# Proliferative and cytotoxic effects of mildly oxidized low-density lipoproteins on vascular smooth-muscle cells

Nathalie AUGÉ,* Marie-Thérèse PIERAGGI,† Jean-Claude THIERS,* Anne NÈGRE-SALVAYRE* and Robert SALVAYRE*‡

Laboratory of Biochemistry, Metabolic Disease Laboratory (*INSERM CJF-9206 and †CNRS URA-74), Faculty of Medicine in Rangueil, University Paul Sabatier, 31054 Toulouse Cédex, France

We have investigated the role of low-density lipoprotein (LDL) oxidation in the proliferative effect of LDLs on cultured bovine aortic smooth-muscle cells and compared it with their effect on bovine aortic endothelial cells. The following conclusions were reached. (1) Non-toxic doses of mildly oxidized LDLs elicit a proliferative effect on smooth-muscle cells significantly higher than that of native LDLs or lipoprotein-depleted serum. The proliferative effect is dependent on time (relatively slow), dose (high doses are cytotoxic) and the level of LDL oxidation. (2) The proliferative effect on smooth-muscle cells is counterbalanced at high concentrations of mildly oxidized LDLs (or at high oxidation levels) by their cytotoxic effect. (3) The same dose of mildly oxidized LDLs exhibits no proliferative effect on endothelial cells but rather a cytotoxic one. Endothelial cells may therefore be intrinsically more susceptible to the cytotoxic effect of mildly oxidized LDLs than are smooth-muscle cells. (4) The proliferative effect of native LDLs on smooth-muscle cells results (at least in part) from cell-induced LDL oxidation during cell culture as suggested by (i) the progressive LDL oxidation over the 3 days of contact between LDLs and smooth-muscle cells and (ii) the concomitant inhibition of LDL oxidation and proliferative effect by butylated hydroxytoluene. The hypothetical mechanisms and potential involvement in atherogenesis are discussed.

## INTRODUCTION

Smooth-muscle-cell migration and proliferation in the intima of arterial walls are critical events in the development of atherosclerotic plaque [1]. Oxidized low-density lipoproteins (LDLs) are thought to play an important role in atherogenesis [2–6], namely in the recruitment of monocytes and foam-cell formation [2,4,7], the chemotaxis of smooth-muscle cells [8] which accumulate in the subendothelial area [1] and the cytotoxicity to cultured cells [9–13]. A major question is do oxidized LDLs directly promote proliferation of smooth-muscle cells? LDLs have been shown to induce general cellular activation [14] of growth-related responses in smooth-muscle cells. We have reported that oxidized LDLs elicit a sustained increase in cytosolic $Ca^{2+}$, even at non-toxic doses [13]. As a $Ca^{2+}$ signal has often been reported to be associated with the proliferative response [15], it was of interest to investigate the potential proliferative effect of oxidized LDLs compared with unoxidized LDLs. Recent independent reports have shown that native LDLs [16,17] and extensively oxidized LDLs [18] are able to induce proliferation of cultured rabbit aortic smooth-muscle cells, but, to our knowledge, the relative effects of non-oxidized and oxidized LDLs in the mitogenic effect have never been reported.

The present study was designed to investigate the role of LDL oxidation in the proliferative and cytotoxic effects of LDLs on vascular smooth-muscle and endothelial cells. We report that oxidized LDLs exhibit a biphasic effect on vascular smooth-muscle cells; a predominantly proliferative effect at low concentration and a cytotoxic effect at high concentration. In contrast, cytotoxicity was the main effect of oxidized LDLs on cultured endothelial cells. The relevance of the reported data to atherogenesis is discussed.

## MATERIALS AND METHODS

### Materials

Trypan Blue dye was purchased form Sigma (L'Isle d'Abeau-Chesnes, France), [$^3$H]thymidine (5 Ci/mmol) from Amersham (Paris, France), Azure-II Blue from Fluka (St. Quentin Fallavier, France), Isoton-II from Coultronics (Paris, France), RPMI 1640, Phenol Red-free RPMI 1640, Dulbecco's modified Eagle's medium, fetal calf serum (FCS), penicillin and streptomycin from Gibco (Cergy-Pontoise, France) and Hydragel from Sebia (Issy-le-Moulineaux, France). Other reagents and chemicals were obtained from Sigma–Merck (Darmstadt, Germany) or Prolabo (Paris, France).

### Lipoprotein isolation

LDLs ($d$ 1.019–1.063) and lipoprotein-depleted serum (LPDS) were isolated from pooled fresh human sera by sequential ultracentrifugation as described by Havel et al. [19], dialysed, sterilized by filtration (0.2 $\mu$m Millipore membrane) and stored at 4 °C under nitrogen until use (up to 1 week), under previously described conditions [12,20–22]. Electrophoretic mobility was monitored by electrophoresis on Hydragel. Cholesterol and apoprotein B (apoB) concentrations were determined using automated analysers (Kodak Ektachem 700 and Roche Cobas-Bio respectively).

### LDL labelling with [$^3$H]cholesteryl oleyl ether and determination of LDL cellular uptake

LDLs were labelled with [$^3$H]cholesteryl oleyl ether (around $10^5$ d.p.m./mg of apoB) by the procedure of Roberts et al. [23], isolated again by ultracentrifugation, dialysed, sterilized by

Abbreviations used: LDL, low-density lipoprotein; apoB, apoprotein B; LPDS, lipoprotein-depleted serum; TBARS, thiobarbituric acid-reactive substances; PDGF, platelet-derived growth factor; FCS, fetal calf serum; LDH, lactate dehydrogenase.

‡ To whom correspondence should be addressed.

filtration and kept at 4 °C as indicated above. Radiolabelled LDLs were added to the culture medium at the concentration indicated and incubated for 12 h. Cells were then carefully washed three times in PBS, harvested and homogenized by sonication (MSE sonicator) in 1 ml of distilled water. Cell-associated radioactivity was determined on an aliquot of homogenate by liquid-scintillation counting (Packard counter model Tricarb 4530).

### LDL oxidation by UVC irradiation

LDLs (2 mg of apoB/ml) were irradiated with UVC as described previously (254 nm; 0.5 mW/cm$^2$) for 2 h (standard conditions) or, where indicated, for a variable time [12,20–22]. The latter led to the formation of a continuum of LDLs oxidized and altered to various extents. In the present paper, mildly oxidized LDLs are defined as those irradiated for 2 h {moderate lipid peroxidation, around 5 ± 1.5 nmol of thiobarbituric acid-reactive substances (TBARS)/mg of apoB, no significant loss of trinitrobenzenesulphonic acid-reactive amino groups [20] and uptake rate similar to that of unoxidized LDLs}. Extensively oxidized LDLs have higher TBARS content and exhibit structural modifications of apoB (decrease in reactive amino groups, altered electrophoretic mobility, reduced staining by lipophilic stains, reduced uptake via the apoB/E receptor). Lipid peroxidation was evaluated by determining TBARS by the method of Yagi [24] as previously used [20–22].

### Cell culture

Bovine aortic smooth-muscle cells (AG 08133A from the NIA Aging Cells Repository, Camden, NJ, U.S.A.) were grown in RPMI 1640 containing Glutamax, 10% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml). Bovine aortic endothelial cells (GM7372A from the NIG Human Mutant Cell Repository, Camden, NJ, U.S.A.) were grown in Dulbecco's modified Eagle's medium containing Glutamax, 10% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml). After trypsin treatment, cells were seeded at a density of 20000/ml in six multiwell culture plaques (Nunc), in medium containing 10% FCS for 24 h. The medium was then removed, cells were washed once with PBS and grown in a medium containing 10% LPDS and, where indicated, LDLs or mildly oxidized LDLs. Cells were grown in humidified air/$CO_2$ (5%) for 10 days. The medium was replaced with fresh medium every 3 days.

### Cell proliferation and cytotoxicity measurements

Smooth-muscle cells or endothelial cells were harvested by treatment with trypsin and counted after dilution in 20 ml of Isoton by using a Coulter counter. DNA synthesis was evaluated by [$^3$H]thymidine incorporation [25]: cells were labelled for 12 h with [$^3$H]thymidine (0.5 μCi/ml), then washed three times with PBS; after precipitation with 3% perchloric acid, the acid-precipitable material was dissolved overnight in 1 M NaOH and 1% SDS. [$^3$H]Thymidine incorporated was counted by liquid scintillation (Packard Tricarb 4530).

Cytotoxicity was evaluated by two tests: (i) Trypan Blue exclusion by the method of Morel et al. [26] as previously used [11,22]; (ii) lactate dehydrogenase (LDH) release into the culture medium (Roche assay kit, MA kit 10) [22].

Protein concentration was determined by the procedure of Lowry et al. [27].

### Morphological studies

Aortic smooth-muscle cells were fixed for 10 min in phosphate-buffered 3% paraformaldehyde and stained with Azure II Blue by the procedure of Richardson et al. [28] before microscopic examination (Leica light microscope, model Diaplan).

## RESULTS

### Proliferative and cytotoxic effects of mildly oxidized LDLs on cultured endothelial and smooth-muscle cells

In preliminary experiments, optimal conditions (initial seeding,

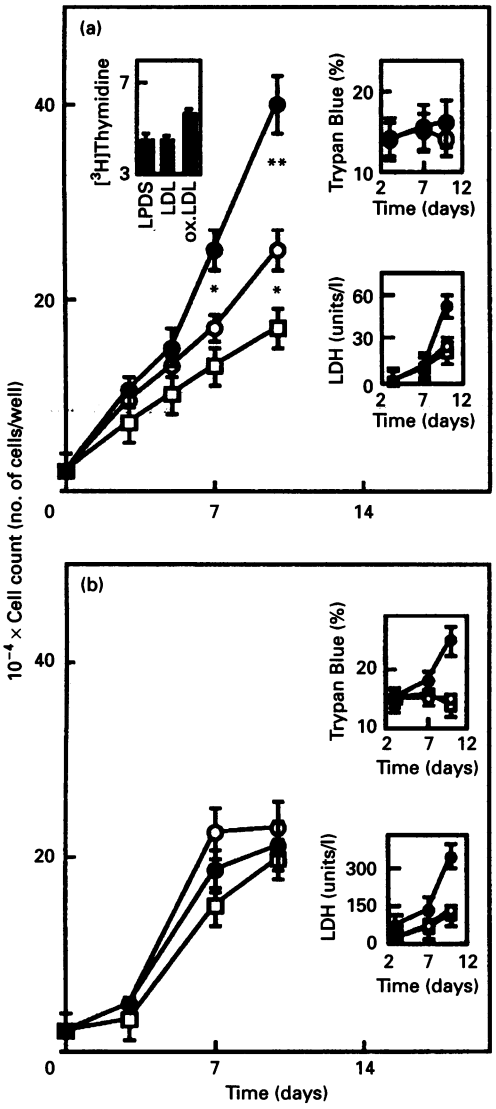


**Figure 1 Effect of LPDS and native or mildly oxidized LDLs on cell growth and viability of cultured aortic smooth-muscle cells (a) and aortic endothelial cells (b)**

Cells (20000/ml; 2 ml/dish) were seeded in 10% FCS. After 24 h, this medium was discarded and replaced with RPMI supplemented with 10% LPDS alone (□) or LPDS (10%) + native LDLs (100 μg of apoB/ml) (○) or mildly oxidized LDLs (100 μg of apoB/ml) (●). Proliferation was evaluated by counting the cells at days 3, 5, 7 and 10. Left inset of (**a**), incorporation of [$^3$H]thymidine: at day 7, cells were labelled for 12 h with [$^3$H]thymidine, then washed three times with PBS, treated with 3% perchloric acid and the acid-precipitable material was counted after overnight extraction with 1 M NaOH and 1% SDS (results are expressed as 100 × d.p.m./mg of cell protein). Right insets of (**a**) and (**b**): estimation of cytotoxicity of the various culture media to smooth-muscle cells, evaluated by the Trypan Blue-exclusion test and by LDH release into the culture medium. Results are means ± S.E.M. of five separate experiments (each performed in triplicate). $^*P < 0.05$; $^{**}P < 0.001$.

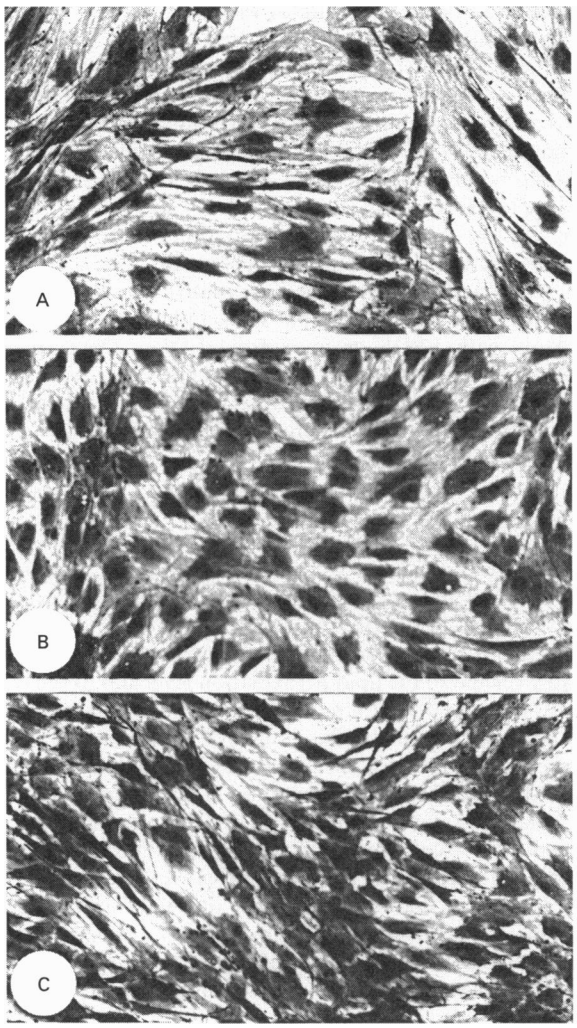


**Figure 2 Micrographs of aortic smooth-muscle cells cultured for 7 days in the presence of 10% LPDS alone (A) or supplemented with native LDLs (100 μg of apoB/ml) (B) or mildly oxidized LDLs (100 μg of apoB/ml) (C)**

Cells were fixed and stained with Azure II Blue as described in the Materials and methods section. Magnification × 250.

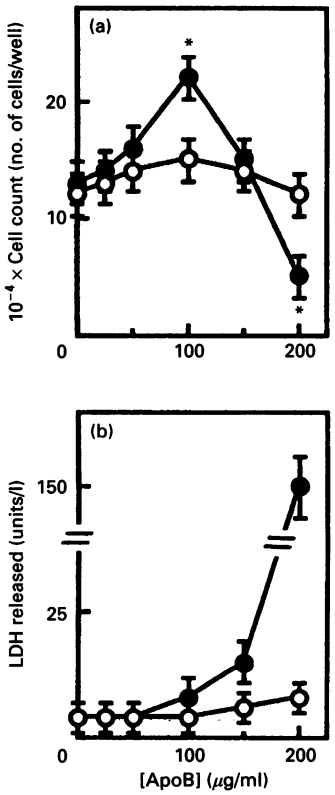


**Figure 3 Dose-dependence of cell growth (a) and cytotoxicity (b) induced by increasing concentrations of native and mildly oxidized LDLs**

(**a**) Cell count of aortic smooth-muscle cells grown for 7 days in the presence of increasing concentrations of native LDLs (○) or mildly oxidized LDLs (●). (**b**) LDH released into the culture medium by aortic smooth-muscle cells grown for 7 days under the same conditions as in (**a**), i.e. in the presence of increasing concentrations of native LDLs (○) and mildly oxidized LDLs (●). Results are means ± S.E.M. of three separate experiments (each performed in triplicate). $^*P < 0.05$, compared with value in the presence of unoxidized LDLs.

cell density, change of culture medium every 3 days) were defined in order to follow the time course of cell proliferation over a relatively long period. Under conditions of maximal proliferation (i.e. in medium supplemented with 10% FCS), confluence was obtained after 10 days of culture (results not shown). We compared the proliferation rates of aortic smooth-muscle cells grown continuously in RPMI containing 10% LPDS alone or supplemented with LDLs or mildly oxidized LDLs. As shown in Figure 1(a), proliferation of aortic smooth-muscle cells was relatively independent of the composition of the culture medium during the first 3 days of culture, but at days 7–10, the differences became highly significant. Mildly oxidized LDLs exhibited a proliferative effect (after 10 days it was about 200% of that in the LPDS control, the latter being considered as inducing the minimal proliferation rate without any loss of cell viability) that was significantly higher than that exhibited by native LDLs (about 130% of the LPDS control). Evaluation of cellular mitogenic activity by [$^3$H]thymidine incorporation (at the seventh day) showed similar results: incorporation was higher with mildly oxidized LDLs than with unoxidized LDLs (inset of Figure 1a). The concentration of mildly oxidized LDLs used in these experiments (100 μg of apoB/ml) was not significantly cytotoxic to aortic smooth-muscle cells (right insets of Figure 1a). Note that the level of released LDH was higher when expressed as units/l but was in the normal range when related to the cell number/dish, suggesting that the higher level at day 10 results from normal LDH release by a larger number of cells (and not from a cytotoxic process). As shown in Figure 2, besides the difference in cell density of aortic smooth-muscle cells, some differences in morphology were observed after 7 days of culture: cells are large and spread out with LPDS, smaller and less spread out with native LDLs and even smaller and distributed in clusters with mildly oxidized LDLs.

In contrast with the proliferative effect on aortic smooth-muscle cells, mildly oxidized LDLs did not influence the growth rate of aortic endothelial cells, being even lower in the presence of mildly oxidized LDLs than with native LDLs (Figure 1b). Moreover, this concentration of mildly oxidized LDLs was more cytotoxic to endothelial cells than native LDLs (insets of Figure 1b).

As demonstrated in Figure 3, the proliferative effect of mildly oxidized LDLs was dose-dependent. Moderate concentrations of mildly oxidized LDLs induced a proliferative effect, maximal at 100 μg of apoB/ml (Figure 3a). At high concentration (200 μg

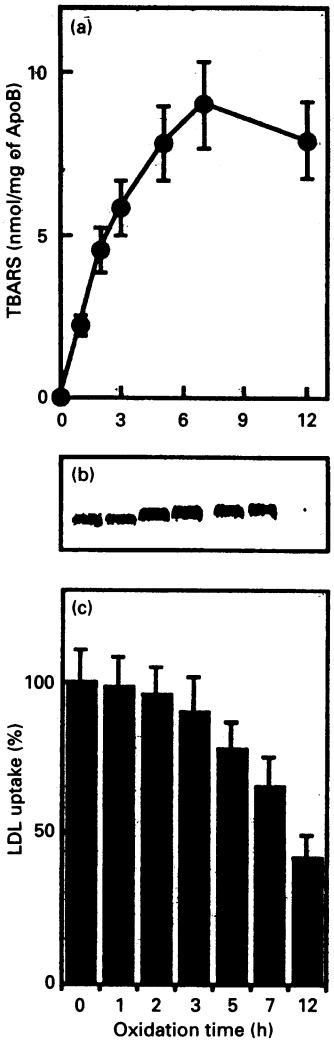


**Figure 4 Determination of oxidation levels of radiolabelled LDLs oxidized for increasing times (0, 1, 2, 3, 5, 7 and 12 h) (a, b) and determination of their uptake by smooth-muscle cells (c)**

Oxidation levels were evaluated by (**a**) TBARS content and (**b**) electrophoretic mobility on Hydragel (note that the 12 h-oxidation sample was not detectable on electrophoresis). The uptake of radiolabelled LDL treated for various UV-irradiation times was determined as indicated in the Materials and methods section (100 $\mu$g of apoB/ml incubated with cells for 12 h); cells were carefully washed three times and cell-associated radioactivity was counted and expressed as percentage of the uptake of unoxidized LDLs (3.1 $\pm$ 0.3 ng of apoB/mg of cell protein) (**c**) Results are means $\pm$ S.E.M. of three separate experiments, except in (**b**) (typical experiment).

of apoB/ml), the lower cell count probably resulted from cytotoxicity, as shown by leakage of cellular LDH (Figure 3b).

These data suggest that moderate doses of mildly oxidized LDLs elicit different effects on the two cell types of the vascular wall: a proliferative effect on aortic smooth-muscle cells and a cytotoxic effect on aortic endothelial cells.

### Dependence of the proliferative effect of mildly oxidized LDLs in cultured aortic smooth-muscle cells on the level of LDL oxidation

When UVC irradiation time was increased, the TBARS content increased to a plateau (Figure 4a) and electrophoretic mobility also progressively increased (Figure 4b). Extensively oxidized LDLs (irradiation longer than 7 h) could not be detected (by Sudan-IV staining) on electrophoresis gels, suggesting considerable alteration of the structure of the LDL particle and apoB. Uptake of mildly oxidized LDLs (up to 2 h irradiation) by smooth-muscle cells (Figure 4c) was not significantly modified, in agreement with our previous studies demonstrating that, under these oxidation conditions, the surface amino groups of apoB were not altered [20]. Uptake of extensively oxidized LDLs was decreased, because modifications of apoB lowered their affinity for the apoB/E receptor.

The effect of the level of LDL oxidation on the proliferative and cytotoxic effects on smooth-muscle cells was next investigated. As shown in Figure 5, the proliferative effect was observed after 7–10 days of incubation with mildly oxidized LDLs (compared with LPDS or unoxidized LDLs). Over an intermediate range of oxidation a simultaneous increase in proliferation and cytotoxicity was observed. More extensive oxidation induced a prevailing increase in cytotoxicity and finally a decrease in cell population.

## DISCUSSION

As shown by using a broad spectrum of LDLs (unoxidized, mildly oxidized and extensively oxidized), oxidized LDLs exhibit a biphasic effect in smooth-muscle cells: a proliferative effect at low doses and a cytotoxic effect at high doses. These effects are probably related to the lipid peroxidation level and to the rate of uptake of oxidized LDLs by cells. Native and mildly oxidized LDLs are taken up at similar rates by smooth-muscle cells, in agreement with the structural features of mildly UV-oxidized LDLs (which exhibit no fragmentation of apoB and no alteration of the surface amino groups) [11,20,29] and of 'minimally modified LDL' (see comment in ref. [30]). In contrast, the uptake of highly oxidized LDLs decreased progressively.

The toxicity of high concentrations of mildly oxidized LDLs and moderate concentrations of extensively oxidized LDLs is consistent with the assumption that the toxic effect becomes apparent above a threshold dose of lipid-peroxidation compounds internalized by the cell [12]. This balance between mitogenic and toxic effects of mildly oxidized LDLs on smooth-muscle cells probably explains the data of Stiko-Rahm et al. [31], who observed a biphasic effect on DNA synthesis (but did not evaluate the cytotoxic effect). Our conclusions generally agree with those of Chatterjee [18], in spite of large differences in the level of LDL oxidation and in the time course of smooth-muscle cell proliferation.

In our experimental model system, the apparent proliferative effect of unoxidized LDLs seems to result (at least in part) from the cell-mediated LDL oxidation in the culture medium because of (i) progressive cell-induced LDL oxidation during the 3 days of contact between LDLs and smooth-muscle cells and (ii) inhibition of the proliferative effect of unoxidized LDLs in the presence of 10 $\mu$mol/l butylated hydroxytoluene (this antioxidant being devoid in itself of any antiproliferative effect) (results not shown). This conclusion is in good agreement with previous reports showing that native LDLs were not really (or only poorly) mitogenic to smooth-muscle cells [32], in spite of their ability to induce cellular events usually linked with cell proliferation (such as translocation of protein kinase C and induction of c-*fos* and c-*myc*) [14,32].

The mechanism of induction of smooth-muscle cell proliferation by mildly oxidized LDLs is not known. Two non-exclusive mechanisms might be proposed involving either direct cellular activation by mildly oxidized LDLs or indirect promotion of cell growth. The hypothesis of direct activation by mildly oxidized LDLs comes from our previous observation that mildly oxidized LDLs elicit a rise in cytosolic $Ca^{2+}$ concentration [11] which is

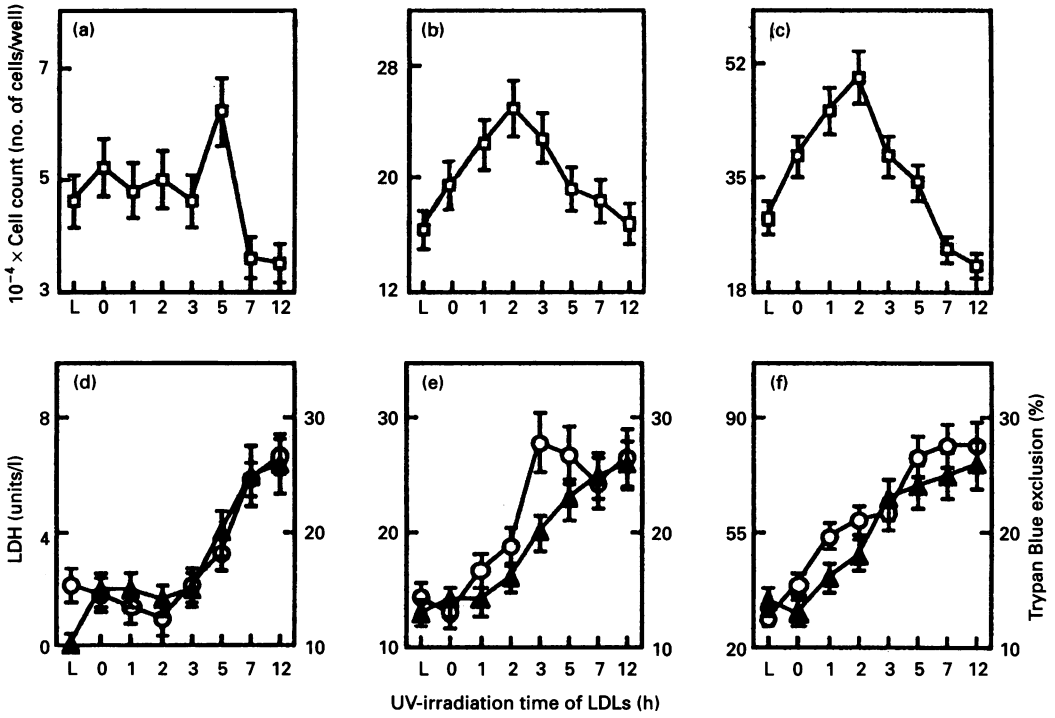


**Figure 5 Effect of progressively oxidized LDLs (as described in Figure 4) on growth and cytotoxicity of smooth-muscle cells at days 3 (a, d), 7 (b, e) and 10 (c, f)**

Cells were grown under standard conditions, in the presence of LPDS (L) or LPDS supplemented with a fixed concentration (100 $\mu$g of apoB/ml) of LDL oxidized by UV for increasing periods of time (0–12 h). Cell growth was evaluated by counting the cells (**a–c**), and cytotoxicity was determined by Trypan Blue exclusion (▲) and LDH release (○) (**d–f**). Results are means ± S.E.M. of three separate experiments.

know to be involved in the progression through the cell cycle and mitosis [16,33]. The second hypothesis is autocrine induction of growth factors by mildly oxidized LDLs. For instance, in our experimental system, mildly oxidized LDLs enhanced platelet-derived growth factor (PDGF)-A mRNA expression in smooth-muscle cells, but not that of PDGF-B mRNA (results not shown), in agreement with previously reported data [31,34]. As PDGF-A is only poorly mitogenic (in contrast with PDGF-BB or AB) [31] and as butylated hydroxytoluene inhibits PDGF-A overexpression [34], but not the proliferative effect of mildly oxidized LDLs, it is unlikely that PDGF plays a major role in our experimental system.

The second major result of the paper is that endothelial cells are more susceptible to the cytotoxic effect of mildly oxidized LDLs than are smooth-muscle cells. This difference in susceptibility and cellular response observed between the two cell types allows speculation that, during atherogenesis, the same level of mildly oxidized LDLs could induce injury of endothelial cells on the one hand and stimulate the proliferation of smooth-muscle cells on the other. In contrast, high concentrations of mildly oxidized LDLs are primarily cytotoxic to both cell types. This may be related to the existence of a gradient of oxidized LDLs in the atherosclerotic plaque, high concentrations of oxidized LDL generating the necrotic centre of the plaque, and lower concentrations of oxidized LDL inducing migration [8] and proliferation ([18]; the present paper) of smooth-muscle cells around the necrotic centre. This hypothesis is also consistent with the focal aspect of atherogenesis.

In conclusion, the reported data strengthen the hypothesis of a direct role of mildly oxidized LDLs in the proliferation of smooth-muscle cells and in progression of the fibroatheroma plaque and suggest that the mitogenic effect can be blocked by antioxidants by two mechanisms: (i) by inhibiting LDL oxidation in the culture medium; (ii) by blocking the mitogenic effect of mildly oxidized LDLs at the cellular level.

We thank Mr. C. Mora and Mr. J. Dumoulin for excellent technical assistance. The financial support of INSERM (CJF-9206), MESR (JE-174), University Paul Sabatier Toulouse-3, Conseil Régional Midi-Pyrénées (RECH. 9308181), European Communities (PL 931790) and CNRS (URA 74) is gratefully acknowledged. N. A. was a fellow of the French MESR.

## REFERENCES

1 Stary, H. C. (1990) Eur. Heart J. **11** (Suppl. E), 3–19
2 Brown, M. S. and Goldstein, J. L. (1983) Annu. Rev. Biochem. **52**, 223–261
3 Ross, R. (1986) N. Engl. J. Med. **314**, 488–500
4 Steinberg, D., Parthasarathy, S., Carew, T., Khoo, J. C. and Witzum, J. L. N. (1989) N. Engl. J. Med. **320**, 915–924
5 Steinbrecher, U. P., Zhang, H. and Lougheed, M. (1990) Free Radical Biol. Med. **9**, 155–168
6 Esterbauer, H., Dieber-Rotheneder, M., Waeg, G., Striegl, G. and Jürgens, G. (1990) Chem. Res. Toxicol. **3**, 77–92
7 Quin, M. T., Parthasarathy, S. and Steinberg, D. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 2805–2809
8 Autio, I., Jaakola, O., Solakivi, T. and Nikkari, T. (1990) FEBS Lett. **277**, 247–249
9 Henricksen, T., Evensen, S. A. and Carlander, B. (1979) Scand. J. Clin. Lab. Invest. **39**, 361–368
10 Hessler, J. L., Robertson, A. L. and Chisolm, G. M. (1979) Atherosclerosis **32**, 213–229
11 Salvayre, R., Nègre, A., Lopez, M. et al. (1990) NATO ASI series A **189**, 249–256
12 Nègre-Salvayre, A., Lopez, M., Levade, T. et al. (1990) Biochim. Biophys. Acta **1045**, 224–232

13 Nègre-Salvayre, A., Fitoussi, G., Réaud, V., Pierraggi, M. T., Thiers, J. C. and Salvayre, R. (1992) FEBS Lett. **299**, 60–65
14 Scott-Burden, T., Resink, T. J., Hahn, A. W. A., Baur, U., Box, R. J. and Buhler, F. R. (1989) J. Biol. Chem. **264**, 12582–12589
15 Lu, K. P. and Means, A. R. (1993) Endocr. Rev. **14**, 40–58
16 Harris-Hooker, S., Sanford, G. L., Montgomery, V., Rivers, R. and Emmett, N. (1992) Cell Biol. Int. Rep. **16**, 433–460
17 Özer, N. K., Palozza, P., Boscoboinik, D. and Azzi, A. (1993) FEBS Lett. **322**, 307–310
18 Chatterjee, S. (1992) Mol. Cell. Biochem. **111**, 143–147
19 Havel, R. I., Eder, H. A. and Bragdon, J. H. (1955) J. Clin. Invest. **39**, 1345–1363
20 Nègre-Salvayre, A., Paillous, N., Dousset, N., Bascoul, J. and Salvayre, R. (1992) Photochem. Photobiol. **55**, 197–204
21 Nègre-Salvayre, A. and Salvayre, R. (1992) Free Radical Biol. Med. **12**, 101–106
22 Nègre-Salvayre, A. and Salvayre, R. (1992) Br. J. Pharmacol. **107**, 738–744
23 Roberts, D. C. K., Miller, N. E., Price, S. G. L. et al. (1985) Biochem. J. **226**, 319–322
24 Yagi, K. (1987) Chem. Phys. Lipids **45**, 337–351
25 Freshney, R. I. (1986) Animal Cell Culture: A Practical Approach. IRL Press, Oxford
26 Morel, D. W., Dicorletto, P. E. and Chisolm, G. M. (1984) Arteriosclerosis **4**, 357–364
27 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
28 Richardson, K. C., Jarret, L. and Finke, E. H. (1960) Stain Technol. **35**, 313–316
29 Dousset, N., Nègre-Salvayre, A., Lopez, M., Salvayre, R. and Douste-Blazy, L. (1990) Biochim. Biophys. Acta **1045**, 219–223
30 Rajavashisth, T. B., Andalibi, A., Territo, M. C. et al. (1990) Nature (London) **344**, 254–257
31 Stiko-Rahm, A., Hultgård-Nilsson, A., Regnstrom, J., Hamsten, A. and Nilsson, J. (1992) Arterioscl. Thromb. **12**, 1099–1109
32 Scott-Burden, T., Resink, T. J., Hahn, A. P. and Buhler, F. R. (1989) Biochem. Biophys. Res. Commun. **159**, 624–632
33 Whitaker, M. and Patel, R. (1990) Development **108**, 525–542
34 Zwijsen, R. M. L., Japenga, S. C., Heijen, A. M. P., Van den Bos, R. C. and Koeman, J. H. (1992) Biochem. Biophys. Res. Commun. **186**, 1410–1416

Received 15 December 1994/15 March 1995; accepted 27 March 1995