Interleukin-8 Mediates Downregulation of Tissue Inhibitor of Metalloproteinase-1 Expression in Cholesterol-Loaded Human Macrophages

Relevance to Stability of Atherosclerotic Plaque

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Background—The accumulation of macrophage-derived foam cells in atherosclerotic lesions correlates with increased local release of matrix-degrading metalloproteinases (MMPs) and a thin fibrous cap. The activity of these enzymes is controlled by specific tissue inhibitors of metalloproteinases (TIMPs).

Methods and Results—Because oxidized low-density lipoprotein (OxLDL) modulates gene expression, we investigated the effect of these particles on the levels of MMP-1, MMP-3, MMP-9, TIMP-1, and TIMP-2 in the culture media of human monocyte-derived macrophages. OxLDL but not native LDL or high-density lipoprotein reduced the level of TIMP-1 in a dose-dependent manner with maximal effect (60% of control) at \(100\mu\text{g protein/mL}\). In addition, Northern blotting revealed marked reduction in the abundance of TIMP-1 mRNA in OxLDL-treated cells. Evaluation of the effect of oxysterol components of OxLDL on TIMP-1 production revealed that 25-hydroxycholesterol (1 \(\mu\text{g/mL}\)) was the most potent inhibitor (\(\approx 30\%\) of control). Such inhibition was partially mediated by interleukin (IL)-8. Indeed, IL-8 (2.5 ng/mL) induced maximal inhibition of TIMP-1 accumulation (30% of control) in 4 of 6 cell preparations. In addition, the inhibitory effect of OxLDL-treated cells in the presence of an anti–IL-8 neutralizing antibody was partially reversed.

Conclusions—Immunohistochemical analyses of human atherosclerotic plaques revealed the expression of TIMP-1 in some but not all macrophage-rich and IL-8–rich areas. Therefore, IL-8 may play a potential atherogenic role by inhibiting local TIMP-1 expression, thereby leading to an imbalance between MMPs and TIMPs at focal sites in the atherosclerotic plaque. (Circulation. 1999;99:420-426.)

Key Words: atherosclerosis ■ lesions ■ interleukins ■ lipoproteins ■ plaque ■ metalloproteinases tissue inhibitor

Most sudden deaths from acute myocardial infarction are caused by rupture of coronary atheroma, particularly at sites of thinning of the fibrous cap of the lesion; such rupture leads to hemorrhage into the plaque, thereby resulting in a prothrombotic response followed by rapid occlusion of the artery.' The accumulation of macrophage-derived foam cells in vulnerable shoulder regions of atherosclerotic plaques correlates with increased local release of matrix-degrading metalloproteinases (MMPs) and weak fibrous cap tissue.' These findings suggest a potential role of macrophage-derived MMPs in the weakening and ultimate rupture of plaque structure.' Indeed, Henney et al' localized stromelysin mRNA to areas enriched in macrophages in human atherosclerotic plaques; Galis et al' colocated immunoreactive interstitial collag enase (MMP-1), the 72-kDa gelatinase (MMP-2), stromelysin-1 (MMP-3), and the 92-kDa gelatinase (MMP-9) to lesional macrophages. In addition, freshly isolated rabbit aortic macrophage foam cells express immunoreactive MMP-3, MMP-1, and MMP-9 activity.' Together, the proteolytic capacity of the MMPs facilitates degradation of extracellular components present within the fibrous areas of atheromatous plaques. Regulation of MMP activity occurs at 3 levels: The first level is gene transcription.' MMPs are secreted in a latent zymogen form and the conversion of the proenzyme into an active form represents the second level of MMP regulation.' Finally, the activity of MMPs can be controlled by specific tissue inhibitors of metalloproteinases (TIMPs). Four TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been reported to date.' These inhibitors can be produced by different cell types including several of those that participate in the inflammatory response;
among them, human monocyte-derived macrophages (HMDM) can produce TIMP-1 and TIMP-2.14,15

The expression of TIMP genes is distinct. The TIMP-1 gene is highly inducible at the transcriptional level in response to several cytokines and hormones.16,17 Equally, TIMP-3 expression is modulated by several stimuli, including modulation by cell cycle regulation.18 In contrast, TIMP-2 gene expression is largely constitutive,19,20 whereas the regulation of the expression of TIMP-4 is not yet established.13

In addition, cholesterol-loading macrophages induce expression of tissue factor,21 monocyte chemoattractant protein-1,1,22 15-lipoxygenase,23 undefined matrix metalloproteinases,24 and other genes.25 We have presently examined the effect of cholesterol loading on the secretion of MMP-1, MMP-3, MMP-9, TIMP-1, and TIMP-2 by HMDM. Our data indicate that release of TIMP-1 by cholesterol-loaded macrophages is selectively reduced in the absence of an effect on other secreted MMPs or TIMPs. Interleukin (IL)-8 partially accounts for such inhibition. In addition, immunohistochemical studies demonstrate the presence of distinct areas of human atherosclerotic plaques in which macrophages stained for all 3 antigens and in others in which macrophages stained for TIMP-1 and CD68 or for IL-8 and CD68.

Methods

Purification and Modification of Lipoproteins

LDL and HDL were isolated from fresh human normolipidemic sera by sequential preparative ultracentrifugation and dialyzed as described earlier.26,27 Protein concentration was determined by the method of Lowry et al.28 Copper-oxidized LDL (OxLDL) was prepared by incubating 500 μg LDL protein per milliliter in PBS containing 2.5 μmol/L CuCl2 for 48 hours at 37°C.27 Acetylated LDL (AcLDL) was prepared according to the procedure of Basu et al.29 Cholesterol and oxysterols were purchased from Sigma. Endotoxin contamination of lipoproteins and of all culture materials and reagents used were measured with the Limulus amebocyte lysate assay (Kabi Vitrum Diagnostica), and only those free of endotoxin contamination were analyzed.

Isolation of HMDM

Monocytes were isolated from the blood of healthy, normolipidemic volunteers (thrombopheresis residues) and cultured as previously described.26 Cells between 10 and 14 days of culture were positive for the lymphocyte specific marker, CD3 (Dako), as visualized by a streptavidin-biotin immunophosphatase procedure. Monoclonal antibody CD68 (KP1 clone, Dako) was used at 1:500 dilution for macrophage identification. Tissue samples corresponding to a full-thickness resection of the common carotid artery were collected from patients undergoing surgical treatment for atherosclerotic lesions in the Department of Vascular Surgery (Pitié-Salpêtrière Hospital, Paris). After resection, specimens were dissected to preserve representative atherosclerotic wall on which the specific antibody had been replaced by 50 μg/mL final) in PBS-Tween 1% and nonimmune horse serum and then with purified primary antibody (anti-mouse IgG) labeled with peroxidase and exposed to x-ray film; band intensity was estimated by densitometric scanning.

Western Blot Analysis

Cell supernatants were dialyzed against 0.01 mol/L NH4HCO3, lyophilized, and dissolved in 20 mmol/L Tris-HCl, 1% SDS, and 1 mol/L EDTA. Equal amounts of protein (5 μg) were loaded onto SDS-PAGE. Samples (20 μg final) were electrophoresed in Tris-glycine-SDS buffer (pH 8.3) for 90 minutes at 30 mA. Electrophoretic transfer was performed in Tris-glycine-methanol buffer for 90 minutes at 90 V. Nitrocellulose paper was incubated in PBS-Tween-milk (5%) for 30 minutes, washed in PBS-Tween, then incubated overnight at 4°C with each purified monoclonal antibody (2 μg/mL final) in PBS-Tween-milk (1%). The nitrocellulose was rinsed and incubated 10 minutes) with a second antibody (anti-mouse IgG) labeled with peroxidase and exposed to x-ray film; band intensity was estimated by densitometric scanning.

ELISA Procedure

The MMP-1, MMP-3, MMP-9, TIMP-1, and TIMP-2 contents in HMDM culture media were estimated by the ELISA technique (Amersham).

Immunohistochemistry

For immunohistochemistry, the avidin-biotin complex–immunoperoxidase technique was used. Tissue sections were incubated with nonimmune horse serum and then with purified primary antibodies. These were visualized with biotinylated rabbit anti-mouse IgG and a streptavidin-biotin immunoperoxidase procedure with fast red as the chromogen (Dako). Monoclonal antibody CD68 (KP1 clone, Dako) was used at 1:500 dilution for macrophage identification. Monoclonal antibodies of IgG1 isotype to human: TIMP-1 (clone 102D1) and TIMP-2 (clone T2 to 101) (Neomarkers) and IL-8 (a gift from Dr J.M. Cavaillon, Institut Pasteur, Paris) were used, respectively, at 8, 20, and 36 μg/mL. Sections of the vessel wall on which the specific antibody had been replaced by 50 μg/mL of purified murine IgG1 (Dako) served as negative controls.

Statistical Analysis

Wilcoxon test was used for comparison of the amounts of TIMP-1 produced by nonstimulated (control) and by stimulated macrophages (Figures 4, 5, and 6) and for comparison between cells treated with OxLDL and OxLDL plus anti–IL-8 (Figure 7).

Results

Effect of Cellular Cholesterol Loading on Production of MMPs and TIMPs by HMDM

To determine whether cellular cholesterol accumulation might influence the release of MMPs and TIMPs, HMDM were exposed to 100 μg OxLDL protein for 48 hours in serum-free medium. These conditions result in substantial
elevation in the cellular content of both free cholesterol and cholesteryl ester. Western blot analysis of concentrated supernatants for the 92-kDa gelatinase (MMP-9), stromelysin (MMP-3), TIMP-1, and TIMP-2 revealed that only TIMP-1 levels were affected by OxLDL (Figure 1). However, MMP-1 antigen was undetectable, even after 10-fold concentration of cell medium. The Western blot is not quantitative; estimation of MMPs including MMP-1 and TIMPs by ELISA showed that only TIMP-1 levels were reduced by OxLDL (data shown only for TIMP-1). In addition, the activities of 92-kDa gelatinase and MMP-3 were detectable by zymography, and no influence of cellular cholesterol loading on secretion of these MMPs or on their conversion from the latent to the active form was observed (not shown).

Dose-response studies to explore the relation between OxLDL treatment and reduction of TIMP-1 concentration revealed that TIMP-1 protein content in the medium decreased with increasing OxLDL concentration (Figure 2). In 2 different cell preparations, the maximal effect was observed at 100 μg protein/mL, representing ~60% reduction as compared with control cells. Similarly, Northern blotting analysis showed a reduction in the abundance of TIMP-1 mRNA in OxLDL-treated versus control cells (Figure 3). TIMP-1 levels were equally reduced by AcLDL, whereas native LDL, HDL, and dextran sulfate were without effect (Figure 4). Because both OxLDL and atherosclerotic plaques have been shown to contain large amounts of several biologically-active oxysterols,27,33,34 we investigated the effect of specific oxysterols on TIMP-1 secretion. All oxysterols tested induced minor reduction in TIMP-1 concentrations in HMDM culture medium (~10%); by contrast, 25-hydroxycholesterol and 5,6-epoxycholesterol (1 μg/mL) were more potent (~30% diminution) (Figure 5).

Figure 1. Western blot analysis of secreted MMPs and TIMPs by HMDM. Cells cultured for 10 days were washed and incubated in serum-free medium in the presence or absence of OxLDL (100 μg protein/mL) for 48 hours. Supernatants were dialyzed and concentrated as detailed in "Methods." An aliquot (5 μg protein) of each cell supernatant was loaded onto 10% SDS-PAGE, electrophoresed, and transferred to nitrocellulose paper. A purified monoclonal antibody (1 μg/mL) against each MMP or TIMP was added separately to each nitrocellulose membrane. Membranes were rinsed and incubated with a second antibody (anti-mouse IgG) labeled with peroxidase and exposed to x-ray film. In the Western blot for TIMP-2, we observed, in addition to the specific band of 21 kDa, an additional and nonidentified band of ~110 kDa (data not shown).

Figure 2. Dose-response curve of effect of OxLDL on TIMP-1 production by HMDM. HMDM cultured for 10 days were washed and incubated in serum-free medium in the presence of various concentrations of OxLDL for 48 hours at 37°C. Supernatants were removed, centrifuged once to remove any nonadherent cells, and the concentrations of TIMP-1 determined with an ELISA kit. Values are mean ± SD of 2 different cell preparations. Each cell preparation was derived from a single donor.

Figure 3. Northern blot analysis of TIMP-1 mRNA from unstimulated HMDM (control) or HMDM exposed to OxLDL. HMDM cultured for 12 days were washed twice with PBS and incubated in the presence or absence of OxLDL (100 μg protein/mL) for ~48 hours. Total RNA was extracted from the cells by the guanidine isothiocyanate method; samples of 20 μg were electrophoresed and blotted onto a nylon membrane. Northern blot was hybridized with a 32P-labeled human TIMP-1 cDNA probe. Because the signal was weak, the membrane was exposed, between 2 intensifying screens, to x-ray film for several days. The same blot was stripped and rehybridized with 32P-labeled human β-actin cDNA and exposed to x-ray film for a short period.

Figure 4. Effect of lipoproteins and dextran sulfate on TIMP-1 production by HMDM. HMDM were cultured for 12 days, washed, and exposed to LDL (100 μg protein/mL), HDL (300 μg protein/mL), OxLDL (100 μg protein/mL), or dextran sulfate (DS) (10 μg/mL) for 20 hours at 37°C in serum-free medium. Supernatants were removed, centrifuged once, and TIMP-1 concentrations were determined with an ELISA kit. Results are mean ± SD of 3 separate experiments (**P < 0.01). Each experiment was performed in duplicate. The 100% control corresponds to 127 pg/μg cell protein of TIMP-1.
Effect of IL-8 on Production of TIMP-1 by HMDM

AcLDL, OxLDL, and oxysterols are potent stimulants of IL-8 production by macrophages. We therefore assessed the effect of IL-8 on the expression of TIMP-1. IL-8 inhibited TIMP-1 production in HMDM in 4 different macrophage preparations of a total of 6. The maximal effect (30% relative to control) was observed at 2.5 ng/mL at 20 hours of incubation (Figure 6) and was partially reversible when HMDM stimulated with OxLDL were exposed to anti–IL-8 neutralizing antibody. The maximum effect of this antibody was observed at 1:1000 dilution (Figure 7).

Immunostaining of Human Carotid Atherosclerotic Plaques

Macrophage-derived foam cells, strongly stained with anti–CD68, were consistently seen in the intima but were absent from the media (Figure 8) and were either isolated or grouped focally. Expression of TIMP-1 and IL-8 was observed in CD68-rich areas (Figure 8); however, staining for these antigens was limited to specific cells. Interestingly, several macrophages were positive for IL-8 but not for TIMP-1 (Figure 9). We were unable to demonstrate any significant
Native LDL particles, however, were without oxysterol species that are subsequently released into the activity. Equally, macrophages may transform cholesterol to the possibility that oxidative modification of AcLDL by macrophages might give rise to proatherogenic lipids. In addition, we cannot exclude the case of AcLDL, oxysterols might be generated during degradation of the HDL particle. TIMP-1 mRNA and protein production.

We presently demonstrate, for the first time, that OxLDL and electron-dense, chemically-modified LDL (AcLDL) but not native LDL or HDL selectively reduce the production of TIMP-1 by cultured HMDM. Such inhibition was dependent on cholesterol loading, since AcLDL analogues such as dextran sulfate did not influence TIMP-1 levels. OxLDL has been detected in plasma and isolated from atherosclerotic dextran sulfate did not influence TIMP-1 levels. OxLDL has been detected in plasma and isolated from atherosclerotic plaque tissue in humans. Therefore, use of OxLDL in our experimental system is of immediate pathophysiological relevance. Taken together, these findings indicate that cholesterol loading of macrophages by scavenger receptor–mediated endocytosis of OxLDL induces a marked reduction in TIMP-1 mRNA and protein production.

OxLDL contains numerous oxysterols, which may account for the low levels of TIMP-1 in cell media. In the case of AcLDL, oxysterols might be generated during AcLDL preparation. In addition, we cannot exclude the possibility that oxidative modification of AcLDL by macrophages generates oxysterols that may, in turn, exert biological activity. Equally, macrophages may transform cholesterol to oxysterol species that are subsequently released into the medium. Native LDL particles, however, were without effect when incubated for 20 hours with macrophages. The oxidative modification of LDL that may occur under such conditions apparently does not allow recognition and uptake by the scavenger receptor. However, when macrophages were stimulated with native LDL for 48 hours, a weak inhibitory effect on the production of TIMP-1 was observed, thereby suggesting the formation of a minor fraction of highly oxidized LDL that was recognized by scavenger receptors and induced cellular oxysterol accumulation.

The possibility that uptake of oxysterols, present as components of either OxLDL or plaque-derived macrophage foam cells, might induce inhibition of TIMP-1 secretion was evaluated. We exposed HMDM to concentrations of oxysterols similar to that in arterial foam cells (1 μg/mL). A minor and nonsignificant suppression (5% to 10% relative to control) of macrophage TIMP-1 protein secretion after incubation with 7-hydroxycholesterol, cholestane-3β,5α,6β-triol, 7-ketocholesterol, and cholesterol was found. However, 25-hydroxycholesterol and 5,6-epoxycholesterol decreased TIMP-1 levels by 30%. Since some oxysterols have been reported to be cytotoxic, cell viability was measured but revealed no variation between control and stimulated cells.

Expression of the HMG-CoA reductase and LDL receptor genes can be modulated by certain oxysterols, such as 25-hydroxycholesterol. A sterol-regulatory element (SRE) present in the promoter of these genes is involved in their negative regulation in response to cellular sterol levels. However, the human lipoprotein lipase (LPL) gene, which is also negatively regulated by oxysterols, does not contain an SRE, at least not in the promoter region up to −1718 bp relative to the transcriptional start site. Also, sequence analysis of the human TIMP-1 gene promoter region up to −1730 bp has failed to reveal the existence of a SRE. However, such an SRE could be located in a region that remains to be identified; alternatively, sterol loading may not exert a direct effect on the TIMP-1 gene.

IL-8 is a proinflammatory polypeptide produced by a variety of cell types, including monocyte/macrophages and T-lymphocytes. The expression of IL-8 is induced not only by several inflammatory agents but also by either AcLDL- or OxLDL-mediated cholesterol loading of macrophages; equally, IL-8 is expressed by macrophage foam cells in human atheroma. In addition, oxysterols, particularly 25-hydroxycholesterol, stimulate IL-8 production by macrophages. IL-8 production by macrophages presents a proatherogenic factor. The potential atherogenic role of IL-8 appears not only as a prothrombotic but also as a potential proatherogenic factor. The potential atherogenic role of IL-8 may be due, in part, to the diminution of the level of TIMP-1, thereby leading to an imbalance between the activities of MMPs and this inhibitor. Indeed, some macrophages in

**Discussion**

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Our data indicate that 25-hydroxycholesterol and 5,6-epoxycholesterol, which are present in atherosclerotic lesions, may play an important role in the regulation of cytokine secretion and in other macrophage functions. We (present study) and others have shown that IL-8 is expressed in macrophage-rich areas of atherosclerotic lesions. In addition, IL-8 has been reported to increase monocyte procoagulant activity by increasing mRNA, protein content, and surface expression of tissue factor. Taken together, IL-8 appears not only as a proinflammatory but also as a potential proatherogenic factor. The potential atherogenic role of IL-8 may be due, in part, to the diminution of the level of TIMP-1, thereby leading to an imbalance between the activities of MMPs and this inhibitor. Indeed, some macrophages in
CD68-rich areas of human atherosclerotic plaques stained positively for IL-8 but not for TIMP-1. A local shift of the proteolytic balance toward protease activities may therefore be responsible for local extracellular degradation in atherosclerotic plaques and for their ultimate rupture.

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