Oxidized low-density lipoprotein induces the expression of P-selectin (GMP140/PADGEM/CD62) on human endothelial cells

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It is now well established that monocytes adhere to endothelial cells activated by oxidized low-density lipoproteins (LDL). However, the adhesive receptors on endothelial cells involved in binding monocytes, following an insult by oxidized LDL, remains to be elucidated. In this study we have looked at the effect of native or oxidized LDL on the expression of P-selectin. Native LDL (N-LDL) was oxidized by incubation with either endothelial cells (EC-LDL) or copper (Cu-LDL), or in culture medium as a control (C-LDL). Expression of P-selectin was assayed with an anti-P-selectin (CD62) monoclonal antibody (LYP20). Results show that EC-LDL and Cu-LDL, but not N-LDL or C-LDL, induce the expression of P-selectin by human umbilical-vein endothelial cells (HUVECs). Induction of P-selectin by low concentrations (20 μg/ml) of LDL is directly related to the state of oxidation of the LDL particles. In addition, high concentrations (100 μg/ml) of N-LDL also activate HUVECs by inducing P-selectin expression. This expression was sustained for a period of over 1 h on LDL-activated endothelial cells, in contrast with thrombin- or histamine-activated endothelial cells, whose P-selectin levels fall within 15–20 min after induction. E-selectin, in contrast with P-selectin, could not be induced by endothelial cells treated with low or high concentrations of oxidized LDL. Results in this study show that P-selectin expressed by oxidized-LDL-treated endothelial cells are involved in mediating the adhesion of a monocytic cell line (U937) or monocytes in peripheral-blood mononuclear cells. An anti-P-selectin monoclonal antibody (LYP20) inhibited the binding of U937 cells and monocytes. These results strongly suggest that P-selectin is involved in the early stages of atherogenesis.

INTRODUCTION

Recent clinical and experimental studies have firmly established that elevated plasma concentrations of low-density lipoproteins (LDL) are associated with accelerated atherogenesis (Goldstein and Brown, 1977; Steinberg et al., 1989; Ross, 1993). LDL may be readily oxidized by endothelial cells, smooth-muscle cells and monocytes. It is thought that oxidized LDL may be involved in insults and subsequent activation of vascular endothelial cells (Witztum and Steinberg, 1991; Parthasarathy and Rankin, 1992). Activated endothelial cells will allow the adhesion and extravasation of monocytes into the intimal area of the vessel wall. Such extravasated monocytes/macrophages will internalize oxidized LDL more rapidly than native LDL, and in the process be transformed into foam cells, which represent the earliest recognized gross lesion in atherogenesis, the fatty streak. Macrophages loaded with cholesteryl esters are most probably involved in a critical way in the mechanism leading to the further extravasation of monocytes and migration of smooth-muscle cells into the sub-endothelial space. Thus, an understanding of the pathogenesis of atherosclerosis may depend in large part on elucidating the mechanism by which monocytes are recruited to the sub-endothelium space.

Vascular endothelial cells may undergo insults and subsequent activation by a number of factors, acting in concert or on their own, such as hypercholesterolaemia, smoking, diabetes mellitus or autoimmune disorders (Ross, 1993). A number of adhesive receptors (P-selectin, E-selectin, ICAM-1, VCAM-1) are expressed or up-regulated on the surface of activated endothelial cells. Increasing evidence is available to show that these adhesive receptors are involved in the adhesion and extravasation of leucocytes homing to sites of inflammation. However, the identity of adhesive receptors involved in the early stages of atherogenesis remains to be elucidated. A potential candidate that is expressed by endothelial cells, within seconds of activation, is P-selectin (also known as GMP 140, PADGEM, or CD62P). P-selectin is an integral membrane glycoprotein located in platelet α-granules (Stenberg et al., 1985) and Weibel–Palade bodies of endothelial cells (Hattori et al., 1989). The rapid but transient expression of P-selectin, induced by thrombin or histamine, mediates activated endothelial-cell interaction with neutrophils, monocytes, eosinophils and a subclass of T-lymphocytes (Larsen et al., 1989; Geng et al., 1990; McEver, 1991b; McGregor, 1994). P-selectin is a member of an adhesive receptor family known as selectins, which includes E-selectin (also known as ELAM-1) and L-selectin (also known as LAM-1, Mel 14 or TQ8) (McEver, 1991a). Selectins are thought to play an essential role in cell–cell interactions during the haemostatic and inflammatory responses to tissue injury. In this study we have investigated the effect of oxidized and native LDL on the expression of P-selectin and subsequent adhesion of monocytes to endothelial cells. Results obtained show that oxidized LDL can induce P-selectin expression and adhesion of monocytes to endothelial cells, and that this adhesion can be blocked by an anti-P-selectin monoclonal antibody.

MATERIALS AND METHODS

Human umbilical-vein endothelial cells (HUVECs)

Culture of HUVECs was as described by Jaffe et al. (1973). Briefly, cells were extracted from umbilical cords treated with

Abbreviations used: LDL, low-density lipoproteins (N-LDL, native LDL; C-LDL, control LDL; EC-LDL, endothelial-cell-modified LDL; Cu-LDL, copper-oxidized LDL); HUVECs, human umbilical-vein endothelial cells; PBMCs, peripheral-blood mononuclear cells; FCS, fetal-calf serum; PAF, platelet-activating factor.

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collagenase type 2 (Worthington Chemical Co., Freehold, NJ, U.S.A.) and grown to confluence on fibronectin (20 μg/ml) (Boehringer, Mannheim, Germany)-coated flasks (Falcon, 75 cm^2) in culture medium 199 (Gibco–BRL, Cergy-Pontoise, France) at 37 °C in 5% CO_2. The culture medium was supplemented (per 100 ml) with 20% (v/v) decomplemented human serum AB (CTS, Beynost, France), 300 μg of endothelial-cell growth factor (Boehringer), 5 mM $NaHCO_3$, 2 mM glutamine, 100 units of penicillin/100 μg of streptomycin and 1 mM sodium pyruvate (Flow Laboratories, Irvine, Scotland, U.K.). Cultured HUVECs were then seeded on to either 24-well (Falcon Primarae, Meylan, France) or 96-well (Nunc Immunolon, Roskilde, Denmark) plates and left to grow to confluence (2.5×10^5 cells/24-well plate or 40×10^3 cells/96-well plate) before being used in e.l.i.s.a. or adhesion assays.

Isolation of peripheral-blood mononuclear cells (PBMCs)

This was performed under sterile conditions by using a density-gradient centrifugation technique (Boyum, 1968). Briefly, whole blood was drawn from donors on to ACD and carefully layered on a Ficoll/Hypaque 1077 gradient (Sigma, St. Louis, MO, U.S.A.). Isolated mononuclear cells present at the Ficoll/Hypaque interface, after centrifugation at 700 *g* for 30 min at 25 °C, were isolated and washed twice in PBS. Isolated mononuclear cells, showed greater than 90% viability as determined by Trypan Blue dye exclusion. U937 cells, a monocytic cell line (European Collection of Animal Cell Cultures, Porton Down, U.K.) were cultured in RPMI tissue medium (Flow Laboratories) supplemented with 10% fetal-calf serum (FCS) (Gibco-BRL), 2 mM glutamine, 100 units of pencillin/100 μg of streptomycin and 1 mM sodium pyruvate. Cells were resuspended in RPMI medium/1% FCS for adhesion experiments. All cells were labelled by sodium [^{51}Cr]chromate incorporation as described by Kunicki et al. (1988).

Lipoproteins

Native LDL (N-LDL) was purchased from Sigma, solubilized in 0.15 M NaCl containing 0.01% EDTA and stored, between two experiments, at 4 °C in the dark and under nitrogen. This N-LDL was seen to be slightly oxidized. Endothelial-cell-modified LDL (EC-LDL) was oxidized by HUVECs. This was prepared by first washing 2×10^6 cells in 6-well plates twice with serum-free medium 199 and then incubating for 16–24 h with 2 ml of the same medium containing N-LDL (100–200 μg of protein/ml) (Henriksen et al., 1981). Copper (Cu)-oxidized LDL (Cu-LDL) was prepared by incubating 100–200 μg of N-LDL/ml with 10 μM Cu^{2+} in a total volume of 2 ml in medium 199 for 16–24 h at 37 °C (Quinn et al., 1985). Control LDL (C-LDL) was prepared in parallel by using a well without either cells or copper, in 2 ml of medium 199. At the end of the incubations, the LDL medium was removed from each well, cooled, and used directly for experiments. N-LDL (100–200 μg of protein/ml) was diluted in 2 ml of serum-free medium 199 and used with the other preparations of LDL at the beginning of each experiment.

Lipid peroxidation assay

The extent of lipid peroxidation was estimated as thiobarbituric acid-reactive material (TBARS) (Steinbrecher et al., 1984) and by the amount of conjugated dienes (Esterbauer et al., 1989). Medium containing LDL (50 μg of protein) was mixed with 1.5 ml of 0.67% thiobarbituric acid and 0.5 ml of 20% trichloroacetic acid. After heating at 100 °C for 30 min, the solutions were cooled on ice and centrifuged at 22000 *g* for 5 min in a Jouan GT 422 centrifuge. The reaction product was then assayed fluorimetrically by using a Farrand optical spectrofluorimeter with absorption at 535 nm. Freshly diluted tetramethoxypropane, which yields malondialdehyde, was used as a standard, and results were expressed as nmol of 'malondialdehyde equivalents' per mg of LDL tested. The amount of conjugated dienes present in the LDL solutions (50 μg/ml of LDL protein) was calculated from the A_{234} by using the molar absorption coefficient of conjugated dienes ($\epsilon_{234} = 2.95 \times 10^4\ M^{-1} \cdot cm^{-1}$). Results are expressed as nmol of conjugated dienes per mg of LDL tested.

Monoclonal antibodies and PAF (platelet-activating factor)-receptor antagonist

LYP20 is an anti-P-selectin monoclonal antibody that is directed against a disulphide-dependent epitope and is known to inhibit platelet aggregation, platelet–monocyte interaction (Parmentier et al., 1991) and monocyte–endothelial-cell interaction (Murphy et al., 1994). Western-blot analysis has shown that it binds to both human and rat endothelial-cell and platelet P-selectin (Chignier et al., 1994). Anti-E-selectin monoclonal antibody (clone BBIG-E6) was purchased from R&D Systems, Minneapolis, MN, U.S.A. Both antibodies were used as purified immunoglobulin. The PAF-receptor antagonist WEB 2086 (a gift from Dr. F. Pistel and Dr. J. Duttmann, Boehringer, Ingelheim, Germany) is a hetrazepine substance which competes with high affinity for the PAF-binding site of both intracellular and cell-surface membrane PAF receptors (Meade and Heuer, 1990). It has been reported that this antagonist has an IC_{50} of 32 μM in monocyte–endothelial-cell interactions (Murphy et al., 1994).

Selectin expression by endothelial cells

The presence of P-selectin or E-selectin at the surface of resting or stimulated HUVECs was observed by using monoclonal antibodies directed against this adhesive protein by the method of Pigott et al. (1991). Briefly, confluent HUVECs grown in 96-well microtitre plates were activated after incubation with oxidized (EC-LDL, Cu-LDL), control (C-LDL) or native (N-LDL) LDL at concentrations of 20 or 100 μg/ml, for a period of 120 min. The 96-well plates were then gently emptied and washed twice with RPMI medium (Flow Laboratories) containing 2.5% FCS (Gibco–BRL) and 0.1% NaN_3 (RPMI/FCS) to kill the cells. HUVECs were then incubated with LYP20 (8 μg/ml) for 1 h at room temperature, washed three times with RPMI/FCS, fixed for 5 min in PBS (PBS/2.5% FCS) with 0.025% glutaraldehyde, and washed three times with PBS/FCS. Non-specific binding sites were quenched by incubation for 1 h with PBS/FCS. A goat anti-mouse IgG–horseradish peroxidase conjugate (Bio-Rad, Richmond, CA, U.S.A.) diluted to 1/3000 in PBS containing 10% goat serum (Gibco–BRL) was used to detect bound monoclonal antibodies. After three washes with PBS/FCS, the substrate *o*-phenylenediamine diluted in citrated buffer with H_2O_2 was added and incubated for about 20 min, and the reaction was then stopped with 2 M H_2SO_4 and the A_{490} was read. Wells with non-activated HUVECs were used as a reference for the expression of P-selectin on resting HUVECs, whereas wells with HUVECs activated with H_2O_2 (300 μM) for 2 h were used as a positive control. The A_{490} value for LYP20 binding to resting EC was normalized to 100%, and the increase in binding on activated EC is presented as percentage increase.

Adhesion assays

Washed PBMCs or U937 cells were assayed for their adhesion to non-stimulated and stimulated endothelial cells by the method, with minor modifications, previously described (Bevilacqua et al., 1985; Zimmerman et al., 1985; Luscinskas et al., 1991). Briefly, fully confluent HUVECs were stimulated with LDL (20 μg/ml) for a period of 120 min, and the reaction was then terminated by plate inversion and washing. Endothelial cells were then immediately incubated with RPMI/1% FCS in the presence or absence of LYP20 for 10 min at room temperature, followed by addition of PBMCs or U937 cells for 10 min. All experiments were carried out at room temperature or 37 °C under static conditions. Wells were then emptied by aspiration and washed three times with RPMI/1% FCS. Well contents were solubilized with 50 mM Tris/0.1% SDS, pH 8.4, and radioactivity in cell lysates was measured. Non-stimulated and stimulated cells treated with thrombin (2 units/ml for 5–10 min) were used respectively as negative and positive controls. Monocyte adhesion to non-stimulated cells was normalized to 100%, and the increase in adhesion to stimulated cells is presented as percentage increase.

Analysis of data

Each separate experiment was carried out in replicate as described above and the mean was calculated. The final mean with S.D. was calculated from the means from different experiments. The significance of the difference between mean values was determined by Student's t test.

RESULTS

Effect of oxidized LDL on P-selectin expression

Surface expression of P-selectin induced by EC-LDL or Cu-LDL is significantly increased compared with the expression of P-selectin on the surface of non-activated and N-LDL-activated HUVECs (Table 1). EC-oxidized LDL was as effective as H_2O_2 at inducing P-selectin expression. Endothelial cells treated with N-LDL, EC-LDL, Cu-LDL or C-LDL (20 μg/ml) for 4 h did not express E-selectin. In contrast, endothelial cells treated with tumour necrosis factor (200 IU/ml) for 4 h expressed E-selectin (results not shown). The ability of LDL to induce P-selectin expression by endothelial cells at concentrations of 20 μg/ml was dependent on the degree of oxidation of LDL (Table 2). In the absence of endothelial cells or in the presence of copper at high concentrations (10 μM), LDL oxidation did not occur in the cell medium as shown by the results obtained for C-LDL. The two methods used to measure LDL oxidation gave consistent and homogeneous results throughout the course of this study. The amount of oxidation of EC-LDL or Cu-LDL in our hands was less than that reported by other workers (Steinbrecher et al., 1986; Kleinveld et al., 1992). These differences may conceivably be due to the culture medium used (Medium 199), which contains some vitamin E, a well-known anti-oxidant, and a low metal content (such as copper, iron or zinc).

Table 1 Effect of native or oxidized LDL (20 μg/ml) on P-selectin expression

Confluent HUVECs were treated for 2 h with LDL modified by a 24 h incubation with native non-modified (N-LDL), control (C-LDL), endothelial-cell-oxidized (EC-LDL) or copper-oxidized (Cu-LDL) LDL (10 μM). Resting endothelial cells were incubated with medium containing no agonist. Moreover, endothelial cells stimulated with H_2O_2 (300 μM) for 2 h were used as positive control. LYP20 binding to P-selectin was carried out as described in the Materials and methods section. Data are expressed as percentage increase in LYP20 binding to stimulated endothelial cells compared with resting endothelial cells and represent means ± S.D. of triplicate determinations from 9 independent experiments: $^*P < 0.01$, $^{**}P < 0.001$, as determined by Student's t test.

Control or agonist	LYP20 mAb binding (%)
Resting	100 ± 11
C-LDL	122 ± 14
N-LDL	91 ± 17
H_2O_2	176 ± 13
EC-LDL	175 ± 12**
Cu-LDL	152 ± 18*

Table 2 Representative curve showing the amount of conjugated dienes produced and malondialdehyde (MDA) formed for the different types of LDL

The amount of conjugated dienes was calculated from the increase in A_{234} and MDA production was determined by the TBARS assay. Typical values for native (N) LDL, control (C) LDL, EC-LDL and Cu-LDL are shown.

LDL	MDA (nmol/mg of LDL)	Dienes (nmol/mg of LDL)
N	6.5	52
C	7.3	60
EC	13.4	97
Cu	27.1	191

Table 3 (i) Effect of native or oxidized LDL (20 μg/ml) on PBMC adhesion to HUVECs and (ii) blocking of PBMC adhesion to oxidized-LDL-treated HUVECs by an anti-P-selectin monoclonal antibody (LYP20)

(i) Confluent HUVECs were treated for 2 h with native non-modified LDL (N-LDL), control LDL (C-LDL), endothelial-cell-oxidized LDL (EC-LDL) or copper-oxidized LDL (Cu-LDL). Resting endothelial cells were incubated with medium containing no agonist. (ii) Confluent HUVECs were activated with LDL (20 μg/ml), as a 1:1 mixture of EC-LDL + Cu-LDL (Ox-LDL) for 2 h. The HUVEC monolayer was then incubated for 10 min at 37 °C with LYP20 (30 μg), and the adhesion of ^{51}Cr-labelled PBMCs was measured as described in the Materials and methods section. Each value represents the mean ± S.D. of triplicate determinations from 4 independent experiments: $^*P < 0.05$, $^{**}P < 0.005$, as determined by Student's t test.

	Control or agonist	PBMC adhesion (%)
	Resting	100
(i)	C-LDL	81 ± 2
	N-LDL	90 ± 2
	EC-LDL	120 ± 8*
	Cu-LDL	136 ± 18**
(ii)	Ox-LDL	116 ± 10
	Ox-LDL + LYP20	83 ± 7*

Adhesion of PBMCs

Pretreatment of HUVECs for 120 min with EC-LDL or Cu-LDL at 20 μg/ml resulted in increased adhesion of PBMCs to endothelial cells compared with the adhesion of PBMCs to non-activated EC or to EC incubated with N-LDL (Table 3). Previous results have shown that most of the cells in PBMC binding to thrombin-activated endothelial cells are monocytes, as judged by specific markers for monocytes and B- and T-lymphocytes (Murphy et al., 1994). The magnitude of the increase was similar to that seen after treatment of endothelial cells with thrombin (2 units/ml) for 5 min. An anti-P-selectin monoclonal antibody (LYP20) inhibited the adhesion of PBMCs to endothelial cells, activated by oxidized LDL, demonstrating the role of P-selectin in the adhesion of these cells (Table 3).

Table 4 Effect of native or oxidized LDL on U937-cell adhesion to resting, thrombin-treated or oxidized-LDL-treated HUVECs, and inhibition by an anti-P-selectin monoclonal antibody or PAF-receptor antagonist

Confluent HUVECs were treated for 2 h with either endothelial-cell-oxidized LDL (EC-LDL), copper-oxidized LDL (Cu-LDL) (10 μM), native non-modified LDL (N-LDL) or control LDL (C-LDL). (Further abbreviation: OX-LDL, oxidized LDL). Resting endothelial cells were incubated with medium containing no agonist. HUVECs treated with thrombin (THR; 2 units/ml) for 5–10 min were used as positive control. Adhesion of U937 cells, in the absence or presence of monoclonal antibody LYP20 (30 μg) or a PAF-receptor antagonist (WEB 2086) (100 μM), was carried out as described in the Materials and methods section. Each value represents the mean ± S.D. of duplicate or triplicate determinations from five independent experiments: $^{*}P < 0.01$, $^{**}P < 0.001$, as determined by Student's t test.

Control or agonist	U937 cell adhesion (%)
Resting	100
N-LDL	90 ± 6
C-LDL	104 ± 12
THR	152 ± 9
THR + LYP20	114 ± 8
EC-LDL	127 ± 10*
EC-LDL + LYP20	109 ± 10
Cu-LDL	146 ± 9**
Cu-LDL + LYP20	116 ± 4
OX-LDL	146 ± 7
OX-LDL + WEB 2086	134 ± 9

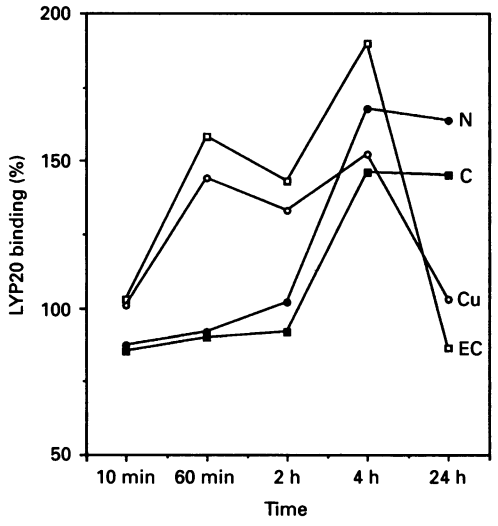

Figure 1 P-selectin expression by HUVECs incubated with oxidized or non-oxidized LDL over a period of 24 h

Confluent HUVECs were treated for different periods of time with native LDL (N) (100 μg/ml), control LDL (C) (100 μg/ml), endothelial-cell-oxidized LDL (EC) (20 mg/ml) or copper-oxidized LDL (Cu) (20 μg/ml). Resting HUVECs were incubated with medium containing no agonist. Endothelial-cell activation was stopped at the different times stated. Results are expressed as percentage of LYP20 binding compared with non-activated HUVECs, and similar values were obtained in two other experiments.

Adhesion of U937 cells

The adhesion of U937 cells to HUVECs activated by oxidized LDL (20 μg/ml) for 2 h supported the results found with mononuclear cells (Table 4). Oxidized LDL induced the adhesion of monocytes to endothelial cells, whereas non-oxidized LDL had no effect at the same concentration (20 μg/ml). Monocyte adhesion induced by LDL was partially dependent on P-selectin, as it was significantly inhibited by the anti-P-selectin monoclonal antibody LYP20, whereas the PAF-receptor antagonist WEB 2086 had no significant inhibitory effect (Table 4). This contrasts with results previously reported by Lorant et al. (1991) and Murphy et al. (1994), which showed that PAF-receptor antagonists (100 μM) inhibited, by over 50%, both neutrophil and monocyte adhesion to endothelial cells activated with thrombin (2 units/ml for 5–10 min). These results suggest that PAF may not be secreted when endothelial cells are activated by LDL.

Effect of high concentration of native LDL on P-selectin expression

Native or oxidized LDL did not induce P-selectin expression, even at high concentrations, when incubated for short periods of time (10 min) on endothelial cells. Activation appeared to be initiated within 1 h for oxidized LDL and 4 h for non-oxidized LDL (Figure 1). After incubation for 1 day, P-selectin expression by endothelial cells incubated with native LDL was observed, but not with those incubated with oxidized LDL (Figure 1). This is probably due to either depletion of stocks resulting from excessive stimulation or cytotoxicity effects.

DISCUSSION

This study shows for the first time that oxidized LDL at a relatively low concentration (20 μg/ml) can induce P-selectin expression by human endothelial cells. In addition, native LDL at much higher concentrations (100 μg/ml) could also induce P-selectin expression by endothelial cells. The effect of oxidized LDL on endothelial cells was specific to P-selectin, but not to E-selectin.

The adhesion of monocytes to endothelial cells activated by oxidized LDL is now well established (Frostegard et al., 1990). Results obtained in the present study show that P-selectin expressed by oxidized-LDL-treated endothelial cells is involved in mediating the adhesion of PBMCs. Monocytes have been shown to be predominantly the cells in PBMC adhering, via P-selectin, to thrombin-activated endothelial cells (Murphy et al., 1994). Moreover, endothelial cells produce a chemotactic factor, known as monocyte chemotactic protein-1 (MCP-1), which is used for the transfer of monocytes through the endothelial barrier (Berliner et al., 1990; Cushing et al., 1990). Release of MCP-1 needs prolonged treatment (24 h) of endothelial cells by LDL and does not occur with native LDL, even at high concentrations (Cushing et al., 1990). In contrast, P-selectin expression by endothelial cells can be induced and sustained for a certain period after a 2 h incubation with oxidized LDL. These results may implicate P-selectin as an important first stage in the mechanism of early-stage atherogenesis.

Endothelial-cell activation by histamine or thrombin results in a rapid expression of P-selectin, which then decreases to basal levels within 15–20 min after induction (McEver, 1991b), in contrast with LDL activation, which requires at least 1 h incubation. LDL may stimulate P-selectin expression by a mechanism different from that of thrombin or histamine. The necessity for at least 1 h incubation suggests that LDL must first be recognized by a membrane receptor and internalized before inducing P-selectin expression. In contrast, thrombin, by cleaving its receptor, stimulates the release of contents of Weibel–Palade bodies in an extremely rapid fashion (within minutes). PAF expression, after stimulation of endothelial cells by oxidized LDL, was not observed when using a PAF antagonist. A previous study also mentions the absence of LDL-induced PAF secretion (Prescott et al., 1990). It is possible that PAF molecules are expressed, after oxidized-LDL treatment of endothelial cells, and

then degraded or internalized during the 2 h incubation leading to the P-selectin assay. Alternatively, the mechanism(s) in endothelial cells inducing P-selectin and PAF co-expression, after thrombin or histamine stimulation (Lorant et al., 1991), may differ from those implicated after oxidized-LDL stimulation.

Agonists such as oxygen radicals and lipid-soluble peroxide induced a sustained expression of P-selectin, similar to that by LDL (Patel et al., 1991). Persisting expression of P-selectin at the cell surface may conceivably be due to an inhibition of P-selectin endocytosis, which starts taking place 15–20 min after stimulation with an agonist such as thrombin. Alternatively, oxidized LDL may induce synthesis *de novo* of P-selectin. Previous work has shown that a mouse endothelial-cell line can be induced to synthesize P-selectin *de novo* after cytokine stimulation (Weller et al., 1992). Both hypotheses imply an endothelial dysfunction induced by LDL at the earliest stage of atherosclerosis.

P-selectin is also expressed for a sustained period of time when native LDL is used at high concentrations (100 μg/ml). It is conceivable that native LDL contains traces, not shown by the lipid-peroxidation assay, of oxidized LDL. Such traces may also be generated when incubating high concentrations of native LDL (100 μg/ml) for 4 h with endothelial cells. Alternatively, these results suggest that hypercholesterolaemia may induce adhesion of monocytes to endothelial cells and then their migration to atherosclerotic sites without any implication of an oxidative factor. Hyperlipidaemia, and especially a high plasma concentration of LDL, is a well-known cardiovascular risk factor. Clinical and angiographic studies have shown that the decrease in plasma LDL is significantly correlated with both the regression of atherosclerotic plaques and the decrease in the cardiovascular mortality (Helsinki Heart Study, 1987; FATS, 1990). The implication of oxidized LDL in atherogenesis is more established under conditions *in vivo*. Oxidized LDL was isolated from human or rabbit atherosclerotic plaques (Ylä-Herttuala et al., 1989). A number of animal studies have shown a decrease in atherosclerotic plaques with the use of antioxidants such as butylated hydroxytoluene or Probucol, which was independent of decreased cholesterol plasma levels (Carew et al., 1987; Sparrow et al., 1992). A recent human study has shown a correlation between the susceptibility of LDL to oxidation and the severity of coronary atherosclerosis (Regnström et al., 1992). Moreover, a randomized prospective study has shown the efficacy of antioxidants in decreasing human coronary disease (Rimm et al., 1993; Stampfer et al., 1993). However, the site at which LDL is oxidized remains to be identified. Oxidized LDL is thought not to circulate in blood, due to the presence of a number of antioxidants (carotene, vitamin E). Detectable levels of cholesterol ester hydroperoxides and phospholipid hydroperoxides were reported in plasma (Parthasarathy and Rankin, 1992). LDL oxidation can conceivably occur in endothelial cells which have LDL receptors, and/or in the sub-endothelium space, which is depleted in antioxidants and where foam cells are able to induce oxidative modifications of LDL (Parthasarathy and Rankin, 1992). The mechanism by which cells oxidize LDL is not clearly understood at this stage, and it could be either via the lipoxygenase pathway (Sparrow et al., 1988) or the generation of oxygen free-radical intermediates.

A working model involving the data obtained in the present study and other results suggest the following working hypothesis. LDL may be oxidized by endothelial cells, as well as other cells present in the sub-endothelial space such as macrophages or smooth-muscle cells. Such oxidized LDL may then be involved in activating endothelial cells and may induce the expression of a number of adhesive molecules involved in monocyte–endothelial-cell interactions. Sustained P-selectin expression stimulated by low doses of oxidized LDL and/or a high dose of native LDL induces monocyte adhesion without necessarily implicating PAF. P-selectin has recently been shown to be present on human atherma plaques (Johnson-Tidey et al., 1994). Moreover, VCAM-1 was found on the endothelium of aortic fatty streaks in a hypercholesterolaemic rabbit (Cybulsky and Gimbrone, 1991), and ICAM-1 was found on human atherosclerotic plaques (Poston et al., 1992). Endothelial cells synthesizing chemotactic factors such as MCP-1 and growth factors (granulocyte/macrophage, macrophage or granulocyte colony-stimulating factors) (Rajavashisth et al., 1990) will induce the migration, the differentiation and the proliferation of monocytes to macrophages and foam cells in the sub-endothelium space and the formation of an atherosclerotic plaque.

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