Oxidised and native low-density lipoproteins induce the release of von Willebrand factor from human endothelial cells *in vitro*

A.D. BLANN[*,‡], G. BURROWS[†] and C.N. McCOLLUM[‡]

[*]*Haemostasis, Thrombosis and Vascular Biology Unit, University Department of Medicine, City Hospital, Birmingham B18 7QH;* [†]*Department of Chemical Pathology, Stepping Hill Hospital, Poplar Grove, Stockport SK2 7JE; and* [‡]*University Department of Surgery, South Manchester University Hospital Nell Lane, Didsbury, Manchester M20 8LR, UK*

Accepted: 1 July 2003

ABSTRACT

Increased low-density lipoprotein (LDL)-cholesterol is a risk factor for atherosclerosis – a disease in which damage to the endothelium is believed to be an important early step. Increased levels of the endothelial marker von Willebrand factor (vWF) in the plasma of patients with hypercholesterolaemia and atherosclerosis probably reflect this process. In this study we seek to link the established observation that oxidised LDL-cholesterol is cytotoxic to human umbilical vein endothelial cells (HUVECs) *in vitro* with the common finding of raised plasma vWF in patients with atherosclerosis by incubating HUVECs with physiological/pathological levels of native and oxidised LDL-cholesterol for up to 48 h. Microphotography revealed morphological changes in the HUVECs within 24 h, becoming severe at 48 h, which was mirrored by increased levels of vWF (ELISA) and the release of preloaded radioactive ^{111}indium tracer into culture supernatants. Our data support and extend the hypothesis that oxidised LDL is directly cytotoxic to HUVECs, and, in addition, provide an important link between *in vitro* studies and clinical studies where endothelial cell markers such as vWF are increased in the plasma of patients with hypercholesterolaemia and atherosclerosis.

KEY WORDS: Endothelium. von Willebrand factor. Oxidised LDL. Atherosclerosis. Hypercholesterolemia.

Introduction

The precise initiating events in the development of atherosclerosis are unclear but are likely to involve injury to the endothelium.[1] Increased plasma levels of von Willebrand factor (vWF), a specific product of the endothelium, are considered by many to be a marker of endothelial dysfunction.[2,3] Clinical and epidemiological studies indicate that plasma levels of vWF are raised in cardiovascular disease and its risk factors[4,5] and predict adverse outcome such as myocardial infarction, stroke and cardiovascular death.[6-8]

The proposed causative (i.e., damaging and/or activating) agents for increased vWF include cigarette smoking, hypertension, diabetes and hypercholesterolaemia, and may also include oxidised low-density lipoprotein (oxLDL).[9,10] In a completely separate laboratory approach, tissue culture studies have shown, using a variety of definitions, that oxLDL is cytotoxic to human umbilical vein endothelial cells (HUVECs) *in vitro*,[11-13] but no studies have shown that oxLDL-damaged HUVECs respond to this insult by releasing vWF.

The current study is designed to link these two approaches of clinical epidemiology and vascular cell biology. Our hypothesis is that cytotoxic oxLDL and unoxidised (i.e., native) LDL (nLDL) both cause damage to HUVECs, which respond by releasing excess vWF into the tissue culture supernatant. To make the study more robust, in addition to the release of vWF, we use three other measures of cell damage: the release of a preloaded radioactive tracer (^{111}indium) from the HUVECs; loss of adhesion to the tissue culture plate; and changes to the morphology of the monolayer. In doing so, we aim to provide a mechanism to link the evidence that, in clinical and population studies, high levels of LDL are a risk factor for atherosclerosis,[9,10] and the finding that increased vWF is not only present in hypercholesterolaemia[4,5] but also predicts an adverse prognosis.[6-8]

Correspondence to: Dr Andrew Blann
Email: a.blann@bham.ac.uk

Materials and methods

Tissue culture

Human umbilical vein endothelial cells were obtained using a standard technique.[14] Briefly, the lumen of the umbilical vein was cannulated, washed free of blood with 0.85% saline and then perfused with 0.1% collagenase type IV (Sigma/Aldrich, Poole, UK) in phosphate-buffered saline (PBS) for 15 min at 37°C. HUVECs were flushed out with Medium 199, centrifuged at 1000 x**g** for 15 min and added to 25-mL flasks (Costar, pre-coated with 1% bovine gelatin [Sigma/Aldrich]) in PBS at 4°C overnight) in RPMI 1690 medium plus 20% (v/v) fetal calf serum (FCS), 150mg/mL endothelial cell growth supplement (Sigma/Aldrich), antibiotics (penicillin, streptomycin) and glutamine. Cultures were incubated at 37°C in an atmosphere of 5%CO_2/95% air. Quality control was assured by >95% positive indirect immunofluorescence staining with a rabbit antiserum to vWF and a fluorescein-conjugated sheep anti-rabbit antiserum (DakoCytomation, Ely, UK).

Preparation of oxidised and native LDL

A single batch of human LDL was prepared from citrated plasma from 10 healthy normolipaemic donors.[11,15-18] Plasma solvent density was adjusted to 1.019 g/mL with a high-density salt solution containing NaCl and KBr (Sigma/Aldrich, Poole, Dorset, UK). After centrifugation for 18 hours at 60,000 rpm and 4˚C in a Beckman L5-75 centrifuge with a 60 Ti rotor, the infranatant was collected. The pooled infranatant was adjusted to a density 1.060 g/mL, re-centrifuged as before, and LDL (density 1.019–1.060 g/mL) was collected. LDL was dialysed at 4˚C for 48–72 h against four changes of a least 50 volumes 0.15 mol/L NaCl with 0.5mmol/L EDTA. Oxidised LDL was prepared through additional dialysis at 4˚C for 48–72 h against four changes of at least 100 volumes dialysate (1 µmol/L $FeSO_4$ in 0.15 mol/L NaCl [pH 7.2–8.0]). Oxidised LDL and nLDL were dialysed at 4˚C for 48–72 h against four changes of at least 100 volumes 0.15 mol/L NaCl, adjusted to 6 mmol/L (defined as total cholesterol [Kodak, dry chemistry]), sterilised through a 0.22 µm Millipore filter and stored in aliquots at –70˚C.

The degree of oxidation of LDL and oxLDL was determined using the thiobarbituric acid (TBA) reactive substances (TBARS) method.[19,20] Briefly, plasma was deproteinated with 12 N sulphuric acid and the clarified supernatant was incubated with freshly prepared TBA (0.67% aqueous TBA in 0.5 mol/L Tris buffer [pH 3.4]) at 95˚C for 1 h. TBARS were determined in the clarified, cooled supernatant in a Shimazdu RF 450 spectrofluorophotometer (515 nm excitation, 553 nm emission), using a standard obtained by reacting bis-malondialdehyde (MDA) with TBA. TBARS in the nLDL and oxLDL fractions were 28 and 390 pmol MDA/mmol LDL-cholesterol, respectively.

Co-culture with LDL fractions

When confluent, primary cultures of HUVECs were split (0.1% trypsin/0.05% EDTA [Sigma and Gibco], 5 min at 37˚C) into tissue culture grade 96-well microtitre plates (Nunc, pre-coated with 1% gelatin in PBS at 4˚C overnight) in 200 µL RPMI/20% FCS. When confluent, usually after 2–3 days, the supernatant was removed, cells (generally, about 10^5/well) were gently washed once with PBS and the monolayer photographed. Cells were then bathed in 200 µL RPMI, supplemented not with FCS but with 1–6 mmol/L nLDL, oxLDL or bovine serum albumin (BSA, Sigma/Aldrich) for 24 and 48 h. All LDL levels were in the corresponding physiological or pathological range *in vivo*.[7,8,21]

Removal of FCS was done to ensure the specificity of any nLDL, oxLDL or BSA effect, and remove the possible effects of factor(s) or co-factor(s) in the FCS. BSA was chosen simply as a comparator molecule to provide some semblance of an inert protein for the HUVECs, because medium alone would have been even less physiological After each 24-hour period, supernatants were removed, centrifuged at 13,000 rpm for 3 min to remove debris of effete HUVECs, and the supernatants were frozen at –70˚C in fresh vials. The wells were gently washed with PBS, and the monolayer photographed. 200 µL supplemented RPMI was then replaced as before.

vWF enzyme-linked immunosorbent assay

This was performed by a modification of a standard method using a rabbit antiserum to vWf (DakoCytomation) and was standardised by reference vWF from NIBSC (Blanche Lane, Potters Bar, Herts, UK).[22] Tissue culture supernatants (50 µL) were added in triplicate, directly into microtitre plates (Immulon II, Dynex, Ashford, UK). In all cases, supernatants were compared to a negative control of BSA/nLDL/oxLDL-supplemented culture medium (i.e., kept in the tissue culture plates but without HUVECs). The level of vWF in supernatants was calculated by subtracting the blank absorbance from the test absorbance obtained from the ELISA reader, compared to reference (NIBSC) vWF, and is presented as miu/mL.

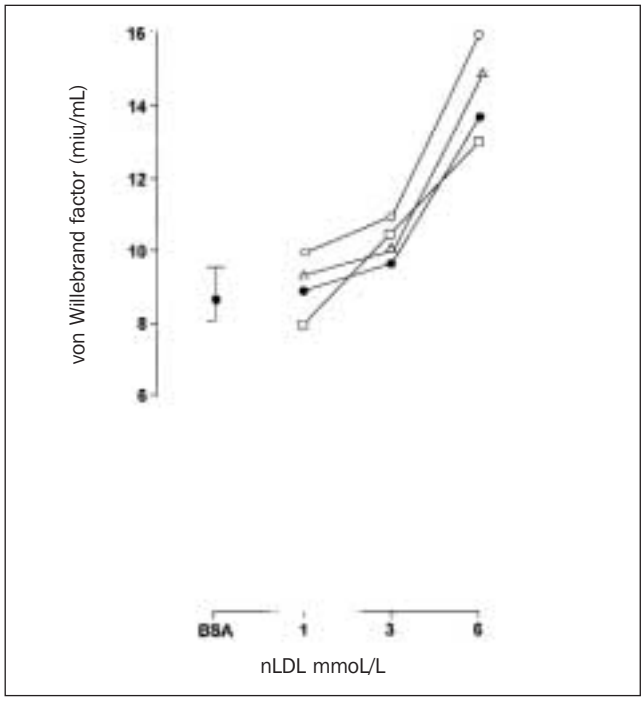

Fig. 1. Dose response curves of the effect of increasing concentrations of nLDL on release of vWF after 24 h culture. Data shows four experiments of mean vWF release from three wells due to increasing concentration of nLDL. Release from control wells incubated with BSA are mean with standard error bars. Levels of vWF are not increased relative to BSA cultures in 1 mmol/L, but increased in trend at higher concentrations (ANOVA $P<0.01$).

Radioisotope assay

HUVECs were harvested and radiolabelled with ^{111}indium as previously described.[23] Briefly, $2x10^6$ –$5x10^6$ HUVECs were incubated in serum-free RPMI medium with 30–40 µL ^{111}indium (The Radiochemical Centre, Amersham, Bucks, UK) at an activity of 3.7 MBq/mL for 4 min at room temperature. After washing (x3), 10^5 cells were dispensed in 200 µL aliquots into wells of a tissue-culture grade 96-well microtitre plate (Nunc) and left in RPMI/20% FCS at 37˚C for 3 h following a brief (5 min) centrifugation at 500 rpm.

After this time, supernatant was gently removed and replaced by 200 µL RMPI supplemented with 4 mmol/L nLDL or oxLDL for 6 h, as described above. Culture beyond this 6-h period proved difficult as non-specific release of isotope increased (data not shown). Some wells were incubated with 1% Triton X-100 (Sigma/Aldrich) to provide a positive control; others were incubated with medium free of lipoproteins but with BSA, to provide a negative control. Supernatant was harvested and released ^{111}indium estimated

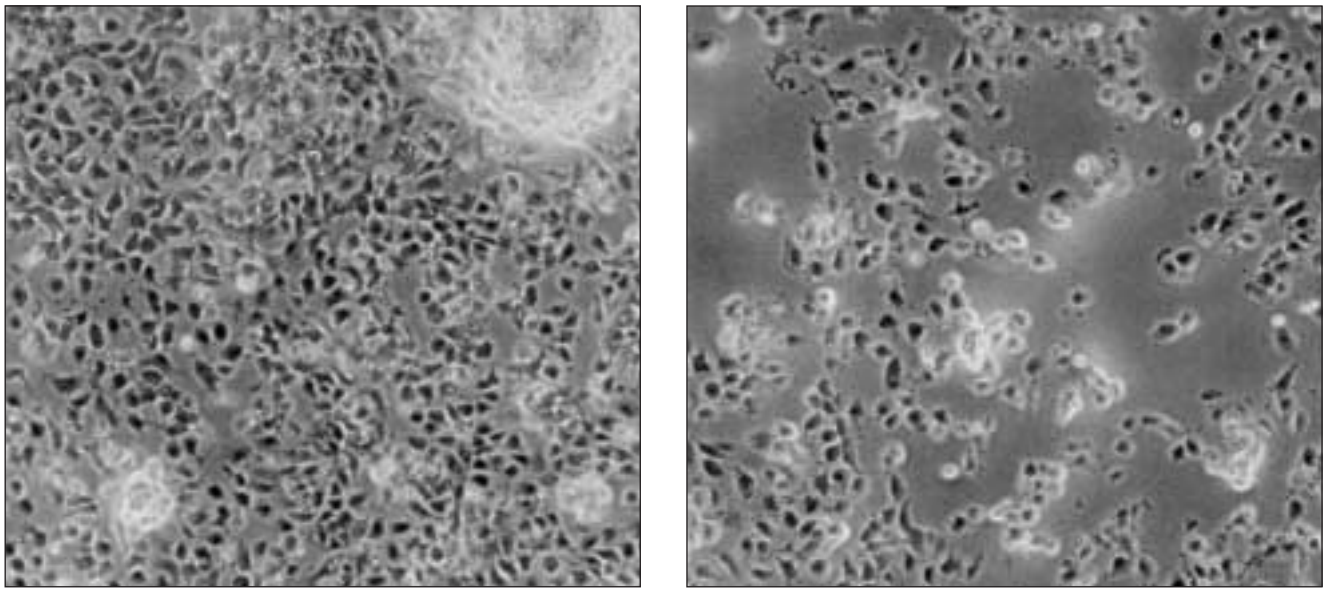

Fig. 2. Low magnification (x40) photomicrographs showing the effect of (left) 24-h exposure of HUVECs to 4 mmol/L nLDL, and (right) 48-h exposure of HUVECs to 4 mmol/L nLDL.

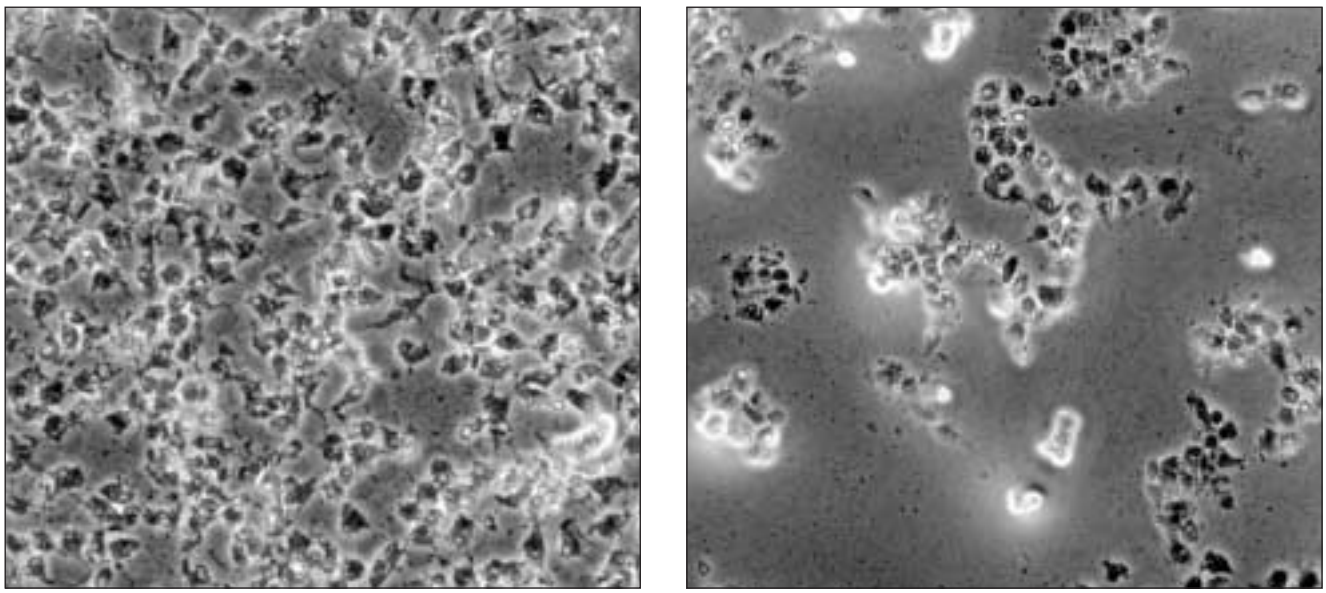

Fig. 3. Low magnification (x40) photomicrographs showing the effect of (left) 24-h exposure of HUVECs to 4 mmol/L oxLDL, and (right) 48-h exposure of HUVECs to 4 mmol/L oxLDL.

in a gamma counter (NE 1600, Nuclear Enterprises, Edinburgh, UK) for 30–90 sec, depending on the specific activity of the isotope.

The supernatant was then stored at –70°C for 10 days to two weeks to allow radioactivity to decay to a background level, and vWF estimated by ELISA. Cytotoxicity was calculated according to the formula: counts per minute (CPM) from experimental wells – CPM from negative control wells (i.e., cells in BSA), divided by CPM from the positive control wells (i.e., cells lysed by detergent) – CPM from the negative control wells, x 100. Units are percent cytotoxicity.

Analysis of data

Data was derived from experiments of triplicate cultures. Experiments were performed on at least three different HUVEC cultures. vWF and radioisotope data were distributed normally and so were analysed by Student's *t*-test and analysis of variance and are presented as mean and standard deviation (SD). All analyses were performed on a Minitab 12 package (Minitab, 3081 Enterprise Drive, State College, Philadelphia PA 16801 USA).

Results

Dose-response experiment

After 24-h culture, supernatant vWF was 9.1±0.8 miu/mL in 1 mm/L nLDL, 10.7±0.8 in 3 mmol/L nLDL, and 14.3±1.7 mIU/ml in 6 mmol/L nLDL (mean±SD of data from four experiments, ANOVA $P<0.01$, Figure 1), indicating dose-dependent injury to the HUVECs by nLDL. This effect was

Table 1. Levels of vWF in tissue culture medium from HUVECs incubated with BSA, nLDL or oxLDL (values are miu vWF/mL of culture medium from three [48, 72 h]) or four [24 h] experiments

	HUVECs Cultured with:			
Time point	BSA	nLDL	oxLDL	*P* value
24 hours	11.7±1.6	13.8±1.9	15.5±0.9	0.022
48 hours	11.2±1.4	6.2±1.2	12.1±0.6	0.227
72 hours	7.9±2.0	4.2±1.8	0.6±0.8	0.004
P value	0.049	0.001	<0.001	

P values from one-way analysis of variance

Table 2. Levels of vWF and release of radioisotope in tissue culture medium from HUVECs (values are miu vWf/mL of culture medium/6 h from three experiments (*four experiments)

	BSA	nLDL	oxLDL	P value
vWF (miu/mL)	1.6±0.5	2.3±0.4	3.0±0.4	0.004
Cytotoxicity (%)	0	7.2±1.7	14.2±3.9	0.001#

P values from one-way analysis of variance (# Kruskal-Wallis test as data non-normally distributed)

Data presented as mean ±SD

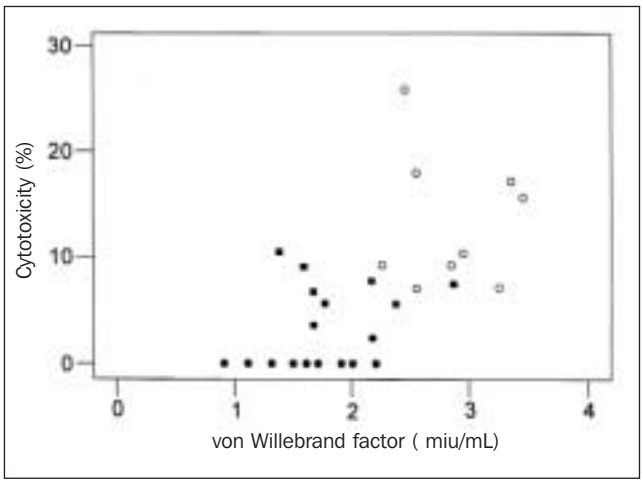

Fig. 4. Correlation between levels of vWF and cytotoxicity. Spearman correlation coefficient $r=0.609$, $P=0.001$ between vWF and the relative release of radioisotope in a particular well (27 experiments). ● release from BSA cultures, ■ from nLDL cultures, and ○ from oxLDL cultures (all at 4 mmol/L).

more pronounced in cultures of HUVECs incubated with the same concentrations of oxidised LDL (i.e., 11.3±1.1 miu/mL at 1 mmol/L, 13.6±1.3 at 3 mmol/L and 17.4±2.1 at 6 mmol/L [$P<0.001$]). HUVECs cultured with BSA at 3 mmol/L for 24 h released 8.7±1.1 miu vWF/mL of culture medium. The amount of vWF released by HUVECs in these experiments was comparable to the amount released by HUVECs cultured in complete tissue culture medium[24] but were much lower than levels in plasma, where the unit is iu/dL.[4-8]

Time course of responses to nLDL and oxLDL

vWF was measured in the HUVEC supernatants after 24, 48 and 72 h culture with 4 mmol/L BSA, nLDL or oxLDL (Table 1, Figures 2 and 3). After 24 h, there was an increase in vWF levels in nLDL and oxLDL cultures, compared to the BSA cultures ($P<0.05$). Figure 2 shows the effect of 24- and 48-h exposure of HUVECs to nLDL. After 24 h the monolayer was still approximately 100% confluent (Figure 2, left), but in many cells the morphology had changed from flattened orthogonal to rounded-up, suggesting loss of adhesion. After 48 h there were clear areas of denudation (Figure 2, right), implying loss of cells from the monolayer, perhaps 60% intact, with very few cells exhibiting a normal morphology.

The pattern was similar at 72 h with approximately 50% of the monolayer remaining (data not shown). Figure 3 (left) shows the effect of 24-h incubation with oxLDL. HUVECs were clearly abnormal, with loss of adhesion and monolayer integrity, and perhaps 80% confluence. However, at 48 h (Figure 3, right) the monolayer was reduced to perhaps 5–10% confluence, indicating considerable loss of cell adhesion. At 72 h only cell debris remained in the culture: all cells had lost adhesion (data not shown). The morphological changes to HUVECs are similar to those presented by others.[15,18,33]

Radioisotope release experiments

Data are presented in Table 2. The negative control was radiolabelled HUVECs cultured with BSA for 6 h. Considerably more isotope was released by cells cultured with 4 mmol/L nLDL and oxLDL, compared to cells in BSA (i.e., both nLDL and oxLDL were cytotoxic toward HUVECs, $P<0.05$). oxLDL was more cytotoxic than nLDL ($P<0.05$). The release of vWF from cells cultured with nLDL was not statistically higher than in cells cultured with BSA, but release from cells cultured with oxLDL was higher than in the BSA cultures ($P<0.05$). Levels of vWF correlated strongly with the degree of cytotoxicity (Spearman $r=0.609$, $P=0.001$; Figure 4).

Discussion

The precise pathogenesis of atherosclerosis has yet to be established, but injury to the endothelium could be an initiating and propagating event.[1] High lipoprotein levels and oxLDL have been identified as the components most likely to be responsible for the greatest degree of damage to the endothelium.[9-13,21,25] For example, the concentration of oxidised lipids identifies a stenosis in cardiac catheterised patients[26] – a group known to exhibit raised plasma vWF. Furthermore, oxLDL induces apoptosis in HUVECs[27] and oxLDL degrades the endothelial surface layer.[28]

Other *in vitro* evidence supporting the hypothesis that lipoproteins are cytotoxic to HUVECs include reduced uptake of nucleotides,[11,13,28] increased release of prelabelled radioisotope,[13,15,29] morphological changes[12,13,15,29] and loss of adhesion.[29-32]

Our current data confirm and extend the three latter techniques by also showing increased release of vWF (an endothelial cell product and marker of damage[2-5] that [in high levels] predicts a poor prognosis[6-8]) from HUVECs due to LDL and oxLDL. Our data also confirm and extend other data, such as that showing cytotoxicity of oxysterols towards HUVECs,[32] and that co-culture of HUVECs with a synthetic

oxygenated cholesterol derivative suffer a loss of adhesion.[33]

Our *in vitro* experiments reflect physiological (i.e., 1–3 mmol/L LDL) and pathological (i.e., 3–6 mmol/L) levels of lipoproteins *in vivo*. After 24 h, vWF release increased in nLDL- and oxLDL- supplemented cultures, in parallel with morphological changes.[15,18,33] We interpret this as nLDL and oxLDL damaging the HUVECs, which respond by releasing more vWF. At 48 h, vWF release in both LDL cultures is reduced, possibly as some cells are already dying and so will have effectively released all their vWF.

High levels of vWF in oxLDL-supplemented cultures may represent release by dying or apoptotic cells,[27,30] as morphological changes are most severe; however, we are unable to say whether the phenomena observed are due to apoptosis or simple necrosis. At 72 h, there were few remaining adherent HUVECs in the nLDL wells, hence the reduction in vWF levels. In the oxLDL wells, there were few remaining cells and, hence, little release of vWF.

We suggest that nLDL slowly but steadily damages HUVECs, which respond with a brief excess production of vWF. However, oxLDL seems to be much more damaging, producing an initial rise, followed by lower levels, reflecting the rapid destruction of the cells in the early stages. The radioisotope assays lasted only 6 h and so were not directly comparable. However, the good correlation between cytotoxicity and vWF release provides support for the concept that nLDL and oxLDL damage HUVECs. The radioisotope release assay seemed to provide better sensitivity as it, and not vWF, discriminated BSA cultures from nLDL cultures.

The approach taken in this study was similar to that used by others, and our findings are generally in agreement with the proposal that oxLDL is cytotoxic to the endothelium.[9-13] Increased vWF release by HUVECs exposed to oxLDL and nLDL complements the widespread finding that high levels of this endothelial marker are found in patients with hypercholesterolaemia and atherosclerosis.[4-8]

vWF is increasingly regarded as a marker of endothelial cell dysfunction/injury and the *in vitro* data presented here support the hypothesis derived from clinical and epidemiological,[4-8] as well as *in vitro* data,[11-13,15-18,25-28,30-33] that hypercholesterolaemia damages the endothelium – one of several potential mechanisms by which it may contribute to the process of atherosclerosis and premature cardiovascular events such as myocardial infarction and stroke.[34] □

References

1 Ross R. Atherosclerosis – an inflammatory disease. *N Engl J Med* 1999; **340**: 115–26.

2 Lip GYH, Blann AD. von Willebrand factor: a marker of endothelial dysfunction in vascular disorders? *Cardiovasc Res* 1997; **34**: 255–65.

3 Pearson JD. Markers of endothelial perturbation and damage. *Br J Rheumatol* 1993; **32**: 651–2.

4 Blann AD, Davis A, Miller JP, McCollum CN. von Willebrand factor and soluble E-selectin in hyperlipidaemia: relationship to vascular disease and the risk factors for atherosclerosis. *Am J Hematol* 1997; **55**: 15–23.

5 Davi G, Romano M, Mezzetti A *et al*. Increased levels of soluble P-selectin in hypercholesterolaemia patients. *Circulation* 1998; **97**: 953–7.

6 Jansson JH, Nilsson TK, Johnson O. von Willebrand factor in plasma: a novel risk factor for recurrent myocardial infarction and death. *Br Heart J* 1991; **66**: 351–5.

7 Thompson SG, Kienast J, Pyke SDM, Haverkate F, van de Loo JCW. Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. *N Engl J Med* 1995; **332**: 635–41.

8 Blann AD, Miller JP, McCollum CN. von Willebrand factor and soluble E-selectin as predictors of cardiovascular disease progression in hyperlipidaemia. *Atherosclerosis* 1997; **132**: 151–6.

9 Leake DS. Oxidised low-density lipoproteins and atherogenesis. *Br Heart J* 1993; **69**: 476–8.

10 Witztum JL, Steinberg D. Role of oxidised low-density lipoprotein in atherogenesis. *J Clin Invest* 1991; **88**: 1785–92.

11 Hessler JR, Robertson AL, Chisholm GM. LDL-induced cytotoxicity and its inhibition by HDL in human vascular smooth muscle cells and endothelial cells in culture. *Atherosclerosis* 1979; **32**: 213–9.

12 Liu B, Sidiropoulos A, Zhao B, Dierichs R. Oxidised LDL damages endothelial cell monolayers and promotes thrombocyte adhesion. *Am J Hematol* 1998; **57**: 341–3.

13 Thorne SA, Abbot SE, Winyard PG, Blake DR, Mills PG. Extent of oxidative modification of low-density lipoprotein determines the degree of cytotoxicity to human coronary artery cells. *Heart* 1996; **75**: 11–6.

14 Jaffe EA, Nachmann RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins: identification by morphological and immunological criteria. *J Clin Invest* 1973; **52**: 2745–56.

15 Evensen SA, Galdal KS, Nilsen E. LDL-induced cytotoxicity and its inhibition by anti-oxidant treatment in cultured human endothelial cells and fibroblasts. *Atherosclerosis* 1983; **49**: 23–30.

16 Kosugi K, Morel DW, DiCorletto PE, Chisholm GM. Toxicity of oxidised low-density lipoprotein to fibroblasts is selective for S phase of the cell cycle. *J Cell Physiol* 1987; **130**: 311–20.

17 Li X, Chen JX, Liao DF, Yu L. Probucol inhibits oxidized low-density lipoprotein-induced adhesion of monocytes to endothelial cells by reducing P-selectin synthesis *in vitro*. *Endothelium* 1998; **6**: 1–8.

18 Morel DW, Hesseler JR, Chisholm GM. Low-density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. *J Lipid Res* 1983; **24**: 1070–6.

19 Asakawa T, Matsushita S. Thiobarbituric acid test for detecting lipid peroxides. *Lipids* 1979; **14**: 401–6.

20 Yagi K. Assay for serum lipid peroxides and its clinical significance. In Yagi K, ed. *Lipid peroxides in biology and medicine*. New York: Academic Press, 1982: 223–42.

21 Steinberg D. The cholesterol controversy is over. Why did it take so long? *Circulation* 1989; **80**: 1070–8.

22 Short PE, Williams CE, Picken AM, Hill FGH. Factor VIII-related antigen: an improved enzyme immunoassay. *Med Lab Sci* 1982; **39**: 351–5.

23 Blann AD, Garner CM. Indium 111 as a label for endothelial cells and fibroblasts used as target cells in cytotoxicity tests. *Med Lab Sci* 1985; **42**: 92–4.

24 Blann AD, Lip GYH. Effects of C-reactive protein on the release of von Willebrand factor, E-selectin, thrombomodulin and intercellular adhesion molecule-1 from human umbilical vein endothelial cells. *Blood Coagul Fibrinolysis* 2003; **14**: 335–40.

25 Penn MS, Chisholm GM. Oxidised lipoprotein, altered cell function and atherosclerosis. *Atherosclerosis* 1994; **108** (Suppl): S21–9.

26 Kummerow FA, Olinescu RM, Fleischer L, Handler B, Shinkareva SV. The relationship of oxidised lipids to coronary

artery stenosis. *Atherosclerosis* 2000; **149**: 181–90.
27 Claise C, Edeas M, Chaouchi N *et al*. Oxidised LDL-induced apoptosis in HUVEC but not in the endothelial cell line EA-hy 926. *Atherosclerosis* 1999; **47**: 95–104.
28 Vink H, Constantinescu AA, Spaan JAE. Oxidised lipoproteins degrade the endothelial surface layer. *Circulation* 2000; **101**: 1500–2.
29 Okajima K, Abe H, Binder BR. Endothelial cell injury induced by plasmin *in vitro*. *J Lab Clin Med* 1995; **126**: 377–84.
30 Lizard G, Monier S, Cordelet C *et al*. Characterisation and comparison of the mode of cell death, apoptosis versus necrosis, induced by 7 beta-hydroxycholesterol and 7-ketocholesterol in the cells of the vascular wall. *Arterioscler Thromb Vasc Biol* 1999; **19**: 1190–200.
31 Morel DW, DiCorleto PE, Chisholm GM. Endothelial and smooth muscle cells alter low-density lipoprotein *in vitro* by free radical oxidation. *Arteriosclerosis* 1984; **4**: 357–64.
32 Zhou Q, Wasowicz E, Handler B, Fleischer L, Kummerow FA. An excess concentration of oxysterols in the plasma is cytotoxic to cultured endothelial cells. *Atherosclerosis* 2000; **149**: 191–7.
33 Pettersen KS, Boberg KM, Stabursvik A, Prydz H. Toxicity of oxygenated cholesterol derivatives toward cultured human umbilical vein endothelial cells. *Arterioscler Thromb* 1991; **11**: 423–8.
34 Stober C, Clarke A, Mulvaney A, Harland J, Neithercul WD. Cholesterol and Lp(a) as risk factors for coronary heart disease. *Br J Biomed Sci* 1994: **51**; 114–8.