Apolipoprotein composition and particle size affect HDL degradation by chymase: effect on cellular cholesterol efflux

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Abstract **Mast cell chymase, a chymotrypsin-like neutral protease, can proteolyze HDL_3. Here we studied the ability of rat and human chymase to proteolyze discoidal preβ-migrating reconstituted HDL particles (rHDLs) containing either apolipoprotein A-I (apoA-I) or apoA-II. Both chymases cleaved apoA-I in rHDL at identical sites, either at the N-terminus (Tyr^{18} or Phe^{33}) or at the C-terminus (Phe^{225}), so generating three major truncated polypeptides that remained bound to the rHDL. The cleavage sites were independent of the size of the rHDL particles, but small particles were more susceptible to degradation than bigger ones. Chymase-induced truncation of apoA-I yielded functionally compromised rHDL with reduced ability to promote cellular cholesterol efflux. In sharp contrast to apoA-I, apoA-II was resistant to degradation. However, when apoA-II was present in rHDL that also contained apoA-I, it was degraded by chymase. We conclude that chymase reduces the ability of apoA-I in discoidal rHDL particles to induce cholesterol efflux by cleaving off either its amino- or carboxy-terminal portion. This observation supports the concept that limited extracellular proteolysis of apoA-I is one pathophysiologic mechanism leading to the generation and maintenance of foam cells in atherosclerotic lesions.**—Lee, M., P. T. Kovanen, G. Tedeschi, E. Oungre, G. Franceschini, and L. Calabresi. **Apolipoprotein composition and particle size affect HDL degradation by chymase: effect on cellular cholesterol efflux.** ***J. Lipid Res.*** **2003.** 44: **539–546.**

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Chymases are neutral proteases whose specificity is chymotrypsin-like: peptide bonds are cleaved on the carbonyl side of *a*) aromatic residues with the order of preference Phe>Tyr>>Trp, and *b*) the branched aliphatic amino acids Val, Ileu, and Leu (1). These enzymes are produced mainly or exclusively by mast cells, and are also found in mast cells of the human arterial intima, the site of atherogenesis (2). In atherosclerotic lesions, the mast cells are activated and release a fraction of their chymase-containing cytoplasmic secretory granules into their microenvironment, where chymase may act on extracellularly located substrates.

Chymase of rat serosal mast cells (rat mast cell protease I) has been found to inhibit cholesterol efflux from macrophage foam cells (3). This finding emerged from studies in vitro concerning the effect of exocytosed rat mast cell granules (i.e., granule remnants) on the functional modification of HDL, and was the first to suggest that a neutral protease present in the arterial intima could block cholesterol transport from this tissue.

The early studies demonstrated that when HDL_3 is incubated with the chymase-containing granule remnants derived from degranulating rat serosal mast cells, it is rapidly proteolyzed and that after even a minimal degree of proteolysis, the net efflux of cholesterol promoted by HDL_3 from macrophage foam cells is significantly inhibited (3). In this in vitro system, degradation by granule remnant-bound chymase of the LpA-I particles present in HDL_3 or in human plasma inhibited the high-affinity component of cholesterol efflux because of specific depletion of the minor subpopulation of $preβ_1$-HDL particles (4, 5). Proteolysis of human plasma by granule remnant-bound chymase also depleted its contents of LpA-IV (5), another minor subpopulation of lipid-poor HDL that is responsible for the high-affinity efflux of cholesterol in apoA-I-deficient plasma (6).

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Abbreviations: rHDL, reconstituted HDL; rh-chymase, recombinant human chymase.

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Recently we demonstrated, using monoclonal site-specific apoA-I antibodies, that degradation of HDL_3 by granule remnant-bound chymase modified several epitopes of apoA-I that are involved in the efflux of cholesterol or in the activation of LCAT (7). Notably, after a minor degradation of HDL_3 with chymase, a truncated form of apoA-I (26 kDa) and small lipid-free peptides had been formed (7). These changes were paralleled by a rapid modification of the apoA-I epitope specific to $pre\beta_1$-HDL (8), and were accompanied by rapid loss of the ability of HDL_3 to act as a cellular cholesterol acceptor. Interestingly, the proteolytically modified HDL_3 retained its ability to induce cholesterol esterification via LCAT activation, suggesting a differential response to apoA-I proteolysis of the two main functions of HDL in reverse cholesterol transport.

The experimental approach of the above studies did not allow us to draw conclusions regarding the specific cleavage sites of apoA-I in the poorly lipidated preβ-migrating species of the HDL_3 fraction by chymase and its effect on their ability as cholesterol acceptors. Here, we performed further studies to characterize the digestion products associated with the chymase-induced modification of the cholesterol acceptor function of the major HDL apolipoproteins using a well-defined system of reconstituted HDL (rHDL) containing POPC and apoA-I or apoA-II as the sole apolipoprotein. These discoidal preβ-migrating rHDL particles are analogs of the precursors of the plasma α-migrating HDL, and have proven useful in studies on the relationships between the structure and function of HDL apolipoproteins and HDL subpopulations (9–11). We now extend the studies initially performed with rat chymase and compare the degradation patterns of apoA-I produced by chymase of rat or human origin. Thus, the present paper concerns: *a*) the cleavage sites produced by rat and human chymase on apoA-I or apoA-II contained in a homogeneous population of discoidal preβ-migrating rHDL, and *b*) the effect of chymase treatment on the cholesterol efflux efficiency of these particles.

MATERIALS AND METHODS

Animals

Adult male Wistar rats and female NMRI mice were purchased from the Viikki Laboratory Animal Center, University of Helsinki.

Rat chymase from mast cell granule remnants

Serosal mast cells were isolated from the peritoneal and pleural cavities of rats. Degranulation was induced with compound 48/80 (Sigma) and the exocytosed granules, i.e., granule remnants, were isolated from the released material by centrifugation as described (12). The quantity of granule remnants is expressed in terms of their total protein content or of their proteolytic activity with BTEE as substrate, as previously described (13). This isolation procedure enables chymase to remain bound to the heparin glycosaminoglycan chains of the granule remnants and so partially protects the chymase from inactivation in the presence of its physiologic inhibitors (14). Hence, granule remnants contain active rat chymase bound to the heparin proteoglycan matrix (heparin chains) of exocytosed granule remnants.

Recombinant human chymase

Recombinant human chymase (rh-chymase) (specific activity 80 BTEE U/μg) expressed in the baculovirus-insect cell system was provided by Teijin Ltd., Hino, Tokyo, Japan. The preparation was dissolved in a buffer containing 150 mM NaCl, 1 mM EDTA, and 5 mM Tris-HCl, pH 7.4, for the HDL degradation assay. The working preparation was stable at 4°C for one week, and was fully inhibited by 100 μg/ml of soybean trypsin inhibitor (SBTI) (Sigma).

Reconstituted HDL

ApoA-I and apoA-II were purified from human blood plasma, as previously described (11). Discoidal preβ-migrating rHDL containing POPC and apoA-I or apoA-II with a POPC-protein weight ratio of 2.5:1 were prepared by the cholate dialysis technique (15). In the final preparation, all the protein was incorporated into stable rHDL, with no lipid-free apolipoprotein remaining. rHDLs containing both apoA-I and apoA-II were obtained as described before (11). The size distribution of rHDL was examined by nondenaturing polyacrylamide gradient gel electrophoresis (16) on precast 8–25% polyacrylamide gels, using the Pharmacia Phast System (Amersham Pharmacia Biotech). After Coomassie Blue staining, the gels were scanned with a BioRad scanner, and the size of the rHDL was calculated with Bio-Rad Multi-Analyst software using thyroglobulin (17.0 nm), apoferritin (12.2 nm), catalase (9.2 nm), lactate dehydrogenase (8.2 nm), and BSA (7.1 nm) as calibrating proteins. The characteristics of the reconstituted particles are summarized in **Table 1**. POPC liposomes were prepared by the same procedure used for the rHDL, but omitting the protein in the starting mixture. The number of apolipoprotein molecules per rHDL particle was determined by cross-linking with dimethylsuberimidate, as described before (9). The phospholipid content of rHDL was determined by an enzymatic method (17), while the protein concentration was measured by the method of Lowry (18), with BSA as standard.

To evaluate effect of particle size on degradation of apoA-I by chymase (Fig. 3), we prepared homogeneous rHDL containing apoA-I and POPC with diameters of 9.6 nm, 12.5 nm, or 17.0 nm containing 2, 3, or 4 apoA-I molecules per particle, respectively, by the cholate dialysis technique (15), as previously described (19).

Chemical modification and radioactive labeling of lipoproteins

Human LDL (d = 1.019–1.050) was isolated from fresh normolipidemic plasma by sequential ultracentrifugation, using KBr (20). LDL was acetylated (acetyl-LDL) by repeated additions of acetic anhydride (21). Acetyl-LDL, radiolabeled by treatment with [^{3}H]cholesteryl linoleate ([1,2(n)^{3}H]cholesteryl linoleate, Amersham Pharmacia) dissolved in 10% dimethylsulfoxide (22), yielded preparations of [^{3}H]cholesteryl linoleate bound to acetyl-LDL with specific activities ranging from 30 dpm/ng to 100 dpm/ng protein. [^{3}H]apoA-I rHDL and [^{3}H]apoA-II rHDL were prepared by labeling the protein component by the Bolton-

TABLE 1. Properties of reconstituted HDL

Type of rHDL	Diameter	POPC:Protein	Molecules/Particle ApoA-I	Molecules/Particle ApoA-II
	nm	*mass ratio*		
ApoA-I rHDL	9.6 ± 0.2	2.15 ± 0.15:1	2	—
ApoA-II rHDL	10.0 ± 0.1	1.96 ± 0.12:1	—	4
A-I/A-II rHDL	10.0 ± 0.2	2.08 ± 0.13:1	1	2

rHDL, reconstituted HDL.

Hunter procedure as described (23). The electrophoretic mobility of the labeled lipoproteins, examined on agarose gels, was not changed after the radioactive labeling.

Proteolysis of rHDLs by granule remnant-bound chymase and rh-chymase

In the standard assay, apoA-I rHDL or apoA-II rHDL (1 mg/ml) was incubated at 37°C in 150 mM NaCl, 1 mM EDTA, pH 7.4, in the absence or presence of either granule remnant-bound chymase (30 μg/ml of granule remnant total protein) or rh-chymase (0.5 μg/ml), both equal to 40 BTEE U/ml, for different periods of time up to 6 h. After incubation, all the tubes were rapidly cooled down by placing them on ice. To remove the granule remnant-bound chymase from the reaction mixtures, the tubes were centrifuged at 4°C at 15,000 rpm for 5 min to sediment the remnants, and the chymase-free supernatants were collected. To fully inhibit rh-chymase, SBTI (final concentration 100 μg/ml) was added to the vials. The incubation mixtures were then subjected to structural and functional studies as described below.

Analysis of proteolytic products

The digestion products were analyzed by SDS-PAGE on 10–16% acrylamide gradient slab gels, using the Tris-tricine buffer system (24). After electrophoresis, the peptide bands in the gels were either visualized with Coomassie Blue or transferred electrophoretically to PVDF membranes. The membranes were then stained with Coomassie Blue, and the bands of interest were carefully cut from the membrane and subjected to automated sequence analysis on a pulsed-liquid sequencer (Applied Biosystems) equipped with a 120A Applied Biosystems PTH analyzer.

The molecular weights of the chymase-generated proteolytic fragments were determined by Flight-Mass Spectrometry (MALDI/TOF-MS) using a Vestec Lasertec MALDI-TOF instrument (Perspective) operating in a linear mode. Ions formed by a pulse UV laser beam (nitrogen laser, $\lambda = 337$ nm) were accelerated through 28 KV. The instrument was calibrated with bovine heart cytochome c (MW = 12,327) and flagellin from *Bacillus subtilis* (MW = 32,626). The calibration proteins and the samples were dissolved in a solution of ferulic acid in 50% acetonitrile: 50% water with 0.1% trifluoroacetic acid to a final concentration of 50 pmol/μl.

Cholesterol efflux assay

Peritoneal cells were harvested from unstimulated mice in PBS containing 1 mg/ml BSA. The cells were recovered after centrifugation and resuspended in DMEM (GIBCO) with 100 U/ml penicillin and 100 μg/ml streptomycin (medium A) supplemented with 20% fetal calf serum, and plated into 24-well plates (Becton Dickinson Labware). After incubation at 37°C for 2 h in humidified CO_2, nonadherent cells were removed. After washing with PBS, the adherent cells (macrophages) were loaded with [^{3}H]cholesterol by incubation for 18 h in the presence of 20 μg/ml of [^{3}H]cholesteryl linoleate acetyl-LDL in medium A containing 20% fetal calf serum. After [^{3}H]cholesterol loading, the macrophages were washed with PBS and then incubated with fresh medium A containing the cholesterol acceptors and the SBTI (100 μg/ml), as required. Efflux experiments were performed by adding to the [^{3}H]cholesterol-loaded macrophage rHDLs in the concentrations described in the figure legends, or by adding POPC liposomes providing the same quantities of phospholipids as in the rHDL. After 4 h, the media were collected and centrifuged at 200 *g* for 5 min. The radioactivity in each supernatant was determined by liquid scintillation counting. Under the conditions used, [^{3}H]cholesterol efflux was found to be linear for up to 4 h of incubation with the [^{3}H]cholesterol-loaded macrophages.

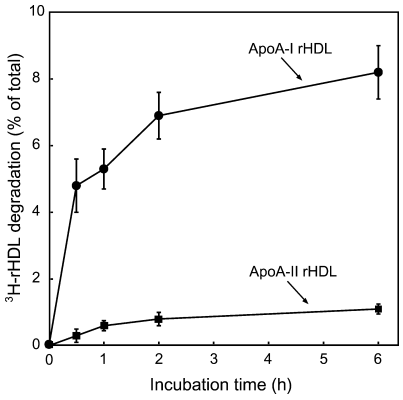

Fig. 1. Time course of generation of trichloroacetic acid-soluble degradation products from radiolabeled apoA-I and apoA-II upon incubation of apoA-I reconstituted HDL (rHDL) and apoA-II rHDL with recombinant human chymase. [^{3}H]apoA-I rHDL (circle) or [^{3}H]apoA-II rHDL (square) (1 mg/ml; 43 or 20 dpm/ng, respectively) were incubated at 37°C in the absence or presence of 40 U/ml of rh-chymase (1 BTEE unit of chymase equals 12.5 ng of protein) in 50 μl of 150 mM NaCl, 1 mM EDTA, 5 mM Tris-HCl, pH 7.4. After the indicated periods of time, the incubations were stopped by adding TCA, and the TCA-soluble radioactivity was measured and expressed as a percentage of the total radioactivity present in the [^{3}H]rHDL at the start of incubation. The TCA-soluble radioactivities of the two nonincubated rHDL preparations were both in the range of 0.5–1.1% and the corresponding blank values were subtracted from the actual values. Values are means ± SD of triplicate incubations.

RESULTS

Effect of proteolysis on the apolipoprotein components of rHDLs

Incubation of rHDL containing apoA-I with rh-chymase resulted in progressive proteolytic degradation of apoA-I, shown by the formation of TCA-soluble peptides (**Fig. 1**). In contrast, incubation of apoA-II with rh-chymase did not generate significant amounts of TCA-soluble material. When the two types of rHDL were incubated with the chymase contained in rat granule remnants, the degradation profiles, as determined by the production of TCA-soluble material, were identical to those observed with the human enzyme (not shown).

The cleavage products generated by digestion of the two rHDLs with either chymase were analyzed on SDS-PAGE. Incubation of apoA-I rHDL (**Fig. 2**, top panels) with rh-chymase for up to 6 h yielded two major bands (2 and 3), corresponding to polypeptides with apparent molecular weights of approximately 26 kDa and 24 kDa. A faint band 2 was visible at 30 min of incubation, and its intensity remained essentially the same over time. A faint band 3 appeared after 2 h of incubation, and at 6 h its intensity had become strong. The same pattern of apoA-I degradation was obtained when rHDLs were incubated with the rat chymase (Fig. 2, top panels). The extent of hydrolysis

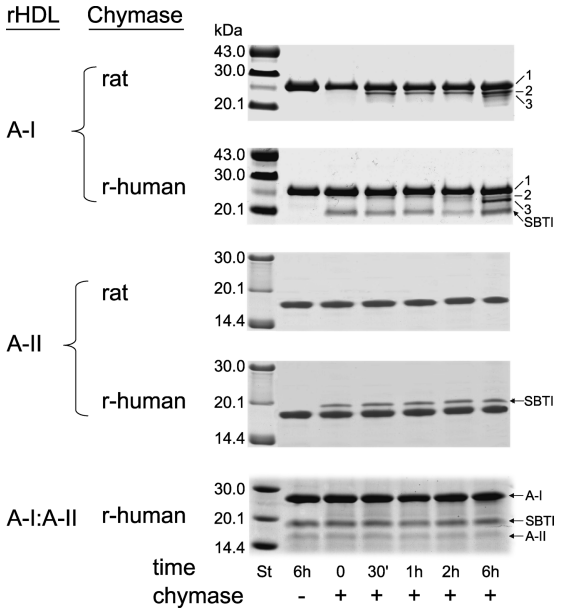

Fig. 2. Pattern of proteolysis of apoA-I rHDL, apoA-II rHDL, and apoA-I:A-II upon incubation with rat chymase or with recombinant human chymase. rHDLs (1 mg/ml) were incubated at 37°C with 40 BTEE U/ml of either granule remnant-bound rat chymase (30 μg/ml of total granule remnant protein) or rh-chymase (0.5 μg/ml). At various times, incubation was stopped by centrifugation at 15,000 rpm for 5 min to sediment the granule remnants or by addition of soybean trypsin inhibitor (SBTI), 100 μg/ml final concentration (SBTI; MW = 20,034 Da, shown by an arrow), to inhibit the rh-chymase, and the digestion products were separated by SDS-PAGE.

of apoA-I at the different incubation times was determined by densitometric analysis of the SDS-PAGE. When incubated with rh-chymase, apoA-I was hydrolyzed by ~15% at 30 min and by ~35% at 6 h of incubation. In the presence of rat chymase, apoA-I is degraded by ~30% after 30 min and by ~40% after 6 h of incubation. In contrast to apoA-I, the apoA-II in rHDL remained intact upon incubation with either chymase for up to 6 h (middle panels). However, when apoA-I was also present in the rHDL (A-I:A-II rHDL), apoA-II, in addition to apoA-I, was degraded by either chymase (results obtained with rh-chymase are shown in Fig. 2, bottom panel). Densitometric analysis revealed that apoA-II was hydrolyzed by about 15% during the 6 h of incubation with either chymase.

Particle integrity over the time course of digestion with chymase was assessed by nondenaturing gradient gel electrophoresis (not shown). No changes in rHDL particle size were detected in control incubations without chymase. ApoA-I rHDL, consisting of a major species with a diameter of 9.6 nm and a minor component of 7.8 nm, did not change in size during the 6 h incubation, despite the extensive degree of protein degradation. No changes were observed in the size of the apoA-II rHDL, which consisted mainly of particles with a diameter of 10.0 nm, or in the size of the A-I:A-II rHDL particles (10.0 nm). No major free polypeptide was found in any preparation.

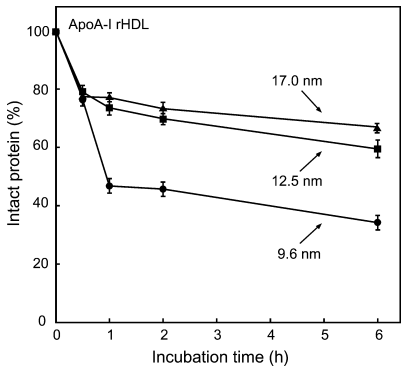

Fig. 3. Time-course of protein degradation in apoA-I rHDL of different sizes upon incubation with recombinant humane chymase. ApoA-I rHDLs with diameters of 9.6 nm (circle), 12.5 nm (square), or 17.0 nm (triangle) were incubated with rh-chymase under the conditions described in the legend to Fig. 2. The results are means ± SD of three different experiments with different rHDL preparations.

Effect of rHDL size on apoA-I degradation by chymase

To evaluate the effect of particle size on the efficiency of degradation of apoA-I by chymase, three homogeneous apoA-I rHDL particles were prepared. The three rHDLs had diameters of 9.6 nm, 12.5 nm, and 17.0 nm, and contained 2, 3, and 4 apoA-I molecules, respectively. When the three rHDL particles were treated with chymase, the same degradation pattern of apoA-I was observed; however, the extent of degradation varied between the three particles, such that apoA-I in the 9.6 nm particles was digested to a greater extent than apoA-I in the 12.5 nm or 17.0 nm particles (**Fig. 3**).

Cholesterol efflux promoted by untreated rHDLs and by rHDLs treated with rh-chymase or with granule remnant chymase

As expected (9), untreated rHDLs caused dose-dependent release of [^{3}H]cholesterol from mouse macrophage foam cell cultures. The addition of increasing concentrations of either apoA-I rHDL or apoA-II rHDL to [^{3}H]cholesterol-loaded macrophages resulted in a steep rise in the release of [^{3}H]cholesterol from the cells, up to about 25 μg/ml; at higher concentrations, the efflux continued to increase, but less steeply. Over the entire concentration range, rHDLs containing apoA-II were able to induce the release of cell cholesterol more efficiently than the apoA-I-containing rHDLs. Addition of increasing concentrations of protein-free POPC liposomes, providing the same amount of phospholipids as were present in the rHDL preparations, led to only a moderate promotion of cell cholesterol efflux (not shown).

Pretreatment of apoA-I rHDL with rh-chymase or with granule remnant-bound chymase at 37°C for different time periods up to 6 h resulted in progressive reduction of cholesterol efflux into the medium (**Fig. 4**, top panel). The inhibitory effect of the two enzymes was similar, a near-maximal effect (about 20% reduction) being observed after 2 h of preincubation. In contrast, apoA-II

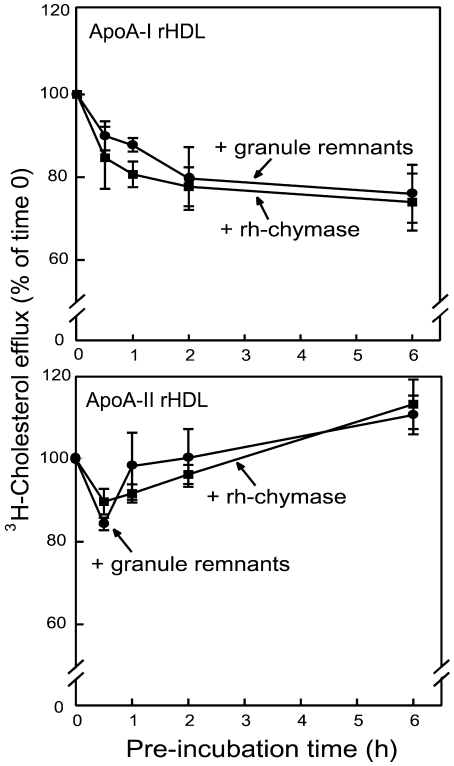

Fig. 4. Effect of preincubation with rat chymase (granule remnants) or recombinant human chymase on the ability of apoA-I rHDL, and of apoA-II rHDL to induce cholesterol efflux. [^{3}H]cholesterol-loaded foam cells were incubated at 37°C in 300 μl of medium A containing 100 μg/ml SBTI and 25 μg/ml of apoA-I rHDL (top panel) or apoA-II rHDL (bottom panel), which had been preincubated in the presence of 40 BTEE U/ml of granule remnant-bound rat chymase (circle) or rh-chymase (square) for periods of time up to 6 h in the standard conditions described in Fig. 2. After incubation with the cells for 4 h, the [^{3}H]cholesterol-associated radioactivity of the medium was determined and plotted as a percentage of the corresponding preincubation time (100% corresponds to the mean values at time 0, which were in the range of 1,052–1,732 and 1,524–2,333 dpm/4 h/well for apoA-I rHDL and apoA-II rHDL, respectively). Values are means ± SD of three experiments.

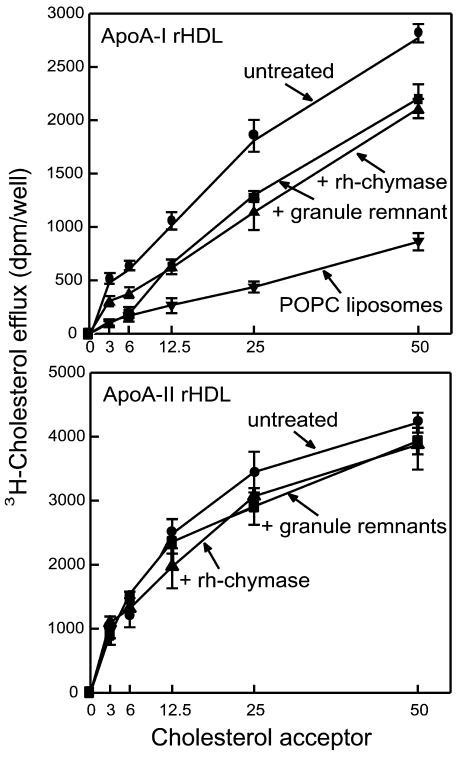

Fig. 5. Dependence of cholesterol efflux on the concentration of rHDL treated with rat chymase (granule remnants) or recombinant human chymase. [^{3}H]cholesterol-loaded foam cells were incubated at 37°C in 300 μl of medium A containing 100 μg/ml SBTI and the indicated concentrations of apoA-I rHDL (top panel) or apoA-II rHDL (bottom panel), which were preincubated for 2 h in the absence (circle) or presence of 40 BTEE U/ml of either granule remnant-bound rat chymase (square) or rh-chymase (upright triangle) in the standard conditions described in Fig. 2. A preparation of POPC liposomes (inverted triangle) providing the amount of phospholipids present in the preparation of A-I rHDL was also added to the cell cultures. After incubation with the cells for 4 h, the [^{3}H]cholesterol-associated radioactivity of the medium was determined and plotted as a function of the protein concentration of the rHDL. Values are means ± SD of three experiments.

rHDL-mediated cholesterol efflux was not inhibited by either chymase (Fig. 4, bottom panel).

In agreement with the above results, investigation of the concentration dependence of cholesterol efflux promoted by apoA-I rHDL (**Fig. 5**, top panel) indicated that pretreatment of apoA-I rHDL with granule remnant-bound chymase or with rh-chymase for 2 h reduced their ability to act as cholesterol acceptors by about 20% at the maximal concentration of the acceptor, and again no difference was observed between the two chymases. The residual cholesterol efflux was still 2-fold higher than the efflux promoted by protein-free POPC liposomes, revealing that the partial proteolysis of apoA-I in the liposomes did not completely abolish the apoA-I-dependent stimulation of cholesterol efflux by the apoA-I rHDL. Preincubation of protein-free POPC liposomes with either chymase did not affect the cholesterol efflux induced by the liposomes (not shown). In contrast to the strong inhibitory effect of chymase on apoA-I rHDL function, preincubation of apoA-II rHDL for 2 h with either chymase failed to produce any significant changes in the cellular efflux of cholesterol (Fig. 5, bottom panel).

Identification of cleavage sites

The cleavage sites in apoA-I were identified after incubation for 2 h of apoA-I rHDL with either type of chymase by *i*) N-terminal sequencing of proteolytic fragments isolated by SDS-PAGE, and *ii*) determination of the molecular mass of the digestion products by mass spectrometry (**Table 2**). The incubation time was selected according to the previous results, which showed that the maximal effect in the chymase-dependent inhibition of the cholesterol efflux could be achieved after 2 h of incubation with either chymase. Sequence and molecular mass analyses indicated that band 2 in the SDS-PAGE gel included two distinct polypeptides, with molecular masses of 26 kDa and 25.98 kDa, their N-terminal sequences starting at Asp^1 and Val^{19}, respectively (Table 2). Therefore, band 2 contains two polypep-

TABLE 2. Identity of fragments obtained by proteolysis of apoA-I rHDL

SDS-PAGE Band[a]	Molecular Mass	N-Terminal Sequence	Fragment
	kDa		
1	28.08	Asp-Glu-Pro-Pro . . .	Asp^{1}-Gln^{243}
2	26.00	Asp-Glu-Pro-Pro . . .	Asp^{1}-Phe^{225}
	25.98	Val-Asp-Val-Leu . . .	Val^{19}-Gln^{243}
3	24.27	Glu-Gly-Ser-Ala . . .	Glu^{34}-Gln^{243}

[a] Labels of fragments refer to their corresponding band positions on SDS-PAGE (at the 2 h-time point), which can be found in Fig. 2. In cases where more than one sequence is present, comigration of fragments is assumed to be due to nonoptimal resolution on SDS-PAGE.

tides generated by cleavage of apoA-I, one in the carboxy-terminal portion (at position 225), and the other in the N-terminal portion (at position 19) of the molecule. Sequence and molecular mass analyses indicated that band 3 contains a single fragment, starting at Glu^{34} (Table 2). The same cleavage sites in apoA-I were observed when apoA-I rHDL of different diameters (9.6, 12.5, and 17.0 nm) were treated with chymase, and also when apoA-I contained in A-I:A-II rHDL was degraded by chymase, demonstrating that the apoA-I cleavage pattern is independent of particle size and apolipoprotein composition (not shown).

DISCUSSION

The present results demonstrate that *i*) apoA-I in rHDL is susceptible to proteolysis by chymase, whereas apoA-II is not, unless the rHDLs also contain apoA-I; *ii*) the digestion pattern of apoA-I by chymase is distinct from that reported for other proteases, and independent of the phospholipid:apoA-I ratio of the particles; and *iii*) proteolysis of apoA-I by chymase reduces the ability of apoA-I rHDL to promote cholesterol efflux from cholesterol-loaded macrophages.

Previous studies have shown that apoA-I in preβ-HDL (25) or in discoidal rHDL (26) is susceptible to digestion by a variety of proteolytic enzymes, including trypsin and chymotrypsin. Various proteases that are found in the arterial intima, such as elastase (25–27), some metalloproteases (28), plasmin, and plasma kallikrein (25, 29) can also proteolyze apoA-I in preβ-HDL. Here we show that human chymase cleaves apoA-I in discoidal preβ-migrating rHDL and generates a proteolytic pattern identical to that produced by rat chymase, supporting the reliability of the rat mast cell granule remnant chymase as a physiological model that mimics apoA-I proteolysis by the human mast cell counterpart.

While apoA-II in plasma HDL is sensitive to proteolytic degradation by rat chymase, trypsin, and elastase (4, 27, 30), it differs from apoA-I in its susceptibility to digestion by the various metalloproteases. Stromelysin degrades almost all of the apoA-I without causing any apparent change in apoA-II, whereas the 92 kDa gelatinase appears to degrade apoA-II only (28). The present results with rHDL containing either apoA-I or apoA-II as a single protein component show that chymase clearly distinguishes between apoA-I, which is degraded, and apoA-II, which is completely resistant to proteolytic digestion by the enzyme. However, when apoA-II is present in rHDL particles containing apoA-I also, it is degraded by chymase, likely reflecting changes in apoA-II conformation in the A-I:A-II rHDL, which expose potential sites of cleavage that are buried in the apoA-II rHDL.

Digestion of apoA-I rHDL by chymase generated three major proteolytic fragments; one cleavage site is located in the carboxy-terminal portion of apoA-I (Phe^{225}), the other two are in the N-terminal region (Tyr^{18} and Phe^{33}). Interestingly, no major fragment corresponding to the size of a double-truncated apoA-I (cleavage at both ends) was formed. This proteolytic pattern is rather unique, as compared with other proteases, and it is independent of the rHDL particle size. A remarkable sensitivity to proteolysis of the central to C-terminal portion of apoA-I, from residue 100 down to the C-terminal end of the protein, has been established in several studies with different proteases (26, 28, 31). This portion of the lipid-bound molecule is arranged in a series of amphipathic α-helices interrupted by surface loops (32), which may be particularly accessible to the protease because of lack of an organized secondary structure (33). In contrast, the structure of the N-terminal region of apoA-I represents a distinct domain having a more globular conformation (34) and tight protein-protein interactions (35). This may explain the more limited susceptibility of this region to proteolytic digestion. In the present study, we show that human and rat chymases uniquely cleave apoA-I either at the carboxy or amino termini. By contrast, treatment of these same apoA-I-containing rHDLs with trypsin, chymotrypsin, or elastase left the amino terminal 119 residues of the protein intact (9). The unique cleavage sites of chymase at the N-terminus of apoA-I suggest that in this region the sites recognized by chymase are more exposed than those recognized by other proteases, or that the interaction between chymase and apoA-I is different from that of other proteases, allowing protein degradation. Interestingly, a recent paper (36) showed that macrophage metalloproteinases also could degrade apoA-I at both the N- and C-termini, and that the N-terminus cleavage is at residue 18, a cleavage site also observed with chymase.

The chymase-digested rHDLs containing apoA-I were less efficient in promoting cholesterol efflux from cholesterol-loaded macrophages than the nonproteolyzed apoA-I rHDL particles. This behavior of the apoA-I rHDL contrasted sharply with the apoA-II rHDLs, which were fully resistant to the proteolytic action of chymase and, consequently, retained their full capacity to induce cholesterol efflux even after chymase treatment. Since the size of rHDLs is known to affect their capacity for cell cholesterol uptake (10), we tested for the effect of chymase on the size of apoA-I rHDL. However, we found that chymase treatment did not modify the size of apoA-I rHDL particles, which indicates that the reduced efficiency of apoA-I rHDL for cell cholesterol efflux must have been due solely to apoA-I digestion. Therefore, it is likely that the portions of apoA-I cleaved by chymase are involved in the interaction of the apoA-I rHDL discs with the cell membrane structures participating in the regulation of cholesterol efflux.

Efflux of cellular cholesterol is considered to occur via two distinct pathways: *i*) interaction between lipid-free apoA-I and specific sites on the cell surface, with membrane microsolubilization and generation of small preβ-migrating HDL; and *ii*) a relatively nonspecific interaction of lipidated apoA-I, particularly of small HDL particles, with the cell, and diffusion of cholesterol from the cell membrane into the particle surface. The first pathway appears to be mediated by the ATP binding cassette transporter (ABCA1) (37), and the second may involve the scavenger receptor class B, type I (SR-B1) (38). The role of distinct apoA-I domains in cellular cholesterol efflux mediated by either lipid-free or lipidated apoA-I has been addressed by using monoclonal antibodies against epitopes distributed along the entire apoA-I sequence, and by using natural or recombinant apoA-I mutants (39–46). Taken together, earlier results suggest that sequences in the central (39–42) and C-terminal (43, 44, 47) regions of apoA-I may facilitate cholesterol efflux to lipidated apoA-I by interacting with specific lipid domains, or proteins in the cell membrane.

In view of the important role of the last apoA-I residues in facilitating cell cholesterol efflux, the cleavage of the extreme C-terminal portion (from Phe^{225}) by chymase could by itself explain the reduced cell cholesterol uptake by chymase-treated native or reconstituted HDL. The effect of cleavage in the N-terminal portion of apoA-I is less clear. However, cleavage of both the N- and C-terminal portions of apoA-I has been found to be necessary for binding of rHDL to SR-BI (48). Our results show that incubation of rHDL up to 2 h with either type of chymase reduced the efflux of cell cholesterol (Fig. 4), and that this reduction in the efflux corresponded to the appearance of a single band in the SDS-PAGE gel (band 2). Notably, this band contained two digestion products of almost identical MW, which were formed by removal either of the first 18 (Tyr^{18}) or the last 18 (Phe^{225}) residues of apoA-I (Fig. 2, band 2; Table 2), and so precludes any conclusions about an independent role of the N-terminal or C-terminal cleavage sites of chymase in the promotion of cholesterol efflux.

In conclusion, the present data show that mast cell chymase proteolyzes apoA-I, but not apoA-II, in rHDLs when apoA-II is the only apolipoprotein present in rHDL. In contrast, when apoA-I was also present in the rHDL, resembling more the HDL particles found in plasma, apoA-II was also degraded. The high degree of cleavage specificity of apoA-I by the rat and human chymase found here, i.e., only one cleavage at the C terminus and two cleavages at the N-terminus, adds this enzyme to the small group of physiologically neutral proteases that are found in plasma or in extracellular fluids and can be used as tools in the analysis of the structure and function of lipid-bound apoA-I.

More importantly, chymase digestion reduces the ability of apoA-I in small nonmature HDL particles to promote cholesterol efflux from macrophage foam cells. Such lipid-poor particles with preβ-mobility turn over very rapidly (49). Therefore, for chymase to have an impact on the preβ-dependent efflux of cellular lipids, the enzyme must also degrade the particles rapidly. Exocytotic secretion of chymase by degranulating mast cells occurs within seconds (2), and both depletion of preβ particles and impairment of cholesterol efflux by chymase are apparent within minutes in vitro (3, 5, 7). Since degranulated chymase-containing mast cells have been observed in human atherosclerotic lesions (50), our findings afford further support for the proposed role of degranulated mast cells in the inhibition of the initial steps of reverse cholesterol transport in vivo. The above observations also provide support for the more general concept that extracellular proteolytic degradation of apoA-I is one pathophysiologic mechanism leading to the generation and maintenance of foam cells in atherosclerotic lesions.

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