Mast Cell Chymase Degrades ApoE and ApoA-II in ApoA-I–Knockout Mouse Plasma and Reduces Its Ability to Promote Cellular Cholesterol Efflux

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Objective—Mast cell chymase is a chymotryptic heparin proteoglycan–bound neutral protease that exerts its activity in extracellular fluids. We studied the effect of chymase on the apolipoprotein compositions and the abilities of plasmas from apolipoprotein (apo)A-I–knockout (A-I-KO) and wild-type (C57BL/6J) mice to stimulate efflux of cellular cholesterol from mouse macrophage foam cells.

Methods and Results—The A-I-KO apolipoproteins compared with the wild-type (apoA-I, apoA-II, apoA-IV, and apoE) showed total lack of apoA-I, unaltered apoA-II, an absence of apoA-IV, and an increase of apoE. Despite these major differences, the 2 plasmas induced similar high-affinity efflux of cholesterol from the foam cells. Quantitative analysis of chymase-treated plasmas revealed (1) in A-I-KO plasma, complete loss of apoE and apoA-II, and (2) in wild-type plasma, slight reduction of apoA-I associated with complete depletion of the minor pre-β-high density lipoprotein fraction, strong reduction of apoA-II, and complete depletion of apoA-IV and apoE. Both proteolyzed plasmas had lost the ability to induce cellular cholesterol efflux with high affinity. Addition of discoidal pre-β-migrating reconstituted high density lipoprotein particles containing human apoA-I or apoA-II to the chymase-treated A-I-KO plasma fully restored its cholesterol efflux–inducing ability, indicating functional replacement of the proteolyzed apoE and apoA-II. Thus, chymase degraded all the nondeleted apolipoproteins of the A-I-KO plasma involved in the high-affinity efflux of cellular cholesterol.

Conclusions—This is the first indication that genetically engineered mice could be used as models for examining the hypothesis that extracellular proteases are involved in the development of atherosclerosis by inhibiting the apolipoprotein-mediated removal of macrophage cholesterol. (Arterioscler Thromb Vasc Biol. 2002;22:1475-1481.)

Key Words: apolipoprotein A-I–knockout mice ■ chymase ■ mast cells ■ reconstituted HDL ■ reverse cholesterol transport

Stimulation of rat serosal mast cells, with ensuing degranulation, leads to exocytosis of their cytoplasmic secretory heparin proteoglycan–containing granules, which, on entering the extracellular fluid, lose their soluble components, such as histamine. The granules also contain 1 neutral endopeptidase, chymase. After exocytosis, chymase remains tightly bound to the heparin proteoglycan matrix of the granules, thus forming protease-proteoglycan complexes, which we have called granule remnants. Chymase in the granule remnants impairs the efflux of cellular cholesterol from mouse peritoneal macrophage foam cells promoted by HDLs and by human plasma.

ApoA-I, the main apolipoprotein in HDL, plays an important role in cellular cholesterol homeostasis by promoting efflux of cholesterol from cholesterol-loaded cells. It has been suggested that HDLs induce cholesterol efflux from cells by 2 fundamentally different mechanisms, the relative importance of which depends on the degree of apoA-I lipidation and on the type of cell. Thus, spherical lipid-enriched α-migrating HDL particles remove cholesterol by nonspecific aqueous diffusion. The passive diffusion requires a concentration gradient of free cholesterol between the cell membrane and the acceptor particle, which is initiated and maintained by the lecithin-cholesterol acyltransferase (LCAT) reaction, and results in cholesterol esterification and in internalization of the formed cholesteryl esters to the HDL core. The other mechanism, known as the apolipoprotein-mediated pathway, requires active intracellular cholesterol transport, and it appears to be controlled by the ATP-binding cassette transporter A1. In this mechanism, the lipid-free apoA-I and small discoidal lipid-poor pre-β-migrating HDL particles interact with the cell membrane through a more efficient process that leads to removal of cholesterol and phospholipids. In line with this concept, we have demonstrated that apolipoprotein integrity constitutes a crucial factor for HDL-mediated stimulation of the high-affinity...
efflux of cellular cholesterol from macrophage foam cells.\textsuperscript{2,3} Importantly, the protease-dependent loss of the high-affinity component of the efflux was due to depletion of the minor, but extremely protease-sensitive, subpopulations of HDL, that contain either only apoA-I (pre-β\textsubscript{-},LpA-I) or only apoA-IV (LpA-IV).\textsuperscript{4} In contrast, the ability of chymase-treated HDL to activate LCAT was not affected.\textsuperscript{5} The above results are consistent with the view that the defective high-affinity removal of cholesterol from macrophage foam cells by the proteolytically modified HDL has resulted from an impairment of the apolipoprotein-mediated pathway.

Further insight into the particular roles of the various HDL apolipoproteins in cholesterol efflux can be obtained from experiments in which cholesterol donor cells are incubated with samples of serum from genetically engineered mice. Such studies have indicated the complexity of the process that determines the efficiency of whole serum to promote cellular cholesterol efflux.\textsuperscript{10–12} Taken together, these studies have highlighted the fact that the concentration of HDL phospholipids and the concentration of the small fraction of particles containing apoA-I (the pre-β fraction) independently contribute to the cholesterol efflux potential of serum. However, besides apoA-I, other apolipoproteins in mouse plasma are also active in inducing cholesterol efflux from cells and can substitute for a specific loss of apoA-I and thus prevent the development of atherosclerosis in the apoA-I–knockout (A-I-KO) mouse.\textsuperscript{13} Indeed, studies with knockout mice have highlighted the physiological redundancy of HDL apolipoproteins.\textsuperscript{14}

In light of the above considerations, the aims of the present studies performed in primary mouse macrophage cultures were to evaluate the following: (1) the effect of mast cell chymase on the residual cholesterol efflux potential of A-I-KO mouse plasma in which the expression of murine apoA-I was abolished by gene targeting (and 2) the ability of discoidal pre-β-migrating reconstituted HDL (rHDL), containing human apoA-I or apoA-II as the sole apolipoprotein, to restore the cholesterol acceptor function of the proteolyzed plasma.

**Methods**

An expanded Methods section can be accessed online at [http://www.atvb.ahajournals.org](http://www.atvb.ahajournals.org).

A transgenic line of mice that does not express murine apoA-I (A-I-KO mice) was kindly provided by Dr Nobuyo Maeda (Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill).\textsuperscript{15} C57BL/6J (wild-type) mice matched for sex and age were used as controls.

Mast cell granule remnants were isolated,\textsuperscript{16} their quantity was expressed in terms of their total protein content, and their proteolytic activity (chymase) was determined, as previously described.\textsuperscript{17} The heparin proteoglycan–bound chymase in granule remnants is partially active in the presence of plasma.\textsuperscript{18}

ApoA-I and apoA-II were purified from human blood plasma, as previously described.\textsuperscript{19} Discoidal pre-β-migrating HDLs containing palmitoyl oleoyl phosphatidylcholine (POPC) and apoA-I or apoA-II were prepared by the cholate dialysis technique,\textsuperscript{20} after which their average diameters\textsuperscript{21} and phospholipid\textsuperscript{22} and protein contents were measured. The preparation yielded rHDLS with a POPC-to-protein ratio of 2.5:1 and average diameters of 9.6 nm (apoA-I rHDL) and 10.0 nm (apoA-II rHDL). Human LDL (density 1.019 to 1.050 g/mL) was isolated by ultracentrifugation and acetylated (yielding acetylated-LDL).\textsuperscript{23} and acetylated-LDL was radiolabeled\textsuperscript{24} to yield \(^{3}H\)cholesterol linolate acetylated-LDL.

Mouse plasma was treated with chymase by incubating it in the presence of granule remnants; the plasma was then subjected to agarose gel electrophoresis for lipoprotein analysis. For apolipoprotein analysis, plasma total lipoprotein fraction was obtained by ultracentrifugation (density <1.21 g/mL), dialyzed, denatured, and loaded onto a 4% to 20% linear gradient SDS-PAGE gel. For measurement of cholesterol efflux, \(^{3}H\)cholesterol-loaded foam cells were incubated with the indicated concentrations of variously treated mouse plasma, supplemented or not with the rHDLS, and the radioactivity in the incubation medium, reflecting efflux of cellular radiolabeled cholesterol, was determined as described previously.\textsuperscript{3}

**Results**

**Cholesterol Efflux Promoted by A-I-KO Plasma After Incubation With Chymase-Containing Granule Remnants**

Mouse peritoneal macrophages were filled with radiolabeled cholesterol, after which increasing concentrations of plasma pools from either the C57BL/6J (wild-type) mouse or the A-I-KO mouse were added to the cells, and the extent of
cholesterol efflux was measured after 4 hours (Figure 1A and 1B). The plasma pools had been previously incubated overnight at 37°C in either the absence (untreated plasma) or the presence (chymase-treated plasma) of granule remnants. We found that compared with the wild-type plasma, the A-I-KO plasma consistently induced only a slightly lower efflux (by ≈15%) of cholesterol from the cholesterol-filled mouse peritoneal macrophages. As judged from the shape of the curves describing cholesterol efflux, both types of untreated plasma induced an efflux of cholesterol, which consisted of 2 components. Thus, on adding increasing amounts of either untreated wild-type or A-I-KO plasma, the rate of cholesterol efflux rose rapidly at low plasma concentrations (up to 0.5%), reflecting a higher-affinity component of the efflux, and then continued to increase less rapidly, reflecting a lower-affinity component of the efflux process. Notably, treatment of either plasma with chymase strongly reduced their cholesterol efflux–promoting ability (by ≈70%), and, more important, abolished the high-affinity component of the cholesterol efflux (Figure 1A and 1B). In control experiments, we found that preincubation of the plasmas at 37°C in the absence of chymase did not influence the efflux kinetics of the plasmas (not shown).

**Effect of Incubation With Granule Remnants on Mouse Plasma Lipoproteins**

To determine whether the chymase-induced inhibition of the ability of plasma to accept cholesterol was related to chymase-induced modification of individual fractions of plasma lipoproteins, aliquots of plasma from the A-I-KO and the wild-type mice were treated with granule remnants overnight and analyzed in agarose gel electrophoresis (Beckman Paragon Lipokit). The results showed that chymase treatment had not modified the relative mobilities of the lipoproteins (not shown). As previously described, the A-I-KO mouse plasma contained relatively low levels of VLDL, LDL, and HDL, and all lipoprotein bands became even more faint after treatment of the plasma with chymase (not shown). Next, we analyzed on the agarose gel the apoA-I–containing particles of the wild-type mouse plasma by immunodetection with a specific antibody against mouse apoA-I. As shown in Figure 2A, the pre-β-HDL band had completely disappeared after the plasma had been treated with chymase.

We then studied the effect of chymase treatment on the apolipoprotein patterns of the 2 kinds of mouse plasma. After incubation of plasma with the chymase-containing granule remnants for 20 hours, the lipoproteins were isolated by ultracentrifugation, and their apolipoprotein profiles were examined on SDS-PAGE (4% to 20%). Sufficient quantities of samples were loaded in each gel to provide reliable visualization of the minor apolipoprotein bands; hence, the gels loaded with the wild-type plasma were overloaded with apoA-I (Figure 2B). ApoA-I, the predominant apolipoprotein in the wild-type plasma, appeared to be degraded only to a small extent, reflecting the extensive degradation of the apoA-I contained in the small pre-β-migrating HDL fraction (see Figure 2A), which was found to constitute only ≈6% to 10% of the total apoA-I in plasma. In addition to the pre-β-migrating apoA-I, some other apolipoproteins also present in minor amounts in the mouse plasma were the main targets of chymase. Thus, in wild-type plasma, we observed total disappearance of the minor bands corresponding to apoA-IV, apoE, and the lower molecular weight band in the apoC zone and, moreover, ≈80% reduction of apoA-II. In the A-I-KO mouse plasma, apoA-IV was not detected, but this plasma exhibited an intense band of apoE, reflecting the typical high apoE content of its HDL. Chymase treatment of the A-I-KO plasma resulted in total disappearance of the apoE and apoA-II bands, thus rendering the plasma practically devoid of HDL apolipoproteins. Incubation without granule remnants did not change the apolipoprotein profile of the native plasma, nor did incubation of the plasma with phenylmethylsulfonyl fluoride–treated granule remnants, in which the chymase is inactive (not shown). Thus, the effect of incubation with granule remnants on the plasma lipoproteins and on their apolipoprotein profiles was shown to be due to the proteolytically active chymase present in the granule remnants.

**Figure 2.** Effect of incubation with chymase-containing granule remnants on mouse plasma lipoproteins. Untreated and chymase-treated plasma from A-I-KO and wild-type mice was prepared as described in Figure 1. A, Aliquots of the untreated and chymase-treated wild-type plasma were applied to agarose gel electrophoresis (Beckman Paragon system), and the apoA-I–treated plasma from A-I-KO and wild-type mice was prepared as described in Figure 1. A, Aliquots of the untreated and chymase-treated wild-type plasma were applied to agarose gel electrophoresis (Beckman Paragon system), and the apoA-I–treated plasma from A-I-KO and wild-type mice was prepared as described in Figure 1. B, Samples of the untreated and chymase-treated wild-type and A-I-KO plasmas were adjusted to a density of 1.21 g/mL, and lipoproteins were isolated from 100 μL in an Air-Fuge (Beckman), desalted on 0.025 μm Millipore membranes, loaded onto SDS-PAGE gel (4% to 20%) and proteins were visualized with Coomassie Brilliant Blue.
Human apoA-II added was chosen to yield a final concentration of approximately 2.5% in the medium of cell culture. We preincubated the various mixtures with [3H]cholesterol-loaded mouse macrophage foam cells in medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 IU/mL hirudin. After 4 hours, the efflux of [3H]cholesterol was determined and expressed as described in Figure 1. Normal distribution of data was confirmed by the Kolmogorov-Smirnov test, and homogeneity of the variance was determined by the Bartlett test, followed by bifactorial analysis and the Duncan multiple range test. The values shown by the columns with different letters differ significantly from each other (P<0.05), whereas those with the same letter do not.

**Effect of ApoA-I rHDL and ApoA-II rHDL on the Ability of Chymase-Treated A-I-KO Plasma to Induce Cholesterol Efflux From Macrophage Foam Cells**

Because treatment of plasma from the A-I-KO mouse with chymase dramatically depleted the HDL apolipoproteins and reduced the ability of the plasma to promote efflux of cellular cholesterol, we then tested whether addition of pre-β-migrating rHDL, containing either human apoA-I or apoA-II, to the chymase-treated plasma would restore the cholesterol efflux potential of the proteolyzed plasma. For this purpose, we added aliquots of apoA-I rHDL or apoA-II rHDL to A-I-KO plasma (2.5% [vol/vol] in medium) that had been preincubated in either the absence (untreated plasma) or presence (chymase-treated plasma) of mast cell granule remnants, as described in Figure 1. Aliquots of the preincubated plasma (final concentration 2.5%) were added to macrophage foam cells in 300 μL of culture medium (DMEM containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 IU/mL hirudin). After 4 hours, the efflux of [3H]cholesterol was determined and expressed as described in Figure 1. Normal distribution of data was confirmed by the Kolmogorov-Smirnov test, and homogeneity of the variance was determined by the Bartlett test, followed by bifactorial analysis and the Duncan multiple range test. The values shown by the columns with different letters differ significantly from each other (P<0.05), whereas those with the same letter do not.

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**Figure 3.** Effect of apoA-I rHDL and apoA-II rHDL on the ability of chymase-treated A-I-KO plasma to induce cholesterol efflux from macrophage foam cells. A-I-KO mouse plasma was preincubated in the absence (untreated) or presence (chymase-treated) of mast cell granule remnants, as described in Figure 1. Aliquots of the preincubated plasma (final concentration 2.5%) in the absence (A) or presence of 40 μg/mL of apoA-I rHDL (B) or 15 μg/mL of apoA-II rHDL (C) were added to mouse macrophage foam cells in 300 μL of culture medium (DMEM containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 IU/mL hirudin). After 4 hours, the efflux of [3H]cholesterol was determined and expressed as described in Figure 1. Normal distribution of data was confirmed by the Kolmogorov-Smirnov test, and homogeneity of the variance was determined by the Bartlett test, followed by bifactorial analysis and the Duncan multiple range test. The values shown by the columns with different letters differ significantly from each other (P<0.05), whereas those with the same letter do not.

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**Effect of ApoA-I rHDL and Protein-Free POPC Liposomes on the Ability of Chymase-Treated C57BL/6J Plasma to Induce Cholesterol Efflux from Macrophage Foam Cells**

Because it had been reported that the cholesterol efflux-inducing ability of plasma was enhanced by the addition of POPC alone,29 we studied the efflux-enhancing effect of POPC on proteolyzed wild-type plasma in which the high-affinity component of efflux sustained by intact HDL apolipoproteins was strongly inhibited. For this purpose, [3H]cholesterol-labeled foam cells were incubated in medium
containing 2.5% of C57BL/6J plasma, which had been preincubated in the absence (untreated C57BL/6J) or presence (chymase-treated C57BL/6J) of granule remnants and supplemented with either apoA-I rHDL or protein-free POPC liposomes, each providing 100 μg/mL of phospholipid to medium. After incubation for 4 hours, the quantity of [3H]cholesterol present in the medium was measured. As shown in Figure 4, chymase treatment reduced the cholesterol efflux induced by the wild-type plasma by ≈60% (panel A), and the addition of apoA-I rHDL to the chymase-treated plasma fully restored its efflux-inducing ability (panel B). In contrast, the addition of POPC liposomes, which resulted in a 4-fold increase of the total phospholipid concentration present in the 2.5% of mouse plasma, only modestly stimulated the efflux of cholesterol and failed to restore the efflux potential of the proteolyzed plasma (panel C). Not even the addition of 300 μg/mL of the POPC liposomes was sufficient to restore the efflux-inducing ability of the apolipoprotein-depleted plasma (not shown). Finally, the addition of POPC liposomes to the untreated plasma (which contained intact apolipoproteins) produced a minor increase in its efflux potential, which was similar to that produced by the addition of apoA-I rHDL to the untreated plasma (compare panels B and C with panel A). The above results revealed that the presence of apoA-I in the rHDL disks, but not the addition of phospholipids (POPCs) alone, compensated for the functional loss in the cholestero-efflux-inducing ability of the chymase-treated wild-type plasma, in which apoA-I, apoA-II, apoA-IV, and apoE were proteolytically depleted to varying extents (see Figure 2).

**Discussion**

In the present study, we demonstrate that plasmas from the A-I-KO mouse and the wild-type mouse promoted similar degrees of cholestero-efflux from mouse peritoneal macrophage foam cells in vitro and that chymase degraded all the apolipoproteins that are efficient cholesterol acceptors, thus inhibiting the high-affinity efflux, which depends on the integrity of HDL apolipoproteins.3,4

The roughly similar cholestero-efflux-promoting ability of both mouse plasmas discussed in the present study contrasts with findings in previous studies using cholestero-labeled Fu5AH rat hepatoma cells, in which cholestero-efflux into the serum of A-I-KO mice was 75% lower than that into the serum of wild-type (C57BL/6) mice.10,11 The reason for the reported differences likely reflects differences in the efflux mechanisms between the 2 cell types. The linear correlation found between cholestero-efflux from Fu5AH cells and HDL phospholipids is consistent with the conclusion that in this cell system (expressing the most scavenger receptor class B type I receptor protein among all of the cells tested), the efflux process largely depends on a nonspecific cholesterol exchange mechanism.7 This view is supported by experimental results indicating that the expression of scavenger receptor class B type I does not increase the efflux of cell cholesterol to lipid-free apoA-I and that apolipoproteins, when incubated with Fu5AH, do not generate HDL.7 In contrast, apolipoproteins directly interact with the cellular surface of mouse macrophages, such as the RAW264 cell line. The induction of ATP-binding cassette transporter A1 expression in these cells is accompanied by increased binding of apoA-I, followed by assembly of pre-β-HDL, when apoA-I is released from its cell surface binding site, and cellular phospholipids and cholesterol are removed with it.8 Moreover, the specific reversible binding of apoA-I to mouse peritoneal macrophages and the release of cellular cholesterol into the medium by extracellular apoA-I are both strongly reduced when the macrophages are isolated from mice treated with probucol.31

When all forms of LpA-I—containing lipoproteins are lacking from human plasma, efflux of cholesterol from fibroblasts into such HDL-deficient human plasma, compared with normal plasma, is decreased by 50%.32 The residual cholesterol efflux potential of serum obtained from subjects with the genetically determined low levels of apoA-I can be explained by the presence of apoA-IV and apoE in their sera.33,34 In previous experiments with normal human plasma, we found that in addition to the degradation by chymase of the quantitatively minute (but most protease-sensitive) fraction of apoA-I (pre-β-LpA-I), LpA-IV was also proteolytically depleted and responsible for the loss of the high-affinity efflux of macrophage cholesterol.4 The role of murine apolipoproteins in cholestero-efflux and the clearance of cholesterol from peripheral sites have been studied extensively in genetically engineered mice.35 Our present finding of similar cholestero acceptor potential of mouse plasma, whether apoA-I was present or not (plasma of the A-I-KO mouse), is compatible with the notion that apoA-I does not play a primary role in the induction of cholestero-efflux by the mouse plasma. This fact may account for the lack of susceptibility to atherosclerosis of the A-I-KO mouse.25 The present data also reveal that like its human counterpart, mouse apoA-I in pre-β-HDL particles is very susceptible to proteolysis by chymase, even in the presence of the high concentration of protease inhibitors found in mouse plasma.36 Indeed, the data suggest that in the mouse, the minor HDL apolipoproteins (apoA-II, apoA-IV, and apoE) are pivotal players in the removal of macrophage cholestero-l. Given the limited contribution that apoA-I seems to confer to the efflux potential of mouse plasma, the strong degradation of apoA-II, apoA-IV, and apoE (besides the full proteolytic depletion of apoA-I in pre-β-HDL) must have been involved in the observed functional loss of the proteolyzed wild-type plasma. Similarly, in the A-I-KO plasma, the extensive degradation of the non-apoB apolipoproteins must have been the reason for the loss of the high-affinity efflux component of cholestero-efflux by this type of mouse plasma.

Taken together, the present data reveal chymase as the first protease that has been demonstrated to proteolytically knock out all the apolipoproteins that are efficient promoters of cellular cholestero-efflux. This fact also provided us with a tool for studying the effect of addition of pre-β-migrating disks containing human apoA-I or apoA-II (ie, apoA-I rHDL or apoA-II rHDL) to plasmas that are deficient in apoA-I, apoA-IV, apoE, and apoA-II. Interest-
ingly, addition of the rHDL disks containing either apoA-I or apoA-II fully reconstituted the efflux potential of both types of mouse plasma that had been proteolytically deprived of the efflux-promoting HDL apolipoproteins. The finding that addition of either apoA-I or apoA-II enhanced the efflux potential of the plasmas suggests that LCAT reaction, which is dependent on activation by apoA-I, was not involved in this effect. Indeed, the concentration-response curves reflecting the addition of apoA-I rHDL to mouse plasma were similar when the efflux experiments were performed in the absence or presence of DTNB, an inhibitor of LCAT (not shown). This finding also provides support for the notion that the nonspecific diffusion-mediated pathway, which partly depends on LCAT activity, was not involved in the rHDL-dependent increase in the cholesterol efflux. The fact that even a 2.5-fold lower concentration of human apoA-II had a higher stimulatory effect than human apoA-I, when added to mouse plasma in discoidal rHDL, was unexpected. However, we made a similar observation when this set of apoA-I or apoA-II in rHDLs was added to plasma-free incubation medium of mouse peritoneal macrophage foam cells (M. Lee, P.T. Kovanen, G. Franceschini, L. Calabresi, unpublished data, 2000). Murine and human apoA-I are immunologically cross-reactive,37 but murine apoA-II differs significantly from human apoA-II regarding primary structure (a monomer instead of a dimer in the latter) and its effect on HDL size. Because human apoA-II is more hydrophobic than mouse apoA-II, we cannot reject the possibility that its incorporation into mouse HDL during the efflux period may have produced displacement of apoA-I from mouse HDL,14 and, by this mechanism, increased the removal of cellular cholesterol.

The present study did not focus on the role of phospholipids in cholesterol efflux. However, in light of the complex interactions of apoA-I with phospholipids in the efflux of cellular cholesterol,7,12,29 we tested the potential of adding either protein-free POPC or apoA-I-containing POPC (ie, apoA-I rHDL) to enhance the ability of wild-type plasma to stimulate cholesterol efflux, and we determined how proteolysis of the plasma would influence such stimulation. Interestingly, apoA-I rHDL and POPC liposomes slightly and equally stimulated the efflux potential of the untreated plasma. In contrast, only the addition of proteoliposomes produced a marked stimulatory effect, leading to full recovery of the efflux potential of the proteolysed plasma from which the apolipoproteins that are essential for this function had been depleted. The finding that even a huge amount of phospholipids (vastly beyond their concentration regarding the nonsupplemented plasma; see Results) was not sufficient to restore the efflux potential of the chymase-treated plasma clearly demonstrated that phospholipids alone are not competent to reverse the impairment in cholesterol efflux produced by proteolysis of HDL apolipoproteins. This finding provides strong support for the notion that chymase treatment of mouse plasma had abolished the apolipoprotein-mediated pathway of cholesterol efflux. It also potentially provides a novel model for the study of the efflux mechanism that is independent on the intactness of HDL apolipoproteins.

In summary, the present data indicate that proteolysis of mouse plasma by mast cell chymase causes functional failure of all the main apolipoproteins involved in the efflux of cellular cholesterol that can be compensated by supplementation of the plasma with discoidal HDL containing human apoA-I or apoA-II. The finding of protease sensitivity of all these apolipoproteins demonstrates that a single protease may effectively incapacitate the whole apolipoprotein-mediated removal of cell cholesterol.

Given the strong similarity between rat granule remnant chymase and human chymase regarding apolipoprotein HDL degradation,4 we hypothesize that in the human arterial intima in which degranulated mast cells have been observed,3 the secreted chymase may partially maintain its activity and thus may retard the efflux of cellular cholesterol by lowering the levels of apoA-I and other high-affinity efflux–promoting apolipoproteins in the intimal fluid.

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Methods (full-length version for online only)

Animals: Adult male Wistar rats and female NMRI mice were purchased from Viikki Laboratory Animal Center, University of Helsinki. A transgenic line of mouse which does not express murine apoA-I (A-I-KO mouse) was kindly provided by Dr. Nobuyo Maeda.\textsuperscript{15} C57BL/6J (wild-type) mice matched for sex and age were used as controls. Fasting blood was collected in precooled plastic tubes on EDTA (1 mg/mL). The blood was centrifuged at low speed at 4°C to obtain plasma. The plasma was placed on ice and used immediately for experiments or stored at –70°C until use.

Isolation of proteolytically active rat 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.\textsuperscript{17} 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.\textsuperscript{16} This isolation procedure does not release chymase from the heparin glycosaminoglycan chains of the granule remnants and partially protects chymase from inactivation in the presence of its physiologic inhibitors.\textsuperscript{18} Hence, reference to "chymase" or to "granule remnants" refers to the active chymase bound to the granule remnant heparin proteoglycan matrix and partially retaining its activity even when added to plasma or other extracellular fluids.

PMSF-inactivated granule remnants: Granule remnants were incubated in 5 mg/mL BSA, 1 mM EDTA, 5 mM Tris-HCl, pH 7.4, containing 250 µg/mL PMSF, at
37 °C for 15 min. The granule remnants were washed with the above buffer without PMSF, and their proteolytic activity was measured as described above. The degree of chymase inactivation in the PMSF-treated granule remnants was > 99%.

**Reconstituted HDL (rHDL).** ApoA-I and apoA-II were purified from human blood plasma, as previously described. Discoidal preβ-migrating rHDL containing palmitoyloleoylphosphatidylcholine (POPC) and apoA-I or apoA-II were prepared by the cholate dialysis technique, yielding particles with a POPC:protein weight ratio of 2.5:1. All of the added protein was incorporated into stable rHDL, and the final preparation contained no lipid-free apolipoprotein. The size distribution of rHDL was examined by non-denaturing polyacrylamide gradient gel electrophoresis on precast 8–25% polyacrylamide gels, using the Pharmacia Phast System. After Coomassie Brilliant Blue staining, the gels were scanned with a BioRad scanner, and the size of the rHDL particles 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 bovine serum albumin (7.1 nm) as calibration proteins. The size analysis revealed that the major populations of apoA-I− or apoA-II−-containing particles had average diameters of 9.6 and 10.0 nm, respectively. POPC liposomes were prepared by the procedure used for rHDL, but omitting the protein in the starting mixture. The phospholipid content of rHDL was determined by an enzymatic method, and the protein concentration was measured by the method of Lowry with bovine serum albumin as standard.

**Chemical modification and radioactive labeling of lipoproteins:** Human LDL (d = 1.019-1.050 g/mL) was isolated from fresh normolipidemic plasma by sequential ultracentrifugation, using KBr. LDL was acetylated (acetyl-LDL) by repeated additions
of acetic anhydride. Acetyl-LDL, radiolabeled by treatment with $^3$H-cholesteryl linoleate (1,2(n)$^3$H-cholesteryl linoleate, Amersham International) dissolved in 10% dimethylsulfoxide, yielded preparations of $^3$H-cholesteryl linoleate bound to acetyl-LDL with specific activities ranging from 30 to 100 dpm/ng protein. The radioactive labeling did not change the electrophoretic mobility of the labeled lipoproteins on agarose gels.

**Proteolysis of mouse plasma by chymase:** Treatment of mouse plasma with chymase was performed by incubating 50 µL of plasma from A-I-KO mouse or C57BL/6J (wild-type) mouse, respectively, at 37°C overnight in the absence (untreated plasma) or presence (chymase-treated plasma) of 50 µg of granule remnants (equivalent to 65 BTEE units of chymase) in 150 mM NaCl, 1 mM EDTA, 5 mM Tris-HCl, pH 7.4, the final concentration of plasma being 50% (vol/vol). The reaction was stopped by centrifugation at 15 000 rpm for 10 min to sediment the granule remnants. The supernatants were then subjected to agarose gel electrophoresis followed by apoA-I immunoblotting for immunodetection of apoA-I subpopulation of particles, or analyzed for their ability to induce cholesterol efflux from cultured mouse macrophage foam cells. Aliquots of supernatants from parallel incubations were adjusted to a density of 1.21 g/mL with solid KBr and the total mouse lipoprotein fraction was isolated from 100 µL of plasma by ultracentrifugation at 90 000 rpm for 3 hours at 4°C in an Air-Fuge (Beckman). Drops containing the isolated lipoproteins were dialyzed on floating 0.025 µm Millipore membranes against 150 mM NaCl, pH 7.4, denatured, and loaded onto a 4 to 20% linear gradient SDS-PAGE gel (BioRad). Electrophoresis of the apolipoproteins was performed for 2 h at 150 V, using Bio-Rad Kalleidoscope MW standards. Proteins were stained with Coomassie Brilliant Blue. To quantitate the apolipoprotein bands after
scanning of the gels, computer-assisted analysis was performed, using Matrox Inspector
image-processing software.

**Cholesterol efflux assay:** Peritoneal cells were harvested from unstimulated NMRI mice
in PBS containing 1 mg/mL BSA. After centrifugation, the cells were recovered 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 onto 24-well plates
(Becton Dickinson Labware). After incubation at 37°C for 2 h in humidified CO₂,
nonadherent cells were removed. The adherent cells (macrophages) were washed with
PBS and loaded with ³H-cholesterol by incubation for 18 h in the presence of 20 µg/mL
of ³H-cholesteryl linoleate acetyl-LDL in medium A containing 20% fetal calf serum.
The ³H-cholesterol-loaded macrophages were washed with PBS and incubated with fresh
medium A containing 10 I.U./mL of recombinant hirudin (CibaGeigy) as anticoagulant,
and the indicated concentrations of mouse plasma. In another set of experiments, mouse
plasma (2.5% v/v in medium) alone or in combination with rHDLs or POPC liposomes,
in the concentrations given in the figure legends, were added to the cells. After 4 h, the
media were collected and centrifuged at 200 g for 5 min. The radioactivity in each
supernatant, reflecting efflux of cellular radiolabeled cholesterol, was determined by
liquid scintillation counting as described previously, and normalized for the cell protein.
Under the conditions used, ³H-cholesterol efflux was found to be linear for up to 4 h of
incubation of plasma with foam cells. All experiments were performed at least twice. The
experimental data points shown are means of triplicate incubations in wells.

**Statistical analysis:** To allow crossed comparisons of multiple means from the
experiments in which different cholesterol acceptors were added to untreated and
chymase-treated plasmas respectively, a multiple-range statistical test was applied to the results shown in Figures 3 and 4. For this purpose, normal distribution of data was confirmed by the Kolmogoroff-Smirnov test, homogeneity of the variance by the Bartlett test, followed by bifactorial analysis and the Duncan multiple range test. In these tests, each mean was compared with the rest of the means, and the results of these comparisons are shown by different letters atop the columns. Among the mean values, the same letter denotes the absence and different letters denote the presence of statistically significant differences at the level of $P < 0.05$. 