De Novo Expression of Macrophage Migration Inhibitory Factor in Atherogenesis in Rabbits

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Abstract—Macrophage migration inhibitory factor (MIF) has been shown to play an important role in macrophage-mediated diseases. We investigate the potential role of MIF in atherogenesis using a hypercholesterolemic rabbit model. New Zealand White rabbits fed with a 2% cholesterol diet developed hypercholesterolemia and early fatty streaks at 1 month. The lesions became advanced at 3 months and were associated with de novo MIF expression by vascular endothelial cells (VECs) and smooth muscle cells (SMCs), as demonstrated by immunohistochemistry, reverse transcriptase–polymerase chain reaction, and in situ hybridization. By contrast, there was no increase in MIF levels in rabbits fed a normal diet. In early atherogenesis, marked upregulation of MIF mRNA and protein by VECs and some intimal cells were closely associated with CD68 monocyte adhesion onto and subsequent migration into subendothelial space. Of significance, the accumulation of macrophages was exclusively localized to areas of strong MIF expression, which may be associated with the macrophage-rich fatty streak lesion formation. Upregulation of MIF by SMCs is transient during atherogenesis. Importantly, strong MIF expression by activated macrophages may be responsible for the development of foam cell-rich lesions. Finally, the ability of MIF to induce intercellular adhesion molecule-1 expression by VECs implicates its pathogenic role in atherogenesis. In conclusion, the present study provides the first demonstration that MIF is markedly upregulated during atherogenesis. Upregulation of MIF by VECs and SMCs may play a role in macrophage adhesion, transendothelial migration, accumulation, and, importantly, transformation into foam cells. Furthermore, strong MIF expression by macrophages may both initiate and amplify the atherogenesis process. (Circ Res. 2000;87:1202-1208.)

Key Words: atherosclerosis ■ migration inhibitory factor ■ macrophages ■ foam cells ■ smooth muscle cells ■ vascular endothelial cells

Monocytes and macrophages have been shown to be key cells involved in the development of atherosclerotic plaques. An early event in atherogenesis is monocyte adherence to activated endothelial cells. After transmigrating across the endothelial cell layer, monocytes mature into macrophages, which phagocytose lipids to become macrophage foam cells forming the early fatty streak. Monocyte chemoattractant protein-1 (MCP-1) and a group of adhesion molecules (intercellular adhesion molecule-1 [ICAM-1], vascular adhesion molecule-1 [VCAM-1], and P-selectin) have been shown to be important in the attachment of monocytes to activated vascular endothelial cells (VECs) in the initial step of atherosclerotic plaque formation. However, there are several fundamental questions remaining in the development of macrophage-rich atherosclerotic plaque, including why the infiltrating macrophages persist for a long time within the lesions and cease migrating within the vessel wall after their transendothelial migration and what factors regulate the infiltrating macrophages to become activated, phagocytose lipids and transform into lipid-laden macrophages (foam cells).

Migration inhibitory factor (MIF) was first described more than 30 years ago as a product of activated T cells, which inhibits the migration of macrophages in vitro and promotes macrophage accumulation in the skin delayed-type hypersensitivity reaction. The recent cloning and characterization of MIF has led to the recognition that this molecule plays a pivotal role in regulating the inflammatory and immune responses, although there is no receptor for MIF identified to date. It has now recognized that MIF is constitutively expressed in a variety of tissues and cells and is a potent macrophage activator. Upregulation of MIF has been demonstrated to be responsible for the recruitment and localization of macrophages and T cells to areas of severe tissue damage in both experimental and human glomerulonephritis and allograft rejection. The use of neutralizing antibodies has confirmed the central role of MIF in the cutaneous response to tuberculin and endotoxic shock.
In addition, anti-MIF treatment significantly inhibits macrophage-dependent progressive renal injury and arthritis. This inhibition was associated with the suppression of blood monocyte recruitment and subsequent macrophage-mediated tissue injury, confirming the important role of MIF in controlling cell-mediated inflammatory and immune responses.

On the basis of the known role of MIF, we postulate that MIF may participate in atherogenesis by promoting macrophage recruitment and localization to form macrophage-rich fatty streaks and their subsequent transformation into lipid-laden foam cells.

Materials and Methods

Animal Model

A rabbit model of atherosclerosis was induced in New Zealand White rabbits fed a standard (control, n = 12) or 2% cholesterol diet for 4 (n = 6) and 12 (n = 6) weeks. The animals were killed at 1 and 3 months. A piece of aortic arch (0.5 cm) was collected from each animal for histology, and thoracic and abdominal aortas (5 cm) and carotid and femoral arteries (2 cm) were collected for reverse transcriptase–polymerase chain reaction (RT-PCT). Serum total cholesterol, triglycerides, HDL, and LDL cholesterol were measured using radioimmunoassay kits (Wako Pure Chemical Industries). Data are expressed as mean±SD for groups of animals.

Antibodies and Probes

The mouse anti-MIF monoclonal antibody (mAb) III.D.9 was used in this study. The specificity of the antibody has been determined previously. Other mAbs used in this study include RAM11, mouse anti-rabbit CD68 (monocytes and macrophages), mouse anti–α-smooth muscle actin (α-SMA), CD31 (VEC), and ICAM-1, goat anti-mouse IgG, mouse peroxidase antiperoxidase complexes, and mouse alkaline phosphatase antialkaline phosphatase complexes. All antibodies were purchased from Dakopatts.

A 420-bp fragment of mouse MIF cDNA was used to prepare a digoxigenin-labeled antisense and sense cRNA probes for in situ hybridization. Other mAbs used in this study include RAM11, mouse anti-rabbit CD68 (monocytes and macrophages), mouse anti–α-smooth muscle actin (α-SMA), CD31 (VEC), and ICAM-1.

Histopathology and Immunohistochemistry

Collected arteries were fixed in 4% formalin and stained with hematoxylin and eosin or periodic acid–Schiff reagent. Atherosclerotic lesions were assessed using an update standard of histological...
classification. One- and two-color immunohistochemical staining was used as previously described microwave-based method. An isotype-matched mAb (73.5) against human CD45R was used as the negative control.

Cell Culture
The endothelial-derived cell line EA.hy926 (generously provided by Dr S.H. Lin, Baker Institute, Melbourne, Australia) was cultured in DMEM containing 20% FCS until subconfluence. Cells were then serum-starved for 24 hours, and rhMIF 0, 10, 25, and 50 ng/mL in the presence or absence of the neutralizing antibody (III.D9, 25 μg/mL) was added into the culture for 0, 3, 6, 12, and 24 hours. Cellular RNA and protein were extracted for RT-PCR and Western blot analysis for ICAM-1 expression.

MIF and ICAM-1 mRNA Detection by RT-PCR and In Situ Hybridization
RNA isolation and RT-PCR for MIF and ICAM-1 were performed as previously described. In situ MIF mRNA expression was detected by a digoxigenin-labeled antisense MIF cRNA probe on 4-μm paraffin sections using a microwave-based protocol. Controls used a sense MIF cRNA or the probe was omitted completely. No staining was seen in either normal or diseased arteries using the sense probe or with no probe at all.

Quantitation of MIF Expression
The number of MIF+ VECs in the areas with or without lesions was counted under high-power field (×400) and expressed as percent positive cells (%). The number of MIF+ smooth muscle cells (SMCs) in the medial areas immediate to the early or advanced atherosclerotic lesions or in the areas without lesions was counted by means of a 0.02-mm² graticule fitted in the eyepiece of the microscope and expressed as cells per mm². All counting was performed on blinded slides, and data are expressed as mean±SD.

Statistical Analysis
Statistical analysis was performed using GraphPad Prism 3.00 (GraphPad Software, Inc). Differences in MIF expression and macrophage accumulation were assessed by Student’s t test.

Results
Hypercholesterolemia
All animals fed with a 2% cholesterol diet developed hypercholesterolemia at 1 month (Figure 1), with up to a 10-fold increase in total serum cholesterol (Figure 1A) and a 14-fold increase in LDL (Figure 1B) compared with the normal-diet control rabbits. However, there was only a 4-fold increase in serum HDL (Figure 1C), resulting in a 3-fold increase in the LDL/HDL ratio compared with the normal control animals (Figure 1D). In contrast, serum levels of triglycerides remained within the normal range throughout the entire experimental time course (0.99 to 1.44 mmol/L).

Constitutive MIF Expression in the Normal Artery
Examination of arteries (aortas and carotid and femoral arteries) from normal-diet control animals showed undetectable fatty streak grossly. In situ hybridization and immunohistochemistry demonstrated that a few VECs exhibited weak positive expression for MIF mRNA (Figures 2A and 3A) but undetectable MIF protein expression and macrophage accumulation (Figures 2B and 3B). In addition, there was undetectable or very little MIF mRNA and protein expression by SMCs (Figures 2A, 2B, 3A, and 3B). Similarly, measurement
of arterial MIF expression by RT-PCR demonstrated constitutive but weak MIF mRNA expression by all normal arteries examined (Figure 4).

De Novo MIF Expression by VECs and SMCs During Atherogenesis

Macroscopically, the fatty streak lesion was apparent in cholesterol-fed rabbits, accounting for 45% to 55% of the aortic surface areas with profound lesions at the branch sites of abdominal aorta. In situ hybridization and immunostaining of the serial sections demonstrated that there was marked upregulation of MIF mRNA and protein by all VECs in the initial microscopic lesions (the earliest stage of fatty streak) in aortas from the hypercholesterolemic rabbits at 1 month after starting the cholesterol diet (Figures 2C and 2D), which was confirmed by double immunostaining with the anti-MIF and CD31 mAb (Figures 5A and 5B). Indeed, de novo MIF expression was associated with many CD68+ macrophages adherent onto the MIF+ VECs (Figures 2D and 5B). In the areas without lesions, few VECs expressed MIF (Figure 6A) and no macrophage adherence to VECs was seen. Importantly, marked upregulation of MIF mRNA and protein also was detected in the subendothelial space, which may be associated with the initiation of CD68+ macrophage transendothelial migration and subendothelial accumulation (Figures 2C, 2D, 5A, and 5B), ultimately leading to the macrophage-rich early fatty streak formation (Figures 2E through 2H and 5E). Within the early fatty streak, marked upregulation of MIF mRNA and protein was found to be associated with macrophage accumulation, as illustrated in the serial sections (Figures 2E through 2H and 5E). De novo expression of MIF mRNA and protein by SMCs was first evident in the medial areas beneath the macrophage-rich early fatty streak (Figures 2E and 2F), which was additionally identified by combined anti-MIF and α-SMA mAb staining (Figure 5C), whereas MIF expression by SMCs in the media without fatty streak lesions remained low (Figure 6B). Interestingly, strong MIF expression by medial SMCs was transient and virtually disappeared as the macrophage-rich early fatty streak developed into foam cell–rich or advanced lesions (Figures 2I through 2L and 5D). In advanced atherosclerotic plaques (Figures 2K and 2L), positive cells for MIF mRNA and protein were reduced, and this was associated with a marked reduction in the number of CD68+ macrophages (Figure 2L). Overall, there are at least 50% of the CD68+ macrophages less in advanced lesions than in the early fatty streak (Figures 2C through 2H). Few SMCs expressed MIF in media in advanced lesions (Figures 2I, 2L, and 5D). In contrast, the number of α-SMA+ cells with characteristic myofibroblast morphology within the advanced lesions were markedly increased compared with the early fatty streak (Figure 5C). Double immunostaining demonstrated that all α-SMA+ cells within the lesions also highly expressed MIF (Figure 5D).

MIF mRNA expression by the atherosclerotic artery was also semiquantitated by RT-PCR. Figure 4 demonstrated that weak constitutive expression of the expected a 0.6-kb MIF mRNA species in normal aortas and carotid and femoral...
arteries was markedly upregulated during atherogenesis, which was consistent with the results seen in situ hybridization.

Expression of MIF by Macrophages and Foam Cells During Atherogenesis

In situ hybridization and double immunohistochemistry showed that all CD68⁺ monocytes and macrophages that adhered onto the MIF⁺ VECs and accumulated within the early fatty streak were strongly positive for MIF expression (Figures 2C through 2L), which was additionally illustrated in Figure 5E. Macrophages within the early fatty streak showed stronger MIF mRNA and protein expression (Figures 2C through 2H and 5E) compared with those with foam cell morphology in the fatty streak (Figures 2I, 2J, and 5F) or with those in the advanced plaque (Figures 2K and 2L). These data suggest that MIF expression by macrophages may be a good indicator of macrophage functional activity within atherosclerotic lesions.

MIF Upregulates ICAM-1 Expression By Endothelial Cells In Vitro

We next tested the potential role of MIF in atherogenesis. Because it is difficult to block MIF with the neutralizing anti-MIF mAb in long-term experiments of atherosclerosis in rabbits, an alternative in vitro study was performed to investigate the functional importance of MIF in upregulation of endothelial ICAM-1 during atherogenesis. RT-PCR and Western blot analysis showed that MIF strongly induced ICAM-1 mRNA and protein expression by endothelial cells (ECs) in both time- and dose-dependent manners, which was abrogated by the neutralizing anti-MIF mAb (Figures 7 and 8).

Discussion

The present study has demonstrated de novo expression of MIF mRNA and protein by intrinsic VECs, SMCs, and inflammatory macrophages and foam cells during atherogenesis in hypercholesterolemic rabbits. Upregulation of vascular MIF may play an important role in the initiation and progression of atherosclerosis.

There are several novel findings in the present study. First, de novo expression of MIF mRNA and protein by VECs was found in the earliest stage of fatty streak formation, and this was associated with monocyte adherence onto the VEC. It is now well-known that the adherence of monocytes onto activated endothelial cells is an initial step of atherogenesis. The expression of VCAM-1, ICAM-1, and P-selectin has been shown to be upregulated on endothelial cells in regions overlying atheromatous lesions, and these molecules play a role in the earliest steps of atherogenesis. The new finding of marked MIF expression and monocyte adherence onto VECs indicates that MIF may play a role in the interaction between monocytes and VECs during early atherogenesis. Furthermore, MIF is a proinflammatory cytokine and is constitutively expressed by a variety of cells, including VECs and monocytes and macrophages. It is possible that hypercholesterolemia or modified LDL can cause the release of MIF from VECs and monocytes. Once released, MIF may be able to induce ICAM-1 and VCAM-1 upregulation by VECs directly or indirectly via MIF-induced tumor necrosis factor (TNF)-α, interleukin-1 mechanism (Reference 33 and C. Metz, unpublished data, September 2000). In the present study, the ability of MIF to induce ICAM-1 mRNA expression by ECs as early as 3 hours strongly indicates that MIF, as a key mediator, directly causes local inflammatory response and thus plays an important role during atherogenesis. This is supported by the recent in vivo study that immunoneutralization of MIF produces a significant inhibition of interleukin-β, ICAM-1, and VCAM-1 expression and macrophage accumulation in an experimental rat model of glomerulonephritis.
Second, marked upregulation of MIF mRNA and protein was found within the intima in the earliest step of atherogenesis. This may contribute to monocyte and macrophage transendothelial migration and subsequent accumulation to form the macrophage-rich early fatty streak. MCP-1, a member of the CC family and a potent chemokine for monocytes, has been found to be highly expressed in macrophage-rich atherosclerotic lesions in both human and animal models5–6 and postulated to be central in monocyte recruitment into the arterial wall. This was confirmed by the recent study that the absence of MCP-1 and its receptor causes a dramatic protection from macrophage recruitment and atherosclerotic lesion formation.34–36

Questions that remain are why macrophages are able to persist within the atherosclerotic lesions and what factors regulate their accumulation, activation for lipid phagocytosis, and transformation into lipid-laden foam cells. Upregulation of MIF within the intima may explain certain of these questions. First, MIF may drive macrophage transendothelial migration via its macrophage activating properties. It is also likely, but not yet proven, that MIF could induce MCP-1 expression, thereby recruiting macrophages via a MCP-1–dependent mechanism. Second, consistent with the known role of MIF, upregulation of MIF within the intima may function to inhibit macrophage movement, ultimately resulting in macrophage-rich fatty streak formation. The ability of MIF to inhibit monocyte and macrophage chemotaxis and random migration induced by MCP-1 suggests the importance of MIF in causing macrophage localization in the early fatty streak. Furthermore, MIF is also a potent macrophage activator, inducing expression of many proinflammatory mediators by macrophages33 and strongly promoting inflammatory and immune response. It is very likely that MIF can activate macrophages to phagocytose lipids, regulating in the macrophage–foam cell transformation. Third, the transient expression of MIF by SMCs after macrophage subendothelial accumulation may be temporally responsible for the additional recruitment of macrophages into the arterial wall, leading to the progressive development of macrophage-rich atherosclerotic lesions. In normal artery, there are undetectable or very few, if any, SMCs that express MIF, which is consistent with our previous finding in normal human vessels.24 De novo MIF expression by SMCs is pronounced within the macrophage-rich atherosclerotic lesion, whereas it becomes minimal or undetectable when the advanced lesions develop. This indicates that macrophage-derived cytokines from the early fatty streak may contribute to the upregulation of MIF by SMCs, which, in turn, recruit more macrophages to the lesions and promote macrophages to uptake lipids and transform to foam cells. This is in contrast to our previous finding that the upregulation of vascular MIF causes macrophage and T-cell accumulation, thereby producing transmural vasculitis in human renal allograft rejection.24 The transient expression of MIF by SMCs observed in the present study may help to limit macrophage-mediated injury and prevent the occurrence of unstable atherosclerotic lesions. In contrast, marked expression of MIF by α-SMA+ cells with characteristic myofibroblast morphology was found within the advanced lesions, indicating that MIF may also be involved in the SMC migration and differentiation during advanced plaque formation. Therefore, SMC-derived MIF may play a role in both progression and regression of atherogenesis.

Finally, an important observation made by this study is that macrophages are an important source of MIF, which may act as a key mediator governing the atherogenesis. Indeed, macrophages have been identified to be a rich source of MIF that is released after stimulation with endotoxin, exotoxin, and cytokines such as TNF-α and interferon-γ.33,38 In macrophage-mediated renal disease, including human glomerulonephritis and allograft rejection,19–25,27 strong MIF expression was found in activated macrophages within areas of severe tissue injury. Only weak or undetectable MIF expression was observed in resting macrophages present in the uninjured areas. All macrophages within the early fatty streak exhibited strong MIF expression, suggesting that they are likely to be activated and play a role in the progression of atherogenesis. In contrast, MIF expression by macrophages was reduced in the advanced lesions. This may reflect their relatively low functional activities in the regressive phase of atherosclerotic lesions.

Local production of MIF by both intrinsic vascular cells and macrophages may be a central mechanism to cause macrophage accumulation, activation, and transformation to foam cells in atherosclerotic plaque. However, the mechanisms responsible for the upregulation of MIF expression by VECs, SMCs, and macrophages remain to be addressed. The accumulation of lipids within the arterial wall, especially cholesterol and LDL, is a distinguishing characteristic of the atherosclerotic lesion and has been shown to be responsible for all stages of plaque development. Indeed, LDL can induce VEC expression of ICAM-1, VCAM-1, and MCP-1.39–41 VLDL has been shown to activate nuclear factor-κB, which induces TNF-α, ICAM-1, and VCAM-1 expression by VECs and SMCs.42 Accordingly, it is likely that LDL may also be a key inducer for MIF expression by VECs, SMCs, and macrophages during atherogenesis.

In summary, this is the first study to identify that VECs, SMCs, and macrophages are a major source of MIF expression in the atherosclerotic vessels of hypercholesterolemic rabbits. Upregulation of MIF expression by intrinsic vascular cells may contribute to local macrophage accumulation, ultimately resulting in macrophage-rich atherosclerotic lesion formation. In addition, MIF production by macrophages within atherosclerotic lesions may initiate autocrine mechanisms, which cause and amplify the inflammatory response, promoting macrophage–foam cell transformation during atherogenesis. However, the pathogenic role of MIF in atherogenesis needs to be additionally investigated in vivo by MIF-blocking studies.

Acknowledgments

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