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Acidity increases the uptake of native LDL by human monocyte-derived macrophages

Riia Plihtari, Petri T. Kovanen, Katariina Öörni*

Wihuri Research Institute, Kalliolinnantie 4, FIN-00140 Helsinki, Finland

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ABSTRACT

The extracellular pH is locally decreased in advanced atherosclerotic lesions, particularly in lipid-rich areas of the lesions. Since accumulation of LDL-derived cholesterol and formation of foam cells are key processes in atherogenesis, we tested here the effects of acidic pH on the uptake of native LDL. First, human monocytes were differentiated into macrophages in the presence of granulocyte–monocyte-colony stimulating factor (GM-CSF) after which native LDL was applied to the monocyte-derived macrophages at pH 5.5, 6.5, or 7.5 and the binding and uptake of LDL by macrophages were determined. The lower the pH was, the higher was the binding and uptake of LDL by macrophages. Also, acidic pH was found to increase the production of cell surface proteoglycans by macrophages and binding of LDL to the glycosaminoglycan chains of the proteoglycans. The acidity-induced increase in the uptake of LDL by macrophages could be inhibited by pretreating the cells with heparinase and chondroitinase as well as by inhibiting the production of proteoglycans with $NaClO_3$. Thus, the observed increase in the uptake of native LDL to macrophages appears to depend on the increased ability of LDL to bind to cell surface proteoglycans at acidic pH. Taken together, our present results indicate that acidity increases the effective concentration of LDL on macrophage surfaces by increasing the amount of cell surface proteoglycans and by enhancing the binding of LDL to them and so promotes LDL uptake with ensuing foam cell formation.

1. Introduction

Atherosclerosis is an inflammatory disease, where accumulation of LDL in the arterial intima plays a crucial role [1,2]. Intracellular accumulation of cholesterol with ensuing foam cell formation is considered to be a critical step in the development of atherosclerotic lesions. Uptake of LDL by cells present in atherosclerotic lesions, particularly by macrophages, has been extensively studied. Indeed, LDL modification has long been considered to be a prerequisite for intracellular cholesterol accumulation [3].

Various characteristics of the lesions, such as hypoxia and pH, are likely to affect foam cell formation. Indeed, when macrophages are exposed to hypoxia, triglyceride-rich foam cells are formed due to reduction in beta-oxidation of fatty acids and increase in both biosynthesis of triglycerides and the expression of ADRP (adipose differentiation-related protein), a protein involved in the formation of cytosolic lipid droplets [4]. Since the arterial intima is an avascular tissue, oxygen can reach the intima only by diffusion from the lumen or from the medial layer of the arterial wall. The diffusion distance of oxygen in tissues is only about 200 μm [5,6], and hypoxic areas develop in the lesions already at this depth [7]. During atherogenesis, the thickness of the intima may increase up to 3300 μm, *i.e.* more than 10-fold the critical diffusion distance of oxygen [8]. Under hypoxia, cells switch to anaerobic metabolism, which leads to production of lactate and secretion of excess H^+ ions. Indeed, increased concentrations of lactate are found particularly in the hypoxic areas of atherosclerotic lesions [9,10]. Also, decreased extracellular pH values have been determined. Thus, visualization of human carotid plaques with pH-sensitive fluorescent dyes indicated heterogeneous pH values ranging from below pH 6 to almost pH 8 and even robust instruments (pH meter tips with a diameter of 750 μm) have revealed pH values as low as pH 6.8 already at a distance of 200 μm from the lumen [10]. A recent report indicates that macrophages generate pericellular pH gradients and pH values as low as pH 5.0 could be measured [11]. As the distance from an acidifying cell grows, the concentration of protons decreases and the extracellular pH tends to approach the neutral pH.

Many proteoglycans have been identified in the arterial intima. Versican, a chondroitin sulphate-rich proteoglycan is the major proteoglycan of the extracellular matrix [12]. On the cell surfaces, the two major proteoglycan families are syndecans and glypicans, which are both composed of heparan sulphate and chondroitin

* Corresponding author. Tel.: +358 9 681 411; fax: +358 9 637 476.
E-mail address: kati.oorni@wri.fi (K. Öörni).

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sulphate, the former being their most abundant glycosaminoglycan chain [13]. LDL binds to proteoglycans via ionic interactions between the positively charged lysine and arginine residues of the LDL apolipoprotein B-100, and the negatively charged sulphate and carboxyl groups of the GAG chains in the proteoglycans [14].

Decrease in pH increases the binding of LDL to proteoglycans [15,16] and, therefore, acidity is likely to enhance the extracellular accumulation of LDL-derived lipids [17]. Since, complexes of LDL and extracellular proteoglycans can also be internalized by macrophages [18], the increased formation of proteoglycan-LDL complexes may also lead to intracellular lipid accumulation. In addition, the interaction of LDL with cell surface proteoglycans may enhance foam cell formation. In fact, activation of macrophages with LPS, which increases the expression of cell surface proteoglycans, also increases the binding and uptake of modified LDL to macrophages [19]. In addition to modified LDL, also native LDL can induce foam cell formation. Thus, Kruth et al. have recently shown that if LDL concentration is high enough, pinocytosis of native LDL can lead to intracellular cholesterol accumulation [20].

We have recently shown that acidity increases the binding and uptake of modified LDL by macrophages [21]. In this study, we examined the effect of pH on the binding and uptake of native LDL by human monocyte-derived macrophages. In addition, the role of proteoglycans in these processes was examined.

2. Methods

2.1. Isolation and modification of LDL

Human LDL (d = 1.019–1.050 g/ml) was isolated from plasma of healthy volunteers (a kind gift from the Finnish Red Cross) by sequential ultracentrifugation in the presence of 3 mM EDTA [22,23]. The amounts of LDL are expressed in terms of their protein concentrations, which were determined by the method of Lowry et al. with bovine serum albumin as standard [24]. Each experiment was performed with at least two different LDL preparations.

2.2. Differentiation of human monocytes into macrophages in culture

Human monocytes were isolated from buffy coats (Finnish Red Cross Blood Transfusion Center, Helsinki, Finland) by centrifugation in Ficoll–Paque gradient as described [25]. Washed cells were suspended in DMEM supplemented with 100 U/ml penicillin and 100 (g/ml streptomycin), counted, and seeded in 24 wells. After one hour, non-adherent cells were removed and the medium was replaced with macrophage-SFM medium (Gibco) supplemented with 1% penicillin–streptomycin and 10 ng/ml of granulocyte macrophage colony stimulating factor (GM-CSF) (Biosite, San Diego, USA). The culture medium was replaced with fresh macrophage-SFM medium after 24 h, and then at 48–65 h intervals. Experiments were started when the monocytes had been cultured for 8 days *in vitro* during which they had been differentiated into macrophages.

2.3. Binding and uptake of LDL by macrophages

Apolipoprotein (apo) B-100 of LDL was labeled with a ^{3}H-labeling reagent according to the Bolton–Hunter procedure [26]. Before the experiments, the culture medium was replaced with custom-made HyQ DME/HIGH Glucose medium (HyClone) having a pH of either 7.5, 6.5, or 5.5 and containing 5% penicillin-streptomycin and L-glutamine. After incubation for 1 h, ^{3}H-radiolabeled LDL was added to cells at each pH value. After incubation for the indicated times, the media and cells were collected for further analysis. No thiobarbituric adic-reactive substances were found in LDL that had been incubated with macrophages for the standard time of 4 h at any of the pH values.

Cell necrosis was not induced by the incubation conditions as determined by Trypan blue exclusion test and by measuring the amounts of lactate dehydrogenase in the culture media. The concentration of lactate dehydrogenase in the culture media did not increase during the 24 h-incubation at any pH. Of the macrophages, at pH 5.5 less than 10% (typically 3–5%) and at pH 6.5 and at pH 7.5 less than 5% (typically 0–2%) were Trypan blue-positive after incubation for 24 h.

To determine the effect of pH on the binding of native LDL by macrophages, 20–200 μg/ml of native LDL was added to the cells and incubated for 3 h at 4 °C. After the incubation, the cells were rinsed three times with PBS and then the cells were lysed with 0.2 M NaOH and their radioactivities were measured.

The uptake of native LDL by macrophages was determined by measuring the cell association and degradation of ^{3}H-labeled native LDL by the macrophages. 100 μg/ml of native LDL was added to the cells and incubated for 1–4 h. In some experiments uptake was inhibited by incubating cells either heparinase and chondroitinase or lactoferrin, or $NaClO_3$ for 1 h before LDL addition. To determine the intracellular amounts of ^{3}H-LDL, the macrophages were first rinsed three times with ice cold 50 mM HEPES, 150 mM NaCl, pH 7.5, after which, heparin (10 mg/ml in10 mM HEPES, 50 mM NaCl, pH 7.5) was added to the cells and the incubation was continued for 1 h at 4 °C to remove any cell-surface-bound LDL particles. The heparin solutions were collected and their radioactivities were measured and the cells were rinsed three times with PBS containing Ca^{2+} and Mg^{2+}. After the final rinse, macrophages were lysed with 0.2 M NaOH and their radioactivities were measured. Lipoprotein degradation was determined by the measurement of trichloroacetic acid-soluble ^{3}H-radioactivity in the incubation media, which measures the amount of radioactive peptides released from LDL particles.

To visualize the formation of foam cells, some macrophages were plated onto 13 mm glass coverslips. After the incubation, the cells were washed with PBS, fixed with 3.7% formalin for 3 min at room temperature, stained with 0.7% Oil Red O, and counterstained with hematoxylin. The coverslips were mounted on glass microscope slides with Aquamount (BDH Laboratory Supplies) and photographed.

2.4. Determination of proteoglycan synthesis, isolation of glycosaminoglycans, and binding of LDL to the isolated glycosaminoglycans

40 μCi/ml of ^{35}S-sulphate was added to the macrophages and incubated for 4 h at 37 °C. The media were collected and the cells were rinsed three times with PBS containing Ca^{2+} and Mg^{2+}. After the final rinse, macrophages were lysed with 0.2 M NaOH and the radioactivities of the media and the cells were measured.

^{35}S-labeled glycosaminoglycans of the cell-associated proteoglycans were isolated by incubating cell lysates with 0.1 mg/ml of pronase [27]. The lysates were centrifuged at 10,000 × g for 10 min and the supernatants were applied to HiTrap Q columns. The bound glycosaminoglycans were eluted using 1 M NaCl. Binding of LDL to the glycosaminoglycans at pH 7.5 and 5.5 was determined using a microtiter well assay as described earlier [16]. Determination of the amounts of ^{35}S-radioactivity of the coated wells indicated that similar amounts of glycosaminoglycans had been attached to the microtiter wells.

2.5. Measurement of fluid uptake

Bovine serum albumin was labeled with a ^{3}H-labeling reagent according to the Bolton–Hunter procedure [28]. 0.25 mg/ml of ^{3}H-