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Oxidized LDL-Mediated Monocyte Adhesion to Endothelial Cells Does Not Involve NFκB

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Oxidised LDL (oxLDL) is a key pathogenic mediator of atherogenesis, exhibiting many proatherogenic properties. We have examined the effect of oxLDL on monocyte adhesion in the endothelial cell line, EA.hy 926. This has included the role of endothelial cell adhesion molecule expression (ICAM-1 and VCAM-1), monocyte chemoattractant protein-1 (MCP-1), and the transcription factor NFκB in this interaction. In response to oxLDL (10–100 μg/ml), monocyte adhesion to cells increased dose-dependently. Adhesion of oxLDL at 100 μg/ml was equivalent to that seen with TNFα (10 ng/ml). Unmodified LDL (nLDL, 100 ug/ml) had no effect. Both oxLDL and nLDL increased MCP-1 mRNA levels. Interestingly, oxLDL had no effect on the expression of ICAM-1 and VCAM-1. In addition NFκB was not activated as shown by western blots of IκB-α degradation and electrophoretic mobility shift assay. In summary these data show that increased monocyte adhesion to EA.hy 926 cells occurs independently of ICAM-1, VCAM-1, and NFκB activation and may involve novel adhesive mechanisms.

Key Words: **oxidised-ldl; NFκB; monocyte adhesion; endothelial cells; cell adhesion molecules.**

Oxidatively modified low density lipoprotein (oxLDL) is a key pathogenic mediator of atherosclerosis. It acts as a monocyte chemoattractant and an inhibitor of macrophage motility (1). It can upregulate expression of numerous pro-atherogenic genes including, M-CSF, MCP-1, IL-1β, tissue factor, LOX-1, and PAI-1 (2–7) and inhibits induction of PDGF, TNFα, and nitric oxide-mediated relaxation (8–10). It also induces smooth muscle cell proliferation (11) and increases selective adhesion of monocytes to endothelial cells (12, 13). Furthermore, antioxidants such as probucol, α-tocopherol and butylated hydroxytoluene (BHT), which inhibit the amount of LDL oxidation, also reduce atherosclerotic lesion formation (14). There also appears to be an inverse relationship between the circulating levels of the antioxidant α-tocopherol and incidence of human heart disease (15). In addition endothelial dysfunction in the coronary circulation and peripheral arteries of hypercholesterolaemic patients appears to be caused predominantly by oxLDL and not by normal, non-oxidised LDL (nLDL). This impairment could be reversed by the antioxidants vitamin E and C (16, 17).

The actions of oxLDL, including both stimulatory and inhibitory effects on gene transcription, have been attributed to lipid peroxides, lysophosphatidylcholine, aldehydes, and oxysterols present in oxLDL preparations (18). High levels of oxidised lipids may induce endothelial oxidative stress by lowering cellular glutathione levels (reducing fatty acid peroxides formed during LDL oxidation), generating reactive oxygen species, and altering the endothelial cell buffering capacity (19). This in turn can overwhelm cellular antioxidant defence mechanisms leading to activation of redox-sensitive transcription factors, including NFκB. Several reports support the activation of NFκB by oxLDL during atherosclerotic lesion formation. Also mildly oxidised LDL or minimally modified LDL activates NFκB in cultured endothelial cells (20). In addition, ICAM-1, tissue factor and activated NFκB colocalize in early and advanced atherosclerotic lesions (21), thereby providing a functional relationship between activation of NFκB, induction of inflammatory genes and development of atherosclerotic lesions. Modulation of expression and function of genes in endothelial and smooth muscle cells by oxidative signals may be an important mechanism through which oxLDL can initiate and propagate atherosclerosis.

Thus the present study was designed to investigate molecular mechanisms of oxLDL-mediated endothelial

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cell-monocyte interactions in the endothelial cell line EA.hy 926.

MATERIALS AND METHODS

EA.hy 926 cell culture. EA.hy 926 cells have been shown to be a reliable model for studying endothelial cell–leukocyte interactions (22–25). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) foetal calf serum (GIBCO BRL), 4 mM glutamine, and HAT (100 μM, hypoxanthine; 0.4 μM, aminopterin; 16 μM, thymidine).

Human mononuclear cell (MNC) preparation. MNCs were isolated from blood, essentially as described by Böyum (26). Briefly, blood was withdrawn by venopuncture into 3.8% sodium citrate (10:1 v/v). Platelet rich plasma was prepared by centrifugation at 750*g* for 10 min. The plasma was replaced with an equal volume of phosphate-buffered saline (PBS), diluted blood was layered over ficoll paque (Sigma, Poole, UK) and centrifuged at 400*g* for 30 min. MNCs were separated in a white band at the interface. Cells were washed twice with PBS to remove platelets and suspended in DMEM. Cells were counted using a haemocytometer and viability was determined by trypan blue exclusion.

Monocyte adhesion assay. This assay is based on the myeloperoxidase (MPO) activity present in monocytes. Cells grown to confluence in 96 well plates were washed twice with DMEM and a mononuclear cell suspension ($2–3 \times 10^5$ cells/well in DMEM) was added. MNCs were allowed to adhere for 30 min at 37°C. Subsequently, plates were washed twice, 300 μl of the MPO assay buffer (0.05 M sodium citrate, 0.2% triton × 100, 10 mg 2,2′-Azino-bis [3-ethylbenzothiazoline]-6-sulphonic acid and 0.0024% H_2O_2, pH 4) was added to each well and incubated for 30 min at 37°C. Absorbance was measured at 405 nm. The number of MNCs bound to endothelial cells in each well was calculated from a standard calibration curve of absorbance against total number of MNCs.

Preparation of oxidised low density lipoprotein. LDL was isolated by sequential density ultracentrifugation (ρ = 1.019–1.210 g/ml) as described by Havel *et al.* (27). LDL was dialysed against 4 L of 150 mM NaCl solution containing 0.01% EDTA (pH 7.4) at 4°C overnight and sterilized by passing through a 0.22 μm filter. Protein concentration was determined using a Biorad (Hemel Hempstead, UK) protein assay kit. LDL preparations contained <5 pg endotoxin/μg LDL (Limulus amoebocyte lysate assay, Pharmacia, Windsor, UK). LDL (1 mg/ml) was oxidised and analysed as previously described (28) by incubating with 50 μM $CuSO_4$ in PBS at 37°C for 18–20 h, further oxidation was prevented by the addition of 100 μM BHT. Preparations were used within 7 days.

Cell adhesion molecule assay. Cells were grown to confluence in 96 well plates. Cells were washed twice with Hank's balanced salt solution (HBSS) and incubated for 1 h with primary antibody ((R & D Systems, Abingdon, UK) 0.5 μg/well in HBSS + 5% FCS) and then with a peroxidase conjugated anti-mouse IgG (Sigma, Poole, Dorset, UK) in HBSS + 5% FCS (1:2000) for 1 h. Antibody binding was measured by the addition of substrate solution (0.04% *o*-phenylenediamine dihydrochloride, 0.05 M sodium citrate, 0.1 M dibasic sodium phosphate, and 0.012% H_2O_2). After 10 min incubation at 37°C, the reaction was stopped by the addition of 3 M HCl (50 μl/well) and absorbance was measured at 490 nm.

Adhesion molecule mRNA analysis. Total RNA was extracted from cells as previously described (29). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using a GeneAmp RNA PCR kit according to instructions supplied (Perkin Elmer, Foster City, CA). The following primers were used: GAPDH (sense primer, 5′-TGAAGGTCGGAGTCAACGGA-3′; antisense primer, 5′-GTGTCGCTGTTGAAGTCAGA-3′) and MCP-1 (sense primer, 5′-CACTCTCGCCTCCAGCATGA-3′; antisense primer, 5′-GGGTAGAACTGTGGTTCAAG-3′). For GAPDH 30 cycles was used, and for MCP-1 35 was used (1 min, 95°C; 1 min, 60°C; 1 min, 72°C). PCR products were separated on a 1% agarose gel and after electrophoresis stained with ethidium bromide.

Cell viability assay (MTT assay). Cells treated as above with LDL and TNFα were assessed for viability using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (30).

Western blot analysis. Cytosolic extracts were prepared by scraping cells into lysis buffer (20 mM β-glycerophosphate, 20 mM sodium fluoride, 2 mM, EDTA, 0.2 mM sodium orthovanadate, 10 mM benzamidine, 0.050 mg/ml phenylmethylsulphonyl fluoride, 0.025 mg/ml leupeptin, and 1 μl/ml β-mercaptoethanol, pH 7.5). Extracts were centrifuged at 10,000*g* for 10 min at 4°C. Protein concentration was measured as described above.

Cytoplasmic extracts were subjected to 10% SDS–PAGE. Fractioned proteins were transferred onto a PVDF membrane in transfer buffer at constant current (250 mA) for 1.5 to 2 h. After blocking with 2% milk protein for 1 h, the membrane was incubated with primary antibody (peptide specific rabbit polyclonal antibody against IκB-α 0.5 μg/ml, in PBS-Tween; Santa Cruz Biotechnology, CA) for 1.5 h. The membrane was then incubated with peroxidase conjugated rabbit IgG (1:1000 dilution) for 1 h. Protein was detected by enhanced chemiluminescence (Amersham, Bucks, UK).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared as previously described (31). Briefly, cells were scraped into PBS and centrifuged at 2000*g* for 10 min at 4°C. The pellet was washed in 5 times the packed cell vol (PCV) of hypotonic buffer (10 mM tris base, 10 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.5 μg/ml protease inhibitor, pH 7.4). After 10 min in HB buffer (3 × PCV) cells were homogenised in a Wheaton tissue grinder using a type "B" pestle (10 strokes) and centrifuged for 10 min at 2000*g*. The cytosol was removed and the nuclear pellet resuspended in 100 μl of microextract buffer (20 mM Hepes (pH 7.8), 450 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 μg/ml leupeptin, 0.5 μg/ml protease inhibitor, 1 μg/ml trypsin inhibitor, 0.5 μg/ml aprotinin, 40 μg/ml bestatin). Nuclei were lysed by freezing and thawing three times. Finally, the extract was centrifuged for 5 min at 14,000*g* and the supernatant (nuclear extract) stored at −70°C.

Double stranded consensus and mutant NFκB oligonucleotides (Santa Cruz Biotechnology) were end labelled by using T4 polynucleotide kinase according to the instructions supplied (Promega, Madison, WI). Nuclear extracts (5 μg) were added to [γ-^{32}P]-labelled oligonucleotide in a reaction mixture containing 4 μl, 40% ficoll, 4 μl; binding buffer (5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–HCl, 0.25 μg/μl poly dIdC, and 25% glycerol) and nuclease free water (total vol of 20 μl) and incubated for 15 min on ice. DNA-protein complexes were separated by a 0.75 mm thick, 4% nondenaturing polyacrylamide gel. Dried gels were autoradiographed (Hyperfilm-MP, Amersham) for 12–18 h at −70°C.

Statistical analysis. All results are expressed as mean ± SE mean and statistical comparisons were made by one-way ANOVA using INSTAT software (GraphPAD Software, San Diego, CA).

RESULTS

Monocyte adhesion in response to oxLDL. Treatment of cells with oxLDL (10–100 μg/ml) for 6 h increased monocyte adhesion in a dose-dependent manner (Fig. 1a, n = 6). Whereas, treatment with nLDL (100 μg/ml), or $CuSO_4$ (5 μM) and BHT (10 μM) (equivalent to that present in 100 μg/ml of oxLDL) for 6 h had no effect (data not shown). Figure 1b (n = 6) shows that percentage increase in the oxLDL (100 μg/ml)-induced monocyte adhesion after 6 h was equivalent to that of TNFα (10 ng/ml), 81 ± 3% and 85 ± 3% respectively. oxLDL had no effect on cell viability.

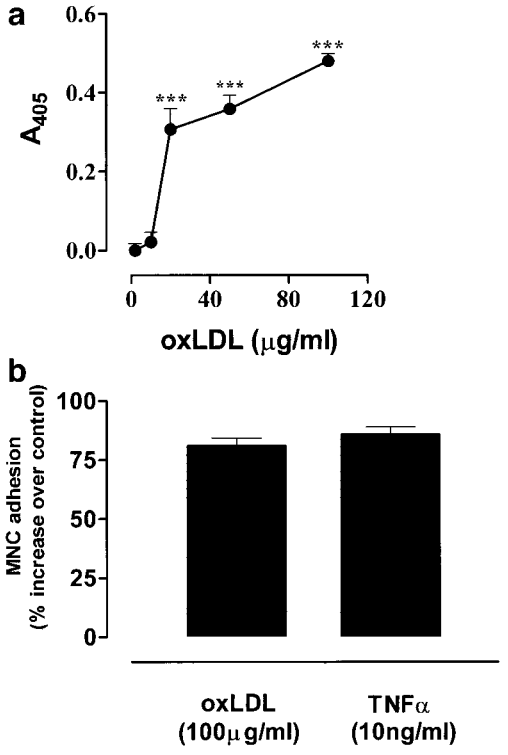

FIG. 1. Monocyte adhesion in response to oxLDL. (a) Cells were incubated with oxLDL (10–100 μg/ml) for 6 h and monocyte adhesion assessed by the MPO assay. Control (untreated) values have been subtracted. (b) MNC binding to endothelial cells after treatment with oxLDL (100 μg/ml) or TNFα (10 ng/ml), expressed as % increase over control. Values are mean ± SE mean (n = 6). ***$P < 0.001$ compared to control.

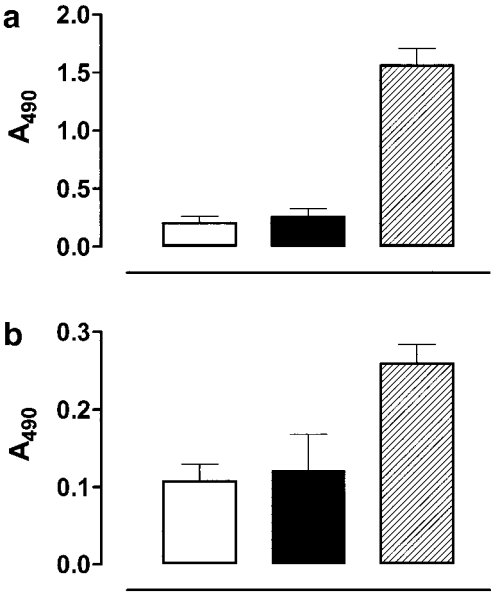

FIG. 2. Effect of oxLDL and TNFα on the expression of and ICAM-1 VCAM-1. Cells were treated with oxLDL (100 μg/ml) or TNFα (10 ng/ml) for 6 h. Subsequently, cells were washed and incubated with primary antibody (0.5 μg/well) for 1 h. Following incubation, cells were washed and incubated with peroxidase conjugated second antibody for 1 h. Binding was measured by the addition of substrate solution. After 10 min incubation, the reaction was stopped with 3N HCl (50 μl/well) and absorbance was measured at 490 nm. (a) ICAM-1; (b) VCAM-1. Clear bars, control; filled bars, oxLDL; hatched bars, TNFα. Values are mean ± SE mean (n = 4).

Role of oxLDL on the expression of cell adhesion molecules. TNFα upregulated the expression of ICAM-1 and VCAM-1 in EA.hy 926 cells after 6 h (Fig. 2). However, incubation with oxLDL had no effect on expression of ICAM-1 and VCAM-1.

Expression of MCP-1 mRNA in response to oxLDL. As shown in Fig. 3, EA.hy cells constitutively expressed MCP-1 mRNA which markedly increased in response to TNFα and oxLDL. nLDL also significantly induced MCP-1 mRNA. Expression of GAPDH was unchanged.

OxLDL has no effect on NFκB activation. Incubation with TNFα completely degraded inhibitor IκB-α within 15 min (Fig. 4a, n = 4), whereas oxLDL had no effect. TNFα-induced degradation of IκB-α was also not affected by oxLDL. Incubation of cells with oxLDL for 60 min did not affect levels of IκB-α (Fig. 4b).

To provide further evidence of the lack of oxLDL effect on NFκB activation, we performed an EMSA. Figure 5 shows that TNFα caused a retardation of a [γ-^{32}P]-labelled DNA NFκB probe (N). A mutant NFκB-binding motif (M) did not bind.

DISCUSSION

Our results show that oxLDL, but not nLDL dose dependently, increased monocyte adhesion to EA.hy 926 cells. oxLDL (100 μg/ml) was as effective as TNFα (10 ng/ml) in stimulating EA.hy 926 cells to bind monocytes. We also looked at the effect of oxLDL on cell adhesion responses by measuring MCP-1, ICAM-1, and VCAM-1 expression. Recently a role for oxLDL in the upregulation of MCP-1 expression has been reported (32–34). Our data further supports this additional pro-

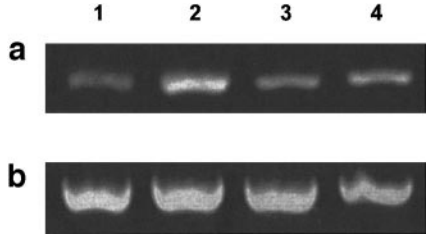

FIG. 3. oxLDL and nLDL increase MCP-1 mRNA expression. Cells were incubated with oxLDL (50 μg/ml), nLDL (50 μg/ml), or TNFα (10 ng/ml) for 6 h. Total RNA was extracted and RT-PCR was performed with specific primers for MCP-1 (panel a) and GAPDH (panel b). Lane 1, control; lane 2, TNFα (10 ng/ml); lane 3, nLDL (50 μg/ml); lane 4, ox LDL (50 μg/ml). Data shown is a representative example from four separate experiments.

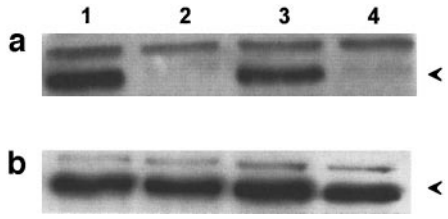

FIG. 4. Effect of oxLDL and TNFα on IκB-α degradation. (a) Cells were treated with oxLDL (50 μg/ml) or/and TNFα (10 ng/ml) for 15 min. Lane 1, control; lane 2, TNFα; lane 3, oxLDL; lane 4, oxLDL + TNFα. (b) Cells were incubated for different time periods (up to 60 min) with oxLDL. Lane 1, 0 min; lane 2, 15 min; lane 3, 30 min; lane 4, 60 min. Subsequently, cytosolic extracts were prepared and IκB-α degradation was measured by Western blot. The arrowhead indicates the position of IκB-α.

atherogenic effect of oxLDL. oxLDL itself is a monocyte chemoattractant and by inducing MCP-1 expression it may amplify monocyte chemotaxis and activation. Additionally, induction of MCP-1 mRNA by nLDL shows that nLDL itself can induce MCP-1 expression and may thereby trigger the initial recruitment of monocytes. Subsequently, this process may accelerate as LDL becomes increasingly oxidised during fatty streak and atherosclerotic lesion formation, thus exacerbating the chemotactic response.

The endothelial cell adhesion receptors, E-selectin, ICAM-1, and VCAM-1, which are present on atherosclerotic lesions (35–38) clearly contribute to the recruitment of monocytes, indeed activation of VCAM-1 expression has been proposed to be an initiating factor in selective recruitment of circulating monocytes (39–41). Surprisingly however, we found that oxLDL had no effect on expression of ICAM-1 and VCAM-1. Thus the monocyte adhesion observed in this study appears to be independent of ICAM-1 and VCAM-1, demonstrating an apparent oxLDL-mediated monocyte adhesion via other adhesion receptor(s). Since C-type-LOX receptor is upregulated by oxLDL and antisense LOX-1 suppresses both MCP-1 expression and monocyte adhesion (32) it is possible that LOX-1 may be involved in EA.hy 926 cell-monocyte interaction. Frostegård *et al.* (12) previously noted that exposure of oxLDL to endothelium increased monocyte adhesion and this increased adhesion was independent of ICAM-1. Furthermore, our EMSA result showed that oxLDL, unlike TNFα, had no effect on NFκB activation in EA.hy 926 cells even after a 60 min incubation. Indeed our EMSA results provided further confirmation of the ICAM-1 and VCAM-1-independent adhesion in EA.hy 926 cells, since these adhesion receptors require NFκB-binding for their transcriptional activation. In contrast, Erl *et al.* (42), Cominacini *et al.* (43) and Takei *et al.* (44) showed that oxLDL increased ICAM-1 and VCAM-1 expression in endothelial cells. Also Cominacini *et al.* (43) and Li and Mehta, (32) showed that oxLDL induced NFκB activation in bovine aortic endothelial cells and human coronary artery endothelial cells respectively. Therefore in EA.hy 926 cells, at least, oxLDL-mediated responses are independent of NFκB and may involve activation of other transcription factor(s). It is currently unclear as to the basis of these differing reports. However differences in the type of endothelial cells used may, in part, be responsible. It is also noteworthy that oxLDL preparations are frequently heterogeneous. Dialyse of LDL after copper oxidation may remove low molecular weight species that may significantly contribute to oxLDL-induced responses. For example Thomas *et al.* (4) showed that dialysis of oxLDL prior to incubation with MNCs rendered it unable to stimulate IL-1β release. Furthermore, the copper ion concentration and the incubation time used for LDL oxidation are variable.

In summary, oxLDL-induced monocyte adhesion is independent of ICAM-1, VCAM-1, and NFκB in EA.hy 926 cells. Thus indicating that oxLDL can also upregulate adhesion responses via NFκB-independent pathways possibly utilising as yet uncharacterised adhesive mechanisms.

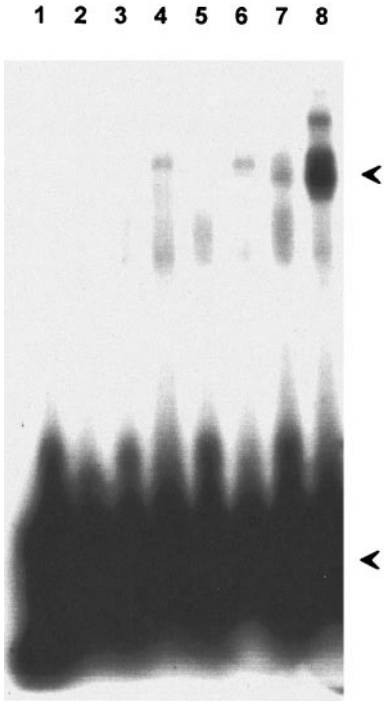

FIG. 5. EMSA gel showing effect of oxLDL and TNFα on NFκB activation. Cells were incubated with oxLDL (50 μg/ml) or TNFα (10 ng/ml) for 1 h. Subsequently, nuclear extract was prepared and NFκB activation was measured by an EMSA using consensus (N) or mutant (M) oligonucleotide. Lane 1, [γ-^{32}P] labelled M probe only; lane 2, [γ-^{32}P] labelled N probe only; lane 3, oxLDL + M probe; lane 4, oxLDL + N probe; lane 5, control cells + M probe; lane 6, control cells + N probe; lane 7, TNFα + M probe; lane 8, TNFα + N probe. Top arrowhead shows the position of the NFκB-oligonucleotide complex. Lower arrowhead indicates free unbound probe. Data shown is a representative example from four separate experiments.

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