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Atherogenic and inflammatory profile of human arterial endothelial cells(HUAEC) in response to LDL subfractions

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Abstract

Background: Electronegative LDL (LDL(−)) is a minor modified LDL fraction present in plasma that has been demonstrated to be inflammatoryin endothelial cells isolated from human umbilical vein (HUVEC).
Methods: A protein array able to measure 42 cytokines, chemokines and related compounds involved in atherogenesis was used to determine theirrelease into the culture medium of human arterial endothelial cells (HUAEC) activated or not by two low-density lipoprotein (LDL) fractions isolated from human plasma by anion-exchange chromatography.
Results: The results of the protein array (confirmed using specific ELISAs for each induced factor) revealed that HUAEC in the absence of stimulireleased small amounts of interleukin 8 (IL-8), monocyte chemotactic protein 1 (MCP-1) and growth-related oncogene (GRO). The major nativeLDL fraction (named LDL(+)) increased the release of these molecules and also those of interleukin 6 (IL-6) and GROα. Compared to LDL(+), the minor modified fraction, named electronegative LDL (LDL(−)), increased all these factors to a greater degree and also induced the release ofgranulocyte–monocyte colony-stimulating factor (GM-CSF) and platelet-derived growth factor B (PDGF-B). These results were confirmed by ELISA.
Conclusions: All these results indicate that, compared to LDL(+), LDL(−) fraction promotes not only the release of proinflammatory factors butalso those of atherogenic factors in endothelial cells of arterial origin, thereby suggesting a new role for LDL(−) in atherogenesis.

Keywords: Electronegative LDL; Arterial endothelial cells; Inflammation; Atherosclerosis

1. Introduction

Increased levels of low-density lipoproteins (LDL) are a major risk factor for cardiovascular disease, and LDL is directly involved in atherogenesis through the induction of an inflammatory phenotype in the arterial wall. This inflammationis mainly exerted by LDL after its chemical modification in thearterial wall or in the bloodstream. Electronegative LDL (LDL (−)) is a minor modified LDL fraction present in plasma that has been demonstrated to be inflammatory [1] in endothelial cells isolated from human umbilical vein (HUVEC). In the same cellmodel, LDL(−) induces the production of chemotactic factors,such as interleukin 8 (IL-8) and monocyte chemotactic protein 1 (MCP-1) [2,3], and enhances the expression of vascular cell adhesion molecule (VCAM) induced by tumor necrosis factor α [4]. Furthermore, increased mononuclear cell adhesion to HUVEC induced by LDL(−) under flow conditions was functionally assessed by Yang et al. [5]. However, these studies were developed in endothelial cells of venous origin, but no data exist on the inflammatory effect of LDL(−) on arterial endothelial cells. This is a matter of particular interest since atherosclerosisoccurs in arteries, and expression profiling of several genes differs in endothelial cells from different blood vessels [6]. Specifically, atherogenic stimuli, such as oxidized LDL, induced an expression profile more atherogenic in endothelial cells of arterial origin than in venous cells [7]. Our purpose wasto screen the effects that LDL fractions (LDL(−) and the non-modified or native form LDL(+)) exert on arterial endothelial cells using a protein array system permitting us to identify the basal and induced profile of a wide range of cytokines, chemokines and related compounds involved in atherogenesis. The release of each induced factor was quantified using specific ELISAs.

2. Methods

2.1. *Lipoprotein isolation*

Plasma EDTA was obtained from normolipemic, normoglycemic and non-smoker subjects and pooled to isolate LDL fractions [3]. Total LDL (1.019– 1.050 g/ml) was isolated by sequential ultracentrifugation and LDL was then subfractioned into LDL(+) (non-modified LDL) and LDL(−) by anion exchange chromatography [3]. LDL fractions were chemically characterized and differences in electronegativity were confirmed by agarose gel electrophoresis, as described[2,3].

2.2. *Cell culture*

Human umbilical artery endothelial cells (HUAEC) were isolated from pooled umbilical arteries by enzymatic digestion with collagenase using an olive-pointed needle. HUAEC were grown in gelatin-coated flasks with endothelial cell EGM-2 medium (Clonetics) containing 2% FCS supplemented with vascular endothelial growth factor, fibroblast growth factor-2, epidermal growth factor,insulin-like growth factor, hydrocortisone, ascorbic acid and antibiotics. The typical morphology, specific positive staining for von Willebrand factor and VE- cadherin and negative staining for smooth muscle cell-specific α-actin confirmed the purity of these endothelial arterial cells (data not shown). Cells at passage 2—4 were seeded in six-well plates at 10,000 cells/cm^2. At confluence, growth medium was replaced with fresh medium containing 5% FCS without other growth factors for 24 h. Thereafter, LDL fractions were added for a subsequent24 h at 150 mg apoB/L in 199 medium containing 1% FCS without growth factors. LDL subfractions were previously dialyzed against 199 medium and filtered through 0.22 μm. Interleukin-1β (IL-1β) (10 μg/L) was used as a positive inflammatory control in all experiments. Culture supernatant aliquots were stored at −80 °C until protein array and ELISA analysis.

2.3. *Protein array*

The RayBio™ Human Cytokine Array III (Ray Biotech) was used. This system permits the measurement of 42 cytokines, chemokines and atherogenicmolecules, as shown in Fig. 1A. Arrays were incubated with undiluted culturemedia and developed following the manufacturer's instructions.

2.4. *ELISA*

Positive results obtained with the protein array for IL-6, IL-8, MCP-1, GROα, GM-CSF and PDGF-B were confirmed and quantified by commercial ELISA (Module Set antibody pairs, Bender MedSystems, except PDGF-B from RayBio and GROα from R&D) according to the manufacturers' instructions. GROγ and GROβ were measured by ELISA with unlabeled and biotinylated polyclonal antibodies (PeproTech) using the procedure recommended by the manufacturer, as described [8]. Culture supernatants of arterial cells incubated with LDL(+) and LDL(−) fractions were diluted 1/30 for IL-6, 1/5 for GM-CSF and GROα, 1/20 for PDGF-B and 1/100 for IL-8, MCP-1, GROγ and GROβ. Supernatants of cells incubated with IL-1β were diluted 10-fold more than supernatants of cells incubated with LDL fractions. Supernatants from six independent experiments were measured by ELISA.

2.5. *Statistical analysis*

Results are expressed as mean±S.D. SPSS 11.5.2 statistical package was used. Inter-group differences were tested with Wilcoxon's *T*-test (for paired data) and Mann—Whitney *U*-test (for unpaired data). A *P*-value b 0.05 was considered significant.

3. Results

Physicochemical characteristics of LDL(+) and LDL(−)were similar to those previously described [2,3]. Briefly, LDL (−) presented increased non-esterified fatty acids (NEFA),apoE, apoC-III, free cholesterol and triglyceride and decreased apoB content compared with LDL(+) (data not shown).

All inflammatory and atherogenic molecules tested with theprotein array used are shown in Fig. 1A, and representative results obtained after incubation of HUAEC with LDL(+), LDL(−) and IL-1β in Fig. 1B. Untreated cells (control) showed a basal production of IL-8, GRO and MCP-1 which clearlyincreased in LDL(+)-treated cells. This unmodified LDL(+) fraction also increased the release of IL-6 and GROα comparedto control cells, whereas LDL(−) promoted to a greater extentthe release of IL-6, IL-8, MCP-1, PDGF-B and GM-CSF compared to LDL(+). Finally, IL-1β increased the production of these molecules (except PDGF-B) and also small amounts of MCP-2 compared to control cells. It should be noted that the GRO family is composed of three members: GROα, GROβ and GROγ; thus, GRO dots in the membrane array refer to the threemembers, whereas GROα dots refer to this molecule alone.

Since the protein array is a semi-quantitative method, the concentration of the molecules whose release appeared inducedin LDL-treated cells in the array system was quantified by ELISA (Fig. 2). Compared to control cells, LDL(+) significantly induced the release of MCP-1, IL-8, IL-6, GM-CSF, GROβ and GROγ, but not those of the atherogenic factor PDGF-B (Fig. 2). In turn, after subtraction of the basal release(control cells), LDL(−) significantly increased the release of IL-8 (1.7-fold), MCP-1 (2.5-fold), IL-6 (2.3-fold), GM-CSF (1.9-fold) and PDGF-B (4.3-fold) compared to LDL(+) fraction. The three members of the GRO family, GROα, GROβ and GROγ increased only marginally (1.2- to 1.5-fold) after incubation with LDL(−). The inflammatory mediator IL-1 β (used as

positive control) was, in general, more active than LDL(−) in inducing the synthesis of all factors produced by treated cells, except MCP-1. However, this cytokine did not increase the release of PDGF-B in HUAEC as did LDL(−)-treated cells.

4. Discussion

A major finding of the current work was that some inflammatory effects induced by LDL(−) on the current venous model of HUVEC [2—5,8] were also reproduced using human arterial cells. This is significant since atherosclerosis occurs in arterialbut not in venous vessels, and it has been demonstrated that endothelial cells from different blood vessels and tissues showdistinct expression profiles and responses to atherogenic stimuli [6,7]. The preferential arterial presence of atherosclerosis is usually attributed to the hemodynamic characteristics of arteries; however, a local inflammatory response that would de- pend on the nature of endothelial cells could also be involved. Our results showed significant differences between arterial cells in the current study and venous cells in a previous study [8] inresponse to LDL(−). Qualitatively, these differences were mainly two. First, in arterial cells, the members of the GRO family were poorly stimulated by LDL(−) compared to LDL(+), suggesting that members of the GRO family could play a minorrole in atherogenesis. Reports focused on GRO members as inflammatory mediators in atherosclerosis are scarce and, to our knowledge, there are no reports describing increased GRO ex- pression in atherosclerotic arterial wall. In fact, the chemotacticactivity exerted by GRO on lymphocytes and granulocytes is efficiently induced by IL-8, a key factor in atherogenesis.However, this possibility should be considered with caution since our observation may be attributed to the high GRO release induced by LDL(+), specifically of β and γ members, rather than a poor stimulation due to LDL(−). In this respect, the induction of GRO members by LDL(+) in cultured HUVEC was only slightly increased compared to control cells [8]; this observation suggests that arterial cells (HUAEC) would be more sensitive to an inflammatory response induced by LDL(+) than the current cell model of HUVEC.

The second qualitative difference between arterial and venous cells was that HUAEC released PDGF-B, an important mediator that was not produced by HUVEC [8] but which has been involved in atherogenesis [9]. Interestingly, its release was significantly increased (4.3-fold) compared to controls and LDL (+)-treated HUAEC in response to LDL(−), but not in responseto the typical inflammatory mediator IL-1β; this could indicatea new atherogenic property for electronegative LDL that is different in arterial and venous cells. This concurs with the study of Deng et al. [7], reporting that other forms of atherogenic LDLs,such as oxidized LDL, stimulate to a greater degree the expression of cell proliferation-related genes in arterial than in venouscells. On the other hand, differences between LDL(+) and LDL(−) on IL-6, IL-8, MCP-1 and GM-CSF release were similar inHUAEC than those previously reported in HUVEC [3,8]. How- ever, on a quantitative basis, the effect of both LDL subfractions on IL-6, IL-8, MCP-1 and GM-CSF was stronger in HUAEC than in HUVEC (approximately 3-fold, 3-fold, 1.5-fold and 10-fold, respectively) [2,8], suggesting that arterial cells are moresensitive and, consequently, a better “in vitro” model to study the inflammatory properties of lipoproteins. Taken together, these results confirm a wide proinflammatory action of LDL(−) on arterial endothelial cells. IL-6 is a central mediator of the acute-phase response and the main determinant of C-reactive protein (CRP) synthesis [10]. IL-8, MCP-1 and GRO membersare chemokines involved in lymphocyte and monocyterecruitment, respectively, during early phases of atherogenesis [11] and GM-CSF can also participate in neutrophil recruitment[12]. In addition, GM-CSF induces monocyte/macrophage differentiation [13] and PDGF-B plays a central role in the modulation of smooth muscle cell growth and migration and inthe angiogenic properties of endothelial cells [14]. Thus, these observations provide evidence that a high proportion of LDL(−) in blood could impair arterial endothelium function, leading to the enhancement of several inflammatory mechanisms involved in different stages of atherosclerosis. Interestingly, non-modified LDL (LDL(+)) could also alter *in vitro* the inflammatory characteristics of arterial endothelial cells, though to a lesser extent. Finally, we report for the first time that only LDL(−) fraction was able to induce significantly the release of PDGF-Bin human arterial cells compared to

LDL(+) and IL-1β. Furtherstudies will be required to assess a more general role of circulatingLDL(−) not only in inflammation but also in atherogenesis.

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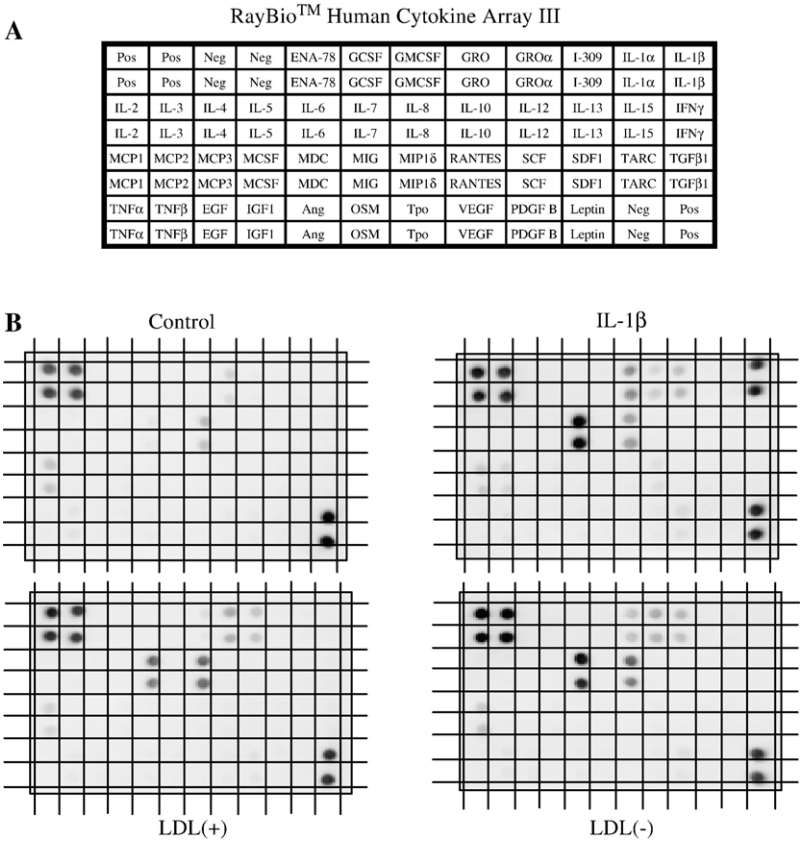

Fig. 1. Simultaneous detection of multiple factors using the RayBio™ Human Cytokine Array III. (A) Distribution of spots in the protein array membrane. (B) Representative experiment of membranes incubated with culture supernatants of untreated cells (control cells) or stimulated with LDL(+) (150 mg/L), LDL(−)(150 mg/L) and IL-1β (10 μg/L) under the conditions described in Methods. Membranes were incubated with undiluted media and revealed as described by the manufacturer.

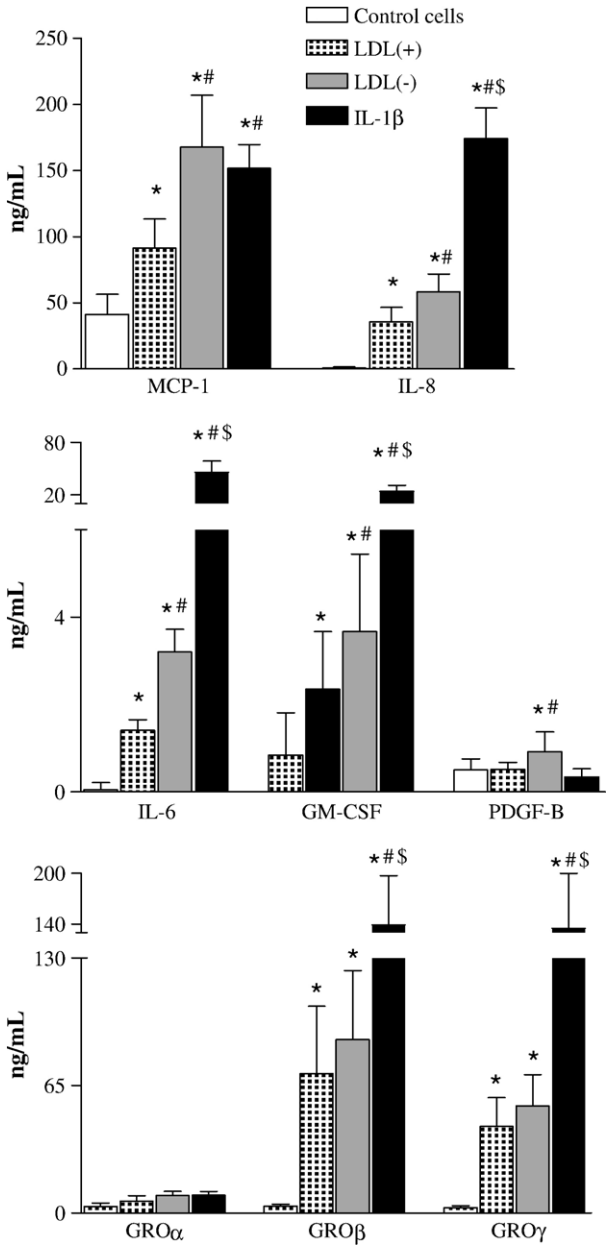

Fig. 2. Release of IL-8, MCP-1, IL-6, GM-CSF, PDGF-B, GROα, GROβ and GROγ by arterial cells (HUAEC). Confluent cells were incubated with LDL(+) (150 mg/L), LDL(−) (150 mg/L) or IL-1β (10 μg/L) and the release of these factors into the culture medium was measured by ELISA, as described in Methods. Data are the mean±S.D. of 6 independent experiments. $^{*}P$ b 0.05 vs control cells; $^{\#}P$ b 0.05 vs LDL(+); $^{\$}P$ b 0.05 vs LDL(−).