Cysteine Protease Cathepsin F Is Expressed in Human Atherosclerotic Lesions, Is Secreted by Cultured Macrophages, and Modifies Low Density Lipoprotein Particles in Vitro*

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During atherogenesis, low density lipoprotein (LDL) particles in the arterial intima become modified and fuse to form extracellular lipid droplets. Proteolytic modification of apolipoprotein (apo) B-100 may be one mechanism of droplet formation from LDL. Here we studied whether the newly described acid protease cathepsin F can generate LDL-derived lipid droplets in vitro. Treatment of LDL particles with human recombinant cathepsin F leads to extensive degradation of apoB-100, which, as determined by rate zonal flotation, electron microscopy, and NMR spectroscopy, triggered both aggregation and fusion of the LDL particles. Two other acid cysteine proteases, cathepsins S and K, which have been shown to be present in the arterial intima, were also capable of degrading apoB-100, albeit less efficiently. Cathepsin F treatment resulted also in enhanced retention of LDL to human arterial proteoglycans in vitro. Cultured monocyte-derived macrophages were found to secrete active cathepsin F. In addition, similarly with cathepsins S and K, cathepsin F was found to be localized mainly within the macrophage-rich areas of the human coronary atherosclerotic plaques. These results suggest that proteolytic modification of LDL by cathepsin F may be one mechanism leading to the extracellular accumulation of LDL-derived lipid droplets within the proteoglycan-rich extracellular matrix of the arterial intima during atherogenesis.

During atherogenesis, lipid droplets accumulate extracellularly within the inner layer of the arterial wall, the intima. Initially, the droplets accumulate subendothelially (1). These droplets, which appear to be derived from low density lipoprotein (LDL)1 particles, are entrapped by the arterial extracellular matrix, especially by its proteoglycans (2). During atherogenesis, proteoglycans form an organized tight network (3) that has the potential to bind apolipoprotein (apo) B-100-containing lipoproteins, notably LDL particles (4–7). Binding of LDL by proteoglycans increases their residence time in the arterial intima and renders the particles more susceptible to various types of modifications, which leads to increased binding strength and accumulation of the LDL-derived cholesterol in the arterial intima (2). The importance of the initial LDL-proteoglycan interaction has been directly assessed with the use of transgenic mice expressing proteoglycan binding-deficient human apoB-100 (8). Thus, despite the accompanying hypercholesterolemia, the binding-deficient LDL caused delayed atherosclerosis as compared with that in control mice expressing normal human apoB-100 (9).

The apoB-100 in the LDL particles isolated from the human atherosclerotic arterial intima is fragmented to variable degrees (10–15). Moreover, when compared with LDL in plasma, arterial lipid droplets have a reduced protein content and contain no immunoreactive apoB-100 (16), and LDL particles, when deposited in human atherosclerotic lesions, lose their apoB-100 immunoreactivity (17), suggesting that apoB-100 in the arterial intima is subjected to proteolytic degradation. Indeed, proteolysis of apoB-100 in vitro has been shown to induce fusion of LDL particles into lipid droplets that resemble those found in atherosclerotic lesions (18–21). On the basis of the above findings, we have proposed that proteolytic modification of LDL particles may be one mechanism leading to LDL fusion and the appearance of the typical extracellular lipid droplets in the arterial intima (2). However, only certain neutral proteases have been shown to be able to trigger aggregation and fusion of LDL particles, these proteases having in common the ability to cause extensive cleavage of apoB-100, i.e. degradation into small peptide fragments, some of which are released from the LDL particles (21). Cultured monocyte-derived macrophages (22, 23) and smooth muscle cells, when stimulated with proinflammatory cytokines (24), have been shown to secrete lysosomal papain-like cysteine proteases. Normally, these cysteine proteases play a major role in intracellular protein degradation and turnover in lysosomes, but they are also capable of degrading proteins extracellularly (25). By degrading the components of the arterial extracellular matrix, the secreted lysosomal cysteine proteases could contribute to the development of atherosclerotic lesions. Indeed, when human arteries were examined for the presence of two cysteine proteases, cathepsins S and K, normal...
arterial segments were found to contain little or none, whereas atherosclerotic lesions contained abundant immunoreactive cathepsins S and K (24). Moreover, cystatin C, a natural extra-cellular cysteine protease inhibitor, was found to be down-regulated in the lesions (26). In addition, atherosclerotic mouse models have provided further support for the view that cysteine proteases play a role in the pathobiology of the arterial wall. Thus, the expression of cathepsins L, K, and S, was found to be increased in an LDL-E-deficient mice (27) and deficiency of cathepsin S was shown to reduce atherosclerosis in LDL receptor-deficient mice (28).

We have now examined the possible role of a newly described lysosomal cysteine protease, cathepsin F (29), in human atherosclerosis and examined the ability of cathepsins to generate lipid droplets from LDL particles. We have tested the effects of human recombinant cathepsins F, S, and K on LDL particles, notably on the degradation of apoB-100, on the aggregation and fusion of LDL particles, and on the retention of LDL particles by human aortic proteoglycans in vitro. We have also followed the expression and secretion of cathepsin F in cultured human monocyte-derived macrophages. Finally, we have also looked for the presence of cathepsin F in normal and atherosclerotic human coronary arteries and compared its localization with that of cathepsins S and K.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin, diamobenzidine, dermatan sulfate, heparan sulfate, and hyaluronan were purchased from Sigma. Chondroitin 4-sulfate and chondroitin 6-sulfate were obtained from Seikagaku Kogyo (Tokyo, Japan). [1,2-3H]Cholesteryl linoleate, r-Butoxy carbonyl l-t[35S]methionine N-hydroxysuccinimidy ester (the 35S labeling reagent), Hitrap SP columns, protein A-Sepharose, and the nucleotides were from Amersham Biosciences (Uppsala, Sweden). In addition, a rabbit polyclonal anti-cathepsin F antibody was generated (mature cathepsin F protein produced in Denmark). A rabbit polyclonal antibody against human recombinant muscle actin (HHF35) and horseradish peroxidase-conjugated anti-methyl green were from Vector Laboratories (Burlingame, CA), and nucleotides were from Amersham Biosciences (Uppsala, Sweden). Cystatin C was from Calbiochem (San Diego, CA). Vectorstar ABC kits were from Vector Laboratories (Burlingame, CA), and anti-human CD68 (PG-M1), anti-human macrophage (HAM56), anti-muscle actin (HHF35) and horseradish peroxidase-conjugated anti-mouse IgG (P0447) antibodies were obtained from Dako (Glostrup, Denmark). A rabbit polyclonal antibody against human recombinant cathepsin F was generated (mature cathepsin F protein produced in Escherichia coli).

In addition, a rabbit polyclonal anti-cathepsin F antibody (SC-13987) from Santa Cruz Biotechnology (Santa Cruz, CA) and a mouse monoclonal anti-cathepsin F antibody from Novacora Laboratories (United Kingdom) were used. Anti-cystatin C antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Alexa-conjugated isotype-specific goat anti-mouse IgG antibodies and DAPI were from Molecular Probes (Leiden, The Netherlands). Microtiter plates (Combi-plate 8, Enhanced Binding) were from Labsystems (Helsinki, Finland). Falcon 12-well cell culturing plates from Becton Dickinson (NJ) and 25-cm2 cell culturing bottles from Naige Nunc International. Dulbecco’s modified Eagle’s medium was purchased from BioWhittaker Europe (Verviers, Belgium), nitrocellulose filters (Transblot Transfer Medium) from Bio-Rad, and Vivaspin concentrators from Vivascience (Hannover, Germany). t-Glutamine, macrophage-SFM medium, Moloney murine leukemia virus reverse transcriptase kit, nonessential amino acids, penicillin-streptomycin, random primers, RPMI 1640, sodium pyruvate, and Ultrapure Agarose were from Invitrogen (Paisley, Scotland). Tsq polymerase, RNase inhibitor, and the lactate dehydrogenase kit were from Roche (Basel, Switzerland), RNeasy minikit and D Nebraska from Qiagen (Hilden, Germany), and human GM-CSF (Leuconax) from Schering-Plough. Cholesteryl ester transfer protein was a kind gift from Drs. Christian Ehnholt and Matti Jauhiainen at the National Public Health Institute, Helsinki, Finland.

Prelabeling of LDL—Human LDL (d = 1.019–1.050 g/ml) was isolated from the plasma of fasting healthy volunteers by sequential ultracentrifugation in the presence of 3 mM EDTA (30, 31).

35S-Bolton-Hunter-LDL was prepared by labeling the protein component of the lipoproteins with a 35S labeling reagent by the Bolton-Hunter procedure (32), as described previously (18). [3H]Cholesteryl linoleate-LDL was prepared by incubating a suspension of LDL and cholesteryl ester transfer protein with solid dispersions of [3H]cholesteryl linoleate on Celite, as described (21). In each experiment, the labeled lipoproteins were mixed with unlabeled lipoproteins. The amounts of LDL are expressed in terms of their protein concentrations, which were determined by the method of Lowry et al. (33) with bovine serum albumin as standard.

**Cathepsins F, S, and K**—Human cathepsin F was produced by the Pichia expression system and purified using a HiTrap SP column, as previously described (29). Human cathepsins K and S were expressed as Pichia pastoris (34) and in SJ9 cells using the baculovirus expression system (35), respectively. Molar concentrations of active cathepsins K and S were obtained by titration with E64 (36), and that of cathepsin F with the irreversible inhibitor, LHVS using the same method as described for E64 (kindly provided by Celera Corp, South San Francisco, CA).

Treatment of LDL with Cathepsins F, S, and K—LDL (0.5 mg/ml) was incubated with 20–100 nM human recombinant cathepsin F, S, or K, respectively, at 37 °C for 20 hr. After 0 (20 mM MES, 150 mM NaCl, 2.5 mM EDTA, 1 mM DTT, pH 6.0) or at 77 °C for the times indicated. When the effect of LDL degradation was studied, the incubations were carried out in either buffer A (20 mM MES, 150 mM NaCl, 2.5 mM EDTA, 1 mM DTT, pH 6.5 or 7.0, or 20 mM Hepes, 150 mM NaCl, 2.5 mM EDTA, 1 mM DTT, pH 7.5. In some experiments, the degradation assays were carried out in the presence of various glycosaminoglycans or proteoglycans. In control samples, LDL was incubated in the absence of proteolytic enzymes.

**Analysis of Proteolyzed LDL**—The degree of proteolytic degradation was determined by measuring the amount of trichloroacetic acid-soluble radioactivity produced (20). The degree of aggregation and/or fusion of proteolyzed [3H]cholesteryl linoleate-labeled LDL was determined by rate zonal ultracentrifugation (26) as described previously (38). Briefly, a linear NaBr gradient (d = 1.096–1.10 g/ml) was layered on top of 50-mL samples of modified [3H]cholesteryl linoleate-LDL in 250 μl of 40% NaBr (w/v) and centrifuged at 33,000 rpm in a SW 40 Ti rotor (Beckman) for 1 h at 20 °C. The gradient was then divided into 500-μl fractions, and the radioactivities were determined using a scintillation counter.

**1H NMR Spectroscopy**—For 1H NMR spectroscopy measurements, LDL samples were prepared at 1 mg/ml concentration. The samples were incubated at 37 °C in the NMR spectrometer in buffer A in the presence and absence (control LDL) of 100 nM cathepsin F, K, or S during data acquisition for 24 h. During the initial 3 h, a spectrum was recorded every 10 min, and subsequently once every 1 h. The spectral width was set to 7008 Hz, comprising 32,000 points yielding a free induction decay of 2.34 s. The recycle delay was 6.4 s. Data were zero-filled eight times and Fourier-transformed. Sodium 3-trimethylsilyl[2,2,3,3-D4]propionate (8 mM) and MnSO4 (0.6 mM), in 99.8% D2O, in a thin coaxial capillary were used as an external chemical shift reference. All the spectra were obtained with a 600-MHz Varian Inova NMR spectrometer at the Institute for Biotechnology NMR Laboratory (Helsinki, Finland).

**Electron Microscopy of LDL**—For thin-section transmission electron microscopy, LDL samples were cast in agarose, and then fixed (39), and stained with the osmium-tinum-acid-paraphenylenediamine technique (40). For negative staining electron microscopy, samples (3 μl) were cast on carbon-coated grids which 3 μl of 2% (w/v) uranyl acetate, pH 7.4, was added and also dried on the grids (41). The samples were viewed and photographed in a JEOL 1200EX electron microscope at the Institute for Biotechnology, Department of Electron Microscopy (Helsinki, Finland).

**Preparation and Characterization of Aortic Proteoglycans**—Proteoglycans from the intima media of human aortas obtained at autopsy within 24 h of accidental death were prepared essentially by the method of Hurt-Camejo et al. (42), as described previously (43). Glycosaminoglycans were determined by the method of Bartold and Page (44), and the amounts of the proteoglycans are expressed in terms of their glycosaminoglycan content.

**Preparation of LDL to Proteoglycans in a Microtiter Well Assay**—The wells in polystyrene 96-well plates were coated with human aortic proteoglycans (50 μg/ml) or with BSA (5 mg/ml) and blocked as described (38). 35S/3H-LDL (10 μg) was incubated with or without recombinant cathepsins F, K, or S (20 nm) in reaction buffer (buffer A containing 1% BSA) for 16 h at 37 °C. The supernatants were removed, and the radioactivity bound to the wells was measured. Specific binding to the proteoglycans was calculated by subtracting the amount of LDL bound to the BSA-coated wells from the amount of LDL bound to the proteoglycan-coated wells.

**Preparation of Macrophage Monolayers**—Human monocytes were isolated from buffy coats (kind gifts from the Finnish Red Cross Blood Transfusion Center, Helsinki, Finland) by centrifugation in Ficoll-Paque gradient as described (45). Washed cells were suspended in...
Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin, counted, and seeded in bottles (25 × 10^6 cells/25 cm²) for Western blotting or in 12-well plates (4 × 10^6 cells/well) for mRNA analysis. After 1 h, nonadherent cells were removed and the medium was replaced with macrophase-SFM supplemented with penicillin-streptomycin and GM-CSF (11 ng/ml).

**mRNA Analysis**—Monocyte-macrophages were cultured for up to 15 days from various time points the total RNA was isolated using a RNeasy minikit (Qiagen) in the presence of DNase. For the isolation, the cells from three different donors were pooled. Total RNA was reverse transcribed into cDNA using a Moloney murine leukemia virus reverse transcriptase kit (Invitrogen) in the presence of an RNase inhibitor. The cDNA obtained was further amplified by PCR using specific nucleotides for cathepsin F: 5'-CAG AGG AGG (sense) and 5'-TAG TCA TCC TTC GTC TCC AGC (antisense) and conditions were 40 cycles, Tc 58 °C. GAPDH-PCR was used for quality control. Primers for GAPDH were 5'-ACC AGC GTA CAT GCC ATC AC (sense) and 5'-TCC ACC ACC CTG CTG TA (antisense). Conditions used were 25 cycles, Tc 58 °C. The PCR products were separated on a 1.4% agarose gel, stained with ethidium bromide, and quantified with a Gel Doc 2000 gel documentation system.

**Western Blot Analysis of Monocyte-Macrophage Media and Lysates**—Monocyte-macrophages were cultured for up to 13 days, and at various time points the medium was replaced with RPMI 1640 supplemented with penicillin-streptomycin and t-glutamine (2 mM). The cells were further cultured for 2 days, after which the media were collected. Lactate dehydrogenase activity in the media and in the cells was measured from parallel incubations using a commercial kit. The level of lactate dehydrogenase activity in the media varied between 5 and 10% of the total cellular activity and did not increase during the 15-day culture period. For each sample, media from three different donors were pooled. Nonadherent cells were removed from the media by centrifugation, protease inhibitors (1 mM PMSF, 2 mM benzamidine, 5 mM EDTA) were added, and the samples were concentrated into 1/20 using Vivaspin concentrators. 20 μl of reducing SDS-PAGE buffer (0.25 mM Tris-HCl, pH 6.8, 4% SDS, 0.02% bromphenol blue, 40% glycerol, 1% β-mercaptoethanol) was added to 20 μl of the sample. Whole cell lysates were prepared by lysing the cells with reducing SDS-PAGE buffer. For each sample, cells from three different donors were pooled. Proteins were separated by SDS-PAGE using 4–20% gradient gels, after which the samples were transferred to nitrocellulose filters. The filters were blocked by incubation in 3% BSA in 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, and 0.1% Tween 20 (TTBS) for 1 h. Cathepsin F was detected using anti-human cathepsin F monoclonal antibody (1:50 in 1% BSA-TTBS) and horseradish peroxidase-conjugated anti-mouse antibody (1:2000 in 1% BSA-TTBS). The bands were detected by using a commercial enhanced chemiluminescence kit (Amersham Biosciences).

**Detection of Active Cathepsin F in Macrophage-conditioned Media**—Monocyte-derived macrophages were cultured for 13 days as described above; cells were then cultured 2 days in the absence and presence of cystatin C (15 μg/ml) in serum-free RPMI supplemented with penicillin-streptomycin and t-glutamine, after which the media were collected as described above. The media were pre-precipitated with 50 μl of protein A-Sepharose for 1 h at 4 °C, after which the media were first incubated with 2 μl of anti-cystatin C antibody for 1 h at 4 °C and then with 50 μl of protein A-Sepharose for 2 h at 4 °C. The bound proteins were eluted with 20 μl of reducing SDS-PAGE sample buffer, loaded into a 4–20% SDS-polyacrylamide gel, and immunoblotted with cathepsin F monoclonal antibody as described above.

**Immunohistochemistry**—Coronary samples were obtained, with permission from the Ethical Committee of Helsinki University Central Hospital, from hearts discarded during heart transplantation. The samples were fixed in 10% formalin and embedded in paraffin, using standard procedures. Paraflin (8 μm) sections were cut, and the sections were deparaffinized, rehydrated, microwaved at high power for 4 × 10 min in 10 mM citrate buffer (pH 6.0), and immunostained with ABC Elite kits from Vector Laboratories according to the instructions from the manufacturer. Trichloroacetic acid was used as the protease substrate. The sections were then counterstained with methyl green, dehydrated, and mounted with Permount. The sections were also double immunostained for cathepsin F and various cell type markers by incubating the samples first with a combination of a monoclonal anti-cathepsin F (IgG2a) antibody and monoclonal cell type markers (IgG1) followed by fluorescence-conjugated species-specific secondary antibodies, and in some samples the nuclei were stained with DAPI. The samples were photographed with a Nikon E600 fluorescence microscope equipped with a cooled CCD camera (Spot RT, Diagnostic Instruments). The primary antibodies used were rabbit anti-human cathepsin F anti-
was incubated with 100 nM cathepsin F, K, or S at pH 6.0 to optimize the activities of the proteases (see Fig. 1A). First, the turbidity of control LDL and of LDL incubated with 100 nM cathepsin F, K, or S for 24 h was measured at 430 nm. There was no change in the absorbance values of the LDL samples treated with cathepsins S or K. However, during treatment of LDL with cathepsin F, the absorbance of LDL increased from 0.052 to 0.330, indicating lipoprotein aggregation with or without ensuing fusion of the particles.

Next, we analyzed the size of the cathepsin-treated LDL particles by rate zonal flotation. Increased flotation velocity of the particles in an ultracentrifugal field indicates an increase in particle size, either through particle aggregation or fusion, or both (48). Control LDL and LDL incubated with cathepsins F, K, or S were subjected to ultracentrifugation in a linear NaBr gradient. In the gradient system used in this experiment, spherical particles having diameters >75 nm float in the top fractions of the tube and, as shown in Fig. 2, untreated LDL resides in a single layer near the bottom of the centrifuge tube (fractions 16–22). In each of the cathepsin-treated samples, some of the radiolabeled apoB-100 peptides were released from the LDL particles and were recovered at the bottom of the centrifuge tube (fractions 21–24). The LDL particles (radiolabeled cholesteryl linoleate) treated with cathepsin S or K floated at a similar rate to that of control LDL and were recovered in fractions 16–22. In contrast, LDL particles proteolyzed with cathepsin F were recovered from the top fractions of the tube. Thus, proteolysis with cathepsin F, but not with cathepsins S or K, must have resulted in aggregation and/or fusion of the proteolyzed particles.

To detect enlargement in the size of individual LDL particles, i.e. particle fusion, we next used a fusion assay based on 1H NMR spectroscopy (49). In this assay, fusion of lipoprotein particles is detected by size-dependent chemical shifts in the 1H NMR resonances of lipoprotein lipids. Fig. 3 shows the terminal fatty acid methyl-CH3 at approximately −0.8 ppm in control LDL and in LDL incubated with 100 nM cathepsin F. In the control LDL, no shift in the -CH3 resonance was observed. Similarly, no shift in the -CH3 resonance was observed during treatment with cathepsin S or K (data not shown). In contrast, during treatment with cathepsin F, the -CH3 resonances shifted toward higher frequencies (Fig. 3), indicating lipoprotein fusion (49). In addition, the area of the -CH3 resonance in LDL progressively decreased, which was likely because of lipoprotein aggregation.

The morphology of the cathepsin-treated LDL particles was next analyzed by transmission electron microscopy. The size distribution of the proteolyzed LDL particles was determined from negatively stained samples, and the morphology of the LDL particles was studied in thin-section electron microscopy. As shown in Fig. 4, the LDL particles incubated with either cathepsin K or cathepsin S resembled control LDL, the average diameters of both control LDL and cathepsin K-treated LDL being 22 nm (± 1 nm, median 22 nm) and the average diameter of cathepsin S-treated LDL being 22 nm (± 2 nm, median 22 nm). In contrast, LDL particles incubated with cathepsin F were larger (30 ± 8 nm, median 29 nm). The largest particles had diameters of 70 nm. The morphology of the fused particles is seen in thin-section electron micrographs of the cathepsin F-treated LDL samples. These micrographs also show membranous material associated with the enlarged lipoproteins, resembling LDL proteolyzed extensively with α-chymotrypsin (50).

Glycosaminoglycans have been shown to stabilize cysteine proteases (51–53). Therefore, we tested whether the proteolytic degradation of apoB-100 by the cathepsins would be affected by the presence of glycosaminoglycans. We found that proteolysis with cathepsin S was unaffected by the presence of glycosami-
 apoB-100 were modified by 1,2-cyclohexanedione, which blocks the binding of LDL to glycosaminoglycans (55). Cyclohexanedione-LDL, in contrast to control LDL, was degraded identically in the absence and presence of glycosaminoglycans (Fig. 5). Thus, cyclohexanedione modification of LDL, which inhibits the binding of LDL to glycosaminoglycans, also inhibited glycosaminoglycan-induced increase in LDL degradation by cathepsin F, a finding revealing that binding of LDL to glycosaminoglycans is essential for the enhanced degradation by cathepsin F.

Aggregation and fusion of LDL particles has been shown to increase the binding strength of LDL to human aortic proteoglycans (20). Therefore, the capacity of proteoglycans to bind cathepsin-treated LDL particles was next analyzed on microtiter wells coated with human aortic proteoglycans. As shown in Fig. 6, proteoglycans bound cathepsin F-treated LDL approximately 5-fold more than untreated LDL or LDL treated with cathepsins S or K. In accordance, cathepsin F treatment also increased the binding strength of LDL particles to human aortic proteoglycans on an affinity column (data not shown). Thus, treatment with cathepsin F, which induced aggregation and fusion of LDL particles, also increased the ability of LDL to bind to the proteoglycans.

Although cathepsins are primarily targeted to lysosomes, it has been demonstrated that, in culture, monocyte-derived macrophages and smooth muscle cells secrete various cathepsins, such as cathepsins S and K. We next examined the expression and secretion of cathepsin F in human monocyte-derived macrophages. For this purpose, peripheral blood monocytes were cultured for up to 15 days to induce their conversion into macrophages and, at the indicated time points (Fig. 7), mRNA expression for cathepsin F was determined. As shown in the figure, the expression of cathepsin F increased with time. Similarly, Western blot analyses (Fig. 8A) showed increasing amounts of both pro-cathepsin F (~53 kDa) and mature cathepsin F (~34 kDa) in whole cell lysates derived from the monocyte-derived macrophages. Most importantly, increasing amounts of pro-cathepsin F and mature cathepsin F were also detected in the cell-free supernatants from the monocyte-derived macrophages (Fig. 8A), indicating secretion of the protein from the macrophages. To examine, whether the mature cathepsin F in cell-free media was active, we next added cystatin C, a cysteine protease inhibitor that binds to the active site of cysteine proteases, to 13-day- old monocyte-derived macrophages and after incubation for 2 days, immunoprecipitated the media with anti-cystatin C antibody. The cystatin-C-bound proteins were then immunoblotted with cathepsin F antibody. As shown in Fig. 8B, cystatin C that was added to the cell culture, was able to form complexes with cathepsin F that had been secreted into the culture medium by the monocyte-derived macrophages. Because cystatin C forms complexes only with active cysteine proteases, this finding indicates that cathepsin F is either secreted in its active form or is activated in the culture medium.

Finally, we examined samples of human coronary arteries for the presence of cathepsin F. Using a polyclonal anti-cathepsin F antibody, we found that the intima of normal human coronary arteries contained only a few immunopositive cells (Fig. 9, left panel). Immunostaining for CD68 revealed that the normal intima was devoid of macrophages (data not shown), suggesting that cathepsin F is expressed by some of the smooth muscle cells. Moreover, some of the smooth muscle cells in the medial layer of the normal arterial wall were found to be weakly positive for cathepsin F (Fig. 9B). The right panels of Fig. 9 show cathepsin F immunostaining of an advanced atherosclerotic lesion containing a large necrotic lipid core sur-
rounded by an inflammatory infiltrate (partly within the rectangle). The inflammatory infiltrate stained for cathepsin F uniformly and strongly, whereas the core was essentially negative. Moreover, some smooth muscle cells in the tunica media and in the fibrous cap were positive for cathepsin F. Of note, the cells most strikingly positive for cathepsin F were the macrophages (based on CD68 immunoreactivity; data not shown) that were devoid of any apparent intracellular lipid accumulation (arrows). The expression of cathepsin F by various cell types present in human atherosclerotic lesions was confirmed by double immunofluorescent staining (Fig. 10). We found that most macrophages were strongly positive for cathepsin F. Of the smooth muscle cells, only a minor fraction was positive and the intensity of the staining was heterogeneous (Fig. 10). Moreover, some endothelial cells appeared positive whereas T lymphocytes were negative for cathepsin F (data not shown). Taken together, the above findings showed that, in the human coronary atherosclerotic lesion, the immunoreactivity for cathepsin F was significantly enhanced. In accordance with the above findings, cathepsin F mRNA was also detected in extracts from atherosclerotic, but not from normal human coronary arteries (data not shown).

**DISCUSSION**

In atherosclerosis, LDL particles become modified and fuse into lipid droplets during their retention within the arterial intima. There is evidence that LDL particles become proteolytically modified in the arterial intima, but it appears that only a few of the proteases that are known to be present in the arterial intima are able to degrade apoB-100 extensively enough to trigger particle fusion (2). In fact, among the many proteases that we have studied, only mast cell chymase was able to induce LDL fusion in vitro (2). The data in this study provide suggestive evidence that cathepsin F may also participate in the formation of lipid droplets in the human arterial intima. Thus, as shown in this study, 1) proteolysis of LDL...
particles with human recombinant cathepsin F led to formation of fused lipid particles with enhanced ability to bind to arterial proteoglycans, 2) monocyte-derived macrophages were found to produce and secrete active cathepsin F in culture, and 3) cathepsin F was found in atherosclerotic lesions. Immunohistochemical analysis revealed that, in normal arteries, cathepsin F was present in only a few cells, but in atherosclerotic lesions it was readily detectable. In the lesions, cathepsin F was expressed by macrophages, as shown earlier for cathepsins S and K (24). Although cathepsins are primarily intracellular enzymes, in the human atherosclerotic arterial intima with an abundance of macrophages, some cathepsin F also appeared to be located extracellularly. Supportive of the notion of plaque macrophages actively secreting the enzyme was our in vitro observation that, upon phenotypic differentiation of blood-derived monocytes into macrophages, the content of cathepsin F gradually increased in the cells, and, importantly, also in the culture medium. The possibility that some of the extracellular enzyme may have originated from dead cells is not favored by the finding that, throughout the experiment, the proportion of pro-cathepsin F/mature cathepsin F was higher in the culture medium than in the cells. Additionally, secretion of other lysosomal cysteine proteases has been demonstrated previously in macrophages (22, 23) and also in vascular smooth muscle cells (24). Taken together, these findings are compatible with the view that in human atherosclerotic lesions, the expression of the newly described cathepsin F, like K and S, is increased both intra- and extracellularly and that this increase is closely associated with the appearance of macrophages in the lesions.

Cathepsin F was found in vitro to degrade apoB-100 very...
extensively, as shown by the abundant generation of trichloroacetic acid-soluble apoB-100 fragments. The extensive degradation of apoB-100 in LDL by cathepsin F was accompanied by formation of aggregated and fused particles, as demonstrated by the increased turbidity of the samples, by the increased flotation of the particles in ultracentrifugation, by 1H NMR spectrometry, and by electron microscopy. Interestingly, electron microscopy revealed that the proteolyzed particles resembled the lipid droplets found in the atherosclerotic human arterial intima (56–58).

Finally, the aggregated and fused cathepsin F-treated LDL particles had an increased ability to bind to human aortic proteoglycans, a key feature of atherogenesis. Cathepsin F-induced degradation of apoB-100 was enhanced by the presence of glycosaminoglycans and proteoglycans even though cathepsin F does not bind to glycosaminoglycans (54). If the binding of LDL to the glycosaminoglycans was blocked by modification of apoB-100, the glycosaminoglycan-induced increase in the degradation of LDL by cathepsin F was inhibited. Thus, the enhanced degradation depends on the binding of LDL to the glycosaminoglycans and is likely to result from glycosaminoglycan-induced changes in LDL particles. Indeed, glycosaminoglycans have been shown to induce irreversible changes in the conformation of apoB-100 (59), and to increase the rate of LDL proteolysis by trypsin (60) and by α-chymotrypsin (61).

A major question regarding the ability of cathepsins to degrade extracellularly located LDL particles in the arterial intima is whether local conditions in the extracellular fluid in atherosclerotic lesions allow the protease to remain catalytically active. Unlike most of the lysosomal cysteine proteases, cathepsin S has a broad pH optimum and is also active in neutral pH (35). However, cathepsin F requires a slightly acidic environment for optimal activity. Thus, in the normal intima, where the pH is near neutral to slightly alkaline, cathepsin F is likely to be rapidly inactivated. However, atherosclerosis is characterized by chronic inflammation (62) and, in inflammatory sites, the pH of the extracellular fluid is known to be acidic (63). Indeed, a recent study demonstrated pH heterogeneity of human and rabbit atherosclerotic plaques. Naghavi et al. (64) showed significantly lower pH values in lipid rich areas (acidic pH) when compared with calcified sites of atherosclerotic and normal human umbilical arteries (alkaline pH). The proton concentrations in the most acidic areas were 10–12 times higher than in the most alkaline areas corresponding to pH differences of more than one pH unit. The finding that the pH in atherosclerotic lesions is decreased is also supported by studies showing that the lactate concentration in such lesions is higher than in normal arteries (65), and that the lesions show signs of hypoxia (66) and neovascularization (67). It is thought that metabolically active macrophages efflux high amounts of lactate, which acidifies the extracellular matrix. De Vries et al. (68) have shown that monocyte-derived macrophages can lower their environmental pH to as low as 5.5 in the presence of oxidized LDL, a pH condition optimal for most cathepsins including cathepsin F. Interestingly, the acidification of the pericellular environment of macrophages is frequently coupled with the secretion of several cathepsins (23). Furthermore, lipolytic modifications of lipoproteins in the arterial intima, by producing free fatty acids, may also participate in the generation of an acidic microenvironment (69), and finally, the negatively charged glycosaminoglycan chains of proteoglycans may decrease the pH locally by attracting positively charged hydrogen ions (46).

As shown in this study, cathepsin F is present in coronary atherosclerotic lesions, especially in macrophage-rich areas. The localization of cathepsin F closely resembles the localization of cathepsins S and K. Similarly with cathepsins S and K, monocyte-derived macrophages secrete cathepsin F, at least in culture. During the progression of atherosclerosis, the extracellular pH will decrease, thus making it possible for cathepsin F to proteolyze the arterial LDL particles. Because the ability of cathepsin F to degrade proteoglycan-bound LDL is enhanced, the enzyme is likely to attack the LDL particles that are retained by the arterial proteoglycans. The cathepsin F-induced aggregation, and fusion would then further enhance the binding of the modified LDL particles to proteoglycans, leading to their accumulation within the extracellular matrix. Thus, the present data are consistent with the proposal that cathepsin F is one of the few proteases responsible for the generation and accumulation of extracellular lipid droplets in the arterial intima, a key feature of atherogenesis.

Acknowledgments—We gratefully acknowledge the excellent technical assistance of Elina Kaperi, Mari Jokinen, Anna Lyly, Suvi Makkinen, and Laura Vatanen. The Wihuri Research Institute is maintained by the Jenny and Antti Wihuri Foundation.

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J. Biol. Chem. 2004, 279:34776-34784.
doi: 10.1074/jbc.M310814200 originally published online June 7, 2004

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