Mast Cell–Mediated Inhibition of Reverse Cholesterol Transport

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Net cholesterol efflux from cholesterol-loaded macrophages, i.e., foam cells, was induced by incubating the foam cells with high density lipoprotein (HDL$_3$). However, when the incubation system included rat serosal mast cells stimulated to trigger exocytosis of their cytoplasmic secretory granules, the ability of HDL$_3$ to induce cholesterol efflux was largely lost. This loss was found to be due to the proteolytic action of chymase, the neutral protease of the granules, which degraded the apolipoproteins of HDL$_3$, so rendering them unable to mediate cholesterol efflux from the foam cells. The observation defines a novel cell-dependent mechanism that blocks the initial steps of reverse cholesterol transport and suggests a role for mast cell chymase in cellular accumulation of cholesterol, an early stage in atherogenesis. (Arteriosclerosis and Thrombosis 1992;12:1329-1335)

KEY WORDS • chymase • exocytosis • granules • high density lipoproteins • atherosclerosis

In atherogenesis, cholesterol accumulates in the intima, the innermost layer of the arterial wall. The first histological sign of atherosclerosis is the formation of foam cells, that is, subendothelially located monocyte-derived macrophages filled with low density lipoprotein (LDL)–derived cholesteryl esters. For foam cell formation to occur, the LDL particles must first be modified or bound to bodies that the macrophages will ingest. Then LDL uptake leading to cholesterol accumulation ensues by mechanisms that are not under negative-feedback control by the internalized LDL cholesterol. In addition to macrophages, mast cells are present in the arterial intima and are sometimes detected in the close vicinity of typical foam cells. The coexistence of these two cell types suggests that mast cells may be involved in the transformation of macrophages into foam cells. Indeed, recent in vitro studies with rat serosal mast cells have shown that these cells may induce a 50-fold increase in the rate of LDL uptake by macrophages, the critical component being the extracellular mast cell granule remnants, which bind LDL and carry it into the macrophages. These remnants are formed when the cytoplasmic secretory granules of stimulated mast cells are exocytosed and come in contact with the extracellular fluid. There they lose their soluble components, such as histamine, a fraction of their heparin proteoglycans, and all of the chondroitin sulfate proteoglycans but retain their two neutral proteases, chymase and carboxypeptidase A, which remain tightly bound to the residual heparin proteoglycan. The resulting granule remnants consist of a network of heparin proteoglycans in which the two neutral proteases are embedded.

In addition to LDL particles, high density lipoprotein (HDL) particles constantly pass from the bloodstream into the arterial intima. According to the "cholesterol balance hypothesis" of atherogenesis, these HDL particles prevent LDL-derived cholesterol from accumulating in the intima by carrying it back to the bloodstream. In this cholesterol-removing process called "reverse cholesterol transport," the initial step is the induction of cholesterol efflux from cholesterol-filled macrophage foam cells. Cholesterol efflux has been observed in studies in vitro on addition of human HDL$_3$ to foam cell cultures. In the present in vitro studies we show that stimulated mast cells, by proteolytically modifying the HDL$_3$ particles, markedly inhibit the transfer of cholesterol from foam cells to HDL$_3$.

Methods

Materials and Animals

[1α,2α(n)-3H]cholesteryl linoleate (30–60 Ci/mmol) and Na$^{125}$I (15–16 mCi 125I per microgram of iodine) were from Amersham International; Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, penicillin, and streptomycin were from GIBCO; bovine serum albumin (BSA), compound 48/80, soybean trypsin inhibitor, and potato carboxypeptidase A inhibitor were from Sigma; Biogel A-0.5m was from Bio-Rad; PD-10 columns prepacked with Sephadex G-25M were from Pharmacia; and plastic Petri dishes (35×10 mm, No. 1008) were from Falcon. Male Wistar rats (300–500 g) were from the Laboratory Animal Center of the University of Helsinki, and female NMRI mice (20–30 g) were purchased from Helmi Poikkijoki (Kuuskangas, Finland).

Preparation of Macrophage Monolayers

Peritoneal cells were harvested from unstimulated mice in phosphate-buffered saline (PBS) containing 1 mg/ml BSA. The cells were pooled, centrifuged, and resuspended in medium A (DMEM containing 20%...
fetal calf serum, 100 IU of penicillin per milliliter, and 100 μg of streptomycin per milliliter). The cells were plated at 4–6x10^4 cells per dish. After incubation for 3 hours in humidified CO_2 (5%) at 37°C, the cells were washed three times with 2 ml PBS, and the macrophage monolayers were used for subsequent experiments.

Preparation of Mast Cell Suspensions, Stimulation of Mast Cells, and Isolation of Mast Cell Granule Remnants

Mast cells were isolated from peritoneal and pleural cavities of rats and stimulated with compound 48/80, and from the released material exocytosed granule remnants were isolated as previously described.10 The quantity of granule remnants is expressed in terms of remnant protein.

Preparation and Radioactive Labeling of Lipoproteins

Human LDL (d = 1.019–1.050 g/ml) and HDL_3 (d = 1.125–1.215 g/ml) were fractionated by sequential ultracentrifugation. LDL was acetylated (acetyl-LDL) by repeated additions of acetic anhydride.16 Acetyl-LDL was radiolabeled with [³H]cholesterol linoleate dissolved in dimethylsulfoxide and yielded preparations of [³H]cholesterol linoleate bound to acetyl-LDL. [³H]cholesterol linoleate–acetyl-LDL with specific activities ranging from 30 to 100 x 10^3 dpm/μg protein. HDL_3 was iodinated by the iodine monochloride method as described.18 The concentrations of acetyl-LDL and HDL_3 are given in terms of their protein contents.

Loading of Macrophages With Cholesterol Esters and Induction of Cholesterol Efflux

Macrophage monolayers were incubated in medium A containing 20 μg/ml [³H]-labeled acetyl-LDL at 37°C for 18 hours. After incubation the medium was removed, and the cells were rinsed three times with 2 ml PBS. The monolayers then received 1 ml medium B (DMEM containing 100 IU of penicillin per milliliter and 100 μg of streptomycin per milliliter) containing 25 μg/ml HDL_3 or granule-treated HDL_3 and were incubated for 1–8 hours to study the rate of efflux of [³H]cholesterol into the culture medium. After incubation the [³H]radioactivity of the medium was determined, and from each value the corresponding blank value (no addition of HDL_3) was subtracted. Each point in the figures represents the average of duplicate incubations. In those experiments in which mass movements of cellular cholesterol were studied, unlabeled acetyl-LDL was used, the concentration of HDL_3 in the medium was 50 μg/ml, and incubations were carried out in triplicate.

Proteolytic Degradation of HDL_3 by Granule Remnants

In a standard assay, 1 mg/ml of [¹²⁵I]HDL_3 (500–8,000 cpm/μg) was incubated with 170 μg/ml of granule remnants in a mixture of 5 mM tris(hydroxymethyl)aminomethane-hydrochloride, 150 mM NaCl, and 1 mM EDTA (pH 7.4) at 37°C for 30 minutes. The granule remnants were then centrifuged at 20,000g for 5 minutes, and aliquots of the supernatants were applied to macrophage monolayers to give final concentrations of HDL_3 as indicated in the figure legends. After incubation, aliquots taken from the incubation media were treated with 10% trichloroacetic acid to determine the amount of trichloroacetic acid–soluble radioactivity. The concentration of HDL_3 is expressed in terms of the total HDL_3 protein, which was determined before proteolytic degradation of the lipoprotein to ensure equimolar concentrations of the variously proteolyzed HDL_3 particles.

Extraction of Sterols From Culture Media and From Macrophage Monolayers

To study the [³H]cholesterol efflux, the culture medium was collected and extracted after incubation,20 and the [³H] radioactivity of the medium was measured with a liquid scintillation counter. Thin-layer chromatography showed that 95% of the radioactivity was present in the free cholesterol fraction. For measurements of the cellular cholesterol mass, the macrophage monolayers were extracted with hexane/isopropanol (3:2, vol/vol), and an aliquot was taken for fluorometric cholesterol assay.20 After extraction of the lipids, the cells in the monolayers were dissolved in 0.2 M NaOH for protein determination.

Other Assays

Lipoprotein triglyceride and phospholipid contents were determined by using commercial kits (Triglycerides GPO-PAP, Boehringer-Mannheim; Phospholipids B, Wako Chemicals). Protein content was determined by the procedure of Lowry et al,21 with BSA as the standard. Histamine content was determined fluorometrically as described.18 The extent of lipid peroxidation of HDL_3 was measured as thiorbituric acid-reactive materials and expressed as malondialdehyde equivalents.22 Chymase and carboxypeptidase A activities were measured spectrophotometrically as described.23 Agarose gel electrophoresis was performed with the Paragon system for lipoprotein electrophoresis (Beckman). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli24 in 15% acrylamide gels on nonreduced samples.

Results

Mouse peritoneal macrophages were loaded with radiolabeled cholesterol by incubating them with acetyl-LDL in which the cholesteryl ester component had been labeled with [³H]cholesterol linoleate. In the resultant foam cells the cholesteryl ester content was increased, on average, 20-fold (from 5.9±4.3 to 113±37 μg/mg cell protein; mean±SD), and the cholesteryl esters were radiolabeled (20–50 x 10^3 dpm/μg total cellular cholesteryl esters). Incubation of these foam cells with HDL_3 resulted in a rapid and steady flux of [³H] radioactivity into the incubation medium (Figure 1A). When mast cells were added to the incubation medium together with HDL_3 and stimulated with compound 48/80, the ability of the HDL_3 to induce cholesterol efflux was greatly reduced (Figure 1A). The mast cell effect was already evident at 1 hour. Thereafter, only minimal rates of cholesterol efflux could be detected. To ascertain whether the stimulated mast cells blocked efflux of the labeled cholesterol by acting on the foam cells, medium with fresh HDL_3 was added to the foam cells, and [³H]cholesterol efflux was measured. As shown in Figure 1B, the rates of cholesterol efflux from control...
FIGURE 1. Effect of stimulated mast cells on high density lipoprotein (HDL)-induced efflux of [3H]cholesterol from macrophage foam cells. Panel A: Macrophages were incubated in 1 ml medium A (see "Methods") containing 20 μg [3H]cholesteryl linoleate--acylated low density lipoprotein (acyt-LDL) for 18 hours, and then each monolayer was washed and received 5 X 10^6 mast cells in 1 ml medium B (see "Methods") containing 25 μg/ml HDL3. The mast cells were then stimulated by addition of 5 μg/ml compound 48/80. After incubation at 37°C for the indicated times the medium was removed, aliquots were taken for measurements of the amounts of [3H] radioactivity and histamine, and the results were plotted as a function of time. Of the total cellular histamine, 60% was released into the incubation medium, reflecting 60% degranulation of the mast cells. No mast cells were added to the control dishes. Panel B: The macrophage foam cells from the experiment in panel A were washed three times with each of the following: 2 ml of phosphate-buffered saline (PBS) containing 10% fetal calf serum, then 2 ml of PBS containing 10 mg/ml bovine serum albumin, and finally 2 ml of PBS. The monolayers then received fresh medium B containing 25 μg/ml HDL3, 5 mg/ml bovine serum albumin, and 100 μg/ml soybean trypsin inhibitor and were incubated at 37°C for the indicated time periods. After incubation the amount of [H] radioactivity in the medium was measured and plotted as a function of time. Panel C: The medium from the experiment in panel A was centrifuged at 12,000g for 5 minutes to sediment the mast cells and exocytosed granule remnants and was added to fresh macrophage foam cells prepared in the same way as in panel A. After incubation at 37°C for the indicated time periods, the medium was removed and the amounts of [H] radioactivity were measured and plotted as a function of time. ○—○, Without mast cells; △—△, with mast cells.

Since the efflux-blocking principle depended on mast cell stimulation, i.e., degranulation, we next studied whether granule remnants derived from exocytosed cytoplasmic granules would reduce cholesterol efflux from foam cells. For this purpose, the apolipoproteins of HDL3 were radiolabeled with 125I, and this 125I-HDL3 was then incubated at 37°C for 30 minutes with granule remnants isolated from the incubation medium in which mast cells had been stimulated. After incubation, the granule remnants were sedimented. No cosedimentation of the 125I-HDL3 was observed, indicating that the HDL3 particles were not bound to the granule remnants. However, incubation of 125I-HDL3 with granule remnants led to degradation (6%) of the radiolabeled apolipoproteins, as measured by the formation of 125I-labeled trichloroacetic acid--soluble products. Finally, granule remnant--treated HDL3 was added to monolayers of [3H]cholesterol-labeled foam cells. Both untreated 125I-HDL3 and remnant-treated 125I-HDL3 induced steady effluxes of [3H]cholesterol from the foam cells during the 8-hour incubation period. However, the ability of the remnant-treated HDL3 to induce efflux of [3H]cholesterol from the foam cells was reduced by 75% (Figure 2).

The two main proteases of granule remnants, chymase and carboxypeptidase A, act in concert to degrade apolipoproteins.23 To study their respective roles in the observed proteolytically induced functional modification of HDL3, we incubated 125I-HDL3 with granule remnants in which one or the other enzyme had been selectively inhibited. In this experiment, incubation with proteolytically active granule remnants for 30 minutes led to 8% degradation of the radiolabeled apolipoproteins and approximately 50% inhibition of [3H]cholesterol efflux from the foam cells. When chymase was inhibited with soybean trypsin inhibitor, thus leaving carboxypeptidase A active, no degradation of 125I-HDL3 was observed, and the ability of the HDL3 particles to induce cholesterol efflux was fully retained. In contrast, when carboxypeptidase A was inhibited with potato carboxypeptidase A inhibitor, thus leaving chymase active, 4% of the 125I-labeled apolipoproteins of HDL3 were degraded, accompanied by about 40% inhibition of the cholesterol efflux. Thus, the proteolytic modification of the HDL3 particles leading to reduced efflux of cholesterol was due to the granule chymase. In such granule remnant--modified HDL3 particles, the contents of unesterified cholesterol, cholesteryl esters, phospholipids, and triglycerides remained unchanged. Moreover, no thiobarbituric acid--reactive material was
Granule remnant-treated HDL₃

**Figure 2.** Line plot showing the abilities of high density lipoprotein₃ (HDL₃) and granule remnant-treated HDL₃ to stimulate [³H]cholesterol efflux from macrophage foam cells. Macrophages were incubated in 1 ml of medium A (see "Methods") containing 20 µg of [³H]cholesterol linoleate-acetylated low density lipoprotein (acetyl-LDL) for 18 hours, and then each monolayer was washed and received 1 ml of medium B (see "Methods") containing 25 µg/ml of either HDL₃ (•—•) or HDL₃ that had been incubated in the presence of isolated mast cell granule remnants (A—A) (see "Methods"). After incubation at 37°C for the indicated time periods, the amount of [³H] radioactivity in the medium was measured.

formed during incubation of HDL₃ with the granule remnants. Taken together, the above experiments demonstrated that what rendered HDL₃ less effective in inducing efflux of radiolabeled cholesterol from macrophage foam cells was the proteolytic degradation of HDL₃ by the neutral protease chymase of the exocytosed mast cell granules.

To exclude the possibility that the observed decrease in cholesterol efflux was caused by aggregation of the proteolyzed HDL₃ particles, a control experiment was carried out in which we incubated ¹²⁵I-HDL₃ with proteolytically active granule remnants and subsequently subjected the incubation mixture to gel filtration on a Bio-Gel A-0.5m column (1×45 cm). We found that the elution profiles of proteolytically modified HDL₃ and of untreated HDL₃ were identical. Finally, we found that incubation of the granule remnant-treated HDL₃ with foam cells was not accompanied by uptake of the ¹²⁵I-HDL₃ by the foam cells, demonstrating that the observed decrease in cholesterol efflux was not due to loss of proteolyzed HDL₃ particles from the incubation medium.

To quantitatively relate the proteolytic effect of chymase to its ability to block reverse cholesterol transport, we determined the ability of HDL₃ to promote [³H]cholesterol efflux from foam cells as a function of the degree of apolipoprotein degradation. Since the full granule-remnant effect was achieved within 2 hours (see Figure 1A), HDL₃ was incubated with granule remnants for periods of time up to 2 hours. As shown in Figure 3A, incubation of ¹²⁵I-HDL₃ in the presence of granule remnants resulted in its progressive degradation. The more extensively ¹²⁵I-HDL₃ was proteolyzed, the smaller was its capacity to promote the efflux of labeled cholesterol from the foam cells (Figure 3B). Most importantly, even minimal proteolytic modification of ¹²⁵I-HDL₃ strongly inhibited cholesterol efflux from the foam cells. Thus, after only a 2-minute incubation of ¹²⁵I-HDL₃ with the granule remnants, when less than 4% of the apolipoproteins of HDL₃ had been degraded, cholesterol efflux was reduced to about half of the maximal inhibition. Such minimal degradation of HDL₃ left the apolipoproteins (AI and AII) of HDL₃ visually unaltered when analyzed by SDS-PAGE. Moreover, on agarose gel electrophoresis the mobility of HDL₃ was unchanged.

**Figure 3.** Curves for granule remnant-induced proteolytic degradation of high density lipoprotein₃ (HDL₃) (panel A) and the ability of proteolyzed HDL₃ to stimulate [³H]cholesterol efflux from macrophage foam cells (panel B). HDL₃ was incubated with granule remnants according to the standard procedure (see "Methods") for the indicated time periods. After incubation the granule remnants were removed, aliquots were taken for determination of trichloroacetic acid–soluble material (panel A), and the remaining incubation medium was added to macrophage foam cells loaded with [³H]cholesterol as described in the legend to Figure 1. After incubation at 37°C for 8 hours, the amount of [³H] radioactivity in the medium was measured (panel B).

Figure 4 compares the relation between the concentration of HDL₃ and the rate of [³H]cholesterol efflux from foam cells. If native HDL₃ was added to the incubation medium, the rate of cholesterol efflux rose sharply, with increasing concentrations of HDL₃ up to 25 µg/ml (Figure 4A); at levels above this concentration, the efflux continued to increase but considerably less steeply. In contrast, addition of increasing concentrations of granule remnant–treated HDL₃ to the incubation system led to only a moderate increase in cholesterol efflux. Kinetic analysis of these data indicated
FIGURE 4. Relation of \(^{3}H\) cholesterol efflux from macrophage foam cells to the concentrations of high density lipoprotein (HDL) and granule remnant-treated HDL. Panel A: Macrophages were incubated in 1 ml of medium A (see "Methods") containing 20 \(\mu\)g of \(^{3}H\) cholesterol linoleate-acetylated low density lipoprotein for 18 hours, and then each monolayer was washed and received 1 ml of medium B (see "Methods") containing the indicated concentrations of either HDL (---) or HDL that had been incubated in the presence of isolated mast cell granule remnants (---) (see "Methods"). After incubation at 37°C for 8 hours, the amount of \(^{3}H\) radioactivity in the medium was measured and plotted as a function of time. Panel B: Double-reciprocal plot of the data in panel A. The apparent \(V_{\text{max}}\) and \(K_{m}\) values for control and granule remnant-treated HDL were 450 dpm versus 1,000 dpm/\(\mu\)g cell protein, and 23 versus 45 \(\mu\)g/ml, respectively.

that the reduction in the rate of cholesterol efflux resulted from both a decrease in the apparent \(V_{\text{max}}\) values of the efflux system and an apparent decrease in the affinity of HDL for this system (apparent \(K_{m}\) values) (Figure 4B).

To study whether the observed HDL-induced \(^{3}H\)-cholesterol efflux and its inhibition by granule remnants reflected changes in the mass transfer of cholesterol from the foam cells, we measured the cholesterol content of foam cells before and after incubation with either native or granule remnant-treated HDL. First, the macrophage monolayers were incubated with acetyl-LDL for 18 hours as in the previous experiments. When the foam cells were then incubated in the presence of native HDL, the cellular content of cholesteryl esters decreased by 75% within 24 hours (Figure 5). In the presence of HDL, that had been treated with granule remnants for 2, 5, or 30 minutes and in which the extent of apolipoprotein degradation was 2%, 7%, and 11%, respectively, the rate of cholesterol mass transfer from foam cells to HDL progressively declined.

Discussion

We describe a mechanism by which exocytosed secretery granules of rat serosal mast cells modify HDLs. The critical component in this process is the neutral protease chymase, a component of the granule remnants that proteolyses the apolipoproteins of HDL and so blocks the HDL-induced efflux of cholesterol from foam cells. Previously, we have shown that another granule-remnant component, heparin proteoglycan, is also involved in the mast cell–mediated formation of macrophage foam cells. Thus, ionic interaction between the apolipoprotein B of LDL and the heparin of the granule remnants leads to binding of LDL particles to the remnant surfaces and initiates a process in which remnants carry LDL into macrophages. Moreover, we have found that chymase, by degrading the apolipoprotein B component of remnant-bound LDL and by inducing fusion of LDL particles on the remnant surfaces, greatly enhances the capacity of the granule remnants to bind LDL and carry it into the macrophages. Thus, these multiple actions of granule remnants on lipoprotein metabolism all appear to favor accumulation of cholesterol in macrophages and so

FIGURE 5. Plot showing effect of granule remnant treatment on the ability of high density lipoprotein (HDL) to stimulate mass transfer of cholesterol from macrophage foam cells. Macrophages were incubated in 1 ml of medium A (see "Methods") containing 20 \(\mu\)g of acetylated low density lipoprotein (acetyl LDL) for 18 hours (■---), and then each monolayer was washed and received 1 ml of medium B (see "Methods") containing 50 \(\mu\)g/ml of either untreated HDL (■--■) or HDL that had been incubated for 2, 5, or 30 minutes in the presence of isolated mast cell granule remnants (■---) (see "Methods"). After incubation at 37°C for 24 hours, the monolayers were washed and their contents of esterified cholesterol were measured.
render the stimulated mast cells potentially powerful inductors of cholesterol accumulation in macrophages.

The herein-described mast cell–dependent proteolytic inhibition of the ability of HDL₄ to promote efflux of cholesterol from macrophage foam cells provides an experimental tool for determining whether proteins are critical in the initial steps of reverse cholesterol transport. Indeed, a major controversy exists today about the role of protein components in cholesterol efflux between cells and HDL. The results obtained by some laboratories suggest that net cholesterol efflux does depend on specific interaction between the apolipoproteins of HDL (either Al or AII) and specific HDL binding sites on the plasma membrane of the cholesterol donor cells, whereas results from other laboratories suggest that interaction between the aforementioned protein components is of no importance for net cholesterol efflux. The observation that even a minimal degree of chymase-induced modification of HDL₃ strongly inhibits the ability of HDL₂ to remove cholesterol from macrophage foam cells over a wide range of acceptor concentrations (see Figures 3A and 3B) clearly indicates that, at least in this experimental model of reverse cholesterol transport with cholesterol-loaded macrophages, efficient net removal of cellular cholesterol requires not only the presence of the apolipoproteins of HDL₂ but also their integrity.

For maintenance of cholesterol balance in the arterial intima, the rate of cholesterol efflux from macrophages must equal that of cholesterol influx into them. The abundance of HDL particles in the interstitial fluid, which greatly exceeds the concentration required for saturation of the cholesterol efflux system, should provide sufficient vehicles for the removal of cholesterol from the intimal macrophages. It is not known what triggers the highly localized disturbances in the sensitive balance of intimal cholesterol metabolism and so leads to the formation of foam cells at certain predilection sites of the arterial tree. One possible explanation for such localized disturbances of intimal lipoprotein metabolism is the presence in the arterial intima, sometimes adjacent to macrophages, of mast cells with their peculiar system of exocytosis of proteolytically active cytoplasmic organelles, the granules.

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