Matrix metalloproteinases-3, -7, and -12, but Not -9, Reduce High Density Lipoprotein-induced Cholesterol Efflux from Human Macrophage Foam Cells by Truncation of the Carboxyl Terminus of Apolipoprotein A-I

PARALLEL LOSSES OF PRE-β PARTICLES AND THE HIGH AFFINITY COMPONENT OF EFFLUX*

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Matrix metalloproteinases (MMPs) have been suggested to function in remodeling of the arterial wall, but no information is available on their possible role in early atherogenesis, when cholesterol accumulates in the cells of the arterial intima, forming foam cells. Here, we incubated the major component responsible for efflux of cholesterol from foam cells, high density lipoprotein 3 (HDL₃), with MMP-1, -3, -7, -9, or -12 at 37 °C before adding it to cholesterol-loaded human monocyte-derived macrophages. After incubation with MMP-3, -7, or -12, the ability of HDL₃ to induce the high affinity component of cholesterol efflux from the macrophage foam cells was strongly reduced, whereas preincubation with MMP-1 reduced cholesterol efflux only slightly and preincubation with MMP-9 had no effect. These differential effects of the various MMPs were reflected in their different abilities to degrade the small pre-β migrating particles present in the HDL₃ fraction. N ε-terminal sequence and mass spectrometric analyses of the apolipoprotein (apo) A-I fragments generated by MMPs revealed that those MMPs that strongly reduced cholesterol efflux (MMPs-3, -7, and -12) cleaved the COOH-terminal region of apoA-I and produced a major fragment of about 22 kDa, whereas MMPs-1 and -9, which had little and no effect on cholesterol efflux, degraded apoA-I only slightly and not at all, respectively. These results show, for the first time, that some members of the MMP family can degrade the apoA-I of HDL₃, so blocking cholesterol efflux from macrophage foam cells. This expansion of the substrate repertoire of MMPs to include apoA-I suggests that these proteinases are directly involved in the accumulation of cholesterol in atherosclerotic lesions.

One key factor in the prevention of atherosclerosis is considered to be the ability of high density lipoproteins (HDL) to remove excess cholesterol from cholesterol-loaded macrophage foam cells. This is the initial step of reverse cholesterol transport, along the pathway by which cholesterol is carried from the peripheral tissues back to the liver (1, 2). The major component of HDL is apoA-I, which, in addition to a structural role, also has a biological function, playing the key role in the initiation of reverse cholesterol transport (3). The mature form of the apoA-I, a 243-amino acid protein with a molecular mass of 28 kDa, can combine with lipids to form stable structures of three types: (i) small lipid-poor complexes; (ii) flattened discoidal particles containing only polar lipids; and (iii) spherical particles containing both polar and nonpolar lipids (as reviewed in Ref. 4). In the lipid-poor complexes with lipid contents of 10–40%, each particle contains only one apoA-I molecule, and this has a unique conformation with a much lower α-helical content than the apoA-I in particles of the other types. The discoidal particles consist of a single lipid bilayer with repeating 22-amino acid helices of apoA-I which are suggested to run around the disc in a belt-like fashion. In the spherical particles, in contrast, the organization of apoA-I is suggested to be disordered; β turns of different apoA-I molecules may be in contact with each other on the surface of the sphere.

The HDLs are a highly heterogeneous family of particles that have been classified into several subgroups according to their density (HDL₂, HDL₃), apolipoprotein (apo) composition (apo-A-I without A-II, apoA-I with apoA-II), or electrophoretic mobility (α-migrating HDL, pre-β-migrating HDL) (5). In recent years, much attention has been focused on the small fractions of lipid-poor HDL particles (the small lipid-poor complexes; see above) that exhibit electrophoretic pre-β mobility (pre-β HDL), in contrast to the major spherical components of HDL, which exhibit α mobility. The total efflux of cholesterol from cells is believed to depend on two components: one that is nonspecific, based on aqueous diffusion of cholesterol from the cell’s plasma membrane to fully lipidated apoA-I, such as the mature HDL₄ particles, and the other that is specific and depends on interaction between lipid-poor apoA-I (pre-β HDL) and particular domains or proteins of the cell membrane (6, 7). The pre-β has been suggested to act as a shuttle, transporting free cholesterol from the plasma membrane to the fully lipidated mature HDL species (8–10).

A prerequisite for the action of HDL particles as efficient acceptors of cellular cholesterol is their integrity. Thus, cholesterol transfer protein; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

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* The abbreviations used are: HDL, high density lipoprotein; PAGE, polyacrylamide gel electrophoresis; apo, apolipoprotein; CETP, cholesterol ester transfer protein; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MMP, matrix metalloproteinase; PLTP,
ical modifications of HDL in vitro, such as copper-mediated oxidation, treatment with trypsin or pronase, or glycation, all reduce the efficiency with which HDL induce cholesterol efflux from cells (11–13). In a more physiological system, we have observed that mast cells reduce a high affinity component of the cholesterol efflux promoted by HDL₃ from cholesterol-loaded macrophages in culture (14–16). This mast cell-mediated mechanism was found to be due to proteolytic degradation of HDL₃ apoproteins by the neutral protease, which is present in the exocyted mast cell granules also found in atherosclerotic lesions. Most importantly, HDL₃ function was lost after only a minimal degree of proteolysis, suggesting that the high affinity process involves a minor subtraction of HDL particularly susceptible to proteolytic cleavage (14).

Matrix metalloproteinases (MMPs) are also present in atherosclerotic lesions. These proteinases are a family of at least 16 distinct, but structurally related, neutral proteases which are specialized in degrading the various components of the extracellular matrix (17–19). MMPs can be divided, according to their substrate specificity, into four subgroups: collagenases, stromelysins, gelatinases, and membrane-type MMPs. Here, we have examined the effects of some members of the first three subgroups of MMPs: the fibroblast type collagenase (MMP-1) of the group of collagenases; stromelysin (MMP-3), matrilysin (MMP-7), and human metalloelastase (MMP-12) of the group of stromelysins (or stromelysin-like MMPs); and 92-kDa gelatinase (MMP-9) of the group of gelatinases (18, 19).

Lipid-laden macrophages in atherosclerotic lesions have been shown to produce several members of the MMP family. These include MMPs-1, -2, -3, -7, -9, -12, and -13 (20–23). As demonstrated by Galis et al. (23), at least some of these MMPs are proteolytically active in atheromas. However, nothing is known about their possible involvement in cellular cholesterol accumulation. Here we show that MMPs-1, -3, -7, and -12, but not MMP-9, can degrade the apoA-I of HDL₃, and so block cholesterol efflux from cholesterol-loaded human macrophages in vitro.

**EXPERIMENTAL PROCEDURES**

**Cells**—Human monocytes were separated from buffy coat cells, allowed to differentiate to macrophages, and loaded with cholesterol, as described before (15).

**Lipoproteins and Purification of ApoA-I**—Human low density lipoprotein (d = 1.019–1.063 g/ml) and HDL₃ (d = 1.215–1.310 g/ml) were isolated from fresh plasma of normal healthy donors by sequential ultracentrifugation using KBr (24, 25). The low density lipoprotein was acetylated by repeated additions of acetic anhydride (26). [3H]Cholesteryl linoleate, 30–60 Ci/mmol; Amersham Pharmacia Biotech) was incorporated into the acetylated low density lipoprotein, as described (27), yielding preparations in which [3H]cholesteryl linoleate/nɡ of protein ranged from 30 to 100 ument. ApoA-I was purified from the HDL fraction by delipidation with ethanol:ether extraction, followed by separation of apoA-I by anion exchange chromatography on a HiTrap Q column (Amersham Pharmacia Biotech) (28, 29).

**Matrixproteinases and Their Activation**—Recombinant MMPs-3, -7, and -12 were expressed as active forms and purified as described (30–32). MMPs-1 and -9 were purified (33) and activated with phenylmucuric chloride (34), and activities were measured by the [14C]gelatin/min/g of enzyme at 37 °C.

**Proteolysis of HDL₃ by Metalloproteinases**—HDL₃ (1.5 mg/ml) were incubated with MMP-1, -3, -7, -12 (each 20 μg/ml), or MMP-9 (40 μg/ml) in 300 μl of 200 mM NaCl, 10 mM CaCl₂, 50 mM Tris, pH 7.6, in the presence of 0.02 mM butylated hydroxytoluene at 37 °C for the indicated time periods. Proteolysis was stopped by adding EDTA to give a final concentration of 15 mM. Aliquots of the incubation mixtures were added to macrophage foam cells, and other aliquots of the remaining were delipidated (28) for mass analysis of the cleavage products.

**Measurement of [3H]Cholesteryl Efflux from Macrophage Foam Cells**—Samples of proteolyzed or control HDL₃ (10 μg of total cholesterol/μl; each corresponding to 25 μg of protein in control HDL₃/μl) were incubated for 6 h in dishes containing [3H]cholesterol-loaded human monocyte-derived macrophage foam cells, as described previously (15). Under these conditions, the efflux of [3H]cholesterol was linear. After incubation, the medium was collected, and its [3H]radioactivity was measured by liquid scintillation counting.

In preliminary experiments, the samples of HDL₃ were incubated with the MMPs for 72 h, the MMPs were then incubated with EDTA, and a sample of each incubation mixture was subjected to gel filtration (Superose 12 HR 10/30-column, Amersham Pharmacia Biotech) to separate the HDL₃ from the enzymes. The cellular cholesterol efflux induced by the treated HDL₃ was similar, irrespective of whether the MMPs had been separated from the HDL₃, or not. Therefore, in the experiments shown, the separation step was omitted.

**NH₂-terminal Sequencing**—Protein samples were separated in a 15% SDS-PAGE followed by electroblotting onto a ProBlott PVDF membrane in 10 mM CAPS, pH 11, containing 10% methanol (36). Proteins were visualized by staining with Coomassie Brilliant Blue, and bands of interest were cut out and loaded onto the sequencer.

**Mass Spectrometry**—Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a Biflex™ time-of-flight instrument (Bruker-Franzen Analytik) equipped with a nitrogen laser operating at 337 nm. The delipidated HDL₃ and the MMP-generated degradation products of HDL₃, proteins were analyzed in the linear positive ion delayed extraction mode, using sinapinic acid as the matrix solution (1% trifluoroacetic acid, 30% acetonitrile, 0.1% acetic acid, 30% acetonitrile, and mixed 1:1 with matrix solution. One μl of the mixture was spotted on the target plate, and dried in a stream of warm air. All mass spectra were calibrated internally, using the [M + H]⁺ and [M + 2H]⁺ signals (m/z 28080.9 and 14040.5, respectively) of apoA-I.

**Other Assays**—15% SDS-PAGE was performed in nonreducing conditions according to Laemmli (37) and transferred to a nitrocellulose membrane (38), and apoA-I was immunolocalized with monoclonal antihuman apolipoprotein (Roche Molecular Biochemicals). A low molecular weight marker from Amersham Pharmacia Biotech was used as a standard. Cellulose acetate electrophoresis was performed with the system of Helena Laboratories for lipoproteins (Titan III Lipo membranes). The protein content was determined by the method of Lowry et al. (39) with bovine serum albumin as standard. The cholesterol and phospholipid contents of HDL₃ were measured using commercial kits (Cholesterol CHOD-PAP, Roche Molecular Biochemicals, and phospholipids B, Wako Chemicals).

**RESULTS**

To generate [3H]cholesterol ester-containing foam cells, human monocyte-derived macrophages were incubated with [3H]labeled acetylated low density lipoprotein (25 μg/ml) for 48 h. During incubation, the content of cholesterol esters in the macrophages increased from ~3 μg to ~90 μg/mg cell protein. Subsequent incubation of the cells with HDL₃ resulted in a constant efflux of [3H]radioactivity into the incubation medium for at least 6 h, the time interval chosen for the efflux experiments. We then preincubated HDL₃ with MMP-3, -7, or -12 for up to 2 h or with MMP-1 or -9 for up to 72 h, and added the variously treated HDL₃ preparations to human macrophage foam cells (Fig. 1). After pretreatment with any of the first three MMPs for only 30 min, HDL₃ rapidly lost its ability to induce cholesterol efflux, which had fallen to about 20% of the initial value. In contrast, the effect of MMP-1 on the efflux-inducing function of HDL₃ was much weaker. After incubation for 2 h, cholesterol efflux was only slightly reduced, and the rate of efflux was significantly reduced only after 72 h. Finally, when HDL₃ was pretreated with MMP-9, its ability to promote cholesterol efflux did not decrease even after prolonged treatment with the active enzyme (12.5 units/300 μl). When the variously pretreated HDL₃ particles were added to cholesterol-loaded mouse peritoneal macrophages, the results were identical (not shown).

Fig. 2 compares the relation between the concentration of HDL₃ and the rate of [3H]cholesterol efflux from macrophage foam cells. If native HDL₃ was added to the incubation medium, the rate of cholesterol efflux rose rapidly at low concen-
trations of HDL₃. In striking contrast, addition of MMP-3-treated HDL₃ to the incubation system led to only a minimal increase in cholesterol efflux, and this increase was not dose-dependent at the HDL₃ concentrations tested. Thus, proteolytic treatment of HDL₃ appeared to abolish the high affinity component responsible for the rapid efflux of cholesterol at low HDL₃ concentrations.

Gel filtration of HDL₃ on a Superose 12 column and analysis of the eluate showed not only a major HDL peak, but also a minor peak of small-sized particles (Fig. 3A). In cellulose acetate electrophoresis, these smaller particles showed pre-β mobility, and immunoblotting of the isolated particles after SDS-PAGE confirmed that they contained apoA-I (not shown). We then treated aliquots of HDL₃ for 2 h with each of the MMPs separately. Treatment with MMP-3, -7, or -12, i.e., with those MMPs that had caused loss of the cholesterol efflux-inducing capacity of HDL₃, also resulted in disappearance of the small particles (Fig. 3A). MMP-1, which had only a slight effect on the cholesterol efflux-inducing capacity of HDL₃ caused a slight shift to the right in the elution of the small particles, reflecting an apparent decrease in their size. But MMP-9, which did not reduce the cholesterol efflux-inducing capacity of HDL₃, also failed to reduce the quantity of the small particles. In a separate experiment (Fig. 3B), HDL₃ and MMP-3-treated HDL₃ were electrophoresed on a cellulose acetate membrane, and this experiment also revealed the absence of pre-β particles from the MMP-3-treated HDL₃ (Fig. 3B, lower panel). Finally, when purified apoA-I was treated with MMP-7, which, like MMP-3, caused complete loss of the small particles, its ability to promote cholesterol efflux was rapidly and strongly reduced (Fig. 4).

Gel filtration study of HDL₃ revealed that treatment of HDL₃ with those MMPs (MMPs-3, -7, and -12) that cause loss of its cholesterol efflux-inducing capacity also caused loss of the minor peak having pre-β mobility. We therefore made a separate study of the capacities of the major and the minor peak to induce cholesterol efflux from macrophage foam cells. As shown in Table I, with the material eluting in the minor peak (1750 dpm) the ability to induce cholesterol efflux was slightly less than that of the material eluting in the major peak (3990 dpm). The ability of the material in the minor peak to induce cholesterol efflux was particularly high, considering that in the HDL₃ fraction the mass ratio of the minor to the major peak was 1:10, and accordingly, in the incubation medium, the protein concentration of the material derived from the minor peak was 2 µg/ml and that of the material derived from the major peak was 20 µg/ml. This result also demonstrated that, despite its high specific activity, the material eluting in the minor peak did not account for the full efflux capacity of the HDL₃ fraction. Furthermore, as shown in Table I, treatment of the material from the minor and the major peaks with MMP-3 reduced their ability to induce cholesterol efflux to similar extents. SDS-PAGE analysis of the major and the minor peaks revealed that MMP-3 treatment resulted in almost complete degradation of

**Fig. 1.** Ability of metalloproteinases to inhibit HDL₃-induced cholesterol efflux from human monocyte-derived macrophage foam cells. [³H]Cholesterol-loaded foam cells were incubated with HDL₃ (25 µg/ml) which had been treated with MMP-1, -3, -7, -9, or -12 for the indicated times. After incubation of the cells with the various HDL₃ preparations at 37 °C for 6 h, the [³H] radioactivity found in the culture media were determined and plotted as functions of exposure to HDL₃ for each MMP. Each point represents a mean of triplicate incubations of macrophages obtained from three different buffy coats.

**Fig. 2.** Relation of [³H]cholesterol efflux from macrophage foam cells to the concentrations of HDL₃ and MMP-3-treated HDL₃. [³H]Cholesterol-loaded foam cells were incubated with the indicated concentration of control HDL₃ or with HDL₃, which had been treated with MMP-3 (0.02 mg/ml) for 2 h. After incubation of the cells with the HDL₃ preparations at 37 °C for 6 h, the [³H] radioactivities found in the culture medium were determined and plotted as functions of HDL₃ concentration. Values are mean ± S.E. (n = 6).

**Fig. 3.** Analysis of matrix metalloproteinase-treated HDL₃ by gel filtration (A) and cellulose acetate electrophoresis (B). HDL₃ were incubated for 2 h with matrix metalloproteinases as indicated under "Experimental Procedures" and applied to a Superose 12-gel filtration column and eluted at a flow rate of 0.4 ml/min (A). In a separate experiment, material of control HDL₃ and MMP-3-treated HDL₃ was electrophoresed on cellulose acetate and stained with Ponceau S (B). Upper panel, control HDL₃; lower panel, MMP-3-treated HDL₃.
MMPs Reduce HDL-induced Cholesterol Efflux

HDL<sub>3</sub> was fractionated on a Superoxide 12 column into a major and a minor peak (see Fig. 3). The materials from the major and the minor peaks (each 1 mg/ml) were incubated separately for 2 h at 37 °C in the absence (control HDL<sub>3</sub>) or presence of MMP-3 (MMP-3-treated HDL<sub>3</sub>). [3H]Cholesterol-loaded foam cells were then incubated with material from the major peak (20 μg/ml) or the minor peak (2 μg/ml), i.e. using quantities reflecting their approximate proportion in the bulk HDL<sub>3</sub>. After incubation at 37 °C for 6 h, the 3H radioactivity found in the culture medium was determined. Each value represents a mean ± S.E. of triplicate incubations of macrophages obtained from two different culture media. Each point represents a mean of triplicate incubations.

To study further this unexpected observation that apoA-I degradation of the major peak, even though of minor degree, caused a significant loss (by half) of its cholesterol inducing ability, we reanalyzed the material from the major peak of the control samples by cellulose acetate electrophoresis and found that, in addition to the α band, it also contained a pre-β band, revealing regeneration of pre-β particles. In sharp contrast, no pre-β band was observed in the MMP-3-treated material collected from the major peak. The regeneration of pre-β particles by the material eluting in the major peak, and loss of this pre-β regenerating ability after MMP-3 treatment, provide plausible explanations for the observation that MMP-3 treatment caused a dramatic loss of the cholesterol efflux inducing capacity, which equaled the loss observed when the material eluting in the minor peak was treated with MMP-3.

SDS-PAGE analysis of MMP-treated HDL<sub>3</sub> revealed rapid degradation of apoA-I during incubation with MMP-3, -7, or -12, i.e. with the enzymes that inhibited HDL<sub>3</sub>-dependent cholesterol efflux from foam cells (Fig. 6). These enzymes degraded apoA-I (28 kDa) to forms with apparent molecular mass of 24–26 kDa and to smaller fragments of approximately 14 and 6 kDa. In contrast, when HDL<sub>3</sub> was incubated with MMP-1, degradation products appeared much less rapidly and, when HDL<sub>3</sub> was incubated with MMP-9, no degradation products appeared. In another experiment, proteolysis with the various MMPs was continued for 72 h. SDS-PAGE analysis of the degraded HDL<sub>3</sub> preparations revealed the following bands (Fig. 7): MMP-3 had degraded almost all of the apoA-I without causing any apparent change in apoA-II, the -14-kDa fragment being strongly accumulated. Similarly, the -14-kDa fragment was enriched when HDL<sub>3</sub> was incubated with two other efflux decreasing MMPs (7 or 12). MMP-1, which decreased the HDL<sub>3</sub>-induced efflux only after prolonged incubation with HDL<sub>3</sub>, also caused enrichment of the -14-kDa fragment, and, in addition, smaller degradation products became visible. In contrast to MMPs-3, -7, and -12, MMP-1 also appeared to degrade apoA-II. MMP-9, which had no effect on HDL<sub>3</sub>-induced efflux even after incubation for 72 h (see Fig. 1), appeared to degrade only apoA-I. A similar degradation pattern was seen with another gelatinase, MMP-2, which also failed to decrease HDL<sub>3</sub>-induced cholesterol efflux significantly after incubation with HDL<sub>3</sub> for 72 h (not shown). In summary, the SDS-PAGE analysis suggests that apoA-II is more vulnerable to gelatinases (MMP-2 and MMP-9) than apoA-I, but that this proteolysis alone does not significantly reduce HDL<sub>3</sub>-induced choleste-
terol efflux from macrophage foam cells.

Inspection of the time course studies of degradation and reduction in cholesterol efflux ability shows that it is the first cleavages converting apoA-I into 24–26-kDa fragments that lead to loss of efflux ability. We therefore chose the larger fragments for more detailed analysis. Using strategies based on MALDI-TOF mass spectrometry combined with NH2-terminal sequencing, we identified the MMP cleavage sites in apoA-I that produce fragments larger than 14 kDa. The major fragments of apoA-I (14–26 kDa) from the MMP-3, -7, and -12-treated HDL3, when subjected to NH2-terminal sequencing, were all found to have the NH2-terminal sequence of apoA-I (DEPPQ . . . ) intact, thus confirming that, with these MMPs, breakdown of apoA-I occurs at its COOH terminal. Mass analysis (Table II) of MMP-3-treated HDL3 yielded major signals at three masses over 14,000: m/z 28,080, 22,220, and 14,723 [M + H]+, corresponding to intact apoA-I, apoA-I cleaved at the Glu191-Tyr192 bond, and apoA-I cleaved at the Glu125-Leu126 bond, respectively. Mass analysis of MMP-7-treated HDL3 yielded major signals at four masses over 14,000: m/z 28,080, 24,650, 23,452, and 14,259 [M + H]+, corresponding to intact apoA-I and apoA-I cleaved at the Asp213-Leu214, His199-Leu200, Glu191-Tyr192, and Pro121-Leu122 bonds, respectively. Mass analysis of MMP-12-treated HDL3 yielded major signals at six masses over 14,000: m/z 28,079, 25,989, 24,637, 23,155, 22,211, and 14,254 [M + H]+, corresponding to intact apoA-I and apoA-I cleaved at the Phe225-Lys226, Asp213-Leu214, His199-Leu200, Glu191-Tyr192, and Pro121-Leu122 bonds, respectively.

Using gel filtration through a Superose 12 column, we then isolated from HDL3 the minor peak of small sized particles, which showed pre-β mobility in cellulose acetate electrophoresis (see Fig. 3, A and B), and separately treated aliquots of this fraction with MMP-3 for 2 h. Importantly, after incubation for as little as 15 min, 90% of the intact apoA-I in these small particles had been converted into 24-kDa sized fragments, as judged by SDS-PAGE analysis (Fig. 8). Mass analysis of the MMP-3-treated pre-β particles yielded major signals at three masses over 14,000: m/z 28,080, 22,220, and 14,223 [M + H]+, corresponding to intact apoA-I, apoA-I cleaved at the Glu191-Tyr192 bond, and apoA-I cleaved at the Glu125-Leu126 bond, respectively. The spectrum was identical with that of MMP-3-treated total HDL3. In sharp contrast, free apolipoprotein A-I isolated from lipoproteins was rapidly degraded by MMP-3 without apparent preference of cleavage sites (not shown).

Since proteolytic breakdown occurred at the COOH terminus of the apoA-I, determination of the cleavage sites by direct sequence analysis was not possible. The cleavage sites had to be deduced from the combined information afforded by the NH2-terminal sequences and the masses of fragments. The assignment of the cleavage sites determined by MALDI-TOF mass spectrometry can be considered reliable, since the accuracy of the instrument in the mass range studied is better than 0.05% (error less than ± 14 Da for 28 kDa protein). Thus, a combination of the known NH2-terminal sequences, the protein sequences, and the fragment masses with the information that the apoA-I did not contain post-translational modifications provides sufficient data for deducing the COOH-terminal sequences by mere calculation (see Table II).

DISCUSSION

The present study shows that several members of the MMP family are able to degrade human HDL3, thereby dramatically decreasing its capacity to promote cholesterol efflux from cholesterol-loaded human monocyte-macrophages. MMPs-3, -7, and -12, which have broad substrate specificity (18), were the most efficient in degrading apoA-I; MMP-1, which has a more restricted substrate specificity, did so far less efficiently, and MMP-9, which also possesses restricted substrate specificity, was unable to degrade apoA-I even during prolonged incubation. The present study also demonstrates that the matrix metalloproteinases act chiefly on the COOH-terminal region of apoA-I. The first degradation products split off by the enzymes, which reduce HDL3-induced cholesterol efflux, are 22–26-kDa fragments, and correspond to losses of the final 18–52 amino acids (see Table II). This susceptibility of the COOH terminus to proteolysis accords with previous findings in which limited degradation by other enzymes (chymotrypsin, trypsin, elastase, subtilisin, Staphylococcus V8 protease, and arginine C endopeptidase) produced 22-, 24-, and 26-kDa NH2-terminal fragments of apoA-I (40–42). The similarities in kinetics between the degradation of apoA-I and the loss of the reverse cholesterol transport activity of HDL3 particles suggest that apoA-I was already inactive by these first cleavages in the COOH-terminal region. Similarly, Marcel and Frank (43), using lipid-free or lipided human apoA-I mutants, concluded that the COOH-terminal domain of apoA-I is especially important for promoting cholesterol efflux from cholesterol-loaded macrophages. Another study using lipid-free apoA-I mutants has shown that induction of phospholipid efflux and subsequent cholesterol efflux from HepG2 cells requires the presence of the 21 COOH-terminal amino acids (44). The COOH terminus has also been shown to be essential for the structural integrity of the adjacent regions of apoA-I (45). Similarly, studies with the natural apoA-I (Pro165 → Arg) variant have shown that this variant of apoA-I is a poor acceptor of cholesterol from macrophages and adipocytes (46, 47). Von Eckardstein et al. (47) suggested that the Pro → Arg substitution in position 165 eliminates a β turn between two adjacent α-helices and so changes the orientation of all subsequent α-helices. Since these mutational changes in the COOH-terminal portion of apoA-I lead to structural changes in the adjacent parts of the molecule, it has not been possible to determine whether the reduction in cholesterol efflux is caused by changes in the COOH terminus itself or by secondary effects on the residual apoA-I molecule. Similarly, we cannot decide whether the event responsible for the observed functional changes was cleavage of the COOH-terminal region of apoA-I or possible secondary changes in apoA-I structure. This study adds a new substrate and potentially a new biological function for MMPs-3, -7, and -12. These MMPs were able to cleave the apoA-I in HDL3 at a substantial rate; incubation of HDL3 at a concentration of 1.5 mg/ml with any of the above MMPs at a concentration of 0.02 mg/ml led to 70% loss of its cholesterol efflux promoting ability within 15 min. It should be noted, however, that such incubation does not lead to 70% loss of intact apoA-I from the HDL3 particles. This discrepancy is not due to changes in the lipid composition of these particles.
**TABLE II**

*Fragmentation of apoA-I by MMPs-3, -7, and -12*

Using strategies based on MALDI-TOF mass spectroscopy combined with NH$_2$-terminal sequencing, MMP cleavage sites in apoA-I that produced fragments larger than 14 kDa were identified.

| Fragment mass | Assignment | Calculated | Counterpart mass | Observed |
|---------------|------------|------------|-----------------|----------|
| 28,080        | D1-Q243    | 28,080     |                 |          |
| 22,220        | D1-E191    | 22,223     | 5,875           | 5,876    |
| 14,723        | D1-E125    | 14,727     | 13,371          |          |
| 28,070        | D1-Q243    | 28,080     |                 |          |
| 24,850        | D1-D213    | 24,645     | 3,453           |          |
| 23,452        | D1-T202    | 23,462     | 4,636           |          |
| 14,259        | D1-P121    | 14,259     | 13,839          |          |
| 28,070        | D1-Q243    | 28,080     |                 |          |
| 25,988        | D1-F225    | 25,998     | 3,453           |          |
| 23,155        | D1-E191    | 23,160     | 4,937           |          |
| 24,637        | D1-D213    | 24,650     | 4,640           |          |
| 14,254        | D1-P121    | 14,259     | 13,839          |          |

**Fig. 8.** Proteolytic degradation by MMP-3 of material contained in HDL$_3$ and eluting from a Superose 12 column as a minor peak. The material from the minor peak (0.9 mg/ml) was incubated at 37 °C with MMP-3 (0.02 mg/ml) for the indicated time periods. Aliquots were electrophoresed on 15% SDS-PAGE under nonreducing conditions.

For, in the MMP-modified HDL$_3$ particles, the contents of total cholesterol and phospholipid remained unchanged (data not shown). Nor is it dependent on lecithin:cholesterol acyltransferase, since inhibition of lecithin:cholesterol acyltransferase during proteolysis by MMP-7 did not have any further effect on efflux. The HDL$_3$ samples, which are not PLTP or CETP activity, as measured by radiometric assays (48). The proteolyzed fragments of apoA-I may also have competed for removal of cholesterol. To test this possibility, we mixed intact HDL$_3$, either with the entire MMP-7-treated HDL$_3$ or with the MMP-7-treated minor peak of HDL$_3$, and added these two mixtures separately to macrophage foam cells. As compared with the ability of intact HDL$_3$ alone to induce cholesterol efflux, the effects of the mixtures were in both cases additive, revealing that neither the proteolized HDL$_3$ as a whole nor the proteolyzed minor peak inhibited the activity of the intact HDL$_3$ (data not shown).

It may be that only a minor fraction of the particles in the HDL$_3$ preparation is capable of accepting cholesterol from the cells, and that this minor fraction is more susceptible to proteolysis than the bulk of the HDL$_3$. Indeed, after Superose 12-gel filtration, we found that the HDL$_3$ fraction contains minute amounts of small particles which showed pre-β mobility on cellulose acetate electrophoresis (see Fig. 3B). The small particles disappeared very rapidly after treatment with cholesterol efflux-reducing MMPs (MMPs-3,-7, and -12). Importantly, this loss of the small particles was accompanied by reduction of the high affinity component of cholesterol efflux from macrophage foam cells (see Fig. 2). This accords with our previous findings that exposure of the LpA-I-containing particles present in HDL$_3$ and in plasma to a minimal degree of proteolysis by the neutral protease chymase from exocytosed rat mast cell granules or from human skin results in reduced high affinity efflux of cholesterol from macrophage foam cells (15, 16). Interestingly, Mendez and Oram (49) have shown that mild trypsin treatment, by proteolyzing a minor trypsin-labile fraction of HDL, almost completely abolished apolipoprotein-mediated cholesterol removal. Similarly, Kunitake et al. (42) showed that apoA-I in the pre-β fraction was more susceptible to proteolysis than in the α particles. Moreover, we found that after incubation with MMP-3 for as little as 15 min, 90% of the intact apoA-I in the small particles had been converted into the 22-kDa sized fragments. Although the loss of the small particles accords well with the rapid inhibitory effect of these MMPs on the cholesterol efflux inducing ability of HDL$_3$, we cannot exclude the possibility that changes were present in other sub-fractions of the ultracentrifugally isolated HDL$_3$ as well, but were not detected by the methods used in this study. A more complete analysis of the proteolyzed subpopulations and correlation with the loss of the cholesterol efflux promoting capacity of HDL$_3$ requires additional experiments.

The pre-β fraction appeared to be in a state of balance with the major HDL$_3$ fraction. In fact, after separation of these two fractions by gel filtration, we repeatedly observed the appearance of a peak containing the small particles in addition to the major HDL$_3$ peak. Even multiple resolation of the major peak failed to produce a fraction without a pre-β band. Only HDL$_3$ that had been floated through a KBr solution (1.21 g/ml) using high g forces (424,000 × g, 16 h), and analyzed immediately after resolation, showed pre-β mobility. But after dialysis, a pre-β band soon reappeared (within 4 h) reflecting the tendency of apoA-I to dissociate from the spherical particles with α mobility (data not shown). Whether apoA-I dissociates spontaneously or in response to trace amounts of pre-β-generating enzymes (PLTP or CETP) cannot be decided here. Since we were unable to detect PLTP or CETP activities in the HDL$_3$ preparations, it likely that apoA-I dissociated spontaneously from the major HDL$_3$ fraction. This notion accords with the suggestion of Mendez and Oram (49) that the apolipoproteins in HDL that are most sensitive to proteolysis are likely to be less tightly bound to phospholipids, and therefore can dissociate more easily from the particle surface and mediate chol-
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terol efflux. From the present findings, we infer that the MMPs preferentially proteolyze the apoA-I molecules, which readily dissociate from α particles and form the pre-β particles. Although the quantity of pre-β particles in the HDL₃ preparations was very small, their regeneration would make them potentially significant cholesterol acceptors. Since pre-β HDL are considered to be the first acceptors of cellular free cholesterol (50), degradation of the pre-β particles and, in particular, inhibition of their regeneration could significantly contribute to the observed inhibition of the cholesterol efflux inducing ability of HDL₃ by the MMPs.

Two reports have shown caseinolytic and gelatinolytic activity in atherosclerotic areas (29, 51). Although the concentrations of MMPs are unknown in the interstitial fluid of atherosclerotic lesions, MMP concentrations have been measured in synovial fluids from arthritic joints, which like atherosclerotic lesions, are inflamed tissues. Thus, in the synovial fluid of patients with rheumatoid arthritis the concentrations of MMPs 1–8, and -9 ranged from 1 to 15 μg/ml (52), that of MMP-3 ranged from 6 to 110 μg/ml, with a mean concentration of 40 μg/ml (53). Importantly, synovial fluid from rheumatoid arthritis patients demonstrated 100-fold higher levels of active MMP-3 than synovial fluid from control subjects (54). We performed additional experiments in which the MMP concentration in the incubation medium was progressively lowered, and found that incubation of HDL₃ with even 2.5 μg/ml (i.e. about 1/10 of that used in the other experiments) of trypsin-activated MMP-3 for 20 h at 37 °C was sufficient for maximal reduction of the efflux promoting ability of HDL₃ from mouse macrophage foam cells. Our previous results with mast cell chymase have also revealed that even a minute degree of proteolysis of HDL₃ can cause a dramatic decrease in its ability to promote cholesterol efflux from macrophage foam cells (14). It is also important to note that the foam cells in the atherosclerotic lesions themselves produce MMPs (22, 55), and that some of the MMPs secreted by the cell remain attached to the cell membrane during cell migration (56). Thus, in the pericellular compartment on or near the cell membrane, the local concentration of MMPs is likely to be high. Also, to be able to induce cholesterol efflux, apoA-I has to come into very close contact with or be attached to the cell membrane. In light of the above considerations, the concentration of MMPs in relation to that of apoA-I may be much higher near the cell membrane than in the bulk of the extracellular fluid in the arterial intima. Thus, the concentration of (active) MMPs would be adequate to prevent apoA-I-dependent cholesterol efflux from the macrophage foam cells in atherosclerotic lesions.

The novel observations made in this study raise two questions. First, does degradation of HDL₃ by MMPs affect any of its other functions apart from its role as a cholesterol acceptor, e.g. its action as an antioxidant, or as a mitogen, or as an anti-inflammatory agent (57–60)? Second, do other proteases present in the aortic intima that are capable of cleaving apoA-I, such as plasmin, thrombin, kallikrein, and elastase (42, 61, 62), also block cholesterol efflux? Interestingly, SDS-PAGE analysis of a major fibril protein purified from amyloid deposits in the aortic intima revealed two broad bands corresponding to ~14 and <10 kDa, which were shown to be NH₂-terminal fragments of apoA-I (63). The mechanism by which these fragments were generated is unknown. One possibility is by degradation of apoA-I by proteases in the intima. In our in vitro studies, proteolysis of apoA-I by matrix metalloproteinases led to formation of fragments of ~14 kDa, and these fragments seemed to be enriched during proteolysis. It is an intriguing possibility that such MMP-derived fragments become enriched and are deposited in the atherosclerotic intima. Taken togethert, elucidation of the effects of matrix metalloproteinases, and possibly of other proteases, on apoA-I and its cholesterol efflux promoting and other properties remains an important challenge for future studies. The proteolytic modifications of apoA-I may turn out to be of major importance in blocking the physiological functions of apoA-I in the arterial intima, such as mediation of the initial steps of reverse cholesterol transport from human atherosclerotic lesions consisting of numerous macrophage-derived foam cells.

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