Expression of Macrophage Migration Inhibitory Factor in Different Stages of Human Atherosclerosis

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Background—Atherosclerosis is a chronic inflammatory response of the arterial wall to injury. Macrophage migration inhibitory factor (MIF), a cytokine with potent inflammatory functions, was thus considered to be important in atherosclerotic lesion evolution.

Methods and Results—We studied the presence and distribution of MIF immunoreactivity (MIF-IR) and MIF mRNA in internal mammary arteries with a normal histology and arteries with plaques in different stages of human atherosclerosis. To address a potential role for the coactivator Jab1 as a cellular mediator of MIF effects in vascular tissue, we correlated the expression of MIF to that of Jab1 by using immunohistochemistry and coimmunoprecipitation. We further sought to determine a potential functional role for endothelium-derived MIF in early atherogenesis by studying the effects of oxidized LDL on MIF expression in cultured human umbilical vascular endothelial cells. The results showed that MIF-IR and Jab1-IR are found in all cell types present in atherosclerotic lesions, that MIF-IR is upregulated during progression of atherosclerosis, that MIF is produced locally in the arterial wall, and that all MIF+ cells are simultaneously Jab1+ 12. Coinmunoprecipitation experiments demonstrated in vivo complex formation between MIF and Jab1 in plaques. MIF expression in human umbilical vascular endothelial cells and a macrophage line was upregulated after stimulation with oxidized LDL.

Conclusions—MIF is produced abundantly by various cells in all types of human atherosclerotic lesions and thus may play an important role in early plaque development and advanced complicated lesions. MIF-Jab1 complexes could serve critical regulatory functions in atherosclerotic lesion evolution. (Circulation. 2002;105:1561-1566.)

Key Words: lesion # atherosclerosis # inflammation # pathology
of MIF in human atherosclerotic tissue with that of Jab1 by using immunohistochemistry and coimmunoprecipitation. Because macrophage accumulation in the vessel wall requires the adhesion and transmigration of blood monocytes across the endothelium and immobilization of cells at the site of injury, we sought to determine a potential functional role for endothelium-derived MIF in early atherogenesis by studying the effects of oxidized LDL (oxLDL) on MIF expression in human umbilical vascular endothelial cells (HUVECs).

Methods

Definitions
Atherosclerotic lesions were graded as previously described.20

Tissue Specimens
Histologically normal internal mammary arteries (IMAs) (n = 10) from patients undergoing aorto-coronary bypass surgery, coronary arteries with adaptive intimal fibrosis (AIF, type I lesions)20 that were taken from the explanted hearts from patients undergoing transplantation for idiopathic dilated cardiomyopathy (n = 8), and aortic tissue specimens with AIF (type I lesions)20 from the thoracic aorta from autopsied individuals (n = 2) served as controls. Aortic fatty streak (FS) lesions (n = 10) were obtained from patients undergoing carotid endarterectomy. Postmortem delay was <12 hours and did not affect the quality nor quantity of staining. Tissue was fixed and prepared by standard methods. Serial sections were stained with hematoxylin and eosin and used for immunohistochemistry. For in situ hybridization (ISH), reverse transcription–polymerase chain reaction (RT-PCR), and coimmunoprecipitations, IMAs specimens with a normal histology and carotid endarterectomy specimens with CPs were snap-frozen after excision.

Immunohistochemistry
We used anti-human MIF IgG (1:200, AF-289-PB, R&D Systems), anti-human Jab1 IgG (1:200, FL 334, Santa Cruz Biotechnology), and anti-human CD3 (1:100, CD3, DAKO) as antibodies against MIF, Jab1, and T lymphocytes, respectively, and monoclonal antibodies against α-actin (1:600, M0851, DAKO) and macrophages (1:100, CD68 (KP1), DAKO). Immunohistochemistry was performed as described.21 For CD3 staining, sections were pretreated with proteinase K; for MIF, Jab1, CD68, and actin staining, antigens were unmasked by pressure cooking in 10 mmol/L citric acid pH 6.5.

In Situ Hybridization
An MIF probe was produced as described22 with primers; sense: 5'-ATG CCG ATG TTC ATC GTA AAC ACC-3', antisense: 5'-TTA GGC GAA GGT GGA GTT GTT CCA GC-3', resulting in a 348 bp product. ISH was performed as described,22 with the use of an anti-digoxigenin antibody-conjugate (1:500, Roche). Controls were derived from hybridization of nonlabeled probes to matched sections and labeled probes to sections incubated with RNase A before hybridization. No hybridization signals were detectable in the controls (not shown). In addition, Northern analysis with the MIF probe revealed a band of the expected size. To identify cell types showing positive ISH signals, double labeling (DL) was performed. After ISH, immunohistochemistry was performed with the ABC method used as described above with anti-actin or anti-CD68 antibodies.

RNA Isolation and RT-PCR
Tissue was homogenized with an Ultraturrax for 20 seconds in 400 μL of Quagen RT Lyse buffer. RNA was isolated with the use of RNeasy (Qiagen). For RT, the M-MLV first-strand synthesis reagent of Invitrogen was used. MIF PCR was performed with the use of 35 cycles (94°C/90°C/72°C) with primers; sense: 5'-ATG CCG ATG TTC ATC GTA AAC ACC-3' and antisense: 5'-TTA GGC GAA GGT GGA GTT GTT CCA GC-3' (product: 348 bp). As internal reference, a GAPDH PCR was performed as described.22 PCR products were analyzed in 1% gels.

Coimmunoprecipitations
Coimmunoprecipitations of native MIF-Jab1 complexes from CPs were performed with the use of a modification of a published procedure.6 Briefly, specimens were resuspended in 600 μL HEPES-NaCl-Triton X-100 buffer and homogenized with an Ultraturrax for 20 seconds. Samples were divided and equal portions used for coimmunoprecipitation of MIF-Jab1 complexes by anti-Jab1 antibody (FL-334, Santa Cruz, 90 minutes) and control incubations. Precipitation of immune complexes was achieved by Protein A Sepharose (2 mL HNT; 3 hours; washes in HNT). MIF was revealed by Western blotting with anti-MIF antibody.

OxLDL Treatment and HUVEC Experiments
Human LDL was prepared as described previously.23 Oxidation of LDL was determined by the FOXII assay23 and yielded 528 μmol oxLDL per mg protein. The degree of oxidation was also assessed by two other methods: increase of mobility in an agarose gel (1.4-fold compared with native LDL) and formation of thiobarbituric acid–reactive substances (4 mmol/L). HUVECs were purchased from Cell Systems and cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/mL), bovine brain extract (3 μg/mL), gentamicin (30 μg/mL), amphotericin B (50 μg/mL), epidermal growth factor (10 μg/mL), and 10% FCS until the third passage. After detachment, cells were grown in 6-cm dishes for 18 hours. HUVECs were stimulated with oxLDL and cellular protein was prepared as described.24 For Western blot analysis, anti-MIF antibody was used at 1:100 and blots were developed by horseradish peroxidase–coupled antibody/ECL (Amersham). Blots were re-probed with anti-actin antibody and quantified by densitometry. RAW 264.7 macrophages were obtained from the American Type Culture Collection and cultured and stimulated with oxLDL as described.3,12,24 Analysis of MIF from the culture supernatants was performed by sandwich ELISA as described.9 The mouse monoclonal IgG, used in the ELISA was a gift from Prof Richard Bucala (Yale University School of Medicine, New Haven, Conn).

Morphometric Analysis and Statistical Methods
With the use of morphometric software, 5 random nonoverlapping microscopic high-power fields of histologically distinct regions of each specimen were examined, scoring all nuclei and cells stained for MIF. A cell was scored positive when the typical cytoplasmic staining was associated with a cell nucleus. Data represent mean ± SD. The Mann-Whitney rank sum test was applied for comparison of data from different plaque types with the controls. Probability values <0.05 were considered significant.

Results

MIF Expression in IMAs With Normal Histology
In the specimens, a weak and diffuse cytoplasmic MIF-IR was present in 10 of 10 tissue specimens in 90% of the ECs and medial VSMCs. Also, we demonstrated the presence of MIF mRNA in 3 of 3 tissue specimens by RT-PCR (Figure 3). Jab1-IR was observed in 10 of 10 tissue specimens in 90% of the ECs and medial VSMCs (Figure 2A). Jab1 staining was present in the cytoplasm but was more prominent in the nucleus. Examination of serial sections showed that all MIF7 cells were simultaneously Jab1+.

Noninflammatory Stage of Atherosclerosis:
Coronary Arteries With Adaptive Intimal Fibrosis
(Type I Lesions)
In the arteries with AIF, MIF-IR was present in 10 of 10 specimens and was localized to ECs (5%), intimal VSMCs...
(α-actin⁺, 5.9±8.0%) (Figure 1B). Jab1-IR was present in most ECs (80%), intimal VSMCs (α-actin⁺, 20.5±5.5%). Whenever present, 80% of the ECs above the FS showed strong MIF-IR (Figure 1C). Jab1-IR was present in most ECs (80%), intimal VSMCs, and medial VSMCs (90%) (Figure 2B). Serial sections showed that cytoplasmic MIF staining never occurred without Jab1 staining.

**Inflammatory Stages of Atherosclerosis I: Aortic Fatty Streaks**

In the FS, MIF was present in 10 of 10 cases in ECs, in intimal macrophages (CD68⁺), intimal VSMCs (α-actin⁺, 24.0±0% of the intimal cells were MIF⁺), and medial VSMCs (α-actin⁺, 20.5±5.5%). Whenever present, 80% of the ECs above the FS showed cytoplasmic MIF staining in few of the medial VSMCs (brown reaction product), B, MIF staining (brown) of coronary artery with AIF showing few scattered intimal and medial VSMCs with MIF expression. C, MIF staining (brown) of FS showing numerous intimal foam cells and medial VSMCs with strong and finely granular cytoplasmic MIF-IR. D, Low-power magnification of CP with large lipid core (LC) separated by a thick fibrous cap from the lumen (L). There are numerous MIF⁺ cells in the zone bordering the acellular lipid core. MIF staining decreases with growing distance from the LC. E, Double immunostaining for MIF (brown) and CD68 (blue) showing numerous MIF⁺ macrophages. F, Double immunostaining for MIF (blue) and CD3 (brown) showing numerous T lymphocytes (TLs) in close association with intimal foam cells that are MIF⁻. Surprisingly, MIF-IR of the TLs is much less intense than MIF-IR of the foam cells and appears as a small rim around the nucleus. G, Double immunolabeling of MIF (brown) and α-actin (blue) showing numerous VSMC-derived foam cells that are MIF⁺. H, ISH with MIF antisense probe revealing that MIF mRNA (black) is produced locally in CD68⁻ intimal macrophages (brown).
Anti-Jab1 IP / 
anti-MIF Western 
+ αJab1 - αJab1 

**Figure 4.** MIF and Jab1 complex formation in atherosclerotic tissue. Coimmunoprecipitation analysis of MIF-Jab1 complex formation by anti-Jab1 pulldown (anti-Jab1 IP) and anti-MIF Western blotting. Specificity was verified by comparing pull-downs with anti-Jab1 antibody (+αJab1) with incubations in the absence of Jab1 antibody (−αJab1). Blot is derived from a plaque lysate of 1 patient with atherosclerosis and is representative of a total of 3 patients. MIF-Jab1 complex formation could not be detected in plaque lysate from one other patient nor in specimens of 4 healthy control subjects.

Acellular lipid core (74.7±21.3% of intimal cells) and to a lesser extent in plaque fibrotic regions and in the media beneath the plaque (9.2±16.2% of medial VSMCs). Luminal ECs and microvascular ECs showed an inconstant MIF-IR (Figure 1D). DL confirmed that macrophages (CD68<sup>+</sup>), intimal VSMCs, medial VSMCs (actin<sup>+</sup>), and T lymphocytes (CD3<sup>+</sup>) were MIF<sup>+</sup> (Figure 1, E through G). Jab1-IR was present in macrophages (CD68<sup>+</sup>), intimal VSMCs, medial VSMCs (actin<sup>+</sup>), and T lymphocytes (CD3<sup>+</sup>, 90% of intimal and medial cells were Jab1<sup>+</sup>) and occurred both in the cytoplasm and nucleus (Figure 2, D through H). DL (MIF/Jab1) revealed that MIF<sup>+</sup> cells were also Jab1<sup>+</sup> (Figure 2, G and H). ISH demonstrated the expression of MIF mRNA in the intima of CPs (5 of 5). As shown by DL (MIF-ISH/CD68), intimal macrophages showed strong MIF hybridization signals. Furthermore, we demonstrated the presence of MIF mRNA in another 6 specimens by RT-PCR (6 of 6) (Figure 3). Coimmunoprecipitation assays from atherosclerotic plaque tissue provided evidence for in vivo complex formation between MIF and Jab1. MIF was detected by Western analysis after immunoprecipitation by anti-Jab1 antibody of endogenous MIF-Jab1 complexes from lysates of atherosclerotic tissue (Figure 4) in 3 of 4 patients, whereas complexes were not detectable in any of the IMAs (0 of 4).

### Statistical Analysis of MIF Immunostaining

In the intima of FS lesions and advanced plaques, significantly more MIF<sup>+</sup> cells were observed compared with the

**Figure 5.** Enhancement of MIF production in HUVECs and macrophages stimulated by oxLDL. A, Time dependence of oxLDL treatment in HUVECs. Left, Anti-MIF Western blot of lysates from HUVECs after stimulation with oxLDL. For standardization, blots were subsequently developed for actin. Right, Quantification of blots by densitometry. Bars represent means of 2 experiments. B, Concentration dependence of oxLDL treatment in HUVECs. Western blots and quantifications were performed as in A. Data represent mean±SD of 3 experiments.

| P Values for Statistical Comparison of MIF Staining Grades in the Intima |
|-----------------|---|---|
|                 | FS | CP |
| AIF             | 0.001 | 0.001 |
| FS              | 0.001 |     |
analysis of the intima of the AIF tissue (type I lesions, Table). Likewise, there were significantly higher numbers of MIF cells in the intima of CPs compared with FS lesions. In summary, a significant upregulation of MIF-IR was seen in the intima of atherosclerotic lesions with chronic inflammation as compared with noninflammatory lesions. Upregulation further progressed with the development of advanced complicated atherosclerotic plaques.

Effect of oxLDL on MIF Expression in HUVECs and Macrophages

As shown in Figure 5, MIF protein in HUVECs was upregulated in a time- and concentration-dependent manner when these cells were incubated with oxLDL. Maximal production was obtained after 24 hours and with 10 μg/mL of oxLDL. OxLDL also led to an enhancement of MIF secretion in macrophages, with maximal induction observed at oxLDL concentrations of ≥45 μg/mL (data not shown). Confirming the coimmunoprecipitations from atherosclerotic plaque tissue (Figure 4), MIF-Jab1 complex formation was also detected in HUVECs (data not shown).

Discussion

We have investigated the expression of the cytokine MIF over different stages of human atherosclerosis. MIF protein is detected in ECs, VSMCs, T lymphocytes, and macrophages of the arterial wall, confirming that MIF differs from other proinflammatory cytokines by exhibiting significant cellular levels at baseline. In line with recent studies of atherosclerotic lesions in rabbits, our evaluation revealed that MIF is present at low levels in ECs and VSMCs of IMAs with a normal histology and, importantly, is upregulated during the progression of atherosclerosis toward inflammatory stages. The immunostaining studies are supported by evidence of local MIF production in the arterial wall by ECs and VSMCs in IMAs and, at least by macrophages, in CPs. The latter observation is further confirmed by oxLDL-mediated induction of MIF, as seen in a macrophage line (this study and Reference 25). Thus, because MIF is produced locally in key cells in all stages of lesion evolution, we conclude that it plays an important role during the progression of human atherosclerosis.

Atherosclerosis is a chronic inflammatory disease characterized by accumulation of blood-derived macrophages and T lymphocytes in the arterial wall. In the early stages, atherosclerosis is also characterized by formation of oxLDL in the vascular wall. OxLDL affects ECs, leading to changes in adhesion molecule expression, promoting the immigration of macrophages and other inflammatory cells. Interestingly, our study demonstrates upregulation of MIF after incubation of HUVECs with oxLDL. Furthermore, recent investigations have indicated that MIF upregulates the expression of intercellular adhesion molecule-1 expression by ECs in vitro. Thus, oxLDL may promote the immigration of macrophages into the vessel wall and the immobilization of these cells in early stages of lesion evolution, directly or through mediation by endothelium-derived MIF.

Uptake of oxLDL by macrophages and VSMCs eventually leads to foam cell formation. In particular, macrophage-derived foam cells produce and secrete proinflammatory cytokines, which then amplify the inflammatory cascade in early stages of plaque evolution and serve to sustain inflammation in advanced plaques. We show that enhanced levels of MIF are released from macrophages on stimulation with oxLDL in vitro and that in vivo, MIF expression is prevalent in regions with chronic inflammation containing VSMC- and macrophage-derived foam cells, indicating that oxLDL also plays an important role in the induction of macrophage MIF in vivo. It is interesting to note that macrophages stimulated with MIF secrete tumor necrosis factor, IL-1β, and IL-8, proinflammatory cytokines abundantly present in atherosclerotic lesions. In addition, MIF upregulates nitric oxide production in macrophages and matrix metalloproteinases in fibroblasts, both key events in lesion evolution. Thus, MIF may play a prominent role in the regulation of the inflammatory response in advanced atherosclerotic lesions.

API proteins are transcription factors with a role in gene regulation, cell proliferation, and mediation of inflammatory signals. AP1 activity is regulated by protein kinases and interaction with the coactivator Jab1. Jab1 was recently identified to specifically bind to MIF and to be a molecular target of MIF action. Jab1 interaction occurs intracellularly and has been demonstrated in macrophages and other cells in vitro. We sought for evidence of an interaction between MIF and Jab1 in human arterial tissue and atherosclerosis in vivo. In fact, immunohistochemistry showed that Jab1 expression is abundant in human arterial tissue, with staining activity mainly occurring in the nuclei of ECs, VSMCs, T lymphocytes, and macrophages. We show that all MIF cells of the vascular wall are simultaneously Jab1. Importantly, coimmunoprecipitation provided specific evidence for an in vivo complex formation between Jab1 and MIF in plaque tissue of patients. MIF-Jab1 complex formation in atherosclerotic tissue indicates that MIF-Jab1 complexes could be a critical regulatory system in atherosclerotic lesion evolution and inflammatory diseases in general. For example, MIF signaling effects in human atherosclerotic tissue could be integrated into the API pathway by Jab1. Depending on the stage of atherosclerosis, the cells involved, and the surrounding inflammatory stimuli present, MIF could serve to either enhance or downregulate inflammation. Also, because API binding sites are present in the promoters of genes regulating cell differentiation and proliferation and in genes controlling expression of E-selectin, collagenase-1, and stromelysins, MIF signaling may contribute to the regulation of expression of these atherosclerosis-associated factors.

In summary, MIF, a cytokine with important inflammation-regulatory activities, is produced abundantly by various cells in all types of human atherosclerotic lesions and may be an important regulator in early plaque evolution and advanced complicated lesions.

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