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Human mast cell neutral proteases generate modified LDL particles with increased proteoglycan binding

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ABSTRACT

Background and aims: Subendothelial interaction of LDL with extracellular matrix drives atherogenesis. This interaction can be strengthened by proteolytic modification of LDL. Mast cells (MCs) are present in atherosclerotic lesions, and upon activation, they degranulate and release a variety of neutral proteases. Here we studied the ability of MC proteases to cleave apoB-100 of LDL and affect the binding of LDL to proteoglycans.

Methods: Mature human MCs were differentiated from human peripheral blood-derived $CD34^+$ progenitors *in vitro* and activated with calcium ionophore to generate MC-conditioned medium. LDL was incubated in the MC-conditioned medium or with individual MC proteases, and the binding of native and modified LDL to isolated human aortic proteoglycans or to human atherosclerotic plaques *ex vivo* was determined. MC proteases in atherosclerotic human coronary artery lesions were detected by immunofluorescence and qPCR.

Results: Activated human MCs released the neutral proteases tryptase, chymase, carboxypeptidase A3, cathepsin G, and granzyme B. Of these, cathepsin G degraded most efficiently apoB-100, induced LDL fusion, and enhanced binding of LDL to isolated human aortic proteoglycans and human atherosclerotic lesions *ex vivo*. Double immunofluoresence staining of human atherosclerotic coronary arteries for tryptase and cathepsin G indicated that lesional MCs contain cathepsin G. In the lesions, expression of cathepsin G correlated with the expression of tryptase and chymase, but not with that of neutrophil proteinase 3.

Conclusions: The present study suggests that cathepsin G in human atherosclerotic lesions is largely derived from MCs and that activated MCs may contribute to atherogenesis by enhancing LDL retention.

1. Introduction

Atherogenesis is initiated at atherosclerosis-susceptible sites of the arterial wall by focal retention of LDL particles within the subendothelial extracellular matrix (ECM), particularly via binding of LDL to proteoglycans (PGs) [1]. Indeed, the atherogenicity of LDL is directly linked to its affinity for arterial wall PGs [1–3], which is related to either the properties of LDL or the PGs. The PG–LDL interaction is ionic and takes place between clusters of positively charged amino acid residues on apoB-100 and negatively charged sulfate and carboxyl groups on the glycosaminoglycan chains of PGs [4]. The principal site in apoB-100, which mediates the PG binding of LDL spans the amino acid residues 3359–3369 (referred to as site B) [2]. However, LDL particles seem to contain additional PG-binding sites as indicated by identification of several regions in apoB-100 that bind glycosaminoglycans, [5]; yet, these binding sites may not be functionally available on native LDL particles [2]. Importantly, various modifications of LDL particles have been shown to increase the binding of LDL to PGs [6–8], implying that additional PG-binding sites may become functional when LDL is

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modified [9,10].

In the arterial intima, LDL particles are subjected to various structural and conformational modifications that promote the formation of LDL-derived lipid droplets and vesicles in the subendothelial extracellular space [7]. Such extracellular lipid particles isolated from human atherosclerotic lesions have features revealing that the apoB-100-containing plasma lipoproteins, which have entered the subendothelial space, have been exposed to proteolysis and lipolysis [11]. Indeed, atherosclerotic lesions contain several proteases capable of hydrolyzing the apoB-100 of LDL *in vitro,* including both plasma-derived and locally synthesized proteases. To the former group belong plasmin, kallikrein, and thrombin, and to the latter group the lysosomal proteases cathepsins D, F, K, and S secreted by intimal macrophages and smooth muscle cells of the lesions [6,12–14].

An additional source of proteolytic enzymes in the arterial intima are mast cells (MCs). This type of inflammatory cell is present in both early and advanced human atherosclerotic lesions [15–17]. The cytoplasm of MCs is filled with secretory granules, which contain high amounts of various neutral proteases [18]. All MCs in atherosclerotic lesions contain tryptase, and a variable fraction contain also chymase [15–17], both of which are virtually MC-specific proteases [19]. A third major neutral granule protease of MCs is carboxypeptidase A3 (CPA3), a zinc-dependent exopeptidase, which has previously been thought to be exclusively expressed in MCs; however, more recently transcripts of CPA3 have been found also in basophils [19]. In addition, human MC neutral granule proteases include two serine proteases that are not unique to this cell type, namely cathepsin G and granzyme B [20–22]. When MCs are activated, large amounts of the granule proteases are released into the extracellular surroundings in a process called degranulation. Unlike most other secreted proteases, the MC granule proteases are stored in fully active form, and, thus, once released they are capable of immediately triggering proteolytic events in the extracellular microenvironment of the tissue in which the activated MCs are present and degranulation occurs.

Recently, we developed a cell culture method for the differentiation of mature MCs from their circulating progenitor cells [23], and showed that mature MCs expressed a wide spectrum of the known MC neutral proteases, namely the tryptases derived from the *TPSAB1*, *TPSB2*, *TPSD1*, and *TPSG1/PRSS31* genes, and chymase, CPA3, cathepsin G, and granzyme B [21]. In the present study, we investigated the ability of these neutral proteases to cleave apoB-100 of LDL, and determined the effects of such proteolysis on the binding of LDL to human aortic PGs.

2. Materials and methods

2.1. Cell preparation and culture

Human MCs were *in vitro* differentiated from buffy coats of healthy volunteers, as previously described [23]. The buffy coats were supplied by the Finnish Red Cross Blood Service (Helsinki, Finland) with the approval of the Ethics Committee of the Finnish Red Cross.

2.2. Preparation of mast cell-conditioned medium (mast cell releasate)

At week 9 of culture, MCs at a concentration of 2×10^6 MCs/ml were activated by 1 μM calcium ionophore A21387 (Sigma-Aldrich) in Dulbecco's phosphate buffered saline (DPBS) in a humidified incubator at 37 °C (5% CO_2). After 30 min of incubation, the MCs were sedimented, and the supernatant containing the released neutral proteases, i.e the MC-conditioned medium, which will be referred to as the MC releasate, was collected and stored at −80 °C until further analysis.

2.3. Protease activity assays and protease inhibitors

The amounts of active neutral proteases in MC releasates were determined through hydrolysis of specific protease substrates, as previously described [21]. In some experiments, some of the proteases in the MC releasates were inhibited as follows: tryptase with 200 μg/ml leupeptin (Sigma-Aldrich), chymase with 670 nM diphenyl Na-benzoxycarbonyl-L-Arg-Glu-Thr-PheP-phosphonate [(RETF-$(OPh)_2$] generously provided by Dr Gunnar Pejler (Uppsala University, Uppsala, Sweden), and CPA3 with 1 μl/100 μl Carboxypeptidase Inhibitor from potato tuber (Sigma-Aldrich).

2.4. Preparation of human aortic proteoglycans

PGs from the intima-media of human aortas were obtained at autopsy within 24 h of accidental death and prepared as described previously [24,25]. The Finnish National Authority for Medicolegal Affairs had approved the study. The amounts of the PGs are expressed in terms of their glycosaminoglycan content, as determined by the method of Bartold and Page [26].

2.5. Isolation and modifications of LDL

Human LDL (d = 1.019–1.050 g/ml) were isolated from plasma of healthy volunteers (supplied by the Finnish Red Cross Blood Service) by sequential ultracentrifugation in the presence of 3 mM EDTA, as described [27,28]. The amount of LDL is expressed in terms of its protein content determined by the method of Lowry and/or BCA Protein Assay Kit (Thermo Scientific), with bovine serum albumin as standard. MC protease-modified LDL was prepared by incubation of LDL (1 or 2 mg/ml) either with MC releasate, or separately with 1.5 μg/ml recombinant human chymase (Sigma-Aldrich), 20 μg/ml recombinant human β-tryptase (a kind gift from Dr. Christian Sommerhoff), 4.5 μg/ml cathepsin G from human neutrophils (Biomol), 10 μg/ml CPA from bovine pancreas (Sigma-Aldrich), or 0.1 μg/ml granzyme B (QuickZyme Biosciences) in DPBS overnight at 37 °C. The protease concentrations were chosen based on those found in the MC releasates. In some experiments, the apoB-100 of LDL was labeled before treatment with MC neutral proteases with a ^{3}H-labeling reagent (*t*-butoxycarbonyl-L-[^{3}H] methionine *N*-hydroxy-succinimidyl ester [Amersham Biosciences]) according to the Bolton-Hunter procedure [29].

The degree of apoB-100 proteolysis was determined by measuring the amount of radioactive peptides released from the LDL particle using the trichloroacetic acid (TCA) precipitation method [30], or by measuring the amount of immunoreactive apoB-100 with apoB ELISA (Mabthech) according to the manufacturer's instructions. SDS-PAGE was performed under denaturing conditions using Novex Bolt Bis-Tris Plus 4–12% precast minigels and Bolt MES-SDS Running buffer (both Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. The extent of oxidation of LDL was determined by measuring the amount of thiobarbituric acid reactive substances (TBARS) [31].

In some experiments plasma of three healthy donors was pooled prior to LDL isolation. The pooled LDL (1 mg/ml) was then incubated overnight with the recombinant human chymase (1.5 μg/ml) or with cathepsin G (4.5 μg/ml) in the presence of human aortic PGs (100 μg/ml), after which cleavage of apoB-100 of LDL was visualized by SDS-PAGE. Finally, a fraction of the pooled LDL was conjugated with Atto 594 N-Hydroxysuccinimide (NHS) ester fluorochrome (Sigma Aldrich), as previously described [32], with the exception of using 0.2 M $NaHCO_3$.

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