Macrophage Migration Inhibitory Factor Deficiency Impairs Atherosclerosis in Low-Density Lipoprotein Receptor–Deficient Mice

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Background—Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine expressed widely by vascular cells. However, scant in vivo evidence supports direct participation of MIF in atherogenesis. Therefore, we investigated whether deficiency of MIF modulates atherosclerotic lesion formation and composition in low-density lipoprotein receptor–deficient (LDLr<-/-) mice.

Methods and Results—MIF<-/-LDLr<-/- and LDLr<-/- mice were generated and consumed an atherogenic diet for 12 or 26 weeks. MIF<-/-LDLr<-/- mice had significantly reduced abdominal aorta lipid deposition and intimal thickening from aortic arch throughout the abdominal aorta compared with LDLr<-/- mice. Marked retardation of atherosclerosis over time in MIF-deficient mice accompanied decreased lesion cell proliferation. At 26 weeks, 20% of MIF-deficient mice developed only early, fatty streak–like lesions, whereas >80% of LDLr<-/- mice developed advanced lesions containing calcification and lipid cores. Analysis of smooth muscle cells from mouse aortae demonstrated that MIF deficiency reduced smooth muscle cell proliferation, cysteine protease expression, and elastinolytic and collagenolytic activities.

Conclusions—Deficiency of MIF reduces atherogenesis in LDLr<-/- mice. These results provide novel insight into inflammatory pathways operating in atheromata and identify a new potential target for modulating atherogenesis.

Key Words: atherosclerosis • cathepsins • smooth muscle cells

Atherosclerosis is an inflammatory disease that involves substantial remodeling of arteries, mediated by proteolytic enzymes and cytokines. Experiments in genetically altered mice have demonstrated a direct role for inflammatory cytokines in atherogenesis.1

Macrophage migration inhibitory factor (MIF), one of the earliest cytokines discovered, inhibits the random migration of peritoneal macrophages.2 Recent studies demonstrated MIF expression in atherosclerotic lesions of humans3 and in animals.4 Furthermore, recent work has implicated MIF in the biological responses to mechanical arterial injury. Carotid arteries of apolipoprotein E–deficient mice injured by wire manipulations and injected with MIF monoclonal antibody showed markedly reduced neointimal macrophages, less foam cell accumulation, and increased quantity of smooth muscle cells (SMCs) and amount of collagen in the intima but no difference in intima thickening.5 In contrast, Chen et al6 observed that immunoneutralization of MIF reduced inflammation, impaired cellular proliferation, and decreased the intima thickening in carotid arteries of LDLr<-/- mice injured in another manner. Such discrepancies could result from differences in animal protocols or incomplete inhibition of tissue MIF activity by antibodies. The present study utilized MIF-deficient mice in a well-characterized mouse model of atherosclerosis to test directly the hypothesis that MIF participates in atherogenesis.

Methods

MIF<-/-LDLr<-/- Mice Generation and Research Design

MIF<+/> mice (backcrossed for 5 generations onto the C57/B16 background) were crossbred with LDLr<-/- mice (C57/B16; The Jackson Laboratory, Bar Harbor, Me) to generate MIF<-/-LDLr<-/- mice and LDLr<+/— littermates. These mice were fertile and showed...
no abnormalities in growth or water/food consumption. Starting at 6 weeks of age, male mice consumed an atherogenic diet for 12 and 26 weeks, followed by lesion characterization.

Atherosclerotic Lesion Characterization

Characterization of mouse atherosclerotic lesions was performed as previously reported. Lesion area was measured as lipid deposition using an en face preparation of abdominal aorta (oil red O staining). Longitudinal sections of aortic arches embedded in OCT were stained for lipids (oil red O), elastin (Verhoeff-van Gieson), collagen (Sirius-Red), macrophages (Mac-3), T cells (CD4), and proliferating cells (Ki67 nuclear antigen) and analyzed as described. To measure serum lipid profiles, blood samples were collected and total cholesterol, HDL, triglyceride, and LDL were determined. The nonparametric Mann-Whitney test was used for statistical analysis.

**Aortic SMC Protease Expression, Activity, and Proliferation**

SMCs were isolated from the aortae of LDLr<sup>−/−</sup> and MIF<sup>−/−</sup> LDLr<sup>−/−</sup> mice. Quiescent SMCs were used for total RNA isolation and real-time PCR analysis. Relative cathepsin and matrix metalloproteinase (MMP) mRNA transcripts were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To examine if absence of MIF impairs cathepsin activity, aortic SMCs were stimulated with interleukin-1β, interferon-γ, tumor necrosis factor-α, basic fibroblast growth factor, or vascular endothelial growth factor (all at 10 ng/mL) for 48 hours followed by cell lysis (30 μg/lane) and exposure to the active site-probe [125I]-JPM. The same cell lysates (10 μg/sample) were utilized to examine the MMP activity by gelatin zymography. For activity assays, SMCs were stimulated with cytokines in the presence of [3H]-elastin (300 μg/well) or fluorescein-conjugated collagen-I (100 μg/well) as previously described.

To assay proliferation, 2-fold serial dilutions of 4×10<sup>3</sup> SMCs were seeded into 96-well plate followed by a 24-hour incubation. Cell numbers were determined by Celltiter 96 AQueous Solution with a standard curve using the same cell types according to the manufacturer (Promega).

**Results**

Reduced Atherosclerosis in MIF-Deficient Mice

Increased MIF expression in human atheroma suggests that MIF deficiency in LDLr<sup>−/−</sup> mice may impair atherogenesis. Indeed, MIF<sup>−/−</sup> LDLr<sup>−/−</sup> mice developed significantly less atherosclerosis in aortic arches and in abdominal aortae after 12 and 26 weeks on an atherogenic diet. Lesional area evaluated as lipid deposition (oil red O % positive area) in an en face preparation of abdominal aorta showed reduced area (mm<sup>2</sup>) compared with those in control mice (10.2±5.5 versus 45.9±10.6, P<0.002; Figure 1A). Intimal lesion sizes (mm<sup>2</sup>) measured on longitudinal sections of aortic arches also demonstrated significant reduction in MIF<sup>−/−</sup> LDLr<sup>−/−</sup> mice (0.1±0.1 versus 0.7±0.2, P=0.001; Figure 1B), although media size (mm<sup>2</sup>) remained unchanged (0.3±0.08 versus 0.3±0.04, P=0.48). Similar reductions of intimal but not medial size were obtained from thoracic (0.003±0.004 versus 0.10±0.03, P=0.02) and abdominal aortae (0.003±0.002 versus 0.398±0.169, P=0.02).

MIF deficiency altered lesion progression as well as initiation. At 12 weeks, the majority of LDLr<sup>−/−</sup> mice developed either fatty streaks (~40%) or early plaques (~60%), whereas MIF<sup>−/−</sup> LDLr<sup>−/−</sup> mice had only subendothelial accumulation of a few macrophages (70%). Similarly, >80% of LDLr<sup>−/−</sup> mice developed advanced plaques containing calcification and lipid cores at 26 weeks, and the remaining animals had early plaques, but only 20% of MIF<sup>−/−</sup> LDLr<sup>−/−</sup> mice developed early plaques; none of them had advanced lesions. Instead, the majority of these mice still had scattered macrophages underlying the endothelial cell layer (50%) or fatty streak (30%) (Figure 1C). Significant reduction of proliferating cells (Ki67 positive) in MIF-deficient intimal lesions (Figure 1D) provided a possible explanation of the impaired lesions. Consistent with this notion, aortic SMCs isolated from MIF<sup>−/−</sup> LDLr<sup>−/−</sup> mice proliferated less than those from LDLr<sup>−/−</sup> mice (Figure 1E), which supports a role of MIF in the regulation of vascular cell proliferation.

Further characterization demonstrated that lesions in MIF<sup>−/−</sup> LDLr<sup>−/−</sup> mice, compared with those in LDLr<sup>−/−</sup> mice, had intact elastic lamellae and contained more macrophages (% positive area) (22.5±10 versus 10.6±3.2, P<0.007), likely because of the formation of macrophage-rich fatty streaks rather than advanced fibrous plaques (Figure 1C). The atherosclerotic lesions in MIF-deficient mice had fewer SMCs and less collagen in 70% of these mice, whereas all control mice developed fibrous caps replete with SMCs and collagen. The statistical analysis did not show significant difference, probably because of the high standard deviations (% Sirius Red, 2.0±3.7 versus 4.0±2.7, P=0.08; α-actin % positive area, 3.5±6.6 versus 6.6±2.5, P=0.1).

Serum cholesterol rose in LDLr<sup>−/−</sup> mice that consumed the atherogenic diet. Deficiency of MIF reduced total serum cholesterol (402.7±72.3 versus 505.8±78.8, P<0.02), LDL (332.8±68.4 versus 420.8±86.3, P=0.04), and triglycerides (129.1±23.1 versus 171.6±17.7, P<0.005) but not HDL levels (44.1±50.7 versus 50.7±9.5, P=0.14) (12 mice/group). Similar results were obtained from mice fed with an atherogenic diet for 12 weeks (not shown).

**MIF Deficiency Impairs Cysteine Protease Expression and Activities**

Prior studies indicated that MIF acts as a proinflammatory cytokine to stimulate fibroblast MMP expression and activities. Immunofluorescent double staining of human atherosclerotic lesions demonstrated colocalization of MIF and cathepsin S in SMCs, macrophages, and endothelial cells (data not shown), which suggests a role of MIF in regulation of cysteine protease expression and activity. To test this hypothesis, aortic SMCs from LDLr<sup>−/−</sup> and MIF<sup>−/−</sup> LDLr<sup>−/−</sup> mice were analyzed for cysteine protease and MMP mRNA levels using real-time PCR. MIF-deficient SMCs had comparable or lower levels of mRNA transcripts encoding most proteases examined (Figure 2A). Active site labeling demonstrated that lack of MIF impaired cathepsins S and L activities (Figure 2B) but not MMP-2 and -9 (zytomgram, not shown). Interestingly, addition of recombinant murine MIF in pathophysiologically relevant concentrations rescued the expression of cathepsins but not MMP (not shown). Consistent with their expression profiles, MIF<sup>−/−</sup> SMCs had lower proteolytic capacity for extracellular elastin (Figure 2C) and collagen (Figure 2D) even after stimulation by inflammatory mediators, which confirms a role of MIF the regulation of protease expression.
Discuss the findings of this study which showed retarded atherogenesis in the absence of MIF. MIF-deficient atherosclerotic mice had reduced intimal thickening in the aortic arch and lesion area in the abdominal aorta, accompanied by diminished SMC proliferation and proteolytic capacity. These observations furnish novel in vivo evidence that MIF participates in diet-induced atherogenesis.

Studies in humans and rabbits have addressed the expression and distribution of MIF in atherosclerosis. MIF protein colocalizes with endothelial cells, SMCs, and macrophages in...
lesions, and these cell types can produce MIF, which raises the possibility that this cytokine contributes to atherogenesis. Emerging evidence supports direct participation of MIF in the biological response to mechanical injury associated with extensive endothelial denudation, medial disruption, and thrombosis. MIF immunoneutralization after wire injury altered the cellular composition of the arterial neointima without a significant change in overall thickening in apolipoprotein E–deficient mice. In contrast, MIF neutralization reduced vascular inflammation, cellular proliferation, and neointimal thickening in LDLr−/− mice after carotid injury. This discrepancy could result from differences in the animal models or from incomplete tissue MIF neutralization, a variable difficult to assess in antibody neutralization experiments. The present study used MIF-deficient mice to show that complete interruption of MIF activity significantly reduced lesion development and neointimal thickening in endothelial-dependent responses to metabolic injury in LDLr−/− mice, animals in which the pathophysiology of atherogenesis recapitulates that of human atherosclerosis with much greater fidelity than arterial injury.

Our study suggests 2 mechanisms by which MIF participates in atherogenesis. First, MIF affects cell proliferation in lesions. Ki67 staining demonstrated significantly fewer proliferating cells in intimal lesions of MIF-deficient mice (Figure 1D). Similar observations were obtained from cultured aortic SMCs. MIF deficiency impaired aortic SMC proliferation (Figure 1E). Second, MIF affects elastolytic/collagenolytic cysteine protease expression. Deficiency of MIF decreased the expression and activity of these cathepsins (Figure 2A through 2D) but not of MMPs (not shown). Pathophysiologically relevant concentrations of recombinant murine MIF rescued cathepsin expression but not MMPs in MIF-deficient SMCs (not shown). Therefore, MIF may act as do other cytokines (eg, tumor necrosis factor-α) to enhance protease expression and vascular cell proliferation, processes that occur during atherogenesis.

Although MIF-null mice had reduced serum lipid levels, the altered lipid profile did not appear to explain the effect on atherosclerosis. Multiple linear regression analysis did not demonstrate a significant confounding effect ($P > 0.6$) of serum cholesterol, triglyceride, HDL, or LDL on either lesion grading or intimal size.

In conclusion, our results support the hypothesis that MIF modulates SMC proliferation and proteolytic activity and contributes to lesion formation in atherosclerosis. Further research may lead to new understanding of the actions of MIF and novel therapies for preventing atherosclerosis and manipulating plaque biology.

**Acknowledgments**

This study was supported by National Institutes of Health grants HL60942 (Dr Shi), HL67249 (Dr Sukhova), AI042310 (Dr Bucala), HL67204 (Dr Chapman), and HL56985 (Dr Libby), and a grant from the Donald W. Reynolds Foundation. The authors thank Eugenia Shvartz for technical assistance and Karen Williams for editorial assistance.
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_Circulation_. 2004;109:3149-3153; originally published online June 14, 2004; doi: 10.1161/01.CIR.0000134704.84454.D2

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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