived until the time of planned sacrifice without bleeding or infection. Animal care and procedures were reviewed and approved by Harvard Medical School Standing Committee on Animals and performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the National Institutes of Health.

Antibody Treatment

Treatments were via intraperitoneal injection of 500 μg monoclonal antibody 1 day before injury and 2 days, 5 days, 8 days, and 11 days after injury. $LDLR^{-/-}$ mice were divided into 2 treatment groups: (1) mouse monoclonal anti-MIF IgG_1 (3.D.9) and (2) mouse monoclonal IgG_1 isotype control (HB49). Monoclonal antibody 3.D.9 was generated in our laboratory and has been previously shown to neutralize murine MIF bioactivity in vitro and in vivo.[23–27]

Lipid Analysis

Blood was collected via retro-orbital puncture into heparin-coated capillary tubes. Plasma cholesterol and triglyceride measurements were performed using commercially available kits (Pointe Scientific, Lincoln Park, Mich).

Tissue Harvesting and Analysis

Seven (n=5; n=5) or 28 days (n=12; n=12) after vascular injury, anesthesia was administered, the chest cavity opened, and the animals euthanized by right atrial exsanguination. A 22-gauge butterfly catheter was inserted into the left ventricle for in situ pressure perfusion at 100 mm Hg with 0.9% saline for 1 minute, followed by fixation with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.3, for 10 minutes. The right and left common carotid arteries were excised and immersed in buffered paraformaldehyde. Spleen and small intestine from 3 animals were harvested as control tissues for immunohistochemistry. All animals received BrdU, 50 mg/kg intraperitoneally, 18 hours and 1 hour before 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 M3/84; 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.[28] The anti-MIF mAb 3.D.9 does not to recognize bovine 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% CO_2 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.[28]

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,

Quantitative Morphometry and Immunohistochemistry

	Control	Anti-MIF	*P* Value
Intimal area (mm^2)			
7 d	0.0057±0.0030	0.0055±0.004	0.94
28 d	0.039±0.022	0.018±0018	0.042
Medial area (mm^2)			
28 d	0.065±0.020	0.058±0.021	0.48
I/M, 28 d	0.57±0.23	0.30±0.26	0.034
External elastic lamina (mm^2)			
7 d	0.102±0.014	0.096±0.051	0.83
28 d	0.215±0.092	0.200±0.086	0.70
BrdU-positive cells (%)			
Media, 7 d	24.0±2.8	10.7±6.3	0.048
Intima, 28 d	5.9±2.2	2.6±1.1	0.046
CD45-positive cells (%)			
Intima, 7 d	49.7±1.6	36.1±5.3	0.010
Intima, 28 d	33.1±8.3	21.2±11.2	0.21
TUNEL-positive cells (%)			
Intima, 7 d	2.8±1.0	5.7±0.9	0.021
Media, 7 d	1.9±0.4	3.4±0.6	0.035

mechanical carotid artery dilation and complete endothelial denudation) in $LDLR^{-/-}$ mice treated with neutralizing anti-MIF (3.D.9) or isotype control monoclonal antibody 24 hours before and then every 72 hours after injury. This model of carotid injury was developed in our laboratory[21] and has been useful in demonstrating that inflammatory cell recruitment and function modulate neointimal formation.[21,22] We performed vascular injury experiments in an atherosclerosis-susceptible strain, because we have observed significantly increased neointimal formation (I/M=0.64±0.37) after carotid injury in mice with LDL receptor–deficient/high-fat diet compared with mice with wild-type/normal chow diet (I/M=0.34±0.13), which is associated with enhanced vascular inflammation and cellular proliferation.[21,22] Monoclonal antibody 3.D.9 was generated in our laboratory and has been shown previously to neutralize murine MIF bioactivity in vitro and in vivo.[23–27]

In mice receiving control antibody, intimal thickening began by 7 days after injury and progressed significantly between 7 days (0.006±0.003 mm^2) and 28 days (0.039±0.022 mm^2) (Table). Immunohistochemical staining of carotid arteries showed enhanced MIF expression within the media 7 days after injury that was attenuated 28 days after injury (Figure 1). Neutralizing anti-MIF antibody reduced intimal thickening at 28 days by 54% (P=0.042) (Figure 2, Table). Anti-MIF treatment did not alter plasma cholesterol (P=0.69) or triglyceride (P=0.26) levels compared with control antibody in $LDLR^{-/-}$ mice. Medial area was unaffected by anti-MIF treatment. I/M at 28 days in control antibody-treated mice was reduced 47% by anti-MIF (P=0.034). 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,[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.[28,29,31] Serum supplementation is known to induce proliferation of quiescent cell populations[32] and, in agreement with a previous study,[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.[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%±1.6% to 33.1%±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,[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[2] or its receptor, CCR2,[3] de-

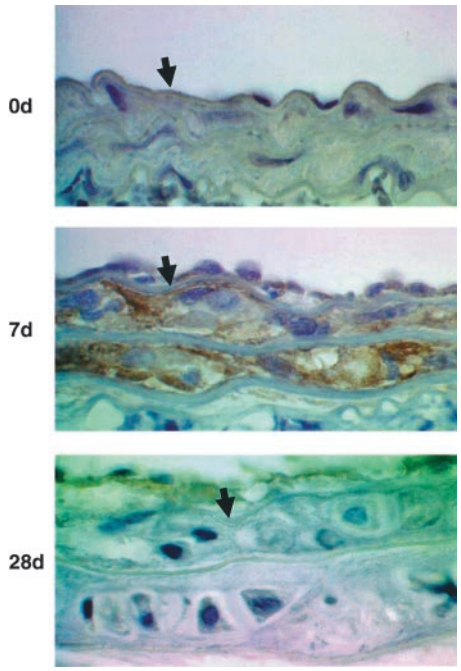

Figure 1. MIF expression after carotid injury. Immunohistochemical staining of carotid artery before (0 days), 7 days, and 28 days after injury showing enhanced MIF staining within the media 7 days after injury (original magnification ×150). Arrow designates the internal elastic lamina.

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,[34] and monocyte number within the vessel wall is associated with cellular proliferation and intimal thickening.[34] 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.[35,36] 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[37] or selective absence[21] of Mac-1 ($\alpha_M\beta_2$, 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),[38] and antibody-mediated blockade of MCP-1[39] and CCR2[40] have reduced neointimal thickening after experimental angioplasty.

MIF is ideally positioned to play an important role during neointimal formation in atherosclerosis and restenosis. Ca-

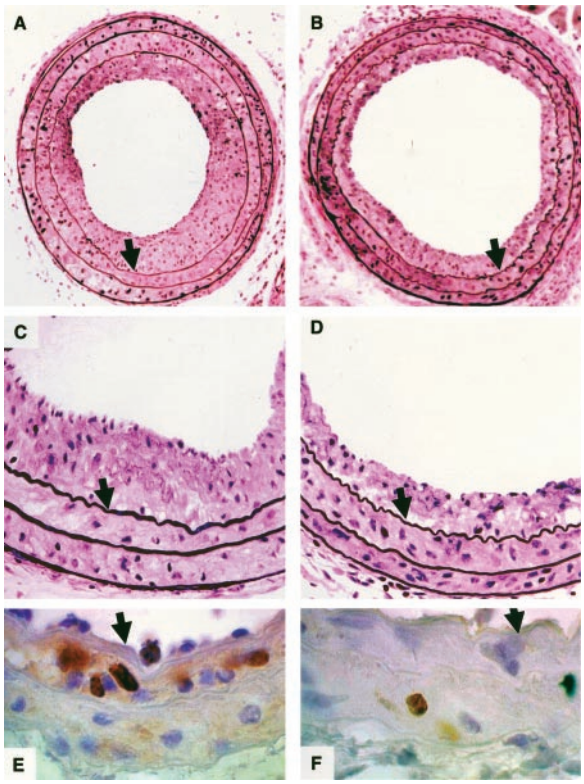

Figure 2. Photomicrographs of mouse carotid arteries after injury. VerHoeff elastin stain 28 days after injury: (a) isotype control; (b) anti-MIF (original magnification ×38); (c) isotype control; (d) anti-MIF (×150). Neointima separates the internal elastic lamina (arrows) from the lumen. Proliferating (BrdU-positive) cells 7 days after injury: (e) isotype control; (f) anti-MIF (×150).

rotid 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.[17–19] 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[13] and in the inflammatory pathologies responsible for arthritis,[14] glomerulonephritis,[15] and acute lung injury.[16] Anti-MIF treatment was associated with suppression of monocyte recruitment, diminished production of tumor necrosis factor, IL-1β, 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.[15] 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,[41] intercellular cell adhesion molecule-1,[42] and vascular cell adhesion molecule-1[43] 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.[29,30] Both

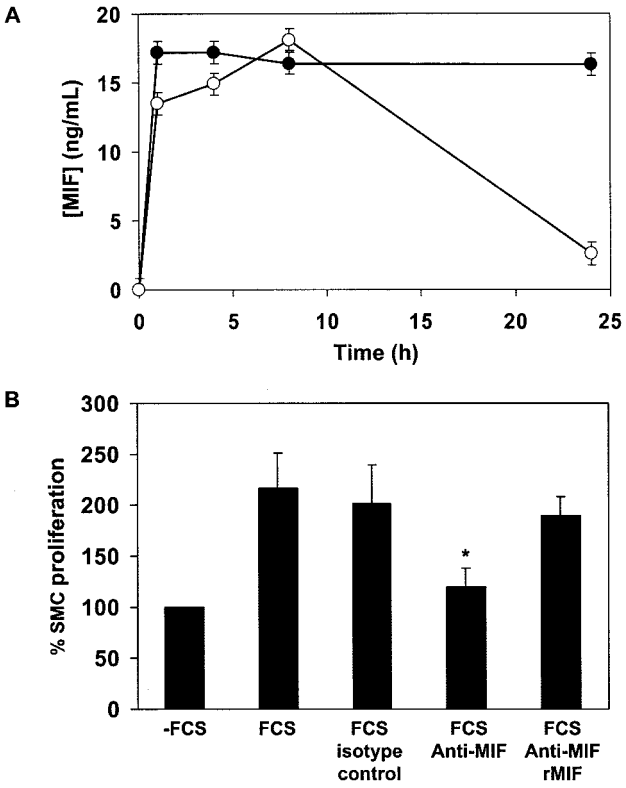

Figure 3. MIF and SMC proliferation. a, Serum and PDGF stimulates MIF secretion from vascular SMC. SMC were incubated overnight in 0.5% serum-containing medium before the addition of 10% serum-containing DMEM (black circle) or PDGF (white circle). Cell supernatants were removed at the indicated times and assayed for MIF content by ELISA. Values are mean±SD, n=3 to 4. b, Serum-stimulated MIF release is responsible in part for serum-induced SMC proliferation. DMEM supplemented with 10% serum was added to quiescent cells together with neutralizing anti-MIF or an isotype control mAb (50 μg/mL). Cellular proliferation was assessed using the WST-1 colorimetric assay, as described in Methods, and is reported as the percent increase relative to quiescent cells. The effect of anti-MIF was largely reversed by the addition rMIF (250 ng/mL). Results shown are the mean±SD of triplicate assays and are representative of 3 separate experiments. *$P<0.01$.

endogenously secreted and exogenously added MIF stimulate the proliferation of mouse fibroblasts via sustained activation of mitogen-activated protein kinase.[28] Furthermore, cells deficient in MIF are defective in growth factor and integrin-induced cyclin D1 expression, Rb inactivation, and E2F-dependent transcription.[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,[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,[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.[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.[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[46] and restenosis,[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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