Overexpression of Tissue Inhibitor of Metalloproteinase 3 in Macrophages Reduces Atherosclerosis in Low-Density Lipoprotein Receptor Knockout Mice

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Objective—Tissue inhibitor of metalloproteinase 3 (TIMP3) is a stromal protein that inhibits the activity of proteases and receptors. TIMP3 is downregulated in metabolic and inflammatory disorders, such as type 2 diabetes mellitus and atherosclerosis, particularly in regions enriched with monocyte/macrophage cells. To investigate the role of TIMP3 in atherosclerosis, we generated a new mouse model in which Timp3 was overexpressed in the atherosclerotic plaque via a macrophage-specific promoter (MacT3). We elucidated any potential antiatherosclerotic effects of TIMP3, including regulation of monocyte/macrophage recruitment within atherosclerotic plaques, in MacT3 mice crossbred with low-density lipoprotein receptor knockout (LDLR−/−) mice.

Methods and Results—MacT3/LDLR−/− mice had an improvement of atherosclerosis and metabolic parameters compared with LDLR−/−. En face aorta and aortic root examination of MacT3/LDLR−/− mice revealed smaller atherosclerotic plaques with features of stability, such as increased collagen content and decreased necrotic core formation. Atherosclerotic plaques in MacT3/LDLR−/− mice contained fewer T cells and macrophages. Furthermore, TIMP3 overexpression in macrophages resulted in reduced oxidative stress signals, as evidenced by lower lipid peroxidation, protein carbonylation, and nitration in atheromas.

Conclusion—Our study confirmed that macrophage-specific overexpression of TIMP3 decreases the inflammatory content and the amplitude of atherosclerotic plaques in mice. (Arterioscler Thromb Vasc Biol. 2012;32:74-81.)

Key Words: atherosclerosis ▪ macrophages ▪ metalloproteinases ▪ inflammation ▪ lipotoxicity
we generated a new mouse model with targeted overexpression of TIMP3 in monocyte/macrophage lineage cells, which results in overexpression of TIMP3 directly at inflammatory sites. This mouse was crossed with the atherosclerosis-prone low-density lipoprotein receptor–null (LDLR−/−) mouse to test the therapeutic role of TIMP3 in atherosclerosis. We confirmed that increased expression of TIMP3 inside the atherosclerotic plaque slows down the progression of vascular damage associated with atherosclerosis.

Methods

Generation of MacT3 Transgenic Mice

The mouse Timp3 gene was amplified by polymerase chain reaction (PCR) using cDNA obtained from mouse muscle as a template. Timp3 cDNA was cloned into a vector containing a CD68 promoter/enhancer and amplified in Escherichia coli strain DH5α. For transgenic mouse generation, the construct was linearized with endonuclease enzymes (EcoRV/StuI) and microinjected into mouse pronuclei by standard methods. Offspring derived from the injections were genotyped by PCR analysis on DNA isolated from tail biopsies performed with primers that amplified a 300-bp fragment of the transgenic construct. PCR results were confirmed by Southern blotting of EcoRV-digested DNA followed by probing of the membrane with the CD68-Timp3 fragment labeled with [α-32P]dCTP. To evaluate hematopoietic cell lineage distribution, peripheral blood was collected from the tail vein and analyzed on a blood cell analyzer (Simply Cell, BPC BioSed, Rome, Italy) to determine cell counts. MacT3 mice used in this study had been backcrossed into the C57BL/6J background for 5 generations. MacT3/LDLR−/− mice were obtained by breeding MacT3 with LDLR−/− mice (Harlan) in a C57BL/6 Background. To induce atherosclerosis, 6-week-old male mice were fed a Western diet (15% fat, 7.4, using a 25-gauge needle. Single-cell suspensions were prepared with RPMI medium containing fetal bovine serum and HEPES, pH 7.4, using a 25-gauge needle. Single-cell suspensions were prepared by repeat pipetting, and the cell preparations were passed through a 70-μm nylon mesh to remove particulate matter. Cells were centrifuged, washed twice in RPMI, and counted. For adoptive transfer experiments, 105 bone marrow cells were depleted of Ly6G+ cells using anti-Ly6G magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Depletion columns (LD) were used according to the MACS separation system protocol provided by the manufacturer (Miltenyi Biotec). Next, Ly6G− cells were incubated with anti-CD11b magnetic beads and Ly6G− CD11b+ cells were obtained using the MACS cell sorting system protocol. Cell purity was confirmed by 2-color flow cytometry analysis of the different cell populations. Antibodies against CD11b (BD Biosciences Pharmingen, San Diego, CA) and Ly6G (Miltenyi Biotec) were used. mRNA was extracted from Ly6G− and Ly6G+ CD11b+ cells, and Timp3 expression was analyzed by real-time PCR. Ly6G− CD11b+ cells were labeled with 5-(and-6)-carboxyfluorescein diacetate (CFDA-SE, Molecular Probes, Carlsbad, CA), and 5×105 cells were resuspended in 200 μL of 0.9% NaCl and then injected retroorbitally into mice that had received a high-cholesterol diet for 16 weeks. Sixty hours after the injection, mice were euthanized by asphyxiation with carbon dioxide. The animals were then gently perfused with 1 mL of heparinized saline via the left ventricle. The base of the heart and the ascending aorta were isolated and formalin-fixed (pH 7.4) for 4 hours, cryoprotected with sucrose, and embedded in OCT blocks. Five serial cryosections (5 μm) spanning 60 μm of the aortic sinus (ie, the aortic portion alongside

Lipoprotein Profiles

To obtain lipoprotein profiles, plasma samples were analyzed by size-exclusion high-performance liquid chromatography on a Superose column (Superose 6PC, Amersham Pharmacia Biotech, Uppsala, Sweden) to separate very-low-density, low-density, and high-density lipoproteins. Total cholesterol levels in the column effluents were continuously measured via in-line mixture with a commercially available enzymatic colorimetric cholesterol detection reagent (Cholesterol TRU, Biomerieux, Marcy l’Etoile, France) followed by downstream spectrophotometric detection of the reaction products at 500 nm absorbance. The first peak of cholesterol eluted from the column was attributed to very-low-density lipoprotein, the second to low-density lipoprotein, and the third to high-density lipoprotein. The area under each peak was calculated using the software provided with the fast protein liquid chromatograph (Chromelone, Dionex, Sunnyvale, CA). To calculate the cholesterol concentration for each lipoprotein fraction, the ratio of the corresponding peak area to total peak area was multiplied by the total cholesterol concentration measured in the sample.

Histology and Quantification of Arterial Lesions

Mice were anesthetized with isoflurane (IsoVet, Schering-Plough, Whitehouse Station, NJ) and bled via cardiac puncture. The heart with the proximal aorta was fixed in 4% paraformaldehyde and embedded in paraffin or OCT blocks for Oil Red O staining. Atherosclerotic lesions were assessed using the method of Paigen et al as previously described. For the en face analysis, the aorta was cut 2 mm distal from the heart, fixed in 4% paraformaldehyde-PBS, opened longitudinally, and pinned flat on a siliconized plate. The en face preparation was then stained with Sudan IV and stored in paraffin. Atherosclerotic lesion area and the total area of the aorta were measured on the dissecting microscope using computer-assisted image analysis software (Optimas 6.5, Bioscan, WA). Immunostaining was performed using antibodies against F4/80, CD3, N-scarboxy methyl lysine, nitrotyrosine, Ki-67, and active caspase-3 (Abcam Inc, Cambridge, MA) following the manufacturer’s instructions.

Migration Analysis of Bone Marrow–Derived Monocytes

Donor mice were euthanized, and their femurs and tibias were removed aseptically. Femur and tibia marrow cavities were flushed with RPMI medium containing fetal bovine serum and HEPES, pH 7.4, using a 25-gauge needle. Single-cell suspensions were prepared by repeat pipetting, and the cell preparations were passed through a 70-μm nylon mesh to remove particulate matter. Cells were centrifuged, washed twice in RPMI, and counted. For adoptive transfer experiments, 106 bone marrow cells were depleted of Ly6G+ cells using anti-Ly6G magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Depletion columns (LD) were used according to the MACS separation system protocol provided by the manufacturer (Miltenyi Biotec). Next, Ly6G− cells were incubated with anti-CD11b magnetic beads and Ly6G− CD11b+ cells were obtained using the MACS cell sorting system protocol. Cell purity was confirmed by 2-color flow cytometry analysis of the different cell populations. Antibodies against CD11b (BD Biosciences Pharmingen, San Diego, CA) and Ly6G (Miltenyi Biotec) were used. mRNA was extracted from Ly6G− and Ly6G+ CD11b+ cells, and Timp3 expression was analyzed by real-time PCR. Ly6G− CD11b+ cells were labeled with 5-(and-6)-carboxyfluorescein diacetate (CFDA-SE, Molecular Probes, Carlsbad, CA), and 5×105 cells were resuspended in 200 μL of 0.9% NaCl and then injected retroorbitally into mice that had received a high-cholesterol diet for 16 weeks. Sixty hours after the injection, mice were euthanized by asphyxiation with carbon dioxide. The animals were then gently perfused with 1 mL of heparinized saline via the left ventricle. The base of the heart and the ascending aorta were isolated and formalin-fixed (pH 7.4) for 4 hours, cryoprotected with sucrose, and embedded in OCT blocks. Five serial cryosections (5 μm) spanning 60 μm of the aortic sinus (ie, the aortic portion alongside
the valves) were cut, stained with anti-Ly-6G- eFluor 625NC antibody (eBioscience, San Diego, CA), and analyzed by fluorescence microscopy (Olympus BX51 equipped with Olympus C-3030 digital camera, Tokyo, Japan). The number of fluorescent leukocytes attached to the intimal surface or to atheromatous plaques was counted in each cross-section of the aortic root. Data were averaged per mouse, and these numbers were used to calculate the mean ± SEM for each group.

For in vitro experiments, $1 \times 10^5$ bEnd.3 cells were cultured on 1.5% gelatin or 4 $\mu$g/cm$^2$ fibronectin-coated Transwell filters for 3 days to allow the formation of confluent monolayers. Following overnight treatment with 20 ng/mL recombinant TNF-α and 200 U/mL mouse interferon-γ (R&D Systems), the inserts were placed into new plates containing 50 ng/mL recombinant mouse monocyte chemoattractant protein-5 (R&D Systems), and $10^5$ CFDA-SE labeled CD11b$^+$ cells were added to endothelial monolayers. After 2 hours at 37°C, the inserts were removed, and the cells that had transmigrated to the bottom of the plates were counted using an inverted microscope.

**Statistical Analysis**
Results of the experimental studies are mean ± SD. Statistical analyses were performed using 1-way ANOVA or the unpaired Student t test as indicated. Values of $P<0.05$ were considered to be statistically significant.

**Results**
To study the protective role of TIMP3 in myeloid cells during the development of atherosclerosis, we generated transgenic...
mice (MacT3) overexpressing TIMP3 under the control of the monocyte/macrophage lineage-specific promoter CD68 (Figure 1A, B). This strategy allowed us to increase TIMP3 expression directly in atherosclerotic plaques where monocytes/macrophages are gradually recruited during disease progression. We found increased expression of TIMP3 at the mRNA level in MacT3 splenocytes and BMDMs compared with the wild-type (WT) mice (Figure 1C). Hematogram results were similar among the littermates of WT and MacT3 with the wild-type (WT) mice (Figure 1D).

To test their physiological function, MacT3 BMDMs and WT BMDMs were stimulated in vitro with lipopolysaccharide.

MacT3 BMDMs showed increased TIMP3 release in the extracellular matrix and enhanced TIMP3 activity compared with WT BMDMs, as assessed by Western blot and reverse zymography, respectively (Figure 1E). Although the basal levels of metalloproteinase activity and TNF-α secretion were not significantly different between WT and MacT3 BMDMs (data not shown), lipopolysaccharide stimulation significantly reduced MacT3 BMDMs responses compared with WT BMDMs (Figure 1F and 1G, respectively). These results demonstrate that MacT3 BMDMs display increased levels of a functional TIMP3 protein when the promoter CD68 is activated.

Next, we investigated whether TIMP3 could reduce the development of atherosclerotic lesions in a mouse model combining genetic predisposition (LDLR−/−) and environmental stress (atherogenic Western diet). To study the infiltration of monocytes into the aortic roots and the extension of atherosclerosis into the aorta surface, MacT3 mice were crossbred with LDLR−/− mice and fed a Western diet. Hematogram results did not differ between LDLR−/− and MacT3/LDLR−/− mice (Supplemental Figure 1, available online at http://atvb.ahajournals.org). After 16 weeks of a Western diet, an analysis of the cholesterol content in circulating lipoproteins revealed that MacT3/LDLR−/− mice had a mild but not significant reduction in total cholesterol and low-density lipoprotein cholesterol compared with LDLR−/− mice (Figure 2A). MacT3/LDLR−/− mice showed a slight but significant reduction in weight and random fed glucose and insulin levels (Figure 2B). Analysis of the expression of inflammatory genes and cytokines in white adipose tissue (WAT) (C) and liver (D). Results are expressed relative to LDLR−/− mice and normalized to expression of 18S rRNA (n = 5 per group, P < 0.05, Student’s t test, data are mean ± SD). LDL indicates low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very-low-density lipoprotein; MCP-1, monocyte chemoattractant protein-1; IL, interleukin.

We then determined whether the rescue effects observed in MacT3/LDLR−/− mice were the result of a diminished recruitment of proinflammatory cells into the aorta. TIMP3 overexpression in macrophages rescued the aortic wall from the accumulation of proinflammatory cells as demonstrated by a reduction of CD3+ and F4/80+ infiltrated cells (Figure 4A). Upregulation of TIMP3 in MacT3/LDLR−/− aortas was coupled with a significant reduction of inflammatory cytokines, such as monocyte chemoattractant protein-1, interleukin-1β, and interleukin-6 (Figure 4B), compared with LDLR−/− aortas. In addition, signs of oxidative stress, such as nitrotyrosine and N-ε-carboxymethyl-lysine, were reduced in MacT3/LDLR−/− mice compared with control mice (Figure 4C).

To evaluate whether the overexpression of TIMP3 changed the cellular composition inside atherosclerotic plaques in vivo, we analyzed markers of cell proliferation and apoptosis. The number of proliferating (Ki67 positive) or apoptotic (caspase-3 positive) endogenous cells in plaque areas did not change, demonstrating that TIMP3 upregulation does not influence atherosclerotic plaque turnover in vivo (Supplemental Figure II). Therefore, we investigated whether the rescue effects observed in MacT3/LDLR−/− mice were partially explained by an impaired migration of MacT3 myeloid cells. We initially performed an in vitro transmigration assay using mouse bEnd.3 endothelial cell monolayers preactivated with proinflammatory cytokines. There was a 2-fold impairment of migration in MacT3 bone marrow–derived CD11b+ cells compared with WT CD11b+ cells, independent of whether gelatin or fibronectin was used for coating (Figure 5A). However, freshly isolated CD11b+ cells, derived from mouse bone marrow, consist of a heterogeneous
population of monocytes/macrophages and granulocytes. Therefore, to investigate the role of monocytes, we obtained a pure population of CD11b<sup>+</sup>/H<sub>11001</sub> cells depleted of neutrophils (CD11b<sup>+</sup>/H<sub>11001</sub> Ly6G<sup>+</sup>/H<sub>11002</sub> cells) (Figure 5B). mRNA analysis confirmed that TIMP3 expression levels were significantly higher in CD11b<sup>+</sup>/H<sub>11001</sub> Ly6G<sup>+</sup>/H<sub>11002</sub> cells than in Ly6G<sup>+</sup>/H<sub>11001</sub> cells (Figure 5C). To investigate in vivo whether the monocyte fraction was the primary mediator of the effects observed in MacT3 mice, we intravenously injected hypercholesterolemic recipient LDLR<sup>+</sup>/H<sub>11002</sub>/H<sub>11002</sub>/H<sub>11002</sub> mice with fluorescently labeled bone marrow–derived CD11b<sup>+</sup>/H<sub>11001</sub> Ly6G<sup>+</sup>/H<sub>11002</sub> cells from donor LDLR<sup>+</sup>/H<sub>11002</sub>/H<sub>11002</sub>/H<sub>11002</sub> or MacT3/LDLR<sup>+</sup>/H<sub>11002</sub>/H<sub>11002</sub>/H<sub>11002</sub> mice. Recipient mice showed no apparent adverse effects, and we isolated aortas 60 hours after injection. Histological sections of the aortic sinuses were analyzed by light and fluorescent microscopy to count the number of labeled monocytes that were adherent or within the atherosclerotic plaques. When injected into recipient LDLR<sup>+</sup>/H<sub>11002</sub>/H<sub>11002</sub>/H<sub>11002</sub> mice, a lower number of fluorescently labeled MacT3/LDLR<sup>+</sup>/H<sub>11002</sub>/H<sub>11002</sub>/H<sub>11002</sub> bone marrow–derived CD11b<sup>+</sup>/H<sub>11001</sub> Ly6G<sup>+</sup> cells infiltrated into the atherosclerotic plaque compared with fluorescently labeled LDLR<sup>+</sup>/H<sub>11002</sub> bone marrow–derived CD11b<sup>+</sup>/H<sub>11001</sub> Ly6G<sup>+</sup> cells (Figure 5D).

**Discussion**

In this study, we analyzed the effects of upregulation of TIMP3 on atherosclerotic plaques in vivo using a transgenic approach. We observed that at the macroscopic level, TIMP3 was able to diminish the lipid deposits in the aorta after 24 weeks of a Western diet. Because lipoprotein levels were not modified by overexpression of TIMP3 in myeloid cells, we hypothesized that increased TIMP3 expression within the atherosclerotic plaques could result in a less severe subendothelial inflammatory infiltrate and diminished macrophage foam cell formation. Reduced TIMP3 expression was associated with reduced accumulation of foam cells in a rabbit model of atherosclerosis, but no functional evidence for a direct role of TIMP3 was provided. More recently, reduced TIMP3 expression was found in a new inflammatory subpopulation of macrophages in the liver. 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effect of TIMP3 on metalloproteinase proteolytic activity. Therefore, we provide functional evidence that overexpression of TIMP3 during atherosclerosis progression stabilizes atherosclerotic plaques in the hypercholesterolemic LDLR-/-/H11002/H11002 mouse model. In a recent study, overexpression of adenovirus encoding TIMP2 but not TIMP1 resulted in regression of atherosclerotic plaques.18 Given the multiple actions exerted by TIMP3 and TIMP2, additional studies are necessary to understand whether the results from this study are due to inhibition of specific proinflammatory proteases or other targets.

When TIMP3 was overexpressed in myeloid cells, the vascular wall was less inflamed and contained a lower number of macrophages, which could inhibit their differentiation into foam cells and their contributing to plaque progression. Our adoptive transfer model suggests that these results may be due to regulation of monocyte recruitment into the atherosclerotic plaque. However, bone marrow–derived CD11b+ cells also include a small fraction of other cell types, such as granulocytes; therefore, despite that they express CD68 at a very low level, we cannot exclude that these cells actively contribute to the observed results.19

TIMP3 also affects cell migration, apoptosis, proliferation, and inflammation.20–24 Overexpression of TIMP3 leads to inhibition of matrix metalloproteinases and induction of apoptotic cell death in a variety of cell types, including smooth muscle cells.23,25–27 However, TIMP3 can also protect cells from apoptosis, and deficiency of TIMP3 induces apoptosis via modulation of matrix metalloproteinase activity.20,28

It was recently reported that TIMP3 can regulate hematopoietic stem cell proliferation in bone marrow.29,30 TIMP3 stimulates hematopoietic stem cell proliferation by causing quiescent hematopoietic stem cells to enter the cell cycle,29 and TIMP3 overexpression results in decreased frequency of B and T lymphocytes and increased frequency of myeloid cells in the blood and bone marrow.30 In our study, MacT3 and MacT3/LDLR-/- hematogram analyses did not reveal

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**Figure 4.** MacT3/low-density lipoprotein receptor knockout (LDLR-/-) mice accumulate significantly lower numbers of macrophages in atherosclerotic lesions compared with LDLR-/- mice. A, Representative sections of aorta, after 16 weeks of a Western diet, analyzed by immunohistochemistry for the presence of inflammatory cells using CD3 or F4/80 antibodies. Quantitative analysis of CD3+ T cells and F4/80+ cells (n=5 per group, *P<0.05, Student t test, data are mean±SD). B, mRNA expression. Results are expressed relative to LDLR-/- mice and normalized to expression of 18S rRNA (n=5 per group, *P<0.05, Student t test, data are mean±SD). C, Representative sections of aorta immunostained with nitrotyrosine or carboxy methyl lysine antibodies (magnification ×250) (n=5 per group, *P<0.05, Student t test, data are mean±SD).
significant differences in the basal state or during the Western diet, possibly because TIMP3 overexpression was restricted to CD68-positive cells and limited to sites of vascular inflammation.

Our in vivo analyses did not reveal any effects of TIMP3 overexpression on proliferation and apoptosis markers in the atherosclerotic plaque as a whole, although we cannot exclude the possibility that TIMP3 regulates these processes in specific cell types during the dynamics of atherosclerotic plaque growth.

We observed a reduction of atherosclerotic lesion development with different techniques at 16 (aortic root analysis) and 24 (en face aorta and in vivo injection of CD11b*Ly6G− cells) weeks after beginning a Western diet. Although our data suggest that TIMP3 overexpression slows the atherosclerotic process at least up until 24 weeks after starting on a Western diet, the reduction in lesion development might not persist at a later time point.

In conclusion, our results reveal that TIMP3 overexpression in monocytes/macrophages reduces the progression of atherosclerosis during a long-term treatment in a genetic model. Whether this effect was only due only TIMP3 expressed by monocytes/macrophages or may result from the interaction of increased TIMP3 with other cell populations must be clarified in future studies.

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Disclosures
None.
References


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Supplement Material

**Supplementary Figure I.** Hematogram analyses demonstrating normal blood cell lineage distributions, including lymphocytes (Lym), monocytes (Mono) and granulocytes (Gran) in MacT3/LDLR^+/− and LDLR^+/− mice fed a Western Diet for 16 weeks (n=3 per group and expressed as mean ± SD).

**Supplementary Figure II.** Representative sections of aortas from LDLR^+/− and MacT3/LDLR^+/− mice after 16 weeks of Western Diet, analyzed by immunohistochemistry for the presence of apoptosis using Anti Active Caspase-3 antibody and proliferation using Anti Ki-67 antibody. Quantitative analysis of positive cells is reported (n = 5 per group, data are mean ± SD).
Supplemental Figure I
Supplemental Figure II