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Electronegative LDL induces MMP-9 and TIMP-1 release in monocytes through CD14 activation: Inhibitory effect of glycosaminoglycan sulodexide

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ABSTRACT

Objective: Electronegative LDL (LDL(−)) is involved in atherosclerosis through the activation of the TLR4/CD14 inflammatory pathway in monocytes. Matrix metalloproteinases (MMP) and their inhibitors (tissue inhibitors of metalloproteinase [TIMP]) are also crucially involved in atherosclerosis, but their modulation by LDL(−) has never been investigated. The aim of this study was to examine the ability of LDL(−) to release MMPs and TIMPs in human monocytes and to determine whether sulodexide (SDX), a glycosaminoglycan-based drug, was able to affect their secretion.

Approach and results: Native LDL (LDL(+)) and LDL(−) separated by anion-exchange chromatography were added to THP1-CD14 monocytes in the presence or absence of SDX for 24 h. A panel of 9 MMPs and 4 TIMPs was analyzed in cell supernatants with multiplex immunoassays. The gelatinolytic activity of MMP-9 was assessed by gelatin zymography. LDL(−) stimulated the release of MMP-9 (13-fold) and TIMP-1 (4-fold) in THP1-CD14 monocytes, as well as the gelatinolytic activity of MMP-9. Co-incubation of monocytes with LDL(−) and SDX for 24 h significantly reduced both the release of MMP-9 and TIMP-1 and gelatinase activity. In THP1 cells not expressing CD14, no effect of LDL(−) on MMP-9 or TIMP-1 release was observed. The uptake of DiI-labeled LDL(−) was higher than that of DiI-LDL(+) in THP1-CD14 but not in THP1 cells. This increase was inhibited by SDX. Experiments in microtiter wells coated with SDX demonstrated a specific interaction of LDL(−) with SDX.

Conclusions: LDL(−) induced the release of MMP-9 and TIMP-1 in monocytes through CD14. SDX affects the ability of LDL(−) to promote TIMP-1 and MMP-9 release by its interaction with LDL(−).

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases crucially involved in the remodeling of the vascular extracellular matrix (ECM) and in the unmasking of several ECM-bound cytokines and growth factors [1,2]. MMP activities are thoroughly orchestrated by control points at transcriptional, post-transcriptional, and post-translational levels, these latter including regulated activation of zymogen pro-forms, as well as inhibition through interaction with endogenous tissue inhibitors of metalloproteinases (TIMP-1, -2, -3, and -4) [1]. MMPs play key roles in the development and regulation of the physiological processes of the cardiovascular system and in the aberrant remodeling of the vasculature and atherosclerotic plaque formation and instability [3].

Besides MMP imbalance, one of the major initiators involved in the development of atherosclerotic plaque is the chemical modification of

Abbreviations: ECM, extracellular matrix; GAG, glycosaminoglycan; IL, Interleukin; LDL, low density lipoprotein; LPS, lipopolysaccharide; LSU, lipasemic unit; MMP, matrix metalloproteinase; SDX, sulodexide; TIMP, tissue inhibitor of metalloproteinase; TLR, toll-like receptor

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lipids. In particular, low density lipoprotein (LDL) modification has been established as an important risk factor in the activation of many atherogenic pathways [4]. In this respect, electronegative LDL (LDL (−)) is a minor modified LDL subfraction present in blood circulation, mainly in patients with high cardiovascular risk factors [5]. LDL(−) differs from electropositive LDL (LDL(+)) in size, density, lipid content, protein composition, and apo-B100 conformation [6,7].

An expanding body of evidence supports the pro-inflammatory role of LDL(−) [5,8–12]. First identified in endothelial cells [13] and later in monocytes [9–12,14,15], a number of studies have described the ability of LDL(−) to induce cytokine release in monocytes [10,11,14] by activating the signaling cascade downstream of CD14/toll-like receptor 4 (TLR4) [9,12,15]. In this respect, it has been reported that MMPs are induced by CD14-TLR activation in monocytic cells [16]; however, although the ability of LDL(−) to stimulate MMP release has been previously investigated in in vitro models of endothelial cells [17], no data are available regarding the modulation by LDL(−) of proteolytic pathways in monocytic cells. The first aim of this study was to evaluate the effects of LDL(−)-treatment on THP1 cells over expressing CD14 in promoting the release of MMPs and their inhibitors.

Another particular feature of LDL(−) is an increased affinity for glycosaminoglycans (GAGs) [6,18,19]. Some GAGs are widely used in vascular medicine for their endothelial protective and anti-inflammatory properties. Sulodexide® (Vessel™, Alfa-Sigma, Italy) is a highly purified mixture of two GAGs, composed of 80% fast-moving heparin (FMH), which has affinity with antitrombin III (ATIII), and 20% dermatan sulfate, which has affinity with the heparin cofactor II (HCII) [20]. Both of these components confer anti-thrombotic and anti-coagulant effects. Moreover, sulodexide (SDX) has endothelial protective and anti-inflammatory properties due to its interaction with the glycocalyx layer of blood vessels and its ability to modulate inflammatory pathways in monocytes [21–23]. Notably, one of the first characterized properties of SDX was its lipoprotein lipase releasing ability [24,25], which endows SDX with cholesterol-lowering properties and the ability to modify lipoprotein catabolism, strengthening the basis for conducting studies on the use of SDX as a potential anti-atherosclerotic agent. Because it is known to increase the affinity of LDL(−) for GAGs [6,18,19], the authors of this study assessed how SDX affects the release of MMPs and TIMPs in monocytes stimulated with LDL(−).

2. Materials and methods

2.1. Materials

Pure grade chemicals and reagents for the MMP multiplex immunoassays were obtained from Bio-Rad (Milan, Italy). Commercial SDX was provided from Alfa-Sigma (Italy). All the reagents for the zymography assays were obtained from Bio-Rad, excluding 90 Bloom gelatin type A, derived from porcine skin, which was obtained from Sigma-Merck (Darmstadt, Germany). An Amplex Red cholesterol kit was obtained from Sigma. THP1 and **THP1-XBlue™-MD2-CD14 cells** were obtained from Invivogen (Toulouse, France).

2.2. Lipoprotein isolation and separation of LDL subfractions

Plasma samples from healthy normolipemic subjects (total cholesterol < 5.2 mM, triglyceride < 1 mM) were obtained in EDTA-containing Vacutainer tubes. Total LDL (1.019–1.050 g/mL) was isolated from pooled plasma by sequential flotation ultracentrifugation at 4 °C in the presence of 1 mM EDTA and 2 μM BHT, and LDL was dialyzed against buffer A (Tris 10 mM, EDTA 1 mM, pH 7.4). Native LDL (LDL(+)) and LDL(−) were separated by preparative anion-exchange chromatography in an ÄKTA-FPLC system (Amersham Pharmacia, Uppsala, Sweden) and characterized as described [26]. The LDL(−) proportion ranged from 4 to 6% of total LDL in all LDL preparations. The physicochemical characteristics and composition of both LDL subfractions were similar to those previously reported [26]. Briefly, LDL(−) contained more triglycerides and non-esterified fatty acids, presented smaller particle size, and demonstrated higher aggregation than LDL(+). No difference in the oxidative level between LDL(+) and LDL(−) was observed (data not shown).

2.3. Cell culture and incubation

THP1-XBlue™-MD2-CD14 cells (Invivogen) (THP1-CD14) are derived from THP1 human monocytic cells over expressing MD2 and CD14 to increase the response to CD14-TLR ligands, such as LDL(−). Cells were grown as previously described [9]. THP1 cells were grown under the same conditions as THP1-CD14 cells, except for the absence of selective antibiotics in RPMI growth medium. LDL(+) and LDL(−) were dialyzed against serum-free RPMI media, filtered in sterile conditions, and added (70 mg apoB/L) to THP1-CD14 cells (400,000 cells/mL) in serum-free media. In order to evaluate the effects of SDX on LDL (−)-induced response, monocytes were co-incubated with SDX (0.12 LSU/mL) and LDL(−) simultaneously. After 24 h of incubation, cell supernatants were collected and stored at −80 °C until analysis.

2.4. Magnetic multiplex immunoassays of MMP-9 and TIMP-1

Levels of MMP and TIMP released in THP1 serum-free culture media were determined with the commercially available Pro™ Human MMP 9-plex Assay and the Pro™ Human TIMP 4-plex Assay. MMP-1, -2, -3, -7, -8, -9, -10, -12, and -13 and TIMP-1, -2, -3, and -4 were analyzed. Multiplex suspension immunomagnetic assays are based on the use of fluorescently dyed magnetic beads covalently conjugated with monoclonal antibodies specific to the target proteins, and these assays were performed according to the manufacturer's instructions (Bio-Plex, Bio-Rad Labs, Hercules, CA, USA). Levels of all molecules were determined using a Bio-Plex 200 array reader based on Luminex X-Map Technology (Bio-Rad Labs, Hercules, CA, USA), which detects and quantifies multiple targets in a 96-well plate with a single small fluid volume (50 μL). Data were collected and analyzed using a Bio-Plex 200 instrument equipped with BioManager analysis software (Bio-Plex Manager Software v.6.1). The protein concentrations (expressed as pg/mL) were calculated with a standard curve. According to the manufacturer's data, the lowest detection limits were 1.0 and 1.6 pg/mL for MMPs and TIMPs, respectively.

2.5. Zymographic analyses

Aliquots of all serum-free media (obtained from a cell density of 400,000 cells/mL) were analyzed by gelatin zymography carried out on 6.5% polyacrylamide gels copolymerized with 3 g/L 90 Bloom Type A gelatin from porcine skin (Sigma). Native samples were loaded with the addition of zymogram sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 4% SDS, 0.01% bromophenol blue) [27]. SDS-PAGE gels were run using a Bio-Rad Mini-Protean Tetra Cell apparatus (Bio-Rad, Hercules, CA) in SDS running buffer (25 mM Tris, 192 mM glycine, and 0.1% w/v SDS) at a constant voltage of 105 V. After electrophoresis, gels were incubated for 40 min at room temperature on a rotary shaker in Triton X-100 2.5% to remove SDS. The gels were washed with distilled water and incubated for 24 h in an enzyme incubation buffer (containing 50 mM Tris, 5 mM $CaCl_2$, 100 mM NaCl, 1 mM $ZnCl_2$, 0.3 mM NaN_3, 0.2 g/L of Brij®-35, and 2.5% v/v of Triton X-100, pH 7.6) at 37 °C. Staining was performed using Coomassie Brilliant Blue R-250 (0.2% w/v Coomassie in 50% v/v methanol and 20% v/v acetic acid). Gels were destained with destaining solution (50% v/v methanol and 20% v/v acetic acid) until clear gelatinolytic bands appeared against the uniform dark-blue background of undigested protein substrate. Gelatinase calibrators (as molecular weight standards) were prepared by diluting 1:15 v:v healthy capillary blood with a non-reducing Laemmli sample buffer. It is important to specify that whole