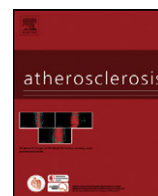




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Mast cell-dependent proteolytic modification of HDL particles during anaphylactic shock in the mouse reduces their ability to induce cholesterol efflux from macrophage foam cells *ex vivo*

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ABSTRACT

Objective: We have found previously that proteolytic modification of HDL by mast cell chymase *in vitro* reduces cholesterol efflux from cultured macrophage foam cells. Here, we evaluated whether mast cell-dependent proteolysis of HDL particles may occur *in vivo*, and whether such modification would impair their function in inducing cellular cholesterol efflux *ex vivo*.

Methods: Systemic activation of mast cells in the mouse was achieved by intraperitoneal injection of a high dose of the mast cell-specific noncytotoxic degranulating agent, compound 48/80. Serum and intraperitoneal fluid were then evaluated for degradation of HDL apolipoproteins and for their potential to act as cholesterol acceptors from cultured mouse macrophage foam cells.

Results: Lysates of isolated mouse peritoneal mast cells containing active chymase partially proteolyzed apoA-I in α - and pre β -HDL particles in mouse serum *in vitro*, and, when injected into the mouse peritoneal cavity, the lysates also degraded endogenous apoA-I in peritoneal fluid *in vivo*. Systemic activation of mast cells in mast cell-competent mice, but not in mast cell-deficient (W-sash c-kit mutant) mice, reduced the ability of serum and intraperitoneal fluid derived from these animals to promote efflux of cellular cholesterol. This inhibitory effect was related to mast cell-dependent proteolytic degradation of apoA-I, apoA-IV, and apoE, i.e., the HDL-associated apolipoproteins that are efficient inducers of cholesterol efflux.

Conclusion: The present results document a role for extracellular mast cell-dependent proteolysis in the generation of dysfunctional HDL, and suggest an inhibitory role for mast cells in the initial step of reverse cholesterol transport *in vivo*.

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1. Introduction

In the intima of human atherosclerotic arteries, mast cells are found in close vicinity to macrophage foam cells [1]. Mast cells are a potent source of neutral proteases in the arterial intima, where they might activate pro-metalloproteinases, thereby destabilizing atheromatous plaques [2]. Among the potential mast cell-activating agents in atherosclerotic lesions are oxidized LDL [3] and components of the complement system, such as C5a [4]. Upon activation, mast cells degranulate, releasing soluble components, such as histamine, and solid-phase proteases, such as chymase, which remain bound to the heparin proteoglycans. *In vitro* studies on rat and human chymases showed that heparin-bound, but not unbound

chymase, remains partially active in serum and intimal fluid, being able to degrade its physiological inhibitor α_1 -antitrypsin [5,6]. Thus, once in the extracellular fluid of the intima, chymase has the potential to proteolyze several protein substrates, among them various HDL apolipoproteins.

HDL particles in the intimal fluid promote efflux of unesterified cholesterol from macrophage foam cells by different mechanisms, as recently reviewed by Rothblat and co-workers [7]. Cholesterol efflux initiates reverse cholesterol transport, counteracting cholesterol accumulation derived from atherogenic lipoproteins, and so tends to retard atherosclerosis [8]. Importantly, the transmembrane protein ABCA1 transporter mediates unidirectional efflux of cholesterol from macrophage foam cells to both lipid-free and lipid-poor species of apoA-I, and also other apolipoproteins containing amphipathic helices [9]. Of note, various proteases found in the human arterial intima reduce the ability of HDL to induce cellular cholesterol efflux from macrophage foam cell cultures [10–12].

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Among them, mast cell proteases are able to impair cellular cholesterol efflux to HDL₃, human plasma, and to human aortic intimal fluid [13,14]. By degrading lipid-poor pre β -migrating HDL, chymase fully blocked ABCA1-mediated cholesterol efflux from J774 mouse macrophages [15,16]. Of note, the secreted chymase does not act on the macrophage plasma membrane proteins involved in cholesterol efflux [17]. Overall, these studies highlight the integrity of the various apolipoproteins of HDL particles to be essential to promote cholesterol efflux, and also support the notion that, besides HDL particle quantity, also particle quality is important for efficient induction of cholesterol efflux.

We have found that local activation of mast cells present in the rat peritoneal cavity efficiently impaired cholesterol release from peritoneal macrophage foam cells generated *in vivo* [6]. Here we explored whether proteolysis of HDL particles present in mouse serum and intraperitoneal fluid would occur after triggering systemic activation of mast cells, and whether such proteolytic modification would compromise their function as cholesterol acceptors. We demonstrated that activation of mast cells *in vivo* inhibited the ability of both serum and intraperitoneal fluid to promote efficient efflux of cholesterol from cultured mouse macrophage foam cells. This effect was due to degradation of HDL-associated apolipoproteins that promote cellular cholesterol removal, so revealing that extracellular proteolysis *in vivo* is able to block the initial step of macrophage-specific reverse cholesterol transport.

2. Materials and methods

2.1. Reagents

Mast cell degranulating compound 48/80 (C48/80) was from Sigma–Aldrich, the chromogenic peptide substrate S-2586 from Chromogenix, medetomidine 1 mg/ml (Domitor) from Orion Pharma, and ketamin 50 mg/ml (Ketalar) from Pfizer Inc. Dulbecco's Phosphate Buffered Saline (PBS), and Dulbecco's Modified Eagle's Medium (DMEM) medium were from Cambrex.

2.2. Animals

NMRI mice were from Viikki Laboratory Animal Center, University of Helsinki, and the mast cell-deficient strain of mice *W-sash c-kit* mutant *Kit^{W-sh/W-sh}* created on the C57Bl/6J background, were purchased from the Jackson Laboratory (Bar Harbor, Maine). The mice (female, 10 to 12 weeks old), housed 5 per cage in conditions controlled for light/dark cycle, temperature and humidity, were provided with a standard chow diet and water *ad libitum*. The experiments were conducted in conformity with Finnish regulations, and the protocols were approved by The National Animal Care and Use Committee of Finland.

2.3. HDL analysis in serum and intraperitoneal fluid

Mouse blood was obtained by cardiac puncture, collected in precooled plastic vials and centrifuged at $400 \times g$ at 4°C to obtain serum, which was used immediately for experiments or stored at -70°C until analysis. Pools of serum were adjusted to a density of 1.21 g/mL and the total lipoprotein fraction was isolated by ultracentrifugation, as described before [18]. Isolated lipoproteins were loaded onto SDS-PAGE 4–20% (BioRad), and proteins were stained with Simply Blue Safe Stain (Invitrogen Corp.). Apparent molecular masses were determined using low MW protein standards. Further analysis of serum lipoproteins was performed by applying 60–70 μL of pooled serum to an FPLC size-exclusion chromatography column (Superose 6HR, GE Amersham Pharmacia Biotech, Newcastle, UK). The column was operated at a flow rate of 0.5 mL/min in

phosphate-buffered saline (PBS, pH 7.4), containing 0.05% Tween 20. Fractions of 0.5 mL were collected and analyzed for cholesterol (Hoffman-La Roche, fully enzymatic Kit # 0722138), for phospholipids (Wako Chemicals GmbH, Neuss, Germany, enzymatic kit # 990-54009), for apoA-I by non-competitive Elisa [19], and for apoE by Western blotting using specific anti-mouse apoE antibody [20]. Intraperitoneal fluid was collected from the peritoneal cavity by gentle aspiration using a plastic pipette, and centrifuged at 4°C at low speed to sediment detached peritoneal cells. Aliquots of serum and intraperitoneal fluid were subjected to agarose electrophoresis followed by immunoblotting with apoA-I polyclonal antiserum (Novus Biologicals), and α - and pre β -HDL bands were visualized by enhanced chemiluminescence (ECL). Semiquantitative analysis (mean $\pm$ SD) of the intensity of the bands was performed by scanning (Bio-Rad Gel Doc 2000 densitometer) and expressed in arbitrary units (AU).

2.4. Preparation of peritoneal mast cell lysates

Peritoneal cells were collected from nonstimulated mice by peritoneal washing with PBS containing BSA 1 mg/mL, and heparin 0.05 mg/mL. Cells were recovered after centrifugation at $200 \times g$ for 10 min, resuspended in DMEM, and cultured at 37°C in humidified CO₂ for 2 h to allow attachment of the peritoneal macrophages. The medium was collected and centrifuged to sediment nonadherent cells, after which the cells were resuspended in PBS. Aliquots of the cell suspension were stained in 1:1 volume of Moore & James staining solution, and counted in a Burkner chamber. Finally, the density of mast cells was adjusted to 1000 mast cells/ μL PBS. The mast cell suspensions were then sonicated on ice for 10 s at a constant duty cycle (Branson sonicator, model 250) to obtain a mast cell lysate.

2.5. Chymotryptic activity in mast cell lysates in the presence of natural inhibitors

Pejler and coworkers have shown that the majority of the chymotryptic activity in mouse peritoneal mast cells is due to the presence of mouse mast cell protease 4 (mMCP-4), and that this protease is a chymase [21]. Hence, when specifically referring to chymotryptic activity of mouse peritoneal mast cells, we will use the term “chymase activity”. Twenty μL of the mast cell lysate was suspended in 100 μL of H₂O followed by addition of 20 μL of the chromogenic substrate for chymotrypsin-like proteases S-2586 (1.8 mM in H₂O), and the change in absorbance was measured at 405 nm at 30 s intervals for 5 min. One arbitrary unit corresponds to a 0.0001 increase in absorbance at 405 nm/min under the described conditions of the assay. To determine the inhibitory effect of the natural inhibitors of chymase present in body fluids, chymase activity derived from 20,000 mast cells was measured in the mast cell lysate after adding serum or intraperitoneal fluid to reach final concentrations up to 80% in 100 μL of reaction mixture. Chymase activity in the various lysate preparations was 1–2 units/1000 mast cells.

2.6. Proteolysis by mast cell lysates of mouse serum HDL *in vitro* and of intraperitoneal endogenous apoA-I *in vivo*

Ten μL of serum was incubated at 37°C in 100 μL 5 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA (TNE buffer), in the absence or presence of mast cell lysates derived from 20,000 peritoneal mast cells. After 30 min the reaction was stopped by centrifugation at $10,000 \times g$ for 10 min at 4°C . Aliquots of the supernatants were subjected to agarose gel electrophoresis and apoA-I Western blotting to visualize HDL subpopulations by ECL. In other experiments, groups of mice were given i.p. injections of mast cell lysates derived from 100,000 or 200,000 mast cells in 100 μL PBS. After 4 h the mice were sacrificed and intraperitoneal fluid was col-

lected, pooled for each group, and apoA-I of intraperitoneal fluid was determined [19].

2.7. Compound 48/80-mediated systemic stimulation of mast cells

Four independent experiments using totally 60 NMRI were carried out. Mice received first subcutaneous injection of a medetomidine (2–4 mg/kg) plus ketamine (150–300 mg/kg), and then they were given an i.p. injection of C48/80 (8 mg/kg) in 100 μ L of NaCl 0.9%, a dose that induces systemic fatal allergic reaction within 1 h [22]. A group of mice matched for weight received corresponding volumes of NaCl 0.9% (control group). The mice were sacrificed 30 min after induction of the anaphylactic response, and control mice were sacrificed at matching times of death. Serum and intraperitoneal fluid were collected from each group, pooled and subjected to electrophoretic analysis, or their function as cholesterol acceptors was evaluated as described below. Cholesterol and protein concentrations were determined in unfractionated sera and in the total lipoprotein fraction ($d < 1.21$ g/mL). Histamine concentration in serum was measured using the 0-phthalaldehyde spectrofluorometric procedure [23]. Following same setup, 8 mg/kg of C48/80 was i.p. injected in 12 mast cell-deficient *sash* mice. Serum and intraperitoneal fluid were collected and used for cholesterol efflux studies.

2.8. Measurement of [^{3} H]cholesterol efflux from macrophage foam cells

Peritoneal macrophages isolated from nonstimulated control mice were loaded with cholesteryl esters by incubation for 18 h in the presence of 20 μ g/mL of [^{3} H]CE-acetyl-LDL (76 dpm/ng protein) in DMEM supplemented with 20% FCS, as described before [15]. Increasing concentrations of serum or intraperitoneal fluid were added to the [^{3} H]cholesterol-loaded macrophage foam cell cultures in the presence of 10 I.U./mL of desirudin (Canyon Pharma, UK) as an anticoagulant. After 4 h of incubation, the cell medium was analyzed for [^{3} H]-radioactivity by liquid scintillation counting. Fractional cholesterol efflux was calculated as $\text{dpm}_{\text{medium}}/(\text{dpm}_{\text{cells}} + \text{dpm}_{\text{medium}}) \times 100$. The results remained essentially the same when cholesterol efflux was expressed per mg cell protein.

3. Results

3.1. Chymase activity in mouse peritoneal mast cell lysates

The specific expression pattern and physiological responses of chymases may vary considerably among mammalian species [21]. Mast cell lysates obtained by sonication rendered preparations that displayed a linear relation between mast cell numbers and chymase activity up to at least 50,000 mast cells per assay (Fig. 1A). Next, we measured the activity of chymase present in mast cell lysates in the presence of its natural inhibitors, i.e. by adding to the lysates increasing concentrations of mouse serum or intraperitoneal fluid, the latter one being a surrogate of extracellular fluid, such as the intimal fluid (Fig. 1B). Consistent with the high concentrations of protease inhibitors in mouse serum [24], a sharp decline ($\approx 70\%$) in activity already occurred at a serum concentration of 10% (v/v), and 50% serum almost completely inactivated chymase. In contrast, after an initial loss of about half of the chymase activity in 10% (v/v) intraperitoneal fluid, no further reduction was observed even when the concentration of intraperitoneal fluid was increased up to 80%. These findings revealed that the mouse peritoneal cavity was a suitable compartment for further study of the physiological consequences of mast cell activation *in vivo*, and suggested

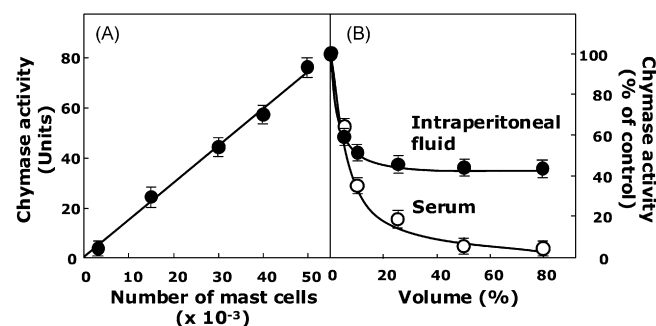


Fig. 1. Chymase activity in mouse peritoneal mast cell lysates in the presence of serum and intraperitoneal fluid. (A) Chymase activity in a cell lysate obtained by sonication of mouse peritoneal mast cells and expressed as a function of mast cells number. (B) Chymase activity of the lysate (20,000 mast cells in the assay) was evaluated in the presence of increasing concentrations of mouse serum and intraperitoneal fluid (% in PBS), and expressed as a percentage of the control (total activity, i.e. in the absence of inhibitors). Results are means of triplicates $\pm$ SD.

that chymase-dependent proteolysis of HDL could occur in other extravascular body compartments, as well.

3.2. Proteolysis of mouse HDL and apoA-I by mast cell lysates

The ability of mouse chymase present in mast cell lysates to degrade HDL particles present in mouse serum was evaluated *in vitro* (Fig. 2A). Incubation of aliquots of lysates derived from 20,000 mast cells in 10% of serum, i.e. at a concentration in which $\approx 30\%$ of chymase activity remains non-inhibited (see Fig. 1B), reduced the

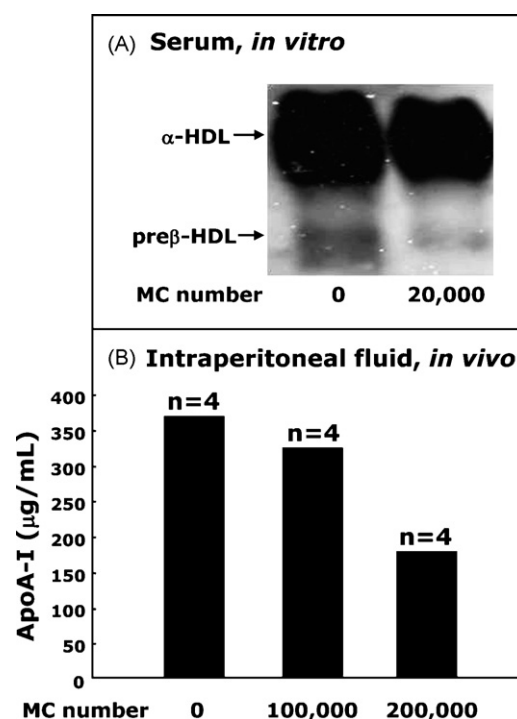


Fig. 2. Degradation of HDL and apoA-I in mouse serum and intraperitoneal fluid by peritoneal mast cell lysates. (A) Mouse serum was incubated for 30 min at 37 $^{\circ}$ C in the absence or presence of mouse mast cell lysate derived from 20,000 peritoneal mast cells. Serum was centrifuged to stop the reaction and aliquots of the supernatants were applied to agarose gel electrophoresis followed by Western blotting for apoA-I. (B) Mice were given i.p. injections of mast cell lysates derived from 100,000 and 200,000 mast cells in 100 μ L PBS. After 4 h mice were sacrificed and intraperitoneal fluid was collected and kept on ice. Analysis of apoA-I in pools of intraperitoneal fluid was carried out by Elisa, as described in Section 2. The specific activity of chymase in the lysate was 18 units/10,000 mast cells. N = number of animals/group.

intensities of both the α - and pre β -HDL bands (by $\approx 12\%$ and $\approx 70\%$, respectively) in apoA-I immunoblots. This result revealed that mouse chymase has a broader specificity towards HDL than its rat and human counterparts, which preferentially degrade lipid-poor HDL particles present in minor concentrations in human serum *in vitro* (reviewed in [6]). Next, to mimic the mast cell degranulation process in the peritoneal cavity, mast cell lysates were injected into the peritoneal cavity of mice and intraperitoneal fluid was collected after 4 h of the injection (Fig. 2B). Quantitation of apoA-I in intraperitoneal fluid demonstrated a dose-dependent degradation of endogenous apoA-I *in vivo* within 4 h of the mast cell lysate injection.

3.3. Effect of extensive systemic mast cell degranulation on plasma lipoproteins

To evaluate whether expanding the mast cell response to all body compartments could exert a systemic effect that modifies HDL particles we induced an acute degranulation of mast cells in mice by administering a high i.p. dose of C48/80 (8 mg/kg), which leads to extensive mast cell degranulation and provides a model of systemic anaphylactic response [22]. Mast cell activation was confirmed by a twofold increase in histamine concentration in serum of the stimulated mice. Furthermore, the chymase activity was 3-fold lower in isolated peritoneal mast cells derived from anaphylactic mice than from control mice (6 ± 2.1 and 17 ± 1.2 units/20,000 mast cells, respectively), reflecting degranulation of mast cells in the latter with ensuing chymase depletion from the mast cells. To analyze the serum apolipoprotein profile, serum total lipoprotein fraction ($d < 1.21$ g/mL) was applied to 4–20% SDS-PAGE electrophoresis (Fig. 3A). Importantly, the major apoA-I band, and the minor apoE and apoA-IV protein components were reduced in serum from the anaphylactic mice. Of note, unfractionated serum of anaphylactic mice contained lower amounts of both α - and pre β -HDL particles ($\approx 40\%$ and 35% reduction, respectively) than did the serum from the matched control mice (Fig. 3B). Also the serum apoA-I levels were lower in anaphylactic than in control mice (0.64 ± 0.13 vs 0.91 ± 0.14 mg/mL). We were unable to detect (not shown) any circulating apoA-I fragments of the anaphylactic serum by Western blot analysis. It is possible that truncated apoA-I fragments had been released from HDL and rapidly eliminated from the circulation.

Further fractionation of mouse lipoproteins was carried out by FPLC size exclusion chromatography analysis, and the eluted fractions were quantitated for cholesterol, phospholipids, apoA-I, and apoE (Fig. 4). Both apoA-I Western blotting (not shown) and Elisa analysis (Fig. 4A, upper panel) revealed two populations of HDL in mouse serum, with substantially reduced apoA-I amounts in the anaphylactic serum. Consistent with the typical mouse lipoprotein profile, both cholesterol (middle panel) and phospholipids (lower panel) were mostly present in the major HDL population. Again, the HDL peaks were significantly smaller in the anaphylactic than in the control serum. Importantly, elution profile for apoB-containing lipoproteins (mostly detected as VLDL) in the anaphylactic serum remained unchanged, confirming that, among the lipoproteins in mouse serum, HDL was a mast cell-specific target. In HDL from anaphylactic mice the reduction of phospholipids was more pronounced than the reduction of cholesterol, changing the cholesterol/phospholipid ratio, e.g. in fraction 30 the ratio was 1.12 in control and 1.74 in anaphylactic HDL. Moreover, a degraded fragment of apoE was clearly visible in immunoblots of the eluted fractions 30–36 from anaphylactic serum (Fig. 4B), indicating that the degraded apoE peptides remained HDL-associated. In the control serum only the native-sized apoE was visible. In summary, systemic mast cell stimulation resulted not only in quantitative reduction of HDL components, but also in altered lipid composition.

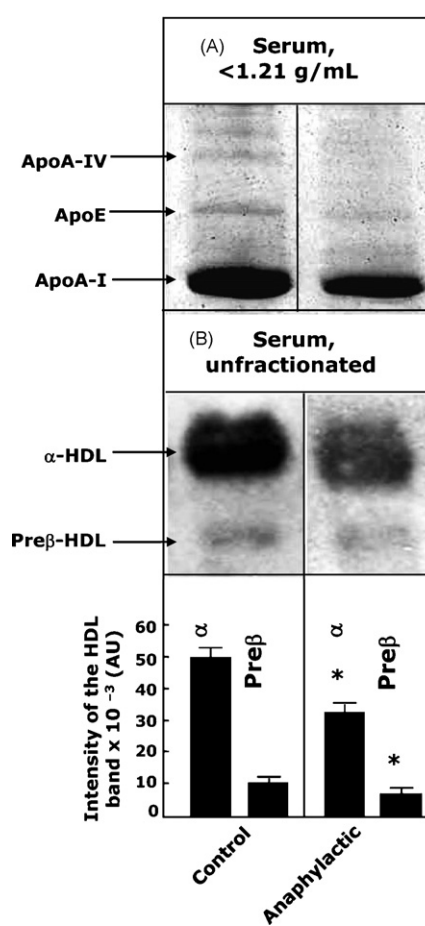


Fig. 3. Apolipoprotein and lipoprotein pattern of serum and intraperitoneal fluid after systemic anaphylaxis of mast cells *in vivo*. Mice were given an i.p. injection of 8 mg/kg body weight of C48/80 (anaphylactic) or corresponding volumes of NaCl 0.9% (control), and after 30 min they were sacrificed. (A) Serum from each group of mice was pooled; lipoproteins were ultracentrifugally isolated ($d < 1.21$ g/mL) and loaded onto a SDS-PAGE gel 4 to 20%. Proteins were visualized with Coomassie Blue. Protein and cholesterol concentrations in the lipoprotein-containing top fractions were 1.2 and 13 mg/mL in the stimulated group, and 1.0 and 14 mg/mL in the control group, respectively. (B) α - and pre β -HDL subpopulation in serum samples from five matched pairs of control and anaphylactic mice were detected by agarose electrophoresis followed by apoA-I Western blotting, and band intensities of was evaluated by densitometry. Statistical significance was determined with two-tailed Student's *t*-test. * $P < 0.05$ vs control. Ten mice were used in each experimental group.

This latter finding of qualitative changes of HDL may be derived from degradation of the phospholipid-binding apolipoproteins of HDL.

3.4. Efflux of cholesterol from macrophage foam cells promoted by serum and intraperitoneal fluid derived from C48/80-treated mast cell-competent and mast cell-deficient mice

Finally, we investigated whether the changes induced by systemic mast cell activation on HDL present in serum and intraperitoneal fluid would impair the abilities of these body fluids to act as cholesterol acceptors *ex vivo* when added to cholesterol-loaded mouse macrophage cultures. For this purpose, both mast cell-competent NMRI mice and mast cell-deficient *W-sash* c-kit mutant mice received a high dose of C48/80 and cellular cholesterol efflux to serum and intraperitoneal fluid was determined (Fig. 5). Importantly, injection of C48/80 into the mast cell competent mice significantly reduced the ability of both serum and intraperitoneal fluid to induce cholesterol efflux (Fig. 5A and B). In sharp contrast, i.p. injection of C48/80 in the mast cell-deficient genotype *sash* mice

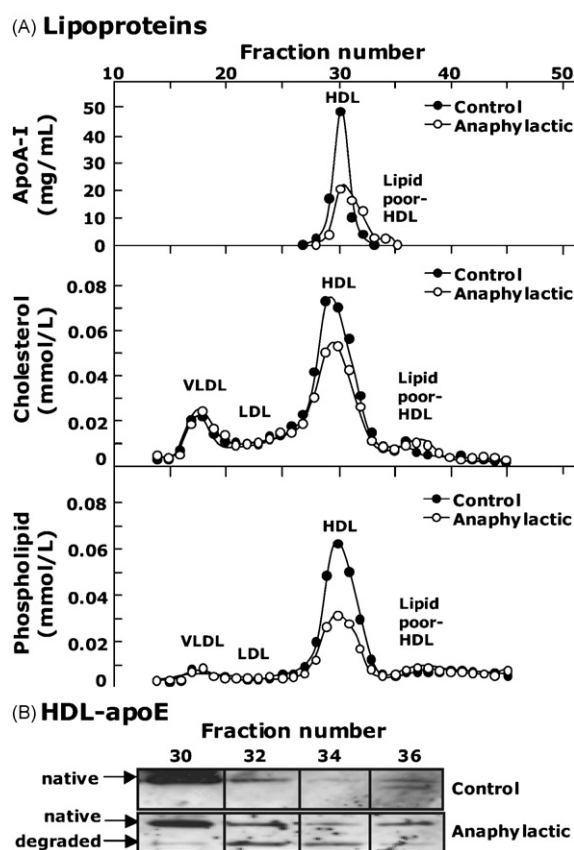


Fig. 4. Distribution of lipids and HDL apolipoproteins within the serum lipoproteins after systemic anaphylaxis of mast cells *in vivo*. Aliquots of serum from anaphylactic and control mice (see Fig. 3) were applied to Superose 6HR size-exclusion chromatography column. The column was run as described in the Section 2, and the eluted fractions were analyzed for apoA-I, cholesterol, and choline-containing phospholipids (Panel A), and apoE degradation was evaluated by Western blotting (Panel B).

failed to reduce the ability of either serum or intraperitoneal fluid to promote cholesterol efflux (Fig. 5C and D), so providing strong evidence for the notion that the observed inhibitory effect on efflux in the mast cell-competent mice was due to an acute mast cell response. Altogether the findings indicate that proteolytic inactivation of HDL by mast cell-derived chymase had occurred in mast cell-competent mice during anaphylactic shock *in vivo*.

4. Discussion

We have previously shown that human and rat mast cell-derived chymases proteolytically degrade HDL particles *in vitro* and so impair their ability to induce cholesterol efflux from macrophage foam cells in cultures (reviewed in [6]). Here, we extended our previous observations to a mouse model, in which full degranulation of mast cells is induced, so leading to systemic anaphylaxis [22]. This model allowed us to evaluate the effect of mast cell activation *in vivo* on the cholesterol efflux-promoting ability of HDL *ex vivo*. Our results demonstrated that serum of the mast cell-stimulated mice was defective in promoting cholesterol efflux from macrophage foam cells, and that the impairment in function was due to a mast cell-dependent proteolytic modification of HDL particles. This is the first indication that protease-modified HDL particles may be generated *in vivo*, giving further support to the notion that the antiatherosclerotic functions of HDL depend not only on the quantitative level of HDL particles but, importantly, also on their quality, i.e. on the preservation of HDL particle integrity

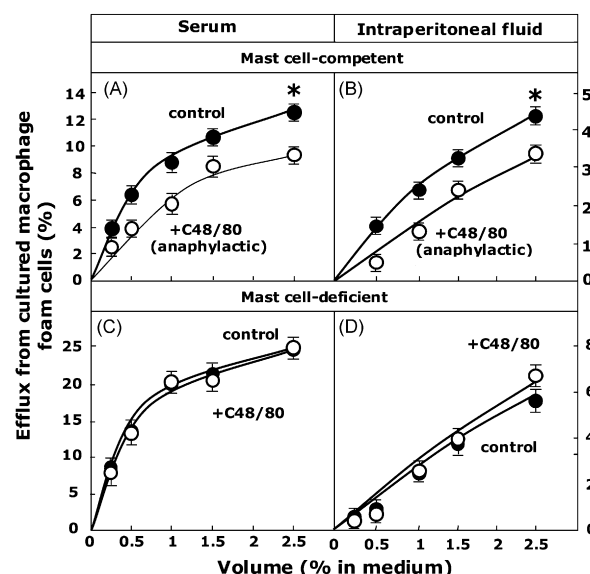


Fig. 5. Cholesterol efflux promoted by serum and intraperitoneal fluid from mast cell-competent and mast-deficient mice after injection of C48/80. The ability of serum and intraperitoneal fluid from mice treated as described in Fig. 3 to promote efflux of cholesterol from ^3H -CE-loaded NMRI macrophage foam cells was determined as described in the Section 2. Panels show concentration-dependent efflux promoted by serum and intraperitoneal fluid from mast cell-competent (NMRI) mice (10 animals/group of a representative experiment) (A and B), and from genetically mast cell-deficient (sash) mice (6 animals/group) (C and D) after injection of PBS (control) or 8 mg/kg body weight of C48/80 (+C48/80). Differences in the absolute levels of efflux in mast cell-competent control mice and mast cell-deficient control mice probably reflect differences related to different mouse strain genotypes. Values are means $\pm$ SD of triplicate wells. *Statistical significance of $P < 0.001$ was found by Student's test for paired samples of control vs stimulated (anaphylactic) serum and intraperitoneal fluid from mast cell-competent mice.

[25]. Besides proteolytic modification of HDL particles [6], also their oxidative (nitrosylation) [26], and inflammatory [27] modifications create dysfunctional HDL *in vivo*.

The differential roles of the various extracellular acceptors in stimulating cellular cholesterol efflux have been recently highlighted [28]. HDL composition directly influences HDL interaction with the two receptor-mediated efflux pathways of macrophage foam cells that promote unidirectional flux of cholesterol via the ABCA1 and ABCG1 transporters. Whereas cholesterol efflux via ABCA1 requires direct or indirect apoA-I binding [29,30], HDL phospholipids modulate the release of cellular cholesterol via ABCG1, as it has been demonstrated by genetic manipulation of mice [31]. Although mere reduction of serum apoA-I levels may explain the reduction in cholesterol efflux observed in the present study, the observed change in cholesterol/phospholipid ratio due to a stronger specific reduction in phospholipids of mast cell-modified HDL particles also partly explains such reduction since phospholipids are required for an effective cholesterol efflux [32]. Thus, both quantitative and qualitative changes in HDL particles are likely to be involved in the observed "dysfunctionality" of HDL after mast cell stimulation. Whether the mast cell-mediated degradation of HDL apolipoproteins was able to induce a loss of phospholipids in HDL, or whether the latter was secondary to a potential activation of e.g. endothelial lipase promoted by the mast cell-derived soluble heparin [33] released during the anaphylactic shock, deserves further investigation.

Of note, the substantial decrease of total circulating apoA-I upon mast cell activation *in vivo* was unexpected, and challenged our previous *in vitro* observations, in which we found preferential degradation of the minor pre β -HDL fraction by the rat and human chymases, so causing only a modest proteolysis of total apoA-I [18,34]. The apparently higher efficiency of the mouse mast

cell chymase to proteolyze HDL found here, both *in vitro* and *in vivo*, may reflect biological variation reported for chymases from different species [21], and may be also related to the specific conditions of the present *in vivo* experimental setup. Indeed, the present study was designed to test the hypothesis that mast cell-dependent HDL proteolysis can occur in an intact animal in which homeostatic mechanisms (protease inhibitors) attenuate proteolysis. Therefore, we used extreme conditions by triggering an anaphylactic shock, i.e. a model that has been used to evaluate other functions of mast cells *in vivo*, such as their role in experimental asthma [22]. This maximal mast cell degranulation resulted in a systemic protease burden, facilitating the mouse chymase a extensive targeting of HDL which in the mouse are present both intra- and extravascularly and normally consists of a single major size distribution of particles approximately 10 nm in diameter. As shown here, even in the presence of 80% (v/v) of intraperitoneal fluid, 40% of mouse chymase remained active (Fig. 1B). We calculated that the whole population of mast cells on the peritoneal surface of the adult NMRI mouse might contain ≈ 200 units of chymase. Provided that upon maximal activation of peritoneal mast cells 200 units were released into the $\approx 30 \mu\text{L}$ volume of peritoneal fluid present in the mice studied here, then ≈ 80 units would be active in the peritoneal fluid. Since the concentration of apoA-I in the mouse peritoneal fluid was $350 \mu\text{g/mL}$, the total amount of apoA-I in the $30 \mu\text{L}$ of intraperitoneal fluid was $\approx 10 \mu\text{g}$. Thus, the chymase activity/apoA-I mass ratio (80/10) in the peritoneal fluid after release of all chymase must have been 8, i.e. 20- to 200-fold higher than the previously used ratios *in vitro* which ranged from ≈ 0.4 [34] to 0.04 [35]. Besides the peritoneal cavity, such high ratio *in vivo* may also apply to other extravascular compartments during an anaphylactic shock in the mouse.

A protease most likely involved in the observed HDL modification was the mMCP-4, derived from connective tissue-type mast cells which are located not only in the peritoneum, but also in the connective tissue of many organs, such as the skin and the heart of the mouse [21]. Another mouse mast cell chymase (mMCP-1) is derived from the gut mucosal surfaces and enters the systemic circulation upon stimulation of mucosal mast cells, but is rapidly complexed with serpins and becomes inactivated [36]. In contrast, as shown here, mMCP-4 which is secreted in complexes with heparin proteoglycans [21], was partially resistant to protease inhibitors, and so can act for prolonged periods of time on apoA-I of which $\approx 50\%$ is present extravascularly [37]. In addition, the histamine released from activated mast cells increases vascular permeability [38], and so facilitates the accessibility of plasma apolipoprotein substrates to the extravascularly located mMCP-4, and also promotes re-entry of the proteolyzed apolipoprotein products to the circulation. Thus, the proteolytic profile of HDL found in blood plasma likely reflects that in the extracellular fluids.

The interest in the pathophysiological role of mast cells in human atherosclerosis is growing [39]. Human mast cells, via secretion of chymase, tryptase, and other active compounds, exert pleiotropic effects in the cardiovascular system, having the potential to contribute to the clinical outcomes of atherosclerosis, such as myocardial infarction and stroke [40]. Interestingly, involvement of G3255A polymorphism of the human mast cell chymase gene with HDL-cholesterol level has been found, rendering it possible that individuals with the rare A3255 allele have a higher potential for reverse cholesterol transport [41]. Studies in animal models of hypertension and atherosclerosis indicate that chymase may also be involved in lipid deposition in the intima [42,43], and chymase inhibitors have been shown to prevent aneurysm development in dogs [44]. Thus, chymase inhibitors may become effective drugs in the prevention of multiple cardiovascular disorders, especially when they act in the arterial wall without having an effect on the systemic blood pressure [45].

By demonstrating that exocytosed mast cell chymase is active against apoA-I in a physiological environment in which its natural inhibitors are present, this study addresses the key question inherent in our hypothesis on the significance of mast cell-dependent proteolytic inactivation of HDL *in vivo*. Interestingly, similar to the present finding of apoE fragmentation in mouse circulation, proteolytic fragments of apoE and apoA-I have been detected in human circulation [46,47]. These observations support the concept that generation of proteolytically modified HDL may occur in conditions of increased protease burden likely to be present in the inflamed intima during atherosclerosis progression. However, since the present model is based on systemic mast cell degranulation in all tissues during anaphylactic shock, further validation of our hypothesis will have to await additional studies under conditions of targeted stimulation of mast cells located in the arterial wall of mice [48].

Acknowledgments

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