

Vitamin C Protects Human Vascular Smooth Muscle Cells Against Apoptosis Induced by Moderately Oxidized LDL Containing High Levels of Lipid Hydroperoxides

Richard C. M. Siow, Justin P. Richards, Kevin C. Pedley, David S. Leake, Giovanni E. Mann

Abstract—Vascular cell death is a key feature of atherosclerotic lesions and may contribute to the plaque “necrotic” core, cap rupture, and thrombosis. Oxidatively modified low-density lipoproteins (LDLs) are implicated in the pathogenesis of atherosclerosis, and dietary antioxidants are thought to protect the vasculature against LDL-induced cytotoxicity. Because LDL oxidative modification may vary within atherosclerotic lesions, we examined the effects of defined, oxidatively modified LDL species on human arterial smooth muscle cell apoptosis and the cytoprotective effects of vitamin C. Moderately oxidized LDL (0 to 300 μg protein/mL), which has the highest content of lipid hydroperoxides, induced smooth muscle cell apoptosis within 6 hours, whereas native LDL and mildly and highly oxidized LDL had no effect. Moderately oxidized LDL increased cellular DNA fragmentation, release of fragmented DNA into the culture medium, and annexin V binding and decreased mitochondrial dehydrogenase activity and expression of the antiapoptotic mediator Bcl- x_L . Treatment of cells with native LDL together with the lipid hydroperoxide 13(S)-hydroperoxyoctadeca-9Z,11E-dienoic acid (HPODE, 200 $\mu\text{mol/L}$, 6 to 24 hours) also induced apoptotic cell death. Pretreatment of smooth muscle cells with vitamin C (0 to 100 $\mu\text{mol/L}$, 24 hours) attenuated the cytotoxicity and apoptosis induced by both moderately oxidized LDL and HPODE. Our findings suggest that moderately oxidized LDL, with its high lipid hydroperoxide content, rather than mildly or highly oxidized LDL, causes apoptosis of human smooth muscle cells and that vitamin C supplementation may provide protection against plaque instability in advanced atherosclerosis. (*Arterioscler Thromb Vasc Biol.* 1999;19:2387-2394.)

Key Words: smooth muscle, vascular ■ low-density lipoprotein, oxidized ■ apoptosis ■ ascorbic acid ■ Bcl- x_L ■ atherosclerosis

Oxidatively modified LDLs are involved in the initiation and progression of atherosclerosis and have been localized within atherosclerotic lesions.¹⁻⁵ Evidence that oxidized LDL induces vascular cell death by apoptosis⁶⁻⁸ and the presence of apoptotic cells in atherosclerotic and restenotic lesions^{7,9,10} has potential clinical implications for atherogenesis and thrombosis. Although smooth muscle cell death may enhance lesion regression,¹¹ it can also result in the accumulation of necrotic debris within the lesion core and instability of the fibrous plaque cap, leading to its rupture and thrombosis.¹⁰ The extent to which LDL becomes oxidized within atherosclerotic lesions has not been well defined, and levels of oxidation may differ within lesions. Because the toxicity of oxidized LDL appears to increase with the degree of oxidative modification,^{12,13} this may contribute to the impaired vascular reactivity in patients with coronary heart disease.¹⁴

Oxidized LDL is cytotoxic to smooth muscle cells,^{12,15} endothelial cells,^{12,16,17} and macrophages¹⁸ and has been shown to induce apoptosis in several vascular cell types.^{6-8,19} Although a mildly oxidized species of LDL (moxLDL) has

been reported to induce endothelial cell toxicity and apoptosis,²⁰ only limited information is available on the effects of moderately oxidized LDL (modLDL), which contains the highest lipid hydroperoxide levels. Dietary antioxidants attenuate the cytotoxic effects of oxidatively modified LDL^{6,16,20,21} and restore endothelial function in patients with coronary artery disease.²² Moreover, a low plasma vitamin C concentration is implicated as an independent predictor of unstable coronary artery disease.²³ In this context, we previously reported that vitamin C protects human vascular smooth muscle cells against oxidized LDL-induced adaptive increases in the synthesis of the key intracellular antioxidant glutathione.²⁴ These results provided the first evidence that vitamin C spares endogenous antioxidant responses in human vascular smooth muscle cells exposed to atherogenic lipoproteins.

In this study, we investigated further the cytoprotective effects of vitamin C by examining LDL-induced apoptosis of human smooth muscle cells. modLDL, which contains the peak level of lipid hydroperoxides, but not highly oxidized

Received February 9, 1998; revision accepted October 3, 1999.

From the Centre for Cardiovascular Biology and Medicine, GKT School of Biomedical Sciences, King's College, London (R.C.M.S., K.C.P., G.E.M.), and Cell and Molecular Biology Research Division, School of Animal and Microbial Sciences, University of Reading, Reading (J.P.R., D.S.L.), UK.

Correspondence to Dr Richard Siow, Division of Cardiovascular Medicine, Department of Medicine, School of Clinical Medicine, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 2QQ, UK. E-mail rcms2@cam.ac.uk

© 1999 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at <http://www.atvbaha.org>

Lipid Hydroperoxide Content and Electrophoretic Mobility of LDL

Degree of LDL Oxidation	Lipid Hydroperoxide Content (nmol/mg protein)	Electrophoretic Mobility (% Increase Relative to nLDL)
nLDL	43±24	
moxLDL	49±21	19±8*
modLDL	615±122*	142±40*
oxLDL	110±47*	347±80*

Human nLDL was oxidized with CuSO_4 as described in Methods to obtain moxLDL, modLDL, and oxLDL. Values are mean±SD, n=30.

* $P<0.01$ compared with nLDL.

LDL (oxLDL), caused smooth muscle cell apoptosis within 6 hours; this apoptosis was attenuated markedly by pretreatment with vitamin C. In addition, cells treated with native LDL (nLDL) and the lipid hydroperoxide 13(S)-hydroperoxyoctadeca-9Z,11E-dienoic acid (HPODE) also underwent apoptosis, which was attenuated by vitamin C. Our findings may have important clinical implications for therapies designed to arrest LDL oxidation, because modLDL appears to be potentially more atherogenic than oxLDL. Moreover, vitamin C supplementation may provide protection against the cytotoxic effects of modLDL and lipid hydroperoxides.

Methods**Reagents**

Smooth muscle cell culture medium (MCDB 131) was obtained from Clonetics; cellular DNA fragmentation ELISA kit, from Boehringer Mannheim; ApoAlert annexin V apoptosis kit, from Clontech; HPODE, from Biomol Research Laboratories; Bcl- x_L polyclonal antibody, from Transduction Laboratories; and the enhanced chemiluminescence kit, from Amersham. All other reagents were of the highest analytical grade and were obtained from Sigma Chemical Co.

Isolation and Oxidation of LDL

LDL was isolated from the blood of healthy volunteers by sequential density ultracentrifugation (1.019 to 1.063 g/mL) in the presence of EDTA.²⁵ nLDL was diluted to 100 μg protein/mL in sterile PBS. nLDL was oxidized with CuSO_4 (5 $\mu\text{mol/L}$ net above the EDTA carried over from the LDL) at 37°C, and the conjugated diene content was determined every 15 minutes by removing an aliquot, measuring its absorbance at 234 nm (A_{234}), and then returning it to the LDL sample. When A_{234} increased by 0.2 above the initial value after 80 to 100 minutes, moxLDL was removed, 1 mmol/L EDTA was added to stop oxidation, and the sample was stored at 4°C. The remaining LDL was further oxidized for ≈ 3 hours until A_{234} reached a peak (an increase of 1.5 to 1.9). We defined this as modLDL, which contains the peak levels of lipid hydroperoxides.²⁶ The level of conjugated dienes closely follows the level of lipid hydroperoxides during the early stages of LDL oxidation.²⁷ A portion was removed, 1 mmol/L EDTA was added, and the sample was stored at 4°C. The remaining LDL was oxidized at 37°C for 24 hours before EDTA (1 mmol/L) was added to obtain what we define as oxLDL. During this time, the lipid hydroperoxides fall, but the later secondary products of lipid and protein oxidation increase.²⁶ Solid KBr was then dissolved in the oxidized LDLs to increase their densities to 1.2 g/mL. Chelex-100 was added as an extra precaution to inactivate transition metal ions. Once the KBr had dissolved, the Chelex-100 was removed by centrifugation at 250g for 10 minutes at 4°C. The oxidized LDL species were then centrifuged at 149 000g for 18 hours at 4°C to concentrate them. The oxidized LDL layer was removed and dialyzed overnight at 4°C in the dark against four 1-L volumes of phosphate buffer (140 mmol/L NaCl, 1.9 mmol/L

NaH_2PO_4 , 8.1 mmol/L Na_2HPO_4 , and 100 $\mu\text{mol/L}$ EDTA, pH 7.4). The oxidized LDL species were then filter-sterilized and stored at 4°C. LDL protein content was determined by a modified Lowry assay; lipid hydroperoxide content was determined by an iodometric method²⁸; and relative electrophoretic mobility was compared with nLDL, as an index of protein modification, using Beckman Paragon Lipo gels. The mobility of the LDL increased with the degree of oxidative modification, whereas the lipid hydroperoxide content was maximal in modLDL and significantly lower in moxLDL and oxLDL (see Table).

Culture of Vascular Smooth Muscle Cells

Human arterial smooth muscle cells (HUASMCs) were cultured from umbilical artery explants in MCDB131 medium supplemented with 10% (vol/vol) FCS, penicillin (100 U/mL), and streptomycin (100 $\mu\text{g/mL}$) at 37°C in a 5% CO_2 /95% air atmosphere. Cells were confirmed as smooth muscle by their typical "hill-and-valley" morphological features and by immunofluorescent staining for smooth muscle α -actin. HUASMCs between passages 3 and 7 were used in all experiments. Confluent, randomly cycling cells were incubated in the absence or presence of up to 300 μg protein/mL nLDL, moxLDL, modLDL, or oxLDL for 3 to 24 hours or nLDL in combination with HPODE (200 $\mu\text{mol/L}$) for 6 or 24 hours. Exposure to LDL was terminated by gently washing the cells twice with warmed PBS.

In experiments examining the effects of vitamin C, cells were pretreated with vitamin C (0 to 100 $\mu\text{mol/L}$) for 24 hours in serum-containing medium, monolayers were washed with warmed PBS, and cells were reincubated for an additional 3 to 24 hours in complete medium containing either defined LDL species or nLDL in combination with HPODE but in the absence of vitamin C.

Measurements of Cytotoxicity

Mitochondrial dehydrogenase activity was used as an index of cell viability and was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay.²⁹ Confluent HUASMCs were pretreated in the absence or presence of vitamin C (100 $\mu\text{mol/L}$, 24 hours), washed with PBS, and then incubated with defined LDL species (3 to 24 hours) in the absence of vitamin C. Culture medium was then replaced with fresh medium containing MTT (500 $\mu\text{g/mL}$), and cells were incubated at 37°C and 5% CO_2 for an additional 1 to 2 hours. DMSO was then added to each well, and the microtiter plates were incubated at room temperature for another 20 minutes before absorbance was measured at 540 nm.

Plasma membrane damage in HUASMCs was also assessed by monitoring the leakage of DNA fragments into the culture medium after treatment of confluent HUASMC monolayers with oxidized LDL for defined time periods. Proliferating cells in 96-well microtiter plates were labeled with 10 $\mu\text{mol/L}$ 5-bromo-2'-deoxyuridine (BrdU) for 24 hours, washed with PBS, and treated with LDL in the absence of BrdU for defined time periods. The culture medium was then collected, and labeled DNA fragments released by the cells were assayed using a DNA fragmentation ELISA (see below). Levels of fragmented DNA were expressed as absorbance at 450 nm.

Measurements of Apoptosis**DNA Fragmentation**

As described previously,³⁰ cellular DNA fragmentation was measured using a commercially available cellular DNA fragmentation ELISA kit. Proliferating cells in 96-well microtiter plates were labeled with 10 $\mu\text{mol/L}$ BrdU for 24 hours, washed with PBS, and treated in the absence of BrdU with nLDL or oxidized LDL for defined time periods. Cells were then washed with PBS and incubated with the kit lysis buffer (BSA, EDTA, and Tween 20) for 30 minutes at room temperature, and soluble BrdU-labeled DNA fragments present in the buffer were quantified using the ELISA. Briefly, an anti-DNA antibody was adsorbed onto the wells of a microtiter plate, and lysis buffer supernatants containing BrdU-labeled DNA fragments were allowed to bind the immobilized anti-DNA antibody. After washing, the immunocomplexed BrdU-labeled DNA fragments were denatured and fixed to the surface of the microtiter plate by microwave irradiation. Labeled fragments

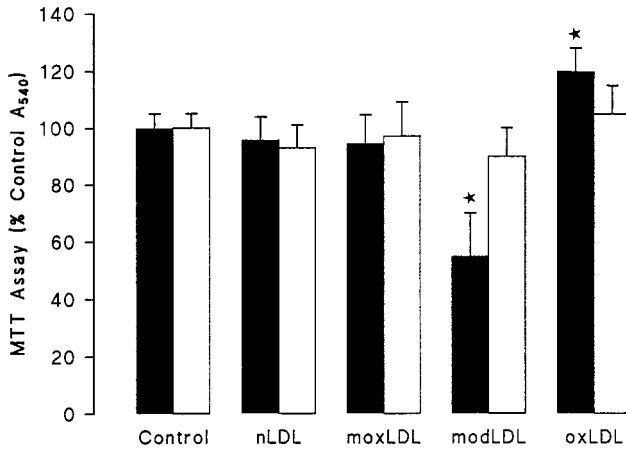


Figure 1. Effects of nLDL and oxidatively modified LDL species on human arterial smooth muscle cell viability and protective effects of vitamin C pretreatment. After pretreatment in the absence (■) or presence (□) of vitamin C (100 μ mol/L, 24 hours), cells were exposed to either nLDL, moxLDL, modLDL, or oxLDL (300 μ g protein/mL) for an additional 24 hours in the absence of vitamin C. Mitochondrial dehydrogenase activity (MTT) was assayed as an index of cell viability. Values are mean $\pm$ SE ($n=3$ to 6 cell cultures). * $P<0.05$ relative to control cells in the absence of vitamin C.

were detected using an anti-BrdU antibody conjugated to horseradish peroxidase. The amount of peroxidase bound to the immune complex was determined by addition of tetramethyl benzidine substrate, and DNA fragmentation was expressed as absorbance at 450 nm.

Annexin V Binding

Active externalization of phosphatidylserine to the cell surface is an early event in apoptotic cell death, and annexin V binding to phosphatidylserine can be used as a marker of early apoptotic events that precede nuclear changes.³¹ In this study, binding of FITC-conjugated annexin V to HUASMCs treated with nLDL, oxidized LDL, or nLDL combined with HPODE was assessed using a commercially available kit (ApoAlert annexin V, Clontech). Cells were also costained with propidium iodide as a marker of cell membrane permeability, which occurs during the later stages of apoptosis and in necrosis. Cells were washed with PBS and incubated with FITC-annexin V (0.5 μ g/mL) or propidium iodide (0.125 μ g/mL). Staining was determined by fluorescence microscopy using dual-filter sets for FITC and rhodamine to visualize annexin V binding and nuclear propidium iodide staining, respectively.

Bcl-x_L Protein Expression

Changes in protein expression of the antiapoptotic mediator Bcl-x_L³² were determined by Western blot analysis in cells treated with LDL. In brief, confluent HUASMCs were treated with oxidized LDL for defined periods, and incubations were terminated by washing cells with ice-cold PBS. Cells were lysed in buffer (2% wt/vol SDS, 10% vol/vol glycerol, 50 mmol/L Tris-HCl, and 1 mmol/L PMSF, pH 6.8), and the lysate was heated at 100°C for 5 minutes. Sample protein content was determined, and samples were heated at 100°C in a mixture of 1% 2-mercaptoethanol and 0.05% bromophenol blue for an additional 3 minutes. Equal protein amounts were separated by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed with a polyclonal rabbit anti-rat Bcl-x_L antibody. A horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was used in conjunction with enhanced chemiluminescence to visualize the 26-kDa Bcl-x_L bands on autoradiographic film, and the level of expression was quantified using image analysis software.

Statistical Analysis

Statistical variance from the mean was determined using the normal distribution and represented by mean $\pm$ SE (n =number of different cell cultures). When 2 values were compared, confidence limits were

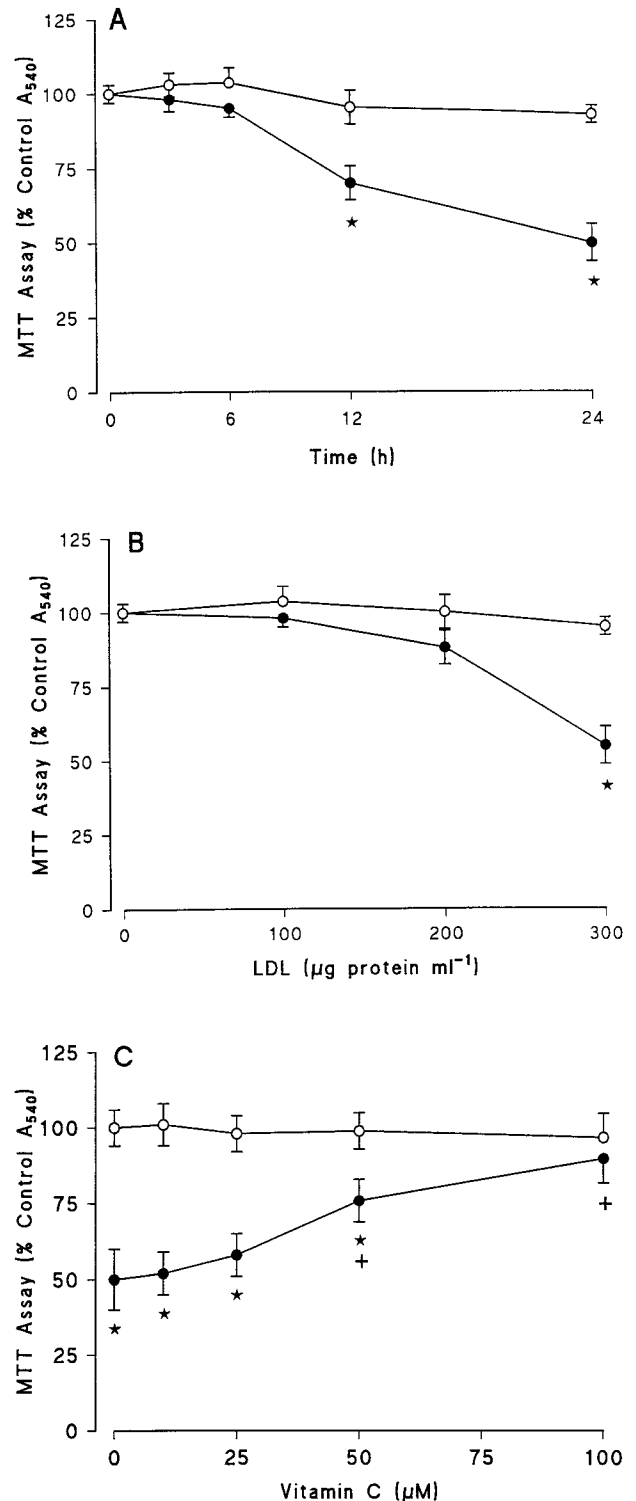


Figure 2. Vitamin C protects against cytotoxic effects of modLDL in human arterial smooth muscle cells. After pretreatment in the absence or presence of vitamin C, cells were exposed to either nLDL or modLDL for an additional 24 hours in the absence of vitamin C. Mitochondrial dehydrogenase activity (MTT) was assayed as an index of cell viability. A and B, Time-dependent (0 to 24 hours, 300 μ g protein/mL) and concentration-dependent (0 to 300 μ g protein/mL, 24 hours) effects of nLDL (○) or modLDL (●). C, Effects of vitamin C (0 to 100 μ mol/L, 24 hours) pretreatment on nLDL-induced (○) or modLDL-induced (●) (300 μ g protein/mL, 24 hours) cytotoxicity. Values are mean $\pm$ SE ($n=3$ to 6 cell cultures). * $P<0.05$ relative to respective values in cells treated with nLDL; + $P<0.05$ relative to cells treated with modLDL in the absence of vitamin C.

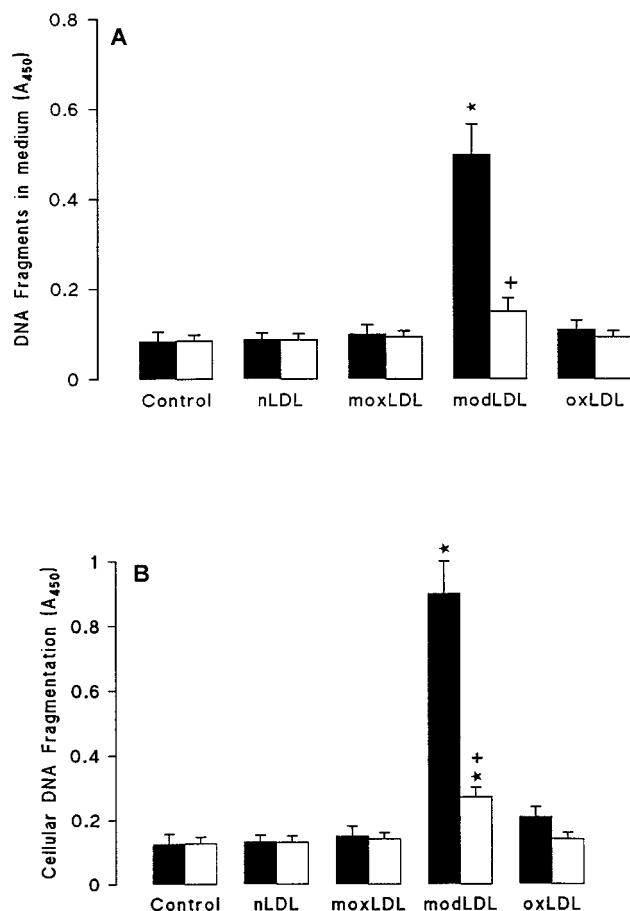


Figure 3. Effects of nLDL and oxidatively modified LDL species on release of fragmented DNA and intracellular DNA fragmentation in human arterial smooth muscle cells. After pretreatment in the absence (■) or presence (□) of vitamin C (100 μ mol/L, 24 hours), cells were exposed to either nLDL, moxLDL, modLDL, or oxLDL (300 μ g protein/mL) for an additional 24 hours, and release of fragmented DNA into the culture medium (A) or intracellular DNA fragmentation (B) was determined by ELISA. Values are mean $\pm$ SE (n=3 to 6 cell cultures). * P <0.05 relative to control cells incubated in the absence of vitamin C; + P <0.05 relative to cells treated with modLDL in the absence of vitamin C.

established using the unpaired Student's t test. ANOVA was used to compare multiple groups. Statistical significance between data sets was established at P <0.05.

Results

LDL-Mediated Cytotoxicity

The activity of mitochondrial dehydrogenases (MTT assay) was significantly decreased after incubation of HUASMCs with modLDL (300 μ g protein/mL) for 24 hours, whereas nLDL and moxLDL had negligible effects and oxLDL increased mitochondrial dehydrogenase activity above basal levels (Figure 1). The decrease in MTT activity induced by modLDL was time-dependent (0 to 24 hours) and concentration-dependent (0 to 300 μ g protein/mL) (Figure 2A and 2B). Pretreatment of cells with vitamin C (100 μ mol/L, 24 hours) before LDL exposure abolished the changes in MTT activity induced by modLDL and oxLDL (Figure 1), with maximal effects observed at vitamin C concentrations >50 μ mol/L (Figure 2C). Plasma membrane permeability, assessed by release of fragmented DNA into the culture medium, was increased in HUASMCs treated with

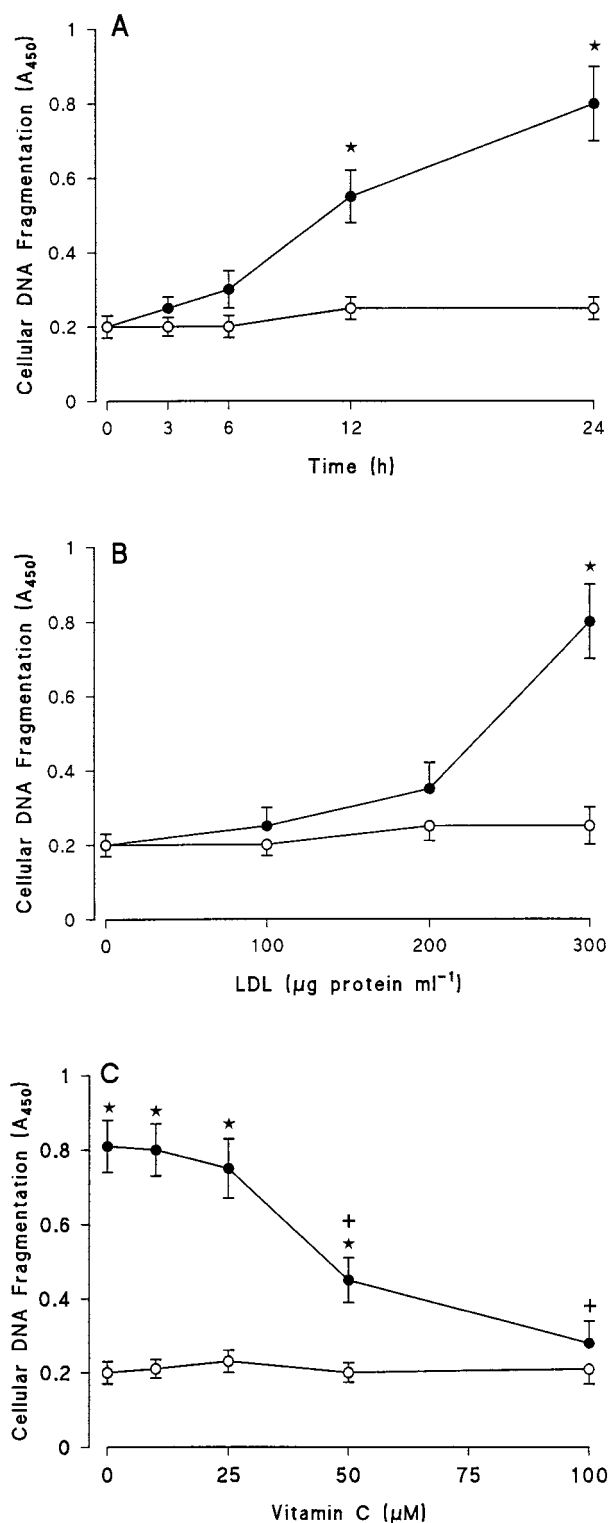


Figure 4. Vitamin C inhibits DNA fragmentation induced by modLDL. After pretreatment in the absence or presence of vitamin C (0 to 100 μ mol/L, 24 hours), cells were exposed to either nLDL or modLDL for an additional 24 hours, and cellular DNA fragmentation was determined by ELISA. A and B, Time-dependent (0 to 24 hours, 300 μ g protein/mL) and concentration-dependent (0 to 300 μ g protein/mL, 24 hours) effects of nLDL (○) or modLDL (●). C, Effects of vitamin C (0 to 100 μ mol/L, 24 hours) pretreatment on nLDL-induced (○) or modLDL-induced (●) (300 μ g protein/mL, 24 hours) DNA fragmentation. Values are mean $\pm$ SE (n=3 to 6 cell cultures). * P <0.05 relative to respective values in cells treated with native LDL; + P <0.05 relative to cells treated with modLDL in the absence of vitamin C.

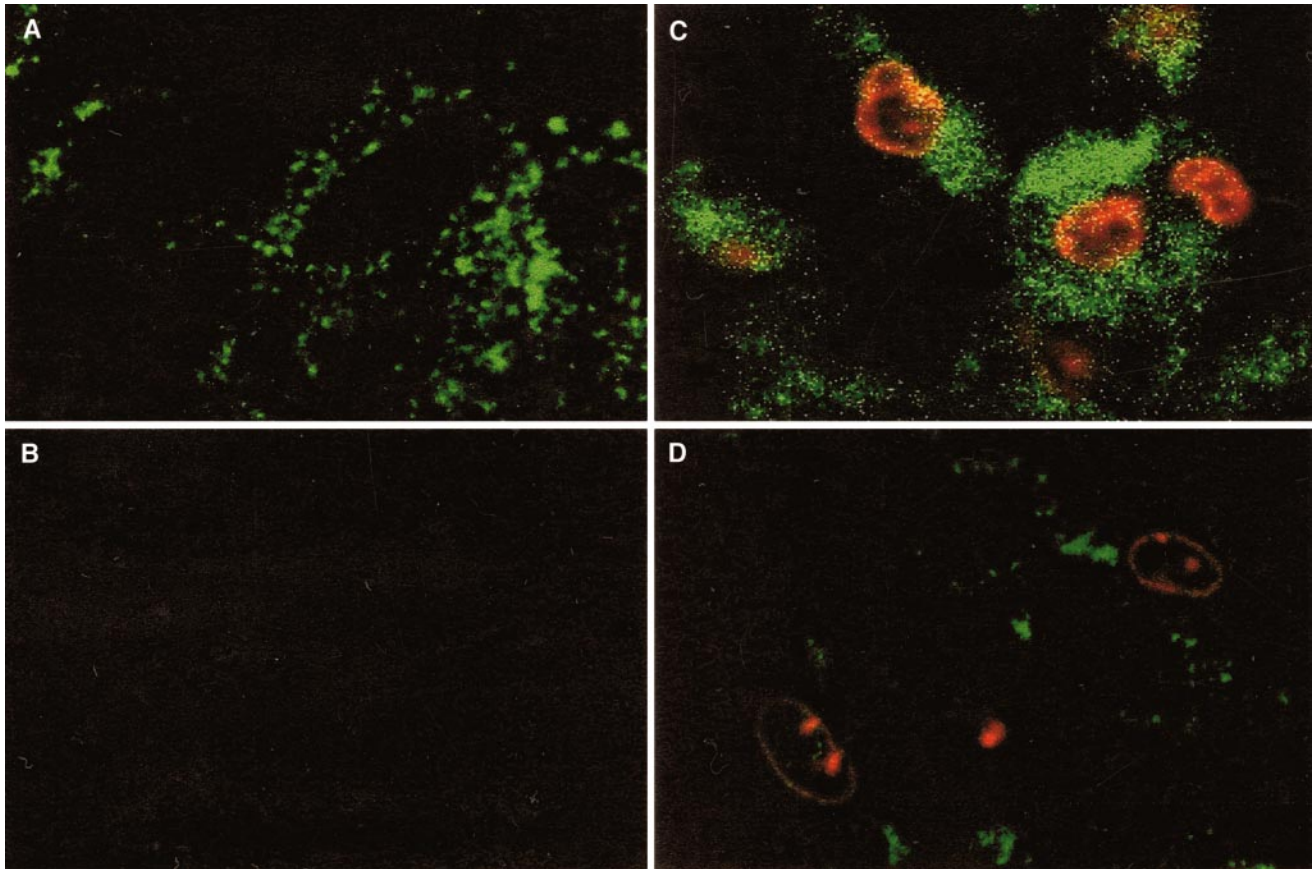


Figure 5. Vitamin C attenuates of annexin V binding and propidium iodide staining induced by modLDL. Cells were pretreated for 24 hours in the absence (A, C) or presence (B, D) of 100 $\mu\text{mol/L}$ vitamin C before exposure to modLDL (300 μg protein/mL) for 6 hours (A, B) or 24 hours (C, D). Annexin V binding (green) and propidium iodide staining (red) were determined by fluorescence microscopy as described in Methods. Micrographs are representative of 3 experiments. Total image width is 230 μm .

modLDL (300 μg protein/mL, 24 hours) but not in those treated with nLDL, moxLDL, or oxLDL (Figure 3A). This release of DNA induced by modLDL was abolished after preincubation of cells with vitamin C (100 $\mu\text{mol/L}$, 24 hours) (Figure 3A).

LDL- and HPODE-Mediated Apoptosis

modLDL (300 μg protein/mL, 24 hours) caused an increase in cellular DNA fragmentation (Figure 3B) that was both time-dependent (Figure 4A) and concentration-dependent (Figure 4B). nLDL, moxLDL, and oxLDL had negligible effects on cellular DNA fragmentation (Figure 3B). Pretreatment of cells with vitamin C for 24 hours attenuated DNA fragmentation induced by modLDL (Figure 3B), with vitamin C providing protection at concentrations >50 $\mu\text{mol/L}$ (Figure 4C). Binding of annexin V, a marker of early apoptosis, was observed in cells treated for 6 or 24 hours with modLDL (300 μg protein/mL) (Figure 5A and 5C), whereas no binding was detected in HUASMCs treated with nLDL, moxLDL, or oxLDL (300 μg protein/mL) (data not shown). Moreover, cells treated with modLDL for 24 hours, but not for 6 hours, were permeable to propidium iodide (Figure 5C), suggesting that cells progressed to a late stage of apoptosis after prolonged exposure to modLDL. Vitamin C pretreatment (100 $\mu\text{mol/L}$, 24 hours) abolished the early (6 hours) annexin V binding (Figure 5B) and attenuated propidium iodide staining (Figure 5D) in cells exposed to modLDL for 24 hours. Moreover, binding of annexin V was also observed

when smooth muscle cells were treated for 6 or 24 hours with nLDL (300 μg protein/mL) together with 200 $\mu\text{mol/L}$ HPODE (Figure 6A and 6C), a level comparable to the lipid hydroperoxide content in 300 μg protein/mL modLDL. In addition, cells treated with nLDL and HPODE for 24 hours, but not for 6 hours, were also permeable to propidium iodide (Figure 6C), indicating late-stage apoptosis. Pretreatment with vitamin C (100 $\mu\text{mol/L}$, 24 hours) abolished the early (6 hours) annexin V binding (Figure 6B) and propidium iodide staining (Figure 6D) in cells exposed to HPODE for 24 hours.

Bcl-x_L Expression

Basal expression of the antiapoptotic mediator Bcl-x_L was unaltered in HUASMCs treated for up to 24 hours with nLDL, moxLDL, or oxLDL (300 μg protein/mL, data not shown), whereas treatment with modLDL for 24 hours significantly decreased Bcl-x_L expression (Figure 7A and 7B). Pretreatment of cells with vitamin C (100 $\mu\text{mol/L}$, 24 hours) prevented the decrease in Bcl-x_L expression induced by modLDL and appeared to slightly elevate Bcl-x_L expression, although this increase was not significant. The lower-molecular-weight band observed may reflect nonspecific cross-reactivity of the antibody with the closely related Bcl-x_S protein, but this was not investigated further.

Discussion

This study established that modLDL and the lipid hydroperoxide HPODE, but neither nLDL, moxLDL, nor oxLDL,

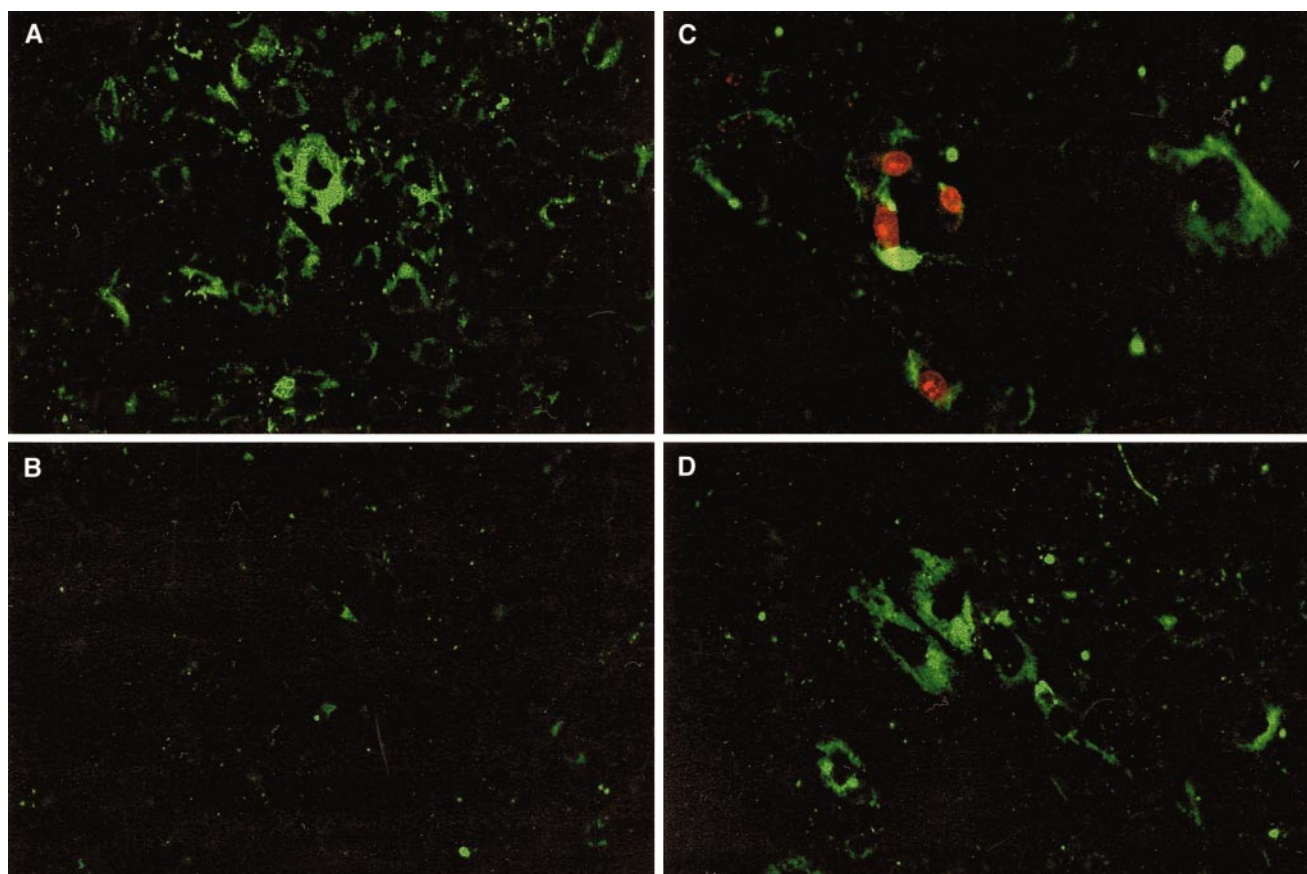


Figure 6. Vitamin C attenuates of annexin V binding and propidium iodide staining induced by nLDL in combination with HPODE. Cells were pretreated for 24 hours in the absence (A, C) or presence (B, D) of 100 $\mu\text{mol/L}$ vitamin C before exposure to nLDL (300 μg protein/mL) and HPODE (200 $\mu\text{mol/L}$) for 6 hours (A, B) or 24 hours (C, D). Annexin V binding (green) and propidium iodide staining (red) were determined by fluorescence microscopy as described in Methods. Micrographs are representative of 3 experiments. Total image width is 460 μm .

induced apoptosis of human vascular smooth muscle cells. Our findings suggest that the high lipid hydroperoxide content of modLDL is more cytotoxic to smooth muscle cells than secondary lipid oxidation products, such as aldehydes, ketones, and lipid alcohols, generated during extended copper oxidation of LDL.^{27,33} Moreover, pretreatment of cells with physiologically relevant vitamin C concentrations attenuated the cytotoxicity induced by modLDL and HPODE. The protection against smooth muscle cell apoptosis provided by vitamin C may reflect that this dietary antioxidant maintained cellular levels of the antiapoptotic mediator Bcl- x_L .³²

Increased apoptotic cell death within atherosclerotic lesions and its contribution to plaque rupture and accumulation of the necrotic lipid core has been established.^{7,34} LDL is oxidized within lesions³⁵ and contributes to the impaired vascular cell function observed in atherogenesis.^{3,4} Although the toxicity of LDL increases with the extent of LDL oxidation,¹³ the methods used to oxidize LDL vary considerably between different laboratories and are often not well defined.³⁶ In this study, we used a well-defined protocol for LDL oxidation^{24,36} to obtain mildly, moderately, and highly oxidized LDL species. The method relies on oxidizing a low concentration of LDL (100 μg protein/mL) with copper; the kinetics of LDL oxidation under these conditions have been characterized in detail elsewhere^{27,37} (see Table). In agreement with our observations in human smooth muscle cells, Kuzuya et al³⁸ reported that the toxicity of LDL to endothelial

cells initially increases and then decreases with time of LDL oxidation. The enhanced mitochondrial enzyme activity observed in human smooth muscle cells treated with oxLDL may have arisen from increased mitochondrial metabolism of oxysterols present in higher amounts in oxLDL.³⁹ Although some studies have reported that oxidized LDL enhances smooth muscle cell proliferation,^{13,17} this seems an unlikely explanation for our findings because protein concentrations were similar in cell monolayers treated with the different LDL species.

Recent studies have identified the presence of epitopes of LDL, oxidized by copper to increasing extents, within animal and human atherosclerotic lesions and in circulating plasma LDL.^{40,41} Chisholm et al⁴² proposed that the cytotoxicity of copper-oxidized LDL to cells is mainly due to cholesterol hydroperoxides, which may be more cytotoxic than hydroxycholesterols or ketocholesterols. We showed that oxLDL, which contains a higher level of hydroxycholesterols and ketocholesterols than modLDL,³³ does not induce human smooth muscle cell apoptosis. Our findings contrast with those of Nishio and Watanabe⁴³ and Harada-Shiba et al,⁸ who reported that oxidized LDL species induced smooth muscle and endothelial cell apoptosis. This discrepancy may reflect the differences in the degree of LDL oxidation and the criteria used to define oxidized LDL. Nevertheless, it is interesting that 7-ketocholesterol and 25-hydroxycholesterol have been shown to induce endothelial and smooth muscle cell apopto-

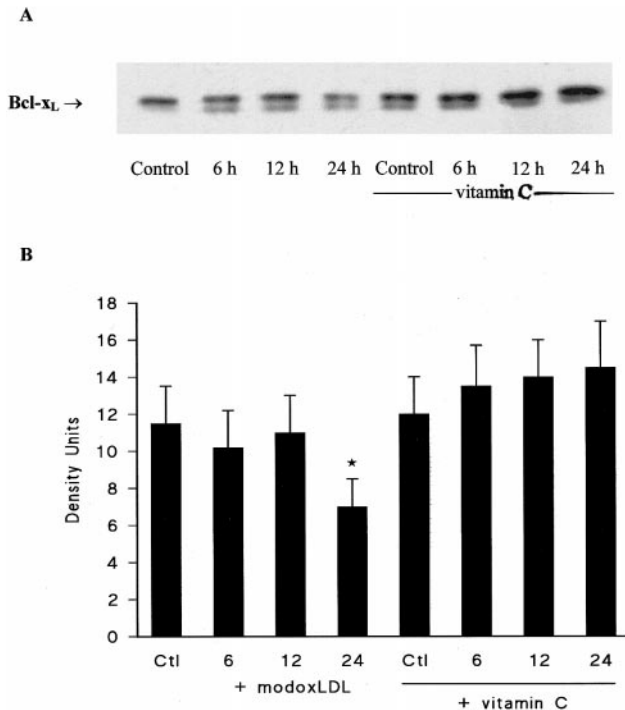


Figure 7. Vitamin C maintains Bcl-x_L expression in human smooth muscle cells treated with oxidatively modified LDL. Cells were pretreated in the absence or presence vitamin C (100 μ mol/L, 24 hours) before exposure to modLDL (300 μ g protein/mL) for 6, 12, or 24 hours or to culture medium alone (control). Bcl-x_L expression was determined by Western blot analysis (A) and quantified by densitometry (B). Values are mean $\pm$ SE (n=3 cell cultures). * P <0.05 relative to untreated control cells.

sis.^{8,43} On the basis of the results of our study, using standardized methods for oxidizing LDL,³⁶ we conclude that cholesterol, cholesteryl esters, or phospholipids with a hydroperoxyl group may be more cytotoxic to human smooth muscle cells than those with a hydroxyl or ketone group. Although studies in rat pheochromocytoma cells⁴⁴ and human T cell lines⁴⁵ have demonstrated that lipid hydroperoxides can induce apoptotic cell death, similar studies in vascular smooth muscle cells are lacking. To our knowledge, our experiments provide the first evidence that HPODE can induce apoptosis of human smooth muscle cells.

Apoptosis of vascular endothelial and smooth muscle cells induced by oxidized LDL species involves activation of caspase-like proteases^{6,43} and downregulation of the antiapoptotic protein Bcl-2.^{43,46} The latter protein is known to act as an endogenous antioxidant, inhibiting lipid peroxidation in apoptosis.⁴⁷ We here report the first evidence that modLDL, in contrast to moxLDL and oxLDL, decreases expression of the closely related antiapoptotic mediator Bcl-x_L³² in human smooth muscle cells (Figure 7). These findings are consistent with the observed apoptosis of smooth muscle cells induced by modLDL (Figures 2, 3, and 4). Vitamin C pretreatment abolished the decrease in Bcl-x_L protein levels and markedly inhibited cellular DNA fragmentation and annexin V binding to smooth muscle cells treated with modLDL. Our results thus provide further mechanistic evidence of the cytoprotective effects of vitamin C against oxidized LDL-induced smooth muscle dysfunction in atherosclerosis. Dimmeler et al⁶ previously reported that a combination of vitamins C and E

prevented the endothelial cell apoptosis induced by oxidized LDL by inhibiting CPP32 activation. Moreover, these vitamins also prevent endothelial cell apoptosis induced by lipopolysaccharide by preserving Bcl-2 protein levels.⁴⁸ Thus, vitamin C could protect against apoptosis induced by oxidative stress and lipid hydroperoxides by modulating mediators of apoptosis. It was recently shown that enhanced expression of Bcl-x_L protects against atherosclerosis and endothelial cell activation,⁴⁹ so maintained or enhanced expression of antiapoptotic genes by vitamin C supplementation may be protective in atherosclerosis.

The extent of LDL oxidation within atherosclerotic lesions remains a controversial issue, yet our findings suggest that protein modifications associated with oxLDL are not necessarily required for LDL-induced cytotoxicity. Although antioxidant dietary vitamins protect against vascular dysfunction and plaque instability^{22,23} and reduce the oxidative modification of LDL,²⁶ it is also possible that LDL modification may be arrested at a more atherogenic "moderately oxidized" level by vitamin supplementation. Thus, modLDL could induce more smooth muscle cells within the lesion to undergo apoptosis than oxLDL. Prolonged dietary antioxidant supplementation may possibly have different effects on advanced atherosclerotic lesions, because initially antioxidants would inhibit oxidative modification of LDL in the arterial wall and limit the formation of lesions. It is beyond the scope of this study to determine whether antioxidants such as vitamin C are proatherogenic through the arrest of LDL oxidation and inhibition of neointimal smooth muscle cell apoptosis. Our study supports the current consensus that antioxidant consumption reduces coronary heart disease,⁵⁰ although additional clinical trials seem warranted to establish the efficacy of vitamin C against accumulation of necrotic debris within the lesion core and instability of the fibrous plaque in atherosclerosis.

Acknowledgments

This work was supported in part by the British Heart Foundation (PG95148), Ministry of Agriculture Fisheries and Food (ANO413), and a Wellcome Trust Research Leave Fellowship (D.S.L.). We thank Dr John Murphy for helpful comments on the manuscript and for providing the Bcl-x_L antibody, Dr V. Moens for taking blood samples, and Dr E. Ruiz for assistance with some of the experiments.

References

- Yla-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witztum JL, Steinberg D. Evidence for the presence of oxidatively modified low-density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest.* 1989;84:1086–1095.
- Witztum JL, Steinberg D. Role of oxidized low-density-lipoprotein in atherosclerosis. *J Clin Invest.* 1991;88:1785–1792.
- Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature.* 1993;362:801–809.
- Steinberg D. Low density lipoprotein oxidation and its pathobiological significance. *J Biol Chem.* 1997;272:20963–20966.
- Hajjar DP, Haberland ME. Lipoprotein trafficking in vascular cells: molecular Trojan horses and cellular saboteurs. *J Biol Chem.* 1997;272:22975–22978.
- Dimmeler S, Haendeler J, Galle J, Zeiher AM. Oxidized low-density lipoprotein induces apoptosis of human endothelial cells by activation of CPP32-like proteases: a mechanistic clue to the 'response to injury' hypothesis. *Circulation.* 1997;95:1760–1763.
- Jovinge S, Crisby M, Thyberg J, Nilsson J. DNA fragmentation and ultrastructural changes of degenerating cells in atherosclerotic lesions and smooth muscle cells exposed to oxidized LDL in vitro. *Arterioscler Thromb Vasc Biol.* 1997;17:2225–2231.
- Harada-Shiba M, Kinoshita M, Kamido H, Shimokado K. Oxidized low density lipoprotein induces apoptosis in cultured human umbilical vein

- endothelial cells by common and unique mechanisms. *J Biol Chem*. 1998;273:9681–9687.
9. Bennett MR, Evan GI, Schwartz SM. Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. *J Clin Invest*. 1995;95:2266–2274.
 10. Bjorkerud S, Bjorkerud B. Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability. *Am J Pathol*. 1996;149:367–380.
 11. Pollman MJ, Hall JL, Mann MJ, Zhang LN, Gibbons GH. Inhibition of neointimal cell *bcl-x* expression induces apoptosis and regression of vascular disease. *Nature Med*. 1998;4:222–227.
 12. 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;75:11–16.
 13. Bjorkerud B, Bjorkerud S. Contrary effects of lightly and strongly oxidized LDL with potent promotion of growth versus apoptosis on arterial smooth muscle cells, macrophages, and fibroblasts. *Arterioscler Thromb Vasc Biol*. 1996;16:416–424.
 14. Cox DA, Cohen ML. Effects of oxidized low-density lipoprotein on vascular contraction and relaxation: clinical and pharmacological implications in atherosclerosis. *Pharmacol Rev*. 1996;48:3–19.
 15. Chatterjee S. Role of oxidized human plasma low-density lipoproteins in atherosclerosis: effects on smooth-muscle cell-proliferation. *Mol Cell Biochem*. 1992;111:143–147.
 16. Schmitt A, Salvayre R, Delchambre J, Negre-Salvayre A. Prevention by alpha-tocopherol and rutin of glutathione and ATP depletion induced by oxidized LDL in cultured endothelial cells. *Br J Pharmacol*. 1995;116:1985–1990.
 17. Auge N, Pieraggi MT, Thiers JC, Negresalvayre A, Salvayre R. Proliferative and cytotoxic effects of mildly oxidized low-density lipoproteins on vascular smooth-muscle cells. *Biochem J*. 1995;309:1015–1020.
 18. Marchant CE, Law NS, Vanderveen C, Hardwick SJ, Carpenter KLH, Mitchinson MJ. Oxidized low-density lipoprotein is cytotoxic to human monocyte-macrophages: protection with lipophilic antioxidants. *FEBS Lett*. 1995;358:175–178.
 19. Nishio E, Arimura S, Watanabe Y. Oxidized LDL induces apoptosis in cultured smooth muscle cells: a possible role for 7-ketocholesterol. *Biochem Biophys Res Commun*. 1996;223:413–418.
 20. Vieira O, Escargueil-Blanc I, Meilhac O, Basile JP, Laranjinha J, Almeida L, Salvayre R, Negre-Salvayre A. Effect of dietary phenolic compounds on apoptosis of human cultured endothelial cells induced by oxidized LDL. *Br J Pharmacol*. 1998;123:565–573.
 21. Guyton JR, Lenz ML, Mathews B, Hughes H, Karsan D, Selinger E, Smith CV. Toxicity of oxidized low-density lipoproteins for vascular smooth muscle cells and partial protection by antioxidants. *Atherosclerosis*. 1995;118:237–249.
 22. Levine GN, Frei B, Koulouris SN, Gerhard MD, Keaney Jr JF, Vita JA. Ascorbic acid reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation*. 1996;93:1107–1113.
 23. Vita JA, Keaney JF, Raby KE, Morrow JD, Freedman JE, Lynch S, Koulouris SN, Hankin BR, Frei B. Low plasma ascorbic acid independently predicts the presence of an unstable coronary syndrome. *J Am Coll Cardiol*. 1998;31:980–986.
 24. Siow RCM, Sato H, Bannai S, Leake DS, Pearson JD, Mann GE. Vitamin C protects human arterial smooth muscle cells against atherogenic lipoproteins: effects of antioxidant vitamins on oxidized LDL-induced increases in cystine transport and glutathione. *Arterioscler Thromb Vasc Biol*. 1998;18:1662–1670.
 25. Wilkins GM, Leake DS. The effect of inhibitors of free radical generating-enzymes on low density lipoprotein oxidation by macrophages. *Biochim Biophys Acta*. 1994;1211:69–78.
 26. Esterbauer H, Gebicki J, Puhl H, Jurgens G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic Biol Med*. 1992;13:341–390.
 27. Esterbauer H, Striegl G, Puhl H, Rotheneder M. Continuous monitoring of in vitro oxidation of human low-density lipoprotein. *Free Radic Res Commun*. 1989;6:67–75.
 28. El-Saadani M, Esterbauer H, El-Sayed M, Goher M, Nassar AY, Jurgens GA. Spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. *J Lipid Res*. 1989;30:627–630.
 29. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods*. 1986;89:271–277.
 30. Ortiz EM, Dusetti NJ, Vasseur S, Malka D, Bodeker H, Dagorn JC, Iovanna JL. The pancreatitis-associated protein is induced by free radicals in AR4-2J cells and confers cell resistance to apoptosis. *Gastroenterology*. 1998;114:808–816.
 31. Martin SJ, Reutelingsperger CPM, McGahon AJ, Rader JA, Vanschrie RC, LaFace DM, Green DR. Early redistribution of plasma-membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med*. 1995;182:1545–1556.
 32. Minn AJ, Boise LH, Thompson CB. Bcl-x_s antagonizes the protective effects of Bcl-x_L. *J Biol Chem*. 1996;271:6306–6312.
 33. Carpenter KLH, Wilkins GM, Fussell B, Ballantine JA, Taylor SE, Mitchinson MJ, Leake DS. Production of oxidized lipids during modification of low-density lipoprotein by macrophages or copper. *Biochem J*. 1994;304:625–633.
 34. Mitchinson MJ, Hardwick SJ, Bennett MR. Cell death in atherosclerotic plaques. *Curr Opin Lipidol*. 1996;7:324–329.
 35. Palinski W, Rosenfeld ME, Yla-Herttuala S, Gurtner GC, Socher SS, Butler SW, Parthasarathy S, Carew TE, Steinberg D, Witztum JL. Low-density lipoprotein undergoes oxidative modification in vivo. *Proc Natl Acad Sci U S A*. 1989;86:1372–1376.
 36. Rice-Evans CA, Leake DS, Bruckdorfer KR, Diplock AT. Practical approaches to low density lipoprotein oxidation: whys, wherefores and pitfalls. *Free Radic Res*. 1996;25:285–311.
 37. Jessup W, Rankin SM, de Whalley CV, Hoult JRS, Scott J, Leake DS. α -Tocopherol consumption during low density lipoprotein oxidation. *Biochem J*. 1990;265:399–405.
 38. Kuzuya M, Naito M, Funaki C, Hayashi T, Asai K, Kuzuya F. Lipid peroxide and transition metals are required for the toxicity of oxidized low-density lipoprotein to cultured endothelial cells. *Biochim Biophys Acta*. 1991;1096:155–161.
 39. Axelsson M, Larsson O. Low density lipoprotein (LDL) cholesterol is converted to 27-hydroxycholesterol in human fibroblasts: evidence that 27-hydroxycholesterol can be an important intracellular mediator between LDL and the suppression of cholesterol production. *J Biol Chem*. 1995;270:15102–15110.
 40. Yla-Herttuala S, Palinski W, Butler SW, Picard S, Steinberg D, Witztum JL. Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL. *Arterioscler Thromb*. 1994;14:32–40.
 41. Palinski W, Horkko S, Miller E, Steinbrecher UP, Powell HC, Curtiss LK, Witztum JL. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice: demonstration of epitopes of oxidized low-density lipoprotein in human plasma. *J Clin Invest*. 1996;98:800–814.
 42. Chisolm GM, Ma GP, Irwin KC, Martin LL, Gunderson KG, Linberg LF, Morel DW, Dicorleto PE. 7-Beta-hydroperoxycholesterol-5-en-3-beta-ol, a component of human atherosclerotic lesions, is the primary cytotoxin of oxidized human low-density lipoprotein. *Proc Natl Acad Sci U S A*. 1994;91:11452–11456.
 43. Nishio E, Watanabe Y. Oxysterols induced apoptosis in cultured smooth muscle cells through CPP32 protease activation and bcl-2 protein down-regulation. *Biochem Biophys Res Commun*. 1996;226:928–934.
 44. Aoshima H, Satoh T, Sakai N, Yamada M, Enokido Y, Ikeuchi T, Hatanaka H. Generation of free radicals during lipid hydroperoxide-triggered apoptosis in PC12h cells. *Biochim Biophys Acta*. 1997;1345:35–42.
 45. Sandstrom PA, Pardi D, Tebbey PW, Dudek RW, Terrian DM, Folks T, Buttkie TM. Lipid hydroperoxide-induced apoptosis: lack of inhibition by Bcl-2 overexpression. *FEBS Lett*. 1995;365:66–70.
 46. Harada K, Ishibashi S, Miyashita T, Osuga J, Yagyu H, Ohashi K, Yazaki Y, Yamada N. Bcl-2 protein inhibits oxysterol-induced apoptosis through suppressing CPP32-mediated pathway. *FEBS Lett*. 1997;411:63–66.
 47. Hockenbery DM, Oltvai ZN, Yin XM, Millman CL, Korsmeyer SJ. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell*. 1993;75:241–251.
 48. Haendeler J, Zeiher AM, Dimmeler S. Vitamin C and E prevent lipopolysaccharide-induced apoptosis in human endothelial cells by modulation of Bcl-2 and Bax. *Eur J Pharmacol*. 1996;317:407–411.
 49. Hancock WW, Buelow R, Sayegh MH, Turka LA. Antibody-induced transplant atherosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes. *Nature Med*. 1998;4:1392–1396.
 50. Tribble DL. Antioxidant consumption and risk of coronary heart disease: emphasis on vitamin C, vitamin E, and β -carotene. *Circulation*. 1999;99:591–595.

Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

Vitamin C Protects Human Vascular Smooth Muscle Cells Against Apoptosis Induced by Moderately Oxidized LDL Containing High Levels of Lipid Hydroperoxides

Richard C. M. Siow, Justin P. Richards, Kevin C. Pedley, David S. Leake and Giovanni E. Mann

Arterioscler Thromb Vasc Biol. 1999;19:2387-2394

doi: 10.1161/01.ATV.19.10.2387

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 1999 American Heart Association, Inc. All rights reserved.

Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://atvb.ahajournals.org/content/19/10/2387>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:
<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
<http://atvb.ahajournals.org/subscriptions/>