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Up-Regulation and Coexpression of MIF and Matrix Metalloproteinases in Human Abdominal Aortic Aneurysms

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ABSTRACT

Abdominal aortic aneurysm (AAA) is a localized dilatation of the arterial wall as a result of extensive breakdown of its structural proteins by matrix metalloproteinases (MMPs). AAA continuously expand and may eventually rupture, causing high mortality rates. The molecular processes underlying expansion and rupture of AAA are only poorly understood. In this study, evidence was sought for a direct involvement of macrophage migration inhibitory factor (MIF) in the pathogenesis of AAA through up-regulating MMPs, with particular reference to macrophages. To this end, expression and cellular localization of MIF were analyzed in human aortic wall samples of stable AAA and ruptured AAA, and compared with control aorta and atherosclerotic aorta (AS). MIF expression was up-regulated in stable AAA and further intensified in ruptured AAA. The increased aneurysmal MIF expression was paralleled by an enhanced expression of specific MMPs, *viz.* MMP-1, MMP-9, and MMP-12, and by a decrease of their inhibitors. Immunohistochemical analysis of AAA and AS showed MIF protein in endothelial cells, smooth muscle cells (SMCs), macrophages, and T cells. MMP-1 (in SMCs and macrophages) and MMP-9 (in macrophages) were colocalized with MIF at the cellular level in ruptured AAA. The up-regulation of aneurysmal MIF/MMP expression was associated with an increased content of cytotoxic T cells. *Antioxid. Redox Signal.* 7, 1195–1202.

INTRODUCTION

By definition, abdominal aortic aneurysm (AAA) is a localized weakening of the vessel wall that results in the formation of an aneurysmal bulge or lesion. A continuing weakening of the bulge area further dilates the vessel wall and may ultimately lead to the life-threatening event of AAA rupture, which is associated with an overall mortality of 80% (1). The molecular processes underlying initiation, maturation, and eventual rupture of AAA are poorly understood, but it is generally assumed that chronic inflammation and enzymatic degradation of extracellular matrix proteins such as collagen and elastin constitute the most prominent characteristics of AAA, and that macrophages play a key role in AAA development (24, 26). There is increasing evidence that macrophages are important regulators of matrix metalloproteinases (MMPs), which are the predominant extracellular matrix protein-degrading enzymes (4, 20, 23).

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine predominantly released from macrophages and T lymphocytes (15). These cells are now considered to be the main regulators of inflammatory processes in the vessel wall (3, 6, 14, 22). Recent observations indicate that the primary function of MIF is not merely immune cell activation, but that it also regulates cell proliferation, neovascularization, angiogenesis, tumor growth, and apoptosis (summarized in 9, 15).

Interestingly, MIF has also been found to directly regulate MMP expression (17, 18). These observations suggest a potential role for MIF in the pathogenesis of AAA, the more so

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because recent studies showed that serum MIF levels positively correlate with AAA size and expansion rate (19). If elevated circulating MIF levels reflect enhanced MIF expression in the aneurysmal vessel wall, this may lead to increased local MMP expression, and consequently to enhanced degradation of structural matrix proteins, thereby destabilizing the aneurysmal wall and ultimately making it prone to rupture.

In the present study, we addressed the following outstanding issues. Firstly, we sought evidence for the hypothesis that elevated levels of circulating MIF in patients with AAA are the result of increased MIF expression in the aneurysmal lesion. To that end, we analyzed MIF protein levels by western blotting and MIF mRNA levels by real-time polymerase chain reaction (RT-PCR) in stable and ruptured aneurysmal human aortic tissue and, for comparison, in human control aorta and human atherosclerotic lesions. A comparison with atherosclerotic lesions was thought to be relevant, because both atherosclerosis (AS) and AAA are inflammatory diseases with a potential involvement of MIF in their pathogenesis (8, 19, 21). Secondly, we evaluated by RT-PCR whether the putatively increased expression of MIF in AAA was accompanied by enhanced expression of (specific) MMPs. A prerequisite for a direct effect of MIF on MMP expression in aneurysmal tissue requires colocalization or close proximity of the two parameters. Therefore, as a third research question, we performed immunohistochemical analysis of different paraffin-embedded tissues, and assigned the occurrence of MIF and specific MMPs to specific cell types. Finally, in an attempt to assess the relationship between MIF/MMP expression and vascular inflammation, we evaluated the macrophage and T-cell content of the tissues by immunohistochemistry (IHC) and RT-PCR, and used this as a measure of inflammatory state.

MATERIALS AND METHODS

Human tissues

Human aortic samples were obtained in accordance with the guidelines of the Medical Ethical Committee of the Leiden University Medical Center. Nonaneurysmal atherosclerotic aortic wall samples (n = 3) containing advanced type IV–VI lesions and AAA wall samples (n = 4) were obtained during elective surgery. Ruptured AAA wall samples (n = 3) were obtained during emergency surgery because of a rupture of an AAA. As normal (control) aortic wall, tissue samples (n = 8) were obtained from the aortic patch of the renal artery immediately after removal of the donor kidney during a heart-beating organ-donor procedure. A longitudinal section of the antherolateral aortic wall was divided in two parallel strips. One strip was fixed in 4% (vol/vol) formaldehyde and prepared for IHC; the other half was snap-frozen in liquid nitrogen and stored at −70°C until used for nucleic acid extraction.

Immunohistochemistry

Fresh tissue samples were fixed in 4% (vol/vol) formalin and embedded in paraffin. Serial 3 μm sections were prepared on silane-coated slides and incubated at 37°C for 18 h. Sections were deparaffinized in xylene, rehydrated in ethanol, and preincubated for 20 min with 0.3% (vol/vol) hydrogen peroxide in methanol to suppress endogenous peroxidase activity.

To block nonspecific antibody binding, samples were incubated with phosphate-buffered saline (PBS) containing 5% (wt/vol) bovine serum albumin (BSA). Next, sections were incubated with PBS containing 5% (wt/vol) BSA and the appropriate specific primary antibody, *viz.* a mouse anti-MMP-1 antibody (Chemicon, Temecula, CA, U.S.A.), a mouse anti-MMP-2 antibody (Neomarkers, Fremont, CA, U.S.A.), a rabbit anti-MMP-9 antibody (TNO, Leiden, The Netherlands), a smooth muscle cell (SMC)-specific mouse anti-α-actin antibody (DAKO, Glostrup, Denmark), a macrophage-specific mouse anti-CD68 antibody (DAKO), an endothelial cell (EC)-specific mouse anti-CD31 antibody (Novocastra, Newcastle, U.K.), a T cell-specific mouse anti-CD3 antibody (DAKO), and a goat anti-MIF antibody (sc-16965; Santa Cruz, Heerhugowaard, The Netherlands). After an incubation for 1 h with biotin-conjugated horse anti-mouse, -rabbit, or -goat IgG, peroxidase-conjugated avidin-biotin complex solution (DAKO) was applied to all sections according to the manufacturer's instructions. Immune complexes were detected with Nova Red (Vector Laboratories, Burlington, CA, U.S.A.) according to the manufacturer's protocol. Nova Red staining was not observed when only the secondary antibodies were used or when the anti-MIF antibody was preincubated with recombinant MIF (100-fold excess). To visualize the gross vascular structure, serial sections were stained with hematoxylin–phloxine–saffron (HPS), which is more effective than the regular hematoxylin–eosin stain described (11). HPS stains the nuclei blue, the cytoplasm, muscle, and myelin red; and the connective tissue yellow.

Western blotting

Aortic tissue was homogenized and prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis separation essentially as described before (12). Then proteins were blotted onto Immobilon-P polyvinylidene fluoride transfer membranes (Milipore, Bedford, MA, U.S.A.), and blots were blocked with 5% (wt/vol) skim milk powder (Merck, Amsterdam, The Netherlands) diluted in 20 m*M* Tris (pH 7.4), 55 m*M* NaCl, 0.1% (vol/vol) Tween-20. Blots were incubated with a goat anti-MIF antibody (sc-16965) or a goat anti-β-actin antibody (sc-1615), followed by horseradish peroxidase-conjugated preadsorbed secondary antibodies. All antibodies were from Santa Cruz and were diluted in 20 m*M* Tris (pH 7.4), 55 m*M* NaCl, 0.1% (vol/vol) Tween-20, 5% (wt/wt) bovine serum. The Super Signal West Dura Extended Duration Substrate (Pierce, St. Augustin, Germany) and the luminescent image workstation (Roche Diagnostics, Almere, The Netherlands) were used for visualization.

Nucleic acid extraction and RT-PCR

Total RNA extraction was performed using RNAzol (Campro Scientific, Veenendaal, The Netherlands) according to the manufacturer's instructions. cDNA was prepared using avian myeloblastosis virus reverse transcriptase (AMV RT) according to the manufacturer's protocol (Promega, Leiden, The Netherlands). cDNA was amplified by RT-PCR with Taqman universal PCR mastermix (Eurogentec, Seraing, Belgium) using an ABI Prism 7700 (Applied Biosystems, Niewerkerk a/d IJssel, The Netherlands) and quantified using the comparative C_T-method according to the manufacturer's protocol. Probe and primer sets were from Bioscource International

(Camarillo, CA, U.S.A.). Glyceraldehyde-3-phosphate dehydrogenase and α2-macroglobulin were used to normalize the signals.

Statistical analysis

All data are presented as means ± SD. Statistical significance between two groups was analyzed with the Student's *t*-test, and a probability value of $p < 0.05$ is considered statistically significant.

RESULTS

MIF expression in aneurysmal lesions

MIF protein levels were found to be markedly elevated in tissue homogenates of stable AAA when compared with homogenates of control aorta samples (Con) obtained from healthy kidney donors (Fig. 1A). A subsequent mRNA expression analysis underscored this observation and revealed that aortic MIF expression is modestly elevated (1.4-fold, $p < 0.05$) in stable AAA, and further intensified (2.5-fold, $p < 0.05$) in ruptured AAA wall samples. In line with previous findings (8), MIF expression was 1.8-fold ($p < 0.05$) increased in AS. These data demonstrate for the first time that MIF gene expression is up-regulated in stable and, even more so, in ruptured AAA, and thus may explain the reported positive correlation between plasma MIF levels and aneurysmal expansion (19).

mRNA expression of MMPs and their inhibitors, TIMPs, in aneurysmal lesions

We next determined, by RT-PCR, the mRNA expression levels of a large number of MMPs and their inhibitors, tissue inhibitor of metalloproteinases (TIMPs), in stable and ruptured AAA and compared these with AS and Con (Table 1).

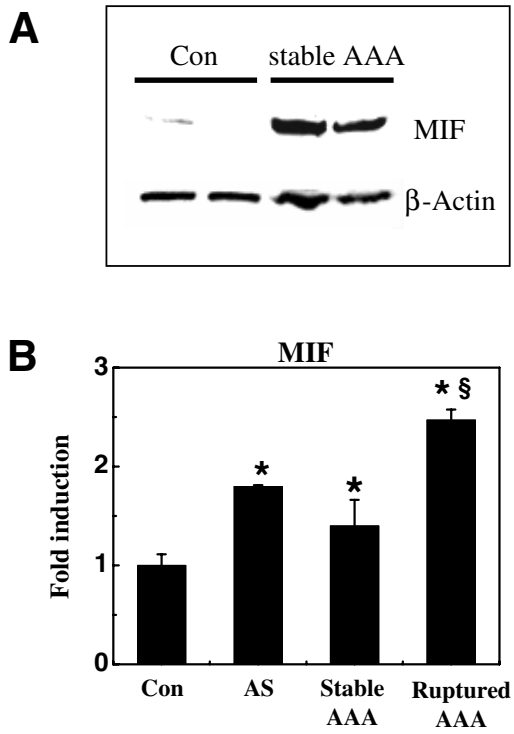

FIG. 1. MIF expression in AAA and AS. (**A**) Western blot analysis of tissue homogenates of control aorta (Con) and stable AAA was executed with an anti-MIF antibody and an anti-β-actin antibody to document equal loading. (**B**) mRNA expression was analyzed in the aortic wall samples of controls ($n = 8$), and patients with AS ($n = 3$), stable AAA ($n = 5$), and ruptured AAA ($n = 3$). The gene expression level of the measured genes in control tissue was set at 1 and relative gene expression levels in diseased tissues are expressed as means ± SD. *$p < 0.05$, statistically significant difference compared with Con; §$p < 0.05$, statistically significant difference between stable AAA and ruptured AAA.

TABLE 1. FOLD DIFFERENCES IN GENE EXPRESSION OF MMPS, TIMPS, AND MIF QUANTIFIED BY RT-PCR

	Control	*Atherosclerosis*	*Stable AAA*	*Ruptured AAA*
MMP-1	1.00 ± 0.21	0.36* ± 0.04	1.92* ± 0.24	4.92*† ± 0.71
MMP-2	1.00 ± 0.23	0.41* ± 0.02	0.52* ± 0.12	0.84† ± 0.05
MMP-3	1.00 ± 0.33	0.07* ± 0.01	11.31* ± 1.94	5.46*† ± 0.72
MMP-7	1.00 ± 0.44	1.03 ± 0.17	0.57* ± 0.21	0.08*† ± 0.02
MMP-8	1.00 ± 0.38	0.53* ± 0.01	0.01* ± 0.01	0.15*† ± 0.01
MMP-9	1.00 ± 0.31	2.65* ± 0.20	15.35* ± 2.19	24.42*† ± 8.01
MMP-12	1.00 ± 0.49	11.12* ± 2.23	6.59* ± 1.22	20.25*† ± 0.88
MMP-13	1.00 ± 0.29	0.61* ± 0.02	0.41* ± 0.03	2.13*† ± 0.30
MMP-14	1.00 ± 0.22	0.97 ± 0.23	0.11* ± 0.02	3.34*† ± 0.18
MMP-16	1.00 ± 0.28	0.93 ± 0.05	3.39* ± 0.48	2.71*† ± 0.55
MMP-17	1.00 ± 0.09	1.29 ± 0.06	9.51* ± 1.26	3.58*† ± 0.33
TIMP-1	1.00 ± 0.55	1.40 ± 0.58	9.78* ± 3.60	3.71† ± 1.49
TIMP-2	1.00 ± 0.11	0.95 ± 0.03	11.08* ± 1.08	2.53*† ± 0.22
TIMP-3	1.00 ± 0.73	0.40* ± 0.01	7.67* ± 5.59	0.38*† ± 0.31

mRNA expression was analyzed in the anterolateral aortic wall of controls ($n = 8$) and patients with AS ($n = 3$), stable AAA ($n = 5$), and ruptured AAA ($n = 3$). The gene expression level of the measured genes in control tissue was set at 1, and relative gene expression levels in diseased tissues are expressed as means ± SD.

*$p < 0.05$, statistically significant difference compared with the control.

†$p < 0.05$, statistically significant difference between stable AAA and ruptured AAA.

The selection of MMPs analyzed was based on their putative involvement in vessel wall degradation, and comprised both collagenases and elastases. Four patterns of MMP expression could be recognized. A first group of MMPs (MMP-1, MMP-9, MMP-12) was elevated in stable AAA and further up-regulated in ruptured AAA. These changes were most marked for MMP-9 and MMP-12 and less pronounced in the case of MMP-1. Interestingly, MMP-9 and MMP-12 were also the only MMPs that were significantly increased in AS and whose expression pattern in the various tissue samples fully paralleled that of MIF (compare Fig. 1B). A second group of MMPs (MMP-3, MMP-16, MMP-17) was highly expressed in stable AAA, but reduced in ruptured AAA, and not significantly changed (MMP-16, MMP-17) or even strongly decreased (MMP-3) in AS. A third group (MMP-13, MMP-14) was down-regulated in stable AAA, but up-regulated in ruptured AAA, and not (MMP-14) or only moderately decreased (MMP-13) in AS. A fourth group (MMP-2, MMP-7, MMP-8) was significantly down-regulated in stable and ruptured AAA and decreased (MMP-2, MMP-8) or not changed (MMP-7) in AS.

Notably, the mRNA expression levels of the MMP inhibitors TIMP-1, TIMP-2, and TIMP-3 were strongly elevated in stable AAA, but had fallen again toward control levels (TIMP-1, TIMP-2) or even below (TIMP-3) in ruptured AAA.

These data indicate that of the various MMPs analyzed, only MMP-9 and MMP-12 and to a lesser extent MMP-1 show the same atherosclerotic and aneurysmal expression pattern as MIF. The decreased TIMP expression in ruptured AAA as compared with stable AAA may contribute to the shift toward enhanced proteolytic activity in the ruptured AAA.

Immunohistochemical analysis of MIF, MMP-1, and MMP-9 expression

Colocalization of MIF and the identified coregulated MMPs (*viz.* MMP-1, MMP-9, and MMP-12) in aortic cells is a prerequisite for a direct role of MIF in the up-regulation of these proteinases. To assign the cell types associated with MIF and MMP occurrence, several series of three consecutive cross-sections were prepared and used for analysis of vascular structure, MIF protein or specific MMP protein, and cellular composition. To that end, a first cross-section was stained with HPS to visualize the gross vascular structure, the second (central) cross-section with an anti-MIF or specific anti-MMP antibody, and the third cross-section with a cell type-specific immunomarker antibody for identification of ECs (EC-specific anti-CD31 surface-antigen staining), SMCs (SMC-specific anti-α-actin staining), macrophages (macrophage-specific anti-CD68 surface-antigen staining), and T cells (T cell-specific anti-CD3 surface antigen staining) (Figs. 2 and 3).

Immunohistochemical analysis showed that in control tissue MIF immunoreactivity (IR) was relatively low and restricted to medial SMCs, whereas in stable AAA MIF-IR was high and localized in ECs of neovascular structures and in SMCs, macrophages, and T cells. MIF expression in ruptured AAA was associated with the same cell types as in stable AAA, but was particularly pronounced in SMCs and in foci of macrophages and T cells. For comparison, MIF-IR in atherosclerotic lesions was observed in ECs, macrophages, and medial SMCs. Of note, all samples of stable AAA showed extensive remodeling of the vasculature and strong neovascularization. In ruptured AAA, the degenerative processes clearly further progressed toward a total abolishment of the vessel wall structure.

Of the group of MMPs that exhibited an aneurysmal expression pattern similar to that of MIF, MMP-1 (a collagenase) and MMP-9 (a gelatinase) were selected for further analysis of their cell type-specific occurrence. MMP-1 was strongly expressed by SMCs and macrophages in both stable and ruptured AAA; a comparably pronounced MMP-1-IR was observed in the same cell types in Con and AS, albeit that the MMP-1 signal in Con was much weaker (Fig. 3A–D). Expression of MMP-9 in stable AAA, ruptured AAA, and AS was strong and fully restricted to macrophages; only a modest macrophage-associated MMP-9 expression was observed in Con (Fig. 3E–H).

Together, these data document that two selected MMPs, *viz.* MMP-1 and MMP-9, with an expression pattern similar to that of MIF are also expressed in the same cell types as MIF in stable AAA, ruptured AAA, and AS.

Coexpression of MIF and MMP-1 and MMP-9 in ruptured AAA

The above findings demonstrate that MIF and MMP-1 and MMP-9 are localized in the same *cell types,* but it remained unclear whether they colocalize in the same *cells*. To investigate their putative colocalization, several series of three consecutive cross-sections of ruptured AAA were prepared. The first cross-section was exposed to an MMP-1-specific antibody, or an MMP-9-specific antibody, the second (central) cross-section to an anti-MIF antibody, and the third cross-section to a cell type-specific immunomarker antibody, as described above (Fig. 4). All MMP-1-expressing SMCs and all MMP-1-expressing macrophages were also MIF-positive (Fig. 4A–C). Likewise, all MMP-9-expressing macrophages were MIF-positive (Fig. 4D–F). These data are in line with a causative role of MIF in the up-regulation of these MMPs. The question remains, however, whether the increased MIF/MMP mRNA levels reflect an increase in MIF/MMP expression per cell or an increase in *number* of MIF/MMP-expressing cells, notably macrophages. To the same token, an abundant infiltration of MIF-expressing T cells could also contribute to and, at least partly, explain increased MIF expression in the aneurysmal wall.

Analysis of the aneurysmal macrophage and T-cell content

To address the above questions, the lesion content of macrophages and T cells, which represent the predominant MIF-expressing infiltrates, was analyzed by IHC and RT-PCR. Figure 5A and B show that macrophages and T cells were sparse in Con, but abundantly present in AS and stable and ruptured AAA. Whereas macrophages were homoge-

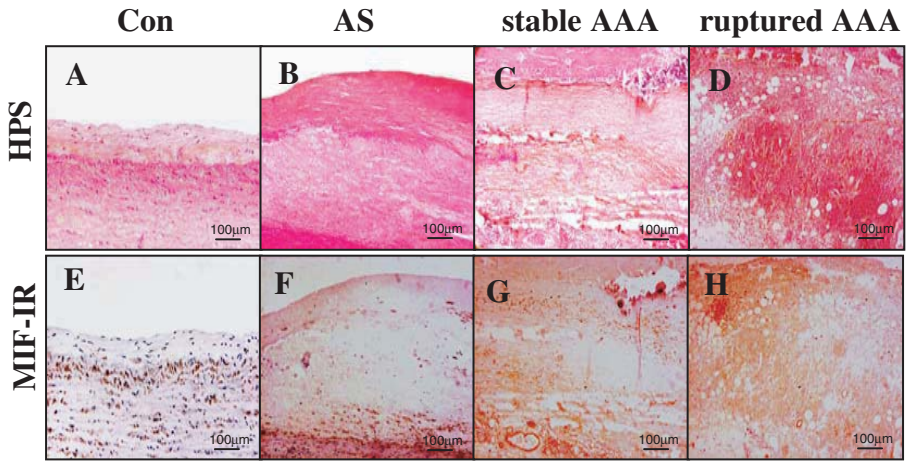

FIG. 2. Immunohistochemical analysis of MIF expression. Representative photomicrographs of HPS staining and MIF-IR in anterolateral aotic wall cross-sections of controls (Con; $n = 8$) (**A** and **E**) and patients with AS ($n = 3$) (**B** and **F**), stable AAA ($n = 5$) (**C** and **G**), and ruptured AAA ($n = 3$) (**D** and **H**). Consecutive cross-sections were stained with a specific anti-MIF antibody, with HPS, or with cell type-specific immunomarkers, *i.e.,* an anti-CD31 antibody for ECs, an anti-α-actin antibody for SMCs, an anti-CD68 antibody for macrophages, and an anti-CD3 antibody for T cells (the staining of immunomarkers is not shown). Bars = 100 μm.

neously distributed throughout the tissues, T cells formed foci, whose number was most markedly increased in ruptured AAA.

To quantify the macrophage and T-cell content, we next determined the mRNA expression level of cell type-specific markers for macrophages (CD68), T_{helper} cells (CD4), and $T_{cytotoxic}$ cells (CD8) (16, 25).

When compared with Con, CD68 mRNA expression was 8.4-fold ($p < 0.05$) and 8.5-fold ($p < 0.05$) increased in stable AAA and AS, respectively, but fell in ruptured AAA (4.5-fold induction; $p < 0.05$) (Fig. 5C). CD4 expression levels were comparably elevated in stable AAA (3.3-fold; $p < 0.05$), ruptured AAA (3.0-fold; $p < 0.05$), and AS (4.3-fold; $p < 0.05$). The expression of CD8 was dramatically increased in stable AAA (26-fold; $p < 0.05$) and even more augmented in ruptured AAA (37-fold; $p < 0.05$), thereby significantly exceeding its expression in AS (11-fold; $p < 0.05$) by far (Fig. 5D). These data suggest that the increased expression levels of MIF, MMP-1, and MMP-9 in (ruptured) AAA are due rather to an elevated expression per cell (notably the macrophage) than to an increase in number of MIF/MMP-expressing cells; in addition, an increase in $T_{cytotoxic}$ cell content, especially in ruptured AAA, is likely to contribute also to increased aneurysmal MIF levels as well.

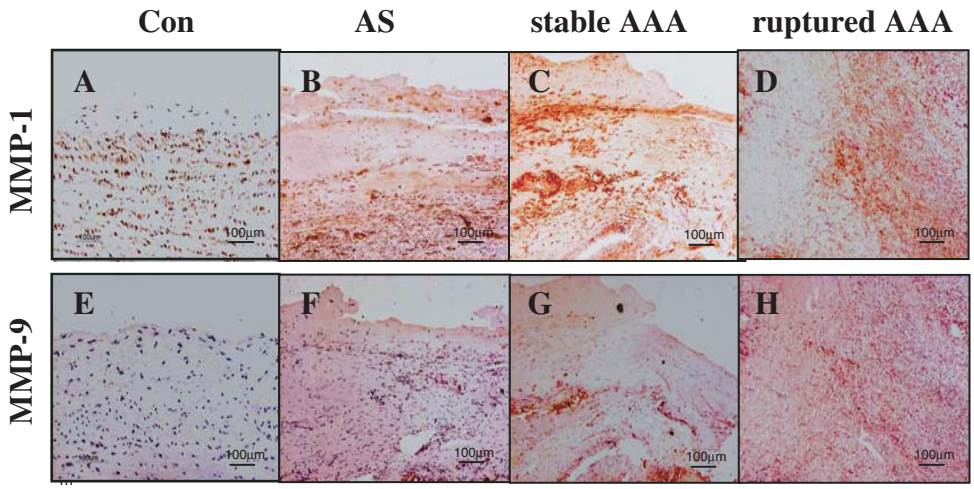

FIG. 3. Immunohistochemical analysis of MMP-1 and MMP-9 expression. MMP-1 expression in control (Con) (**A**), AS (**B**), stable AAA (**C**), and ruptured AAA (**D**) and MMP-9 expression in Con (**E**), AS (**F**), stable AAA (**G**), and ruptured AAA (**H**) were analyzed. Photomicrographs are representative for a total of ($n = 8$) control, ($n = 3$) AS, ($n = 5$) stable AAA, and ($n = 3$) ruptured AAA patients. Consecutive cross-sections were stained with a specific anti-MMP-1 or anti-MMP-9 antibody, with HPS (data not shown), or with cell type-specific immunomarkers, *i.e.,* an anti-CD31 antibody for ECs, an anti-α-actin antibody for SMCs, an anti-CD68 antibody for macrophages, and an anti-CD3 antibody for T cells (staining of immunomarkers not shown). Bars = 100 μm.

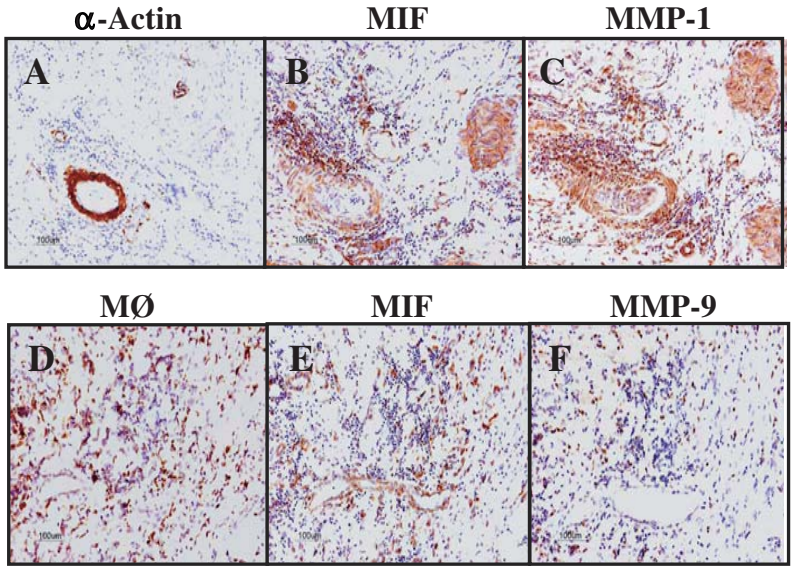

FIG. 4. MIF, MMP-1, and MMP-9 expression in adjacent sections of ruptured AAA. Photomicrographs show MIF-IR and (**B** and **E**), α-actin and CD68-IR (**A** and **D**), and MMP-1 and MMP-9 (**C** and **F**). MMP expression was analyzed in cross-sections adjacent to the cross-sections used for MIF analysis. Photomicrographs are representative for a total of $n = 3$ patients with ruptured AAA.

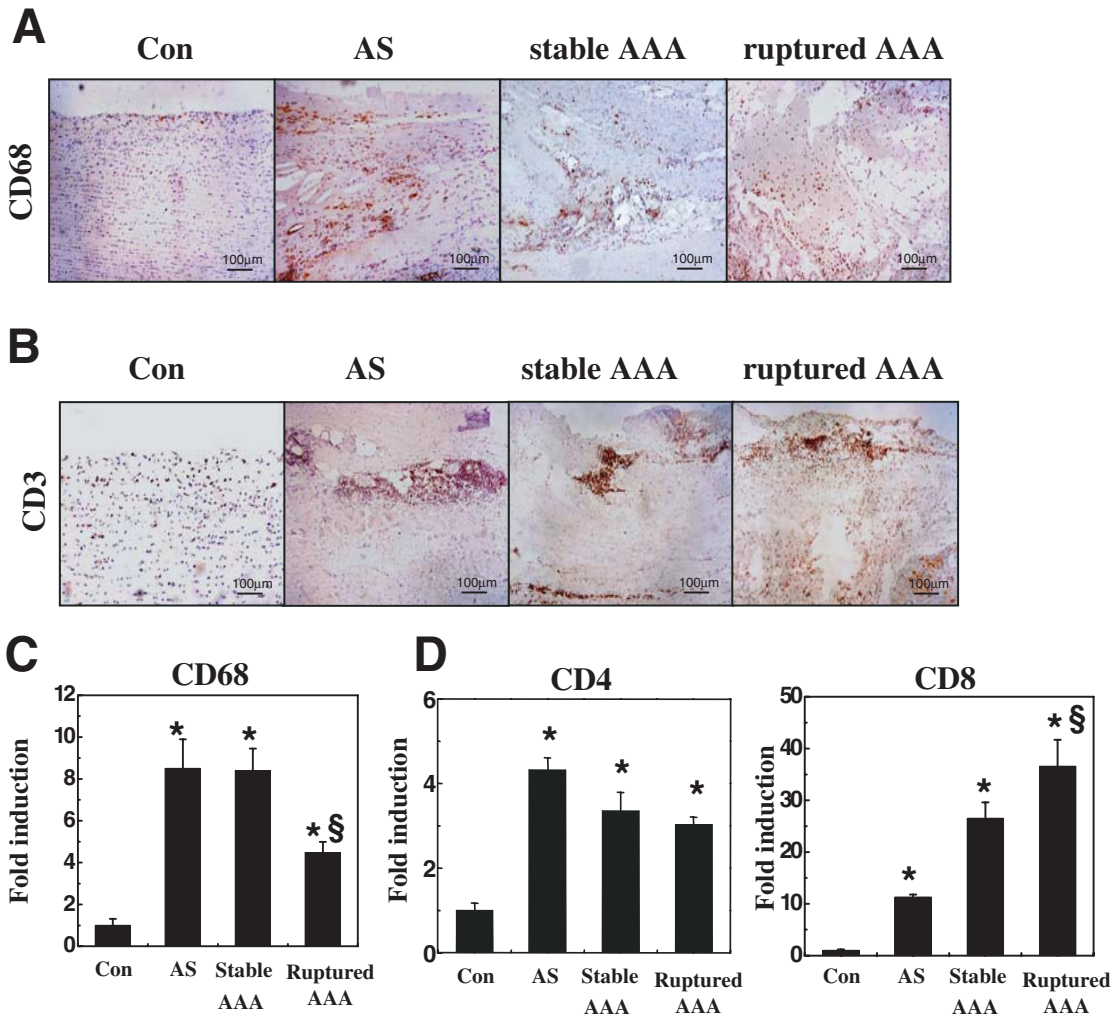

FIG. 5. Increased infiltration of macrophages in AAA determined by IHC and RT-PCR. Cross-sections of anterolateral aortic wall of controls (Con), AS, stable AAA, and ruptured AAA were stained with (**A**) a macrophage surface marker (CD68) or (**B**) a T-cell surface marker (CD3). Photomicrographs are representative for a total of $n = 8$ control, $n = 3$ AS, $n = 5$ stable AAA, and $n = 3$ ruptured AAA specimens. Bars = 100 μm. mRNA expression of (**C**) CD68 or (**D**) T-cell subtype-specific markers (CD4 and CD8) was analyzed in anterolateral aotic wall samples of controls ($n = 8$) and patients with AS ($n = 3$), stable AAA ($n = 5$), and ruptured AAA ($n = 3$) by RT-PCR. Values are expressed as means ± SD. $^{*}p < 0.05$, statistically significant difference compared with the control group; $^{\S}p < 0.05$, statistically significant difference between stable and ruptured AAA.

DISCUSSION

Aneurysmal lesions are regions of arteries that continuously dilate and eventually rupture as a result of extensive breakdown of structural proteins (collagen and elastin) in the arterial wall (1, 4, 20, 23, 24, 26). The molecular processes that contribute to evolution and rupture of AAA are only poorly understood, but probably involve collagenases and elastases of the MMP class. It has been suggested that MIF may contribute to the pathogenesis of the disease because plasma MIF levels positively correlate with aneurysmal size and expansion rate (19).

As demonstrated here for the first time, MIF protein and MIF gene expression are augmented in stable aneurysmal tissue and further increased in ruptured AAA. Immunohistochemical analysis shows that MIF is expressed by vascular ECs, SMCs, macrophages, and T cells. It is likely that MIF, once expressed by these cells in the vessel wall, is secreted (15) and may leak to the circulation, which may explain the reported elevated MIF serum levels in patients with AAA (19).

RT-PCR analysis revealed that the increase in MIF mRNA expression in ruptured AAA as compared with stable AAA is paralleled by an enhanced expression of specific MMPs, notably MMP-1, MMP-9, and MMP-12, all of which are known to contribute to vessel wall matrix degradation and rupture (4, 10, 23). Especially MMP-9 is thought to be the most predominant protease expressed in AAA and causing rupture (23). As MIF can directly induce the expression of MMPs (17, 18), increased aneurysmal MIF levels might thus be directly responsible for the up-regulation of MMPs in AAA. Such a direct effect of MIF on MMP gene expression becomes likely if MIF and MMPs colocalize in the same cells. Immunohistochemical analysis of ruptured aneurysmal tissue for MMP-1 and MMP-9 protein revealed that MIF indeed colocalizes with MMP-1 (in SMCs and macrophages), and with MMP-9 (in macrophages exclusively), findings that are in line with previously reported observations (7, 13, 15). The increase in MIF/MMP expression in (ruptured) AAA without a concomitant increase in number of macrophages, the predominant MIF/MMP-expressing cell type, supports the concept that increased cellular expression of MIF in (activated) macrophages is the crucial factor for stimulated MMP-1 and MMP-9 expression, and thereby for AAA rupture.

Further research should also clarify the contribution of CD8-positive cells ($T_{cytotoxic}$ cells) in this process. The strong increase in $T_{cytotoxic}$ cell number (as deduced from the increased expression of CD8 mRNA) reflects the higher inflammatory state of the vessel wall, and appears to be a discriminating factor between ruptured and stable AAA. Infiltrated vascular $T_{cytotoxic}$ cells could, by producing MIF, indirectly affect MMP expression in neighboring cells such as macrophages (and SMCs). In this respect, it is important to note that AS, an inflammatory process comparable to AAA, is characterized by less $T_{cytotoxic}$ cells, less MIF expression, and limited MMP expression.

Our data demonstrate that the expression of several TIMPs, which are specific inhibitors of MMPs, was strongly up-regulated in stable aneurysmal lesions, which confirms similar observations made for TIMP-1 (5) and TIMP-3 (4) in other human studies. Interestingly, overexpression of TIMP-1 has been shown to prevent the degradation and rupture of AAA (2). The observed concomitant decrease of TIMP-1, TIMP-2, and TIMP-3 expression in ruptured AAA as compared with stable AAA has, to our knowledge, not been reported before, and may be an important factor in the shift of the proteolytic balance relevant for AAA rupture. Of note, TIMP expression was not intensified in AS, which supports the notion that remodeling processes deviate in aneurysmal and atherosclerotic lesions (24).

In conclusion, this study provides a molecular explanation for the relationship between elevated plasma MIF levels and the progression of AAA by demonstrating that aneurysmal MIF expression is augmented and associated with increased levels of matrix protein-degrading MMP-1, MMP-9, and MMP-12. An important, but still elusive, role may also be played by CD8-positive $T_{cytotoxic}$ cells.

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ABBREVIATIONS

AAA, abdominal aortic aneurysm; AS, atherosclerosis; BSA, bovine serum albumin; CD, cluster of differentiation; EC, endothelial cell; HPS, hematoxylin–phloxine–saffron; IHC, immunohistochemistry; IR, immunoreactivity; MIF, macrophage migration inhibitory factor; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; RT-PCR, real-time polymerase chain reaction; SMC, smooth muscle cell; TIMP, tissue inhibitor of metalloproteinases.

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