Endothelin-1 plus oxidized low-density lipoprotein, but neither alone, increase human monocyte adhesion to endothelial cells

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ABSTRACT

Endothelin-1 is a potent vasoconstrictor and mitogenic peptide that is implicated in the atherosclerosis of apolipoprotein E-deficient mice and may promote atherogenesis in humans. We hypothesized that endothelin-1 might promote the adhesion of monocytes to endothelial cells, a key early event in atherosclerosis. We investigated the adhesion of primary human monocytes (isolated by elutriation) to human umbilical vein endothelial cell cultures after incubation with endothelin-1 (0.1 and 0.01 nM; approximately physiological concentrations), copper-oxidized low-density lipoprotein (LDL) (0.1 mg/ml) and a combination of the two. After a 4 h incubation with 0.1 or 0.01 nM endothelin-1 combined with oxidized LDL, adhesion was increased to 120 ‡± 4 ‰ (P < 0.001 compared with control) and 118 ‡± 4 ‰ (P < 0.002) respectively, whereas neither substance alone increased adhesion (92–104 ‰ of control values; not significant). Neither endothelin receptor A blockade nor co-incubation with anti-fibronectin antibody inhibited the pro-adhesive effects of endothelin-1 plus oxidized LDL (115 ‡± 7 ‰ and 115 ‡± 3 ‰ of control compared with 120 ‡± 4 ‰ respectively; not significant). Endothelial cell expression of intercellular adhesion molecule-1, vascular adhesion molecule-1 and E-selectin were unchanged throughout the experiment. Therefore physiological concentrations of endothelin-1 and oxidized LDL may act synergistically to increase the adhesion of human monocytes to endothelial cells, contributing in part to the observed pro-atherogenic effects of endothelin-1.

INTRODUCTION

Endothelin-1 is a vasoconstrictive and mitogenic 21-amino-acid peptide produced by endothelial cells that has been implicated in the pathogenesis of atherosclerosis. Endothelin-1 is present in human atherosclerotic tissue [1], and increased arterial tissue levels of endothelin-1 are found in conditions associated with an increased risk of atherosclerosis, such as hypertension [2,3] and hyper-cholesterolaemia [4]. Furthermore, blockade of the endothelin system partly inhibits atherosclerosis in apolipoprotein E-deficient mice [5] and in cholesterol-fed hamsters [6]. The mechanism(s) by which endothelin-1 might promote atherogenesis, however, remain unknown.

The adhesion of monocytes to endothelial cells represents a key early step in atherogenesis [7]. Adhesion is mediated in part by endothelial cell adhesion molecules

Key words: adhesion, endothelin-1, monocytes, oxidized low-density lipoprotein.
Abbreviations: HBSS, Hanks balanced salt solution; HUVEC, human umbilical vein endothelial cell; ICAM, intracellular adhesion molecule-1; IL-1β, interleukin-1β; LDL, low-density lipoprotein; VCAM-1, vascular cell adhesion molecule-1.
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such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin [8]. Connecting-segment 1 fibronectin has also been reported to play a role in monocyte–endothelial-cell adhesion [9]. Endothelin-1 increases the adhesion of neutrophils to endothelial cells [10,11]. Concentrations of endothelin-1 used in these previous studies (between 1 nM and 1 μM), however, are 1000-fold greater than plasma levels typically found in humans (0.01–0.1 nM) [12]. In the present study, therefore, we have investigated whether physiologically relevant concentrations of endothelin-1 increase the adhesion of human monocytes to human endothelial cells.

Hypercholesterolaemia is a major risk factor for atherosclerosis [13] and has been found to be present in animal models where endothelin-1 has been proatherogenic [5,6]. The oxidation of low-density lipoprotein (LDL) can enhance the cellular uptake of this lipoprotein. LDL found in human atheroma is oxidized, although only to a small degree [14], and exposure to oxidized LDL stimulates endothelin-1 secretion from endothelial cells [15]. He et al. [16], however, reported that oxidized LDL might inhibit endothelin-1 release. Therefore we also addressed whether co-incubation of endothelin-1 with oxidized LDL enhances the pro-adhesive effects of endothelin-1 in endothelial cells. Furthermore, the mechanisms for any such pro-adhesive effects were explored by use of cell adhesion molecule assays, endothelin receptor antagonists and fibronectin-blocking agents.

**METHODS**

**Materials**

RPMI, M199 and endothelin-1 were obtained from Sigma Science. Endothelin receptor antagonists BQ 123 (a selective endothelin A receptor antagonist) and BQ 788 (a selective endothelin B receptor antagonist) were from Peninsula Laboratories, Inc. Anti-fibronectin antibody was obtained from Calbiochem (polyclonal rabbit antibody #341644). Interleukin-1 was obtained from ImmunoKontact. Mouse monoclonal antibodies against human VCAM-1, ICAM-1 and E-selectin were obtained from Becton-Dickinson, and isotype mouse IgG, and IgG2 not directed against endothelial cell antigens were obtained from ICN Immunobiologicals. Sheep anti-(mouse antibody)–horseradish peroxidase conjugate was obtained from Amersham Pharmacia Biotech International.

LDL was prepared from the plasma of fasted normolipidaemic healthy volunteers by density gradient centrifugation, as described previously in detail [17]. Oxidation was performed by incubation of LDL with 20 μM CuCl₂ over 20–24 h at 37 °C under sterile conditions, and was stopped by adding medium containing heat-inactivated human serum. Oxidation was confirmed by the increased relative electrophoretic mobility of the oxidation products (3–4-fold increase compared with native LDL) on an agarose gel. Oxidized LDL was stored at 4 °C and protected from light, and was used within 2 weeks of preparation for assays.

**Endothelial cell culture**

Human umbilical vein endothelial cells (HUVECs) were harvested enzymically from male and female umbilical cords under sterile conditions, as described by Minter et al. [18], and established as primary cell cultures in M199 (Sigma Science) containing 2 mmol/l L-glutamine (ICN Biomedicals), 0.5% endothelial cell growth promoter (Starrate Products), 100 units/ml penicillin, 0.1 mg/ml streptomycin and 20% (v/v) filtered heat-inactivated pooled human serum (Red Cross Blood Bank). Powdered medium was reconstituted with endotoxin-free water and filtered at 0.2 μm.

Endothelial cell monolayers (passages 2–3) were propagated on gelatin-coated flasks in medium, then trypsinized and re-plated on to gelatin-coated 24 mm-diam. tissue culture wells (for adhesion molecule expression studies) or 96-well plates (for adhesion molecule expression studies). Wells were coated with 1 ml/5 cm² Hemaccel (Boehringer-werke AG) diluted 1:250 in PBS and incubated for 15 min at 37 °C, and excess solution was removed before use. Endothelial cells were grown to confluence before treatment with endothelin-1 and/or oxidized LDL. The purity of the endothelial cell monolayers was confirmed microscopically by their cobblestone pattern. At the end of the 24 h treatment, viability was > 95% (by lactate dehydrogenase assay) for each condition.

**Isolation of human monocytes**

White-cell concentrates (Red Cross Blood Bank) were obtained from the peripheral blood of healthy human volunteers, and monocytes were removed within 24 h of collection by density gradient separation of the white blood cells on Lymphoprep (Nyomed Pharma) followed by counterflow centrifugation elutriation at 20 °C, as described previously [19]. This involves the use of a Beckman J2-21 M/E centrifuge equipped with a JE-6B elutriation rotor and a 4.2 ml elutriation chamber (Beckman Instruments, Inc). The elutriation buffer was Hanks balanced salt solution (HBSS) without calcium or magnesium (Sigma) supplemented with EDTA (0.1 g/l) and 1% (v/v) heat-inactivated human serum. The system and tubing were rinsed with 250 ml each of 70% (v/v) ethanol, endotoxin-free water, 6% (v/v) hydrogen peroxide, endotoxin-free water and elutriation buffer, in that order, before the Lymphoprep-derived mononuclear cell fraction was loaded at 9 ml/min into the elutriation rotor.
chamber (2020 rev./min at 20 °C). The flow rate was increased by an increment of 1 ml/min every 10 min, and monocytes were typically eluted at between 15 and 17 ml/min. Collected fractions were examined by a Cytospin system (Shandon) and Wrights’ stain (Diff-Quik; Laboratory-Aids). Monocyte purity of > 90% and viability of > 95% by Trypan Blue exclusion were confirmed by light microscopy, and the monocytes were resuspended in RPMI containing 2% (v/v) human serum and used immediately for adhesion studies.

**Monocyte–endothelial-cell adhesion assay**

Experiments were repeated five times for each condition at each time point. Confluent endothelial monolayers were established in 24 mm-diam. wells before incubation for 4 or 24 h with the following treatments: (1) control wells treated with M199 containing 10% (v/v) heat-inactivated human serum, l-glutamine (2 mmol/l), penicillin (100 units/ml) and streptomycin (0.1 mg/ml); (2) endothelin-1 (0.1 and 0.01 nM) dissolved in M199 (as above); (3) oxidized LDL (0.1 mg/ml) in M199 (as above); (4) endothelin-1 (0.1 nM or 0.01 nM) in combination with oxidized LDL (0.1 mg/ml) in M199 (as above).

The adhesion assay involved the addition of (1.0–1.5) × 10^6 monocytes/ml of RPMI (containing 2% heat-inactivated human serum, and free of endothelin-1 and oxidized LDL) to the endothelial monolayer after removal of the endothelin-1- and oxidized LDL-containing media from the endothelial cell cultures, and subsequent incubation for 1 h at 37 °C under 5% CO2 in air. Non-adherent cells were then removed by gentle, standardized washing with a 1000 μl automatic pipette (Gilson); the suspension was stored on ice for 30–60 min until the cell concentration was determined with an automatic cell counter (Bayer Technicon H2 or Avidia 120; Bayer Diagnostics). The coefficient of variation for this method is 2.13% for white blood cells (Bayer technical information sheet). The percentage of adherent monocytes was calculated by comparison with the initial concentration. This method has been shown to demonstrate maximal basal adhesion after 1 h of incubation [19].

**Expression of endothelial cell adhesion molecules**

The cell-surface expression of adhesion molecules on the endothelial cell monolayers exposed to different treatments was assessed with an ELISA technique. Experiments were repeated five times. Confluent cell monolayers were established in 96-well plates and exposed for 4 or 24 h to treatments with or without interleukin-1β (IL-1β) stimulation (10 units/ml). Wells were then washed twice with HBSS, and monoclonal antibodies to ICAM-1, VCAM-1 and E-selectin, and isotype mouse IgG [0.1 μg in 100 μl of of HBSS containing 10% (v/v) heat-inactivated human serum], were added to separate wells and incubated for 30 min at 4 °C in the dark. The layers were washed three times with HBSS/0.05% (v/v) Tween 20 before a 30 min incubation at 4 °C in the dark with sheep anti-(mouse antibody)–horseradish peroxidase conjugate [1:500 in 100 μl of HBSS containing 10% (v/v) heat-inactivated human serum and 0.05% (v/v) Tween 20]. After four additional washes, 150 μl of ABTS [2,2’-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] substrate (Kirkegaard and Perry Laboratories) was added to each well and allowed to develop for 15 min at 4 °C in the dark. Results were expressed as units of absorbance measured at 414 nm with an ELISA plate reader (Titertek Multiscan; Flow Laboratories).

**Adhesion assay with receptor antagonists**

To block endothelin receptors, endothelial cells grown to confluence were preincubated with the endothelin A receptor antagonist BQ 123 (1 μM) in M199 containing 10% (v/v) heat-inactivated human serum, l-glutamine (2 mmol/l), penicillin (100 units/ml) and streptomycin (0.1 mg/ml), with the endothelin B receptor antagonist BQ 788 (1 μM) in M199 (as above) or with both antagonists for 15 min at 37 °C under 5% CO2 in air. Excess solution was removed, and then the different treatments were added as described above, each containing the respective antagonist (1 μM) to ensure continuous receptor antagonism.

**Adhesion assay with anti-fibronectin antibody**

Polyclonal rabbit anti-fibronectin antibody was used to block fibronectin on endothelial cells. A similar polyclonal antibody was demonstrated to inhibit monocyte–endothelial-cell adhesion with a maximum at 5 μg/ml [20]. Endothelial cells were incubated with anti-fibronectin antibody in M199 containing 10% (v/v) heat-inactivated human serum, l-glutamine (2 mmol/l), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) for 30 min after the initial 4 h incubation with endothelin-1, oxidized LDL or the combination of both. Excess solution was then removed, and the adhesion assay was performed as described above.

**Statistical analysis**

All descriptive data are expressed as means ± S.E.M., and the data were analysed on SPSS for Windows 6.0. Because each experiment involved both endothelial cells and monocytes from different donors, results for the adhesion assays and ELISAs for cell adhesion molecule expression were expressed as a percentage of the control condition within each experiment. Intra-assay variability was 13%, and inter-assay variability was 17%. Groups were
RESULTS

Monocyte–endothelial-cell adhesion studies
Incubation with endothelin-1 (0.1 or 0.01 nM) or with oxidized LDL (0.1 mg/ml) alone for 4 h did not increase the adhesion of monocytes to endothelial cells (Figure 1). The combination of endothelin-1 (0.1 nM) and oxidized LDL (0.1 mg/ml), however, produced a significant increase in monocyte adhesion (Figure 2, lower panel) compared with the control (Figure 2, upper panel) (120±4% compared with 100±3%; *P < 0.001) (see Figure 1) after a 4 h preincubation of endothelial cells. The same effect was observed at an even lower concentration of endothelin-1 (0.01 nM; approximately human plasma levels) in combination with oxidized LDL (118±4% adhesion; *P < 0.001 compared with control). After a 24 h preincubation of endothelial cells, however, no change in monocyte adhesion was observed with any of the study conditions (results not shown). Co-incubation with 0.1 nM endothelin-1 and 0.1 mg/ml native LDL instead of oxidized LDL did not produce increased monocyte adhesion (103±4%; not significant compared with control) (see Figure 1).

After stimulation of endothelial cells with IL-1β for 4 h, basal monocyte adhesion under control conditions rose from 32±1% to 43±3% (*P < 0.002), but treatment with endothelin-1, oxidized LDL or a combination of the two substances did not increase adhesion above that induced by IL-1β. Stimulation of endothelial cells with IL-1β for 24 h increased basal monocyte adhesion from 37±2% to 51±2% (*P < 0.001), but again none of the experimental conditions induced a further enhancement of the adhesion induced by IL-1β.

Expression of endothelial cell adhesion molecules
Expression of all the measured adhesion molecules was increased after stimulation of endothelial cells with IL-1β after 4 h and 24 h of incubation (E-selectin to 220±19% and 174±8% respectively; ICAM-1 to 149±15% and 220±12% respectively; VCAM-1 to 142±20% and 203±12% respectively). However, no increased expression of E-selectin, ICAM-1 or VCAM-1 compared with controls was found after incubation with endothelin-1, oxidized LDL or a combination of the two, with or without IL-1β, at either 4 h or 24 h (Figure 3). Thus the combination of endothelin-1 and oxidized LDL did not lead to enhanced expression of the three principal adhesion molecules, despite significantly increased monocyte adhesion to the same endothelial cells.
Endothelin receptor blocking studies

The increased monocyte adhesion induced by combined incubation with endothelin-1 and oxidized LDL was not significantly reduced by endothelin A receptor antagonism (120±4% to 115±7%; not significant) or by endothelin B receptor antagonism (to 126±6%; not significant) (Figure 4). Combined endothelin A and B receptor blockade also did not reduce the increased adhesion (to 125±7%; not significant).

Fibronectin blocking studies

Blocking of fibronectin on endothelial cells did not significantly reduce the increased monocyte adhesion induced by endothelin-1 plus oxidized LDL (115±3% of control value compared with 120±4%; not significant) (Figure 4).

DISCUSSION

We have found that endothelin-1 and oxidized LDL when added together, but neither substance alone, increased the adhesion of human monocytes to human endothelial cells. The observed increase in adhesion of 20% is of a similar magnitude to that reported previously by our group on exposure of endothelial cells to androgens [21], and represents more than 50% of the pro-adhesion effect of IL-1β, a potent stimulatory cytokine. In the present study, however, neither endothelin-1 nor oxidized LDL had any significant effect alone at the 4 h or at the 24 h time point. We cannot exclude the possibility that the two substances have an effect on monocyte adhesion at individual time points that might be outside the measured incubation times; however, the combination of endothelin-1 and oxidized LDL enhanced monocyte adhesion significantly at the 4 h time point.

Endothelin-1, at concentrations that were orders of magnitude higher (1 μM to 1 nM) than those found in the systemic (1–5 pM) [12,22] or in the coronary (6 pM) [23] circulation, has been shown previously to enhance neutrophil adhesion to endothelial cells [10,11]. The effects of endothelin-1 on the important atherosclerosis-related process of monocyte adhesion to the endothelium, however, have not been investigated previously. We found that endothelin-1 alone at concentrations that resemble those found in human plasma did not increase monocyte adhesion significantly.

Although the concentrations of endothelin-1 in human endothelial cells are not known, it can be assumed that they might be up to 10-fold higher than plasma con-
centrations, due to the polar pattern of endothelin-1 secretion from these cells [24,25]. To represent the estimated physiological levels in endothelial cells, we therefore chose endothelin-1 concentrations for our study to be 10-fold higher than plasma concentrations (0.1–0.01 nM). We found that, at these low concentrations of endothelin-1, when given alone, human monocyte adhesion was not enhanced. In the setting of vascular disease, however, the effects of endothelin-1 might synergize with other potentially pro-atherogenic substances, such as oxidized LDL.

Oxidized LDL might mediate some or even most of the atherosclerotic effects of hypercholesterolaemia: immunoreactivity of lipid-peroxide-modified peptide, a component of oxidized LDL, is found in atherosclerotic lesions [26], and antibody levels to oxidized LDL in plasma correlate with the extent of atherosclerosis [27]. Various studies investigating the effects of oxidized LDL on endothelial cell adhesiveness to monocytes have found increased adhesion [20,28–30]. In contrast, endothelial cell exposure to oxidized LDL at a relatively high concentration alone (which was chosen to mimic the maximal effects of hypercholesterolaemia) did not significantly increase adhesion in the present study. This apparent discrepancy may be explained by the observation that the pro-adhesive effects of oxidized LDL are concentration-dependent, with a maximum effect in a previous study at 10 μg/ml [29]. At higher concentrations, monocyte adhesion declines [29]. The high concentration of 0.1 mg/ml oxidized LDL in our present study might therefore explain why we did not observe increased monocyte adhesion; however, the viability of the endothelial cells was >95% in a representative 24 h incubation experiment, excluding a cytotoxic effect of this concentration of oxidized LDL.

To investigate the underlying mechanism of the increased monocyte adhesion when endothelial cells were co-incubated with endothelin-1 plus oxidized LDL, we measured the expression of endothelial cell adhesion molecules. However, no significant enhancement of E-selectin, ICAM-1 or VCAM-1 expression was observed. Previous studies have found up-regulated expression of E-selectin [11] and ICAM-1 [31] after incubation with high concentrations (10–100 nM) of endothelin-1. This effect of endothelin-1 seems to be concentration-dependent, as demonstrated in previous studies of rat aortic endothelial cells, where significant enhancement of ICAM expression was only seen at endothelin-1 concentrations of 10 nM and higher [31]. Our results are in keeping with these findings, and indicate that approximately physiological concentrations of endothelin-1 (0.01–0.1 nM in our study) probably do not have an impact on adhesion molecule expression.

The adhesion of monocytes to endothelial cells is mediated not only by adhesion molecules such as ICAM-1 and VCAM-1, but also by fibronectin, which acts as a ligand for very-late-acting antigen 4 on monocytes. For example, minimally oxidized LDL (which is a much less extensive method of LDL oxidation, producing a different spectrum of oxidation products) enhances monocyte adhesion via the connecting segment-1 domain of fibronectin, independent of endothelial cell E-selectin, ICAM-1 or VCAM-1 expression [20]. However, we could not block increased adhesion significantly by anti-fibronectin antibodies, indicating that fibronectin does not mediate the pro-adhesive effects of endothelin-1 plus oxidized LDL. The time of maximal adhesion at 4 h might indicate that a relatively rapidly responding mechanism, such as via P-selectin, might be responsible, or an as yet unidentified adhesion molecule.

We were unable to reverse the increased adhesion induced by endothelin-1 plus oxidized LDL by blockade of endothelin A and/or endothelin B receptors. This is consistent with a previous study in which endothelin A receptor blockade only partially blocked neutrophil adhesion, whereas endothelin B receptor or combined A and B receptor antagonism did not have any effect [11]. By contrast, Zhu et al. [32] have suggested that the effects of oxidized LDL on endothelial vasomotor function in pig ciliary arteries may be mediated by endothelin A receptor activation. In the primary human cells utilized in our laboratory, however, endothelin A receptor blockade was insufficient to reverse the enhanced adhesion effect. This result speaks against the possibility that oxidized LDL might have stimulated endothelin-1 secretion from endothelial cells, as has been described previously [24], and that the observed increase in monocyte adhesion is caused by this mechanism. The effects on monocyte adhesion that we observed were dependent on both endothelin-1 and oxidized LDL, because no enhanced adhesion was observed when native LDL was used instead of oxidized LDL. Therefore oxidative modification of LDL is a prerequisite for the increased adhesion induced by endothelin-1 plus oxidized LDL.

Nitric oxide (NO) inhibits the adhesion of monocytes to endothelial cells [19], and oxidized LDL inhibits NO release from endothelial cells [33]. Endothelin-1, in contrast, leads to increased NO formation via activation of endothelin B receptors [34]. Therefore it is unlikely that an overall reduced NO formation accounts for our finding of increased adhesion.

Although we cannot state the exact mechanism by which endothelin-1 together with oxidized LDL induces a pro-adhesive effect on endothelial cells, similar synergistic effects have been described for endothelin-1 and lipopolysaccharide with regard to neutrophil adhesion [11]. Furthermore, cholesterol enhances vasoconstriction in response to endothelin-1 [35], strengthening the notion that endothelin-1 might amplify the properties of pro-atherogenic substances such as oxidized LDL. This hypothesis is further supported by animal studies in apolipoprotein E-deficient mice [5] and cholesterol-fed
hamsters [6], in which antagonism of the endothelin A receptor resulted in a reduced extent of cholesterol-induced atherosclerosis. These findings indicate that an interaction between the endothelin system and LDL seems to exist in vivo.

In summary, endothelin-1 at approximately physiological concentrations in combination with oxidized LDL, but neither agent alone, increases the adhesion of human monocytes to human endothelial cells, but this effect is not mediated by enhanced expression of E-selectin, ICAM-1 or VCAM-1, nor is it preventable by endothelin receptor antagonism or fibronectin blockade. This synergistic action might enhance the deleterious effects of LDL in the early stages of atherogenesis, and potentially explain a pro-atherogenic effect of endothelin-1.

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