Macrophage foam cells from experimental atheroma constituently produce matrix-degrading proteinases

ZORINA S. GALIS, GALINA K. SUKHOVA, ROGER KRANZHOFER, STEPHEN CLARK, AND PETER LIBBY

Vascular Medicine and Atherosclerosis Unit, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115

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ABSTRACT Monocyte-derived foam cells figure prominently in rupture-prone regions of atherosclerotic plaques. Peripheral blood monocytes in culture can produce certain enzymes that degrade extracellular matrix, known as matrix metalloproteinases (MMPs). Lipid-laden macrophages may thus contribute to weakening of extracellular matrix of rupture-prone atherosclerotic plaques. However, the spectrum and regulation of MMP production by foam cells remain unknown. To investigate this issue, we isolated lipid-laden macrophages from rabbit aortic lesions produced by a combination of hypercholesterolemia and balloon injury. Freshly isolated aortic macrophage foam cells, identified using cell-specific antibodies, contained immunoreactive stromelysin and interstitial collagenase, whereas alveolar macrophages isolated from the lungs of same rabbits did not. Macrophages from both tissue sources released gelatinolytic activity consistent with the 92-kDa gelatinase. In vitro, lipid-laden aortic macrophages, but not alveolar macrophages, synthesized de novo and released immunoprecipitable stromelysin and collagenase, with or without stimulation by phorbol ester or bacterial lipopolysaccharide. These stimuli caused foam cells to release additional gelatinolytic activity that migrated faster than a purified preparation of 92-kDa gelatinase in substrate-containing polyacrylamide gels, indicating activation of the 92-kDa gelatinase or induction of the 72-kDa gelatinase. Our results show that lipid-laden macrophages elaborate MMPs capable of degrading the major constituents of vascular extracellular matrix even without further stimulation. Therefore, these cells may contribute to remodeling of the extracellular matrix during atherosclerosis and to the disruption of plaques often responsible for acute clinical manifestations of atherosclerosis.

Rupture of coronary atheroma precipitates most acute myocardial infarctions (1). These plaque ruptures occur predictably at sites of thinning of the lesion's fibrous cap, areas frequently associated with accumulations of monocyte-derived macrophages (2–4), an important cellular component of the atherosclerotic plaques (5–8). Under certain conditions, mononuclear phagocytes can produce enzymes of the matrix metalloproteinase (MMP) family that are capable of degrading components of the extracellular matrix (9, 10). Thus, monocyte-derived foam cells may be the sources of matrix-degrading activity that may contribute to lability of the fibrous cap (2). However, the macrophages present in atherosclerotic plaques differ from the mononuclear phagocytes previously studied in relation to MMP production (11). Notably, lesional macrophages contain substantial amounts of cholesterol esters (12, 13) and assume a foamy appearance, hence the name "foam cells." Whether this special type of mononuclear phagocyte retains the ability to produce MMPs is unknown. Henney et al. (14) localized stromelysin mRNA in areas enriched in macrophages in some of the human atherosclerotic lesions they studied but did not explore the expression of stromelysin protein or of other MMP family members. In human atherosclerotic tissue, we have colocalized immunoreactive interstitial collagenase (MMP-1), the 72-kDa gelatinase (MMP-2), stromelysin (MMP-3), and the 92-kDa gelatinase (MMP-9) with lesional macrophages (15). Yet, it is not clear whether these cells actually produce these enzymes or whether they have phagocytosed MMPs derived from other sources. In light of the potential importance of matrix degradation in plaque pathophysiology, we undertook to establish whether macrophage-derived foam cells produced MMPs. For this purpose, we studied foam cells isolated from atherosclerosis-like lesions produced experimentally in rabbits (16). These cells contain much higher levels of cholesterol than those achievable by usual in vitro loading techniques (13) and likely reflect the functions of lesional macrophages more faithfully than foam cells produced in culture.

MATERIALS AND METHODS

Reagents. Cell types were identified using anti-muscle actin monoclonal antibody HHF-35 (Enzo Diagnostics) and anti-rabbit macrophage monoclonal antibody RAM-11 (Dako). Sheep polyclonal antibodies raised against rabbit interstitial collagenase (or MMP-1) (17) and sheep polyclonal antibodies against human stromelysin (or MMP-3) cross-reacting with rabbit MMP-3 were generously provided by Constance Brinckerhoff (Darmouth Medical School, Hanover, NH). Affinity-purified donkey antimouse antibodies conjugated to fluorescein or anti-sheep antibodies conjugated to rhodamine were from Jackson ImmunoResearch. Phorbol 12-myristate 13-acetate (PMA) and Escherichia coli lipopolysaccharide (LPS) serotype 055:B5 were from Sigma.

Lesion Induction. Ten New Zealand male rabbits (2–3 kg, body weight) were divided into two groups for convenience and handled identically during the two experiments. Aortic macrophages were obtained from rabbits treated to produce aortic lesions rich in macrophage-derived foam cells (13, 16) by using a modification of procedures as described (13, 18, 19). Rabbits consumed a diet supplemented with 0.5% cholesterol and 4.5% (vol/vol) coconut oil. After 1 week, aortic injury was performed under aseptic conditions in rabbits anesthetized with ketamine (35 mg/kg)/xylazine (7 mg/kg), in accordance to a protocol approved by the Standing Committee on Animals of Harvard Medical Area. A 4F Fogarty balloon catheter was introduced through the iliac artery up to the ascending aorta, then inflated with 2 ml of saline, and withdrawn down to the level of the bifurcation. The maneuver was repeated three times. The animals were allowed to recover and consumed the same atherogenic diet for another 12 weeks. Serum cholesterol was measured at the beginning and end of 13 weeks of the diet.

Macrophage Isolation. Rabbit aortae were harvested en bloc under sterile conditions and kept in ice-cold Dulbecco's modified Eagle's medium (DMEM, BioWhittaker) during

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Abbreviations: LPS, lipopolysaccharide; MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate.
processing. Circumferential specimens of the aorta were reserved for immunocytochemical study. The remainder of the aorta was opened longitudinally and the endothelium was removed by several passages of sterilized cotton swabs. The intimal layer was peeled off and several small pieces were reserved to monitor the morphology by microscopy. The balance of the intimal tissue was weighed and minced into 1-mm² pieces by using a McIlwain tissue chopper (Brinkmann). Tissue was then digested for 1 h with collagenase type I (Worthington; 450 units/ml)/elastase (4.7 units/ml)/soybean trypsin inhibitor (Sigma; 1.0 mg/ml)/2 mM CaCl₂ in Hank's balanced salt solution [HBSS; 10 ml/1.0 g (wet weight) of intima]. The dissociated cells were filtered through sterile nylon and undigested tissue was incubated with a fresh enzymatic mixture. The procedure was repeated four times, and the filtrates obtained from each rabbit aorta were pooled separately and fractionated with a step gradient (30%/10%) of metrizamide (13) (Sigma) in HBSS. After a 15-min centrifugation at 1200 × g, foam cells were collected from a band forced close to the top of the gradient and washed repeatedly by HBSS. Aliquots of cells resuspended in HBSS were used for cell counting and for cytological preparations. The remaining cells were resuspended and plated in Opti-MEM (GIBCO/BRL) supplemented with 0.5% fetal calf serum.

Rabbit Alveolar Macrophages. Alveolar macrophages were obtained by alveolar lavage performed on the same rabbits used to isolate aortic macrophages. The trachea was cannulated with sterile plastic tubing (diameter, 4 mm) fitted to a 30-ml syringe and lungs were perfused twice with 25 ml of sterile saline at 37°C. The two aspirates were pooled and centrifuged at 514 × g for 5 min. A portion of the alveolar macrophages resuspended in HBSS was used for cytological preparations. The rest of the cells were plated in Opti-MEM supplemented with 0.5% fetal calf serum.

In Vitro Experiments. After isolation, aortic macrophage foam cells or alveolar macrophages were incubated overnight in Opti-MEM containing 0.5% fetal calf serum. Subsequently, cells were kept for 24 h in serum-free medium and then stimulated for the following 24 h with PMA (50 ng/ml) or LPS (10 μg/ml). Culture medium was collected, and cells were processed for immunocytochemistry or metabolically labeled for 24 h with [³⁵S]methionine and [³⁵S]cysteine in methionine/cysteine-free DMEM (50 μCi/ml; DuPont/NEN; 1 Ci = 37 GBq). Newly synthesized radiolabeled proteins were immunoprecipitated from culture medium or cell lysate obtained using lysis buffer [PBS (10 mM sodium phosphate, pH 7.2/150 mM sodium chloride)/1% Triton X-100/0.1% SDS/0.5% sodium deoxycholate/0.2% sodium azide].

Gelatin Zymography. Gelatinolytic activity in cell culture medium or in extracts of cultured cells was visualized by zymography after SDS/PAGE in gels containing gelatin (1 mg/ml) (20).

Immunoprecipitation. Antibodies raised against stromelysin (MMP-3) or interstitial collagenase (MMP-1) and protein A-Sepharose (Sigma) were incubated at room temperature for 2 h with culture medium or cell lysates from metabolically labeled cells (17). Protein A-Sepharose was washed with three changes of lysis buffer and then once with PBS. Immunoprecipitated proteins were eluted from protein A-Sepharose by heating at 65°C in SDS/PAGE sample buffer. De novo-synthesized proteins in the immunoprecipitates were detected by fluorography of the dried gels (20).

Immunoblot Analysis. Proteins were transferred from minigels onto nitrocellulose by using a semi-dry blotting apparatus (Bio-Rad). Antigens were detected by chemiluminescence by the manufacturer’s instructions (DuPont/NEN). Blocking of nonspecific binding and dilution of the primary and secondary antibodies was in 5% (wt/vol) dry defatted milk/PBS/0.1% Tween 20.

Tissue Sections. Sections were obtained from tissue embedded in OCT (Miles) and frozen in isopentane cooled with liquid nitrogen or fixed in 4% (vol/vol) formaldehyde and embedded in paraffin.

Isolated Cell Specimens. Freshly isolated cells were used for cytological preparations. Aliquots of macrophages resuspended in HBSS were applied to poly(L-lysine)-covered glass slides by centrifugation for 4 min at 700 rpm in a Cytospin centrifuge (Shandon, Pittsburgh), then fixed for 5 min in acetone at −20°C, air-dried, and kept at −20°C until immunostaining. Macrophages cultivated on 8-chamber glass slides (Nunc) were incubated at the end of the stimulation experiment for an additional 3 h with 1 mM menadione to prevent MMP secretion (21), then washed with PBS (37°C), fixed with acetone at −20°C, and kept at −20°C until staining.

Immunocytochemistry. Isolated macrophages were stained by single/double immunofluorescence or by an immunoperoxidase-based method. For double immunofluorescence, cell specimens were treated with 10% (vol/vol) horse serum (HyClone)/1% Triton X-100/PBS and then incubated for 1 h with a mixture of primary antibodies. After washing with PBS, specimens were incubated with a mixture of secondary antibodies coupled to fluorochromes. Nuclei were stained with bisbenzimide (Calbiochem) at 0.5 μg/ml in PBS for 2 min. Staining of rabbit aortic tissue was performed on frozen sections or paraffin sections after deparaffinization with xylene and ethanol. Tissue sections were preincubated with 0.3% hydrogen peroxide in PBS for reduction of endogenous peroxidase activity and then incubated for 60 min at room temperature with primary antibodies diluted in PBS/10% horse serum (HyClone). After washing in PBS and then in 100 mM Tris HCl/150 mM NaCl/2% horse serum, biotinylated secondary antibodies were applied, followed by avidin–biotin peroxidase complexes (Vector Laboratories) and 3-amin-9-ethylcarbazole (Sigma). Sections were counter stained with Gill’s hematoxylin (Sigma). Some specimens were also processed with nonimmune serum as controls.

RESULTS

Lipid-Laden Macrophages Contain Several MMPs. Aortae of rabbits injured by balloon-withdrawal and fed an atherogenic diet developed oil red O-positive lipid-rich intimal lesions (data not shown), as described (13, 16). Immunoactive MMP-1 and MMP-3 colocalized with smooth muscle cells in the fibrous cap and with macrophage-derived foam cells, which predominate in this type of experimental lesions (Fig. 1). Indeed, all foam cells freshly isolated from the intima of these lesions (Fig. 2) and identified immunocytochemically as derived from macrophages (Fig. 3 Left Inset) also stained for interstitial collagenase and stromelysin (Fig. 3). In contrast, alveolar macrophages freshly isolated from the lungs of same animals did not stain for these two MMPs (data not shown). Immunostaining of macrophage foam cells and alveolar macrophages cultured for 2 days on glass slides under basal conditions gave similar results; i.e., lipid-laden macrophages derived from rabbit aorta stained for collagenase and stromelysin (data not shown), whereas alveolar macrophages did not (Fig. 4). However, stimulation with PMA or LPS induced expression of these two MMPs in alveolar macrophages (Fig. 4 Insets). Culture medium of macrophage-derived foam cells either stimulated or exposed to PMA or LPS also contained stromelysin, as detected by Western blot analysis (data not shown). Zymography showed gelatinolytic activity in the culture medium conditioned by alveolar or aortic macrophages (data not shown). The principal gelatinase released by both alveolar macrophages and aortic foam cells had electrophoretic mobility similar to the 92-kDa gelatinase (MMP-9). Additional activity released basally by foam cells or elicited by LPS or PMA stimulation in alveolar macrophages migrated.
faster than MMP-9 and could represent either proteolytically activated MMP-9 or the 72-kDa gelatinase (MMP-2). Species-specific reagents to unambiguously distinguish these two MMPs in rabbits are currently unavailable.

**Macrophage-Derived Foam Cells Produce and Release Interstitial Collagenase (MMP-1) and Stromelysin (MMP-3) Constitutively.** Macrophage-derived foam cells appeared, therefore, to contain MMP-1 and MMP-3 by immunocytochemistry. However, macrophages are "professional" phagocytes, and to evaluate whether macrophages actually synthesize, rather than ingest MMPs from an extracellular source, we investigated whether isolated macrophages can actually produce MMPs de novo. Immunoprecipitation revealed metabolically labeled MMP-1 and MMP-3 released in the culture medium by unstimulated macrophage-derived foam cells (Fig. 5). The double bands in the immunoprecipitates may indicate secretion of both glycosylated and unglycosylated forms of these MMPs (22). Alternatively, they could represent the zymogen and the proteolytically activated forms of the enzymes. LPS or PMA stimulation of foam cells did not appear to affect the synthesis or release of these MMPs. By using alveolar macrophage-conditioned culture medium containing comparable amounts of newly synthesized total protein, anti-MMP-3 antibodies did not immunoprecipitate any labeled protein from unstimulated or stimulated cells. However, the anti-MMP-1 immunoprecipitates of alveolar macrophage culture medium showed faint radiolabeled bands (Fig. 5).

**DISCUSSION**

Both smooth muscle cell- and macrophage-derived foam cells express stromelysin mRNA and immunoreactive MMP in human atherosclerotic lesions (14, 23). The present study investigated the protein expression of interstitial collagenase and stromelysin in a purified preparation of macrophage-
derived foam cells isolated from experimental atheroma. We found that lipid-laden macrophages produced and released MMP-1 and MMP-3 in culture without addition of stimuli, demonstrating constitutive secretion of these enzymes. Immunocytochemical detection of these enzymes within cultured foam cells required incubation with monensin to block MMP secretion (data not shown). In contrast to macrophage foam cells, we found that alveolar macrophages only displayed immunoreactive collagenase orstromelysin after in vitro stimulation (e.g., with LPS or PMA). Likewise under basal conditions, the culture medium of aortic macrophages, but not those of alveolar macrophages, contained newly synthesized collagenase and stromelysin. Thus, unstimulated lipid-loaded macrophages actually produced more MMPs than did alveolar macrophages, another type of resident phagocyte considered more versatile than other mononuclear phagocytes in regard to their MMP production (10, 11).

We considered the possibility that conditions used for dissociation or for culturing induced MMP expression in the isolated macrophage foam cells ex vivo. Immunolocalization of MMPs in foam cells in situ (Fig. 1) and lack of MMP expression in a control preparation of alveolar macrophages subjected to the same experimental manipulation, including incubation with the enzyme mixture and centrifugation in metrizamide, argue against such an artifact due to the isolation procedure. Likewise, expression of MMPs in situ and in freshly isolated macrophage foam cells, but not in alveolar macrophages maintained in identical culture medium, indicates that endotoxin contamination did not account for MMP production by foam cells.

What endogenous signals might thus explain the constitutive expression of MMPs by lesion-derived foam cells but not by alveolar macrophages? Human macrophages at sites of plaque rupture exhibit signs of activation, such as expression of HLA-DR antigen (4), probably induced by interferon γ derived from lesional T cells (24, 25). In addition to activation of macrophages, this lymphokine may aggravate weakening of the lesion's fibrous cap by inhibiting smooth muscle proliferation (26, 27) and collagen synthesis (28). In turn, lipid-laden macrophages of human and experimental atherosclerotic lesions can generate reactive oxygen radicals and oxidize low density lipoprotein (13, 29). Oxidatively modified low density lipoprotein, etc.

**Fig. 3.** Immunofluorescent staining of Cytospin preparation of macrophage-derived foam cells shows that cells freshly isolated from rabbit aorta contain immunoreactive stromelysin (MMP-3) and interstitial collagenase (MMP-1). (Left) Double immunofluorescence for detection of stromelysin (red fluorescence) and identification of cell-type with RAM-11 macrophage-specific antibody (green fluorescence). (Inset) The same field of a double-stained specimen is shown at lower magnification. (Bars = 50 μm.) Nuclei were counter stained with bisbenzimide (blue fluorescence). (Right) Immunodetection of interstitial collagenase. (Inset) Control. Macrophage foam cell preparation was incubated with nonimmune serum (NIS). (Magnifications are as in Left.)

**Fig. 4.** Immunofluorescent staining of alveolar macrophages. (Left) Stromelysin (MMP-3). (Right) Interstitial collagenase (MMP-1). These MMPs were not detectable in cells kept under basal conditions (large images), but in vitro stimulation of cells with LPS (+ LPS) enabled the detection of immunoreactive stromelysin and interstitial collagenase (Insets). Similar results were obtained using a peroxidase-based method of detection (data not shown). The difference in immunofluorescent staining between macrophages from the two tissue sources was observed for all paired preparations processed and photographed under the same conditions (n = 5). (Bar = 50 μm.) Note the smaller size of alveolar macrophages compared with macrophage foam cells shown in Fig. 3.
FIG. 5. Macrophage-derived foam cells constitute release newly synthesized stromelysin (SL) and collagenase (CL). Anti-stromelysin or anti-collagenase antibodies were used to immunoprecipitate these MMP from metabolically labeled culture medium (CM) from unstimulated (−) or LPS- or PMA-stimulated rabbit macrophage (Mf) foam cells or alveolar macrophages. The two macrophage types were isolated from the same rabbits and simultaneously processed. Similar amounts of radio-labeled total protein (trichloroacetic acid-precipitable counts) in the culture medium obtained from different cell culture conditions were used for immunoprecipitation. Arrows indicate the position of migration of immunoprecipitated proteins relative to molecular mass markers.

lipoprotein, also present in atherosclerotic lesions (29, 30), may stimulate macrophages. Other possible inducers of MMP expression include tumor necrosis factor α, interleukin 1, or macrophage colony-stimulating factor (10), cytokines expressed at higher levels in atheroma than in normal arteries (31–34). Whatever the in vivo stimulus, continuous secretion of MMPs by lipid-laden macrophages may provide an ongoing source of matrix-degrading activity within the lesions. Foam cells may also express MMP inhibitors that limit the activity of MMPs, but lack of species-appropriate reagents presently hinders testing this possibility.

Macrophages are sparse in the normal arterial wall but abound in atherosclerotic lesions. Matrix degradation in the shoulders of the plaques, regions that are rich in macrophages and subjected to high circumferential stress (35, 36), may render these areas particularly vulnerable to rupture. Human vascular smooth muscle cells, another major cellular component of the atherosclerotic lesions, probably also contribute to connective tissue degradation since these cells can produce a range of MMPs that as a group digest the main components of the vascular matrix (20). However, smooth muscle cells also produce the bulk of the vascular extracellular matrix (37, 38). Matrix breakdown could thus predominate in areas of macrophage accumulation, compared to lesion areas occupied mainly by vascular smooth muscle cells. Demonstration of constitutive production of matrix-degrading enzymes by lesion-derived lipid-laden macrophages provides a biochemical mechanism by which these cells may contribute to the focal weakening of atherosclerotic tissue at sites of plaque rupture (2, 4), the cause of the majority of acute myocardial infarctions.

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