Mononuclear leukocytes exposed to oxidized low density lipoprotein secrete a factor that stimulates endothelial cells to express adhesion molecules

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Abstract

In animals fed a hypercholesterolemic diet, development of atherosclerosis is preceded by attachment of mononuclear leukocytes to the arterial endothelium. Early lesions begin to develop as monocytes migrate into the intima and ingest lipids. A major part of these lipids is believed to be derived from oxidatively modified low density lipoprotein (LDL). In the present study we demonstrate that human mononuclear leukocytes exposed to low concentrations of copper-oxidized LDL secrete one or several factors that stimulate the expression of intercellular adhesion molecule-1 (ICAM-1, CD54), vascular cell adhesion molecule (VCAM-1) and endothelial selectin (E-selectin-1, ELAM-1), whereas native LDL was found to be without effect. Exposure of endothelial cells to non-conditioned medium containing oxidized LDL did not influence the expression of adhesion molecules. Incubation of endothelial cells with conditioned medium from mononuclear cells grown in the presence of oxidized LDL also resulted in a three-fold increase in the binding of monocytoid U937 cells. The present findings suggest that mononuclear leukocytes exposed to oxidatively modified LDL in early atherosclerotic lesions may stimulate the recruitment of other leukocytes by secreting cytokines which induce the expression of adhesion molecules on the endothelium.

Key words: Atherosclerosis; Oxidized low density lipoprotein; Endothelial cells; Adhesion

1. Introduction

Macrophages are believed to play a key role in the development of atherosclerotic lesions. In animals fed a hypercholesterolemic diet, adhesion of mononuclear leukocytes to the endothelial lining is observed within a few weeks [1]. Subsequent migration of monocytes into the arterial intima where they accumulate cholesterol and become lipid-laden macrophages results in formation of fatty
streaks, which are believed to represent the earliest type of atherosclerotic plaque [2]. It is now generally recognized that a significant part of the lipid content of intimal macrophage foam cells is derived from scavenger receptor-mediated uptake of oxidatively modified low density lipoprotein (LDL) [3]. Based on this background, considerable interest has focused on the role of lipoproteins, and in particular that of oxidatively modified LDL, in the recruitment of intimal mononuclear leukocytes. Studies using cultured cells have demonstrated that oxidized LDL is chemotactic for monocytes [4]. Furthermore, an increased release of chemotactic factors for monocytes [5], as well as an enhanced capacity to adhere to leukocytes [5,6], has been shown in cultures of endothelial cells exposed to oxidized LDL.

Attachment of leukocytes to the endothelium is mediated by cell adhesion molecules (CAMs) present on the surface of both cell types. Three major CAMs of endothelial cells are intercellular adhesion molecule-1 (ICAM-1, CD54), vascular cell adhesion molecule (VCAM-1), both belonging to the immunoglobulin superfamily of adhesion molecules, and endothelial selectin (E-selectin), belonging to the selectin family of adhesion molecules [7]. ICAM-1 binds to the adhesion molecule LFA-1 (CD11a, LeuCAMα) which is present on both mononuclear and polynuclear leukocytes [8–10]. The counterpart of VCAM-1 is VLA-4 (CD49, CD29), an integrin present on monocytes and T lymphocytes that functions as a receptor for both matrix and cells [7,10]. E-selectin binds a carbohydrate ligand on neutrophils [11]. The expression of the endothelial CAMs is regulated by cytokines released from monocytes/macrophages and T lymphocytes such as interleukin (IL) 1 and 4, interferon-gamma (INF-γ) and tumor necrosis factor-alpha (TNF-α) [13–15]. In the present study we have analyzed the effect of native and oxidized LDL on the release of CAM-regulating cytokines from human mononuclear leukocytes.

2. Materials and methods

2.1. Cell culture

Endothelial cells were prepared from human umbilical cords as described in Ref. 6. The endothelial cells were seeded out in gelatin-coated 25-cm² tissue culture flasks and cultured in MEM (GIBCO BRL, Glasgow, UK) with addition of 30% human serum (HS; GIBCO), 50 µg/ml penicillin and 50 U/ml streptomycin at 37°C in an atmosphere of 5% CO₂ in air. Confluent cultures were trypsinized (0.1% trypsin/0.02% EDTA) and seeded out on gelatin-coated 13-mm Theranova cover slips placed in 24-multiwell plates (Costar, Cambridge, MA) for analysis of cell adhesion and in 12-multiwell plates without Theranova cover slips for FACSscan analysis of adhesion molecule expression.

Peripheral blood mononuclear (PBMC) leukocytes were isolated from human buffy coats. The buffy coat was diluted in phosphate buffered saline (PBS) at a ratio of one to four, layered onto Ficoll-Hypaque and centrifuged for 20 min at 1500 x g. Interface cells were washed twice in PBS. They were then counted and resuspended in RPMI 1640 medium supplemented with 50 µg/ml of gentamycin and 10% FCS at a cell concentration of 1 x 10⁶ cells/ml. The PBMC suspensions contained approximately 30% monocytes and 70% T lymphocytes.

Monocytoid U937 cells [16] were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% FCS and 50 µg/ml of gentamycin sulphate. The culture medium was changed twice weekly and cell density was kept at 2–8 x 10⁵ cells/ml.

2.2. Preparation and oxidation of LDL

LDL (1.025–1.050 kg/l) was isolated from plasma of healthy donors by sequential preparative ultracentrifugation in a 50.3 Ti Beckman fixed angle rotor (Beckman L8-80 ultracentrifuge) for 20 h [17]. The LDL protein content was determined as described by Lowry et al. [18]. Oxidation of LDL was performed by incubating LDL (0.2 mg/ml) in F-10 medium containing 10 µM CuSO₄ at 37°C overnight. The efficacy of the oxidation procedure was determined by analyzing the presence of thiobarbituric acid-reactive substances (TBARS; expressed as malondialdehyde equivalents) as described by Yagi [19]. In a typical experiment, native LDL contained 1.8 ± 0.15 MDA eq./mg protein and oxidized LDL contained...
41.0 ± 2.5 MDA eq./mg protein. Iodination of LDL and analysis of LDL degradation in mouse peritoneal macrophages were performed as described in Ref. 20. Endotoxin contamination, determined using the Limulus assay (Kabi, Stockholm, Sweden) was below 2.5 ng/mg LDL protein in both the native and oxidized LDL preparations.

2.3. Analysis of cell adhesion molecule expression

Conditioned medium was prepared by incubating PBMN cells (10^6 cells/ml) in serum-free RPMI medium with or without the addition of native or copper-oxidized LDL for 24 h at 37°C. The PBMN cells were then removed by centrifugation and the conditioned medium was given to confluent secondary cultures of endothelial cells to study the effect of CAM expression. Control cells were given lipoprotein-containing medium which had not been pre-conditioned by PBMN cells. Analysis of CAM expression was performed after 6 h (E-selectin), 8 h (VCAM-1) and 18 h (ICAM-1) to adjust for the differences in lag phase required for maximal induction of the three CAMs. Fluorescent conjugated F(ab)₂ fragments of rabbit antimouse IgG were used as fluorescent antiserum. Non-immune mouse IgG was used as unspecific control. All antibodies were purchased from British Bio-technology, Oxford, UK. Following the incubation with experimental medium, endothelial cells were rapidly trypsinized at room temperature and washed three times in PBS containing 0.1% bovine serum albumin and 0.2% sodium azide (binding buffer). After the last centrifugation the pellet was gently suspended in a minimum volume of binding buffer containing the respective antibodies and placed on ice for 30 min. The cells were then washed three times in binding buffer and flushed gently with a Pasteur pipette to obtain a single cell suspension, and the antigen expression was analyzed in a FACScan from Becton Dickinson. Values were expressed as median log fluorescence intensity. To correct for background binding, each value obtained from staining with specific antibody was subtracted with values from non-specific mouse IgG (Coulter Immunology Hialeah, FL). Similar CAM expression responses were observed in five independent experiments. The significance of the experiments using FACScan was determined by using the Kolmogorov–Smirnov test [21].

2.4. Cell adhesion assay

Secondary or tertiary cultures of endothelial cells were grown to confluence on gelatin-coated Thermanox cover slips in medium containing 30% HS. The cultures were then washed in serum-free medium and exposed to lipoprotein-containing PBMN-conditioned medium for 6 h for E selectin, 8 h for VCAM-1 and 18 h for ICAM-1 at 37°C. The cultures were then washed three times in PBS and 5 x 10⁵ of U937 cells were added to each well for 30 min at 37°C. The cover slips were then removed and washed six times in PBS, and the number of remaining cells was determined in an electronic cell counter (VDA Analyis Instrument, Stockholm, Sweden). The number of adhering U937 cells was calculated by subtracting the mean number of endothelial cells from cover slips without U937 cells [6].

3. Results

Endothelial cells exposed to lipoprotein-free cell culture medium did not express VCAM-1, nor could VCAM-1 expression be induced by incubating endothelial cells with copper-oxidized LDL in concentrations up to 10 μg/ml (Fig. 1). We then studied the ability of native and copper-oxidized LDL to stimulate secretion of CAM-regulating factors from cultured human PBMN. Conditioned medium from PBMN exposed to copper-oxidized LDL for 24 h was found to stimulate endothelial cell expression of VCAM-1. A maximal effect was obtained with conditioned medium from cells exposed to medium containing as little as 1 μg/ml of copper-oxidized LDL. Incubation of PBMN in medium containing up to 10 μg/ml of native LDL did not result in release of factors stimulating endothelial VCAM-1 expression (Fig. 1). A low basal expression of E-selectin could be identified on endothelial cells grown in lipoprotein-free medium (Fig. 2). Conditioned medium from PBMN exposed to copper-oxidized LDL stimulated endothelial expression of E-selectin with a maximum
Fig. 1. Expression of VCAM-1 on HUVEC. The cells were exposed for 6 h to oxidized LDL (■), or conditioned medium from PBMN was exposed to native LDL (○) or oxidized LDL (●) for 24 h. The surface expression of VCAM-1 was determined using a FACScan. The surface expression of VCAM-1 is expressed as median fluorescence. ***P < 0.001.

Fig. 2. Expression of E-selectin on HUVEC. The cells were exposed for 6 h to oxidized LDL (■), or conditioned medium from PBMN was exposed to native LDL (○) or oxidized LDL (●) for 24 h. The surface expression of E-selectin was determined using a FACScan. The surface expression of E-selectin is expressed as median fluorescence. ***P < 0.001.

Fig. 3. Expression of ICAM-1 on HUVEC. The cells were exposed for 18 h to oxidized LDL (■), or conditioned medium from PBMN was exposed to native LDL (○) or oxidized LDL (●) for 24 h. The surface expression of ICAM-1 was determined using a FACScan. The surface expression of ICAM-1 is expressed as median fluorescence. ***P < 0.001.

increase occurring in cells exposed to conditioned medium containing 10 μg/ml of copper-oxidized LDL. In contrast, conditioned medium from PBMN exposed to native LDL or to non-conditioned medium containing copper-oxidized LDL was without effect (Fig. 2). The basal endothelial expression of ICAM-1 was much higher than that of E-selectin (Fig. 3). A maximal increase in ICAM-1 expression was observed in endothelial cells incubated with conditioned medium from PBMN grown in 1 μg/ml of copper-oxidized LDL. ICAM-1 expression was not influenced by direct exposure to copper-oxidized LDL or by incubation with conditioned medium from cells grown in the presence of native LDL (Fig. 3). To study whether or not the increased expression of endothelial CAMs induced by conditioned medium from PBMN exposed to copper-oxidized LDL was coupled to an increased endothelial cell adhesive capacity, adhesion analysis was performed using the monocytic cell line U937. Conditioned medium from PBMN exposed to copper-oxidized LDL was found to enhance U937 cell adhesion to endothelial cells in a dose-dependent manner, whereas conditioned medium from PBMN cultured with native LDL was without effect (Fig. 4).

When 50 μg/ml of polymyxin B was added during incubation of 10 μg/ml oxidized LDL with PBMN, the adhesion of U937 cells to cultured endothelial cells induced by the supernatant was 112% ± 20% of the adhesion seen when only oxidized LDL was present. Polymyxin B binds to lipopolysaccharide (LPS) and inhibits its biologi-
Low density lipoprotein (μg/ml)

Fig. 4. Adhesion of U937 cells to a cultured monolayer of HUVEC previously exposed for 6 h to conditioned medium from PB MN exposed to native LDL (○) or oxidized LDL (●) for 24 h. Each value represents the mean and standard variation of triplicate determinations. \*P < 0.05.

The results thus show that LPS/endotoxin contamination is not likely to be responsible for the oxidized LDL-induced effects.

4. Discussion

In animals fed a hypercholesterolemic diet, development of atherosclerosis is preceded by attachment of mononuclear leukocytes to the arterial endothelium [1]. Migration of monocytes into intima, where they are believed to ingest oxidatively modified LDL, results in the formation of fatty streaks similar to those observed in human arteries [2,3]. Activated T cells also constitute an important component of human atherosclerotic lesions [22]. The present findings demonstrate that human mononuclear leukocytes exposed to low concentrations of oxidized LDL secrete one or several factors which stimulate the endothelial expression of CAMs as well as the ability of endothelial cells to bind monocytic cells, whereas native LDL is without effect in these respects. The endothelin levels in LDL were low and did not differ between preparations of native and oxidized LDL. Addition of polymyxin B, which is known to inhibit the biological effects of endotoxin, did not alter the effect of supernatants from oxidized LDL-treated PB MN on endothelial adhesiveness. It is therefore not likely that the observed effects are due to endotoxin contamination. Oxidized LDL did not in itself stimulate endothelial CAM expression. This finding adds further support to the notion that the observed effects are mediated by molecules other than endotoxins, since endotoxins are known to increase endothelial CAM expression by a direct mechanism.

Several cytokines, such as IL-1, IL-4, INF-γ and TNF-α, are known to stimulate endothelial expression of CAM and the attachment of leukocytes to cultured endothelial cells [13–15]. Recent studies from our group have demonstrated that copper-oxidized LDL stimulates release of IL-1 from human mononuclear leukocytes and activates T cells by a monocyte-dependent mechanism [20]. Accordingly, it is possible that the effect on endothelial CAM expression described in the present report is due to cytokine-release from monocytes and/or T cells activated by oxidized LDL. The conditioned medium from mononuclear leukocytes exposed to oxidized LDL stimulated the expression of all CAMs studied. Human atherosclerotic lesions of all subtypes except fibrous plaques have recently been demonstrated to show an enhanced expression of ICAM-1 [23]. Cybulski and Gimbrone [24] recently reported that in hypercholesterolemic rabbits VCAM-1 is expressed at endothelium covering early atherosclerotic lesions and focally in regions with small intimal accumulations of foam cells, whereas no expression was found at non-lesioned areas or in normocholesterolemic controls. Using immunological techniques it has been possible to demonstrate that macrophages in these lesions contain lipids derived from oxidized LDL [25]. Based on these in vivo studies, a coupling between exposure of intimal macrophages to oxidized LDL and induction of endothelial expression of VCAM-1 can be hypothesized.

The interaction of monocytes/macrophages and oxidatively modified LDL particles may play a key role in the development of atherosclerosis. By its chemotactic properties, oxidized LDL may initiate recruitment of macrophages into arterial tissue [4]. Oxidized LDL may stimulate monocyte–endothelial interactions, not only by stimulating the
release of CAM-inducing cytokines from intimal macrophages, but also by a direct effect on endothelial cells [5,6]. Furthermore, exposure of monocytes to oxidized LDL has been found to result in activation of T lymphocytes [20]. The fact that humans frequently have circulating antibodies against oxidized LDL [26] implies that this activation is due to an immunological process directed against epitopes present on oxidized but not native LDL. IL-1 has been demonstrated to stimulate the growth of vascular smooth muscle cells by activating the endogenous production of platelet-derived growth factor [27]. Accordingly, it is possible that oxidized LDL may also promote intimal smooth muscle cell proliferation by its ability to stimulate release of IL-1 from monocytes/macrophages.

Findings from in vitro studies clearly suggest that oxidation of LDL may play an important role in atherogenesis. This hypothesis has been further substantiated by animal studies demonstrating that probucol, a lipid-lowering drug with antioxidant properties, is a more potent inhibitor of formation of arterial lipid-rich lesions than other equally lipid-lowering drugs [28,29]. There are also some recent reports implicating lipid oxidation as a risk factor for cardiovascular disease in humans. Low levels of antioxidant vitamins have been demonstrated in patients suffering from angina pectoris [30]. In a study on young post-infarction patients, Regnström et al. [31] found a significant association between the susceptibility of LDL to oxidation and the severity of coronary atherosclerosis as assessed by angiography.

In summary, the present findings suggest that monocytes/macrophages exposed to oxidatively modified LDL release cytokines which stimulate the endothelial expression of CAMs, and in particular that of VCAM-1. This adds further support to the hypothesis that oxidation of LDL plays an important role in the development of atherosclerosis.

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6. References

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