0014-4754/96/030234-05$1.50 + 0.20

Oxidative modifications of low-density lipoproteins (LDL) by the human endothelial cell line EA.hy 926

M. A. Pech-Amsellem*, I. Myara, I. Pico[a], C. Mazière[a], J. C. Mazière[a] and N. Moatti

Laboratoire de Biochimie Appliquée, Faculté de Pharmacie, F-92296 Châtenay-Malabry, Fax +33 1 4683 5695; Laboratoire de Biochimie, Hôpital BROUSSAIS, 96, rue Didot, F-75014 Paris; [a]Laboratoire de Biochimie Appliquée, Faculté de Médecine Saint-Antoine, 27, rue Chaligny, F-75012 Paris (France)
Received 18 April 1995; accepted 25 July 1995

Abstract. Modifications of LDL by the EA.hy 926 cell line were compared to those generated by human umbilical vein endothelial cells (HUVEC). Thiobarbituric acid reactive substances (TBARS) index values (TBARS sample/TBARS cell-free control ratio) were 2.64 ± 0.18 ($m \pm SE$, $n = 11$) and 3.12 ± 0.24 ($n = 11$), for HUVEC and EA.hy 926, respectively. The percentage of the most electronegative modified LDL fraction (fraction C), assessed by using an ion-exchange chromatographic method based on fast protein liquid chromatography (FPLC), represented $14 \pm 3\%$ ($n = 34$) and $22 \pm 13\%$ ($n = 10$) of total modified LDL in HUVEC and EA.hy 926, respectively. LDL modified by both cell lines showed increased agarose electrophoretic mobility and apo B100 fragmentation on SDS-PAGE. None of the results were significantly different between the two cell lines. Superoxide anion production was 0.12 ± 0.04 ($n = 11$) and 0.07 ± 0.01 nmol/min/mg cell protein ($n = 11$) in HUVEC and EA.hy 926, respectively. Cell-specific effects on LDL were abrogated in cysteine-free medium. Moreover, cell-modified LDL were similarly degraded by J774 macrophage-like cells. We conclude that EA.hy 926 cells are a good model for investigating endothelial cell-induced modifications of LDL. Advantages include ready availability and less individual variability than with HUVEC.
Key words. LDL peroxidation; low-density lipoprotein; endothelial cell; EA.hy 926; thiol; atherosclerosis; FPLC

Abbreviations. LDL, low-density lipoprotein; HAT, 100 μM hypoxanthine/0.4 μM aminopterin/16 μM thymidine; FCS, fetal calf serum; TBARS, thiobarbituric acid-reactive substances (lipid peroxidation products); MDA, malondialdehyde; FPLC, fast protein liquid chromatography; cpm, counts per minute.

Oxidative modifications of low-density lipoprotein (LDL) play an important role in the initiation and progression of atherosclerosis[1–5]. LDL, the major plasma cholesterol carrier, can be oxidatively modified by the three major cell types of the arterial wall, i.e. macrophages[6], smooth muscle cells[7], and endothelial cells[8–12]. Human umbilical vein endothelial cells (HUVEC) are a convenient model for studying LDL modifications generated by endothelial cells[9–12]. Modified LDL is no longer recognized by the fibroblast apo B/E receptor but is avidly taken up by macrophages via the scavenger receptor pathway[13,14]. This phenomenon leads to cholesteryl ester accumulation by macrophages and to their subsequent transformation into foam cells; the latter form fatty streaks, which are an early state of atherosclerotic lesions. HUVEC are difficult to isolate in large numbers and show marked individual variations. We tested EA.hy 926 cells, a permanent human endothelial cell line, established by fusing HUVEC with the permanent cell line A 549 (derived from a human lung carcinoma), which has the differentiated properties of endothelium[15] and presented no change in the fraction of factor VIII-related antigen-positive cells during more than 100 population doublings[15], to determine whether LDL modifications were similar to those induced by HUVEC.

* Corresponding author.

Materials and methods

Materials. HAT (50×) medium and FCS were from Boehringer Mannheim (Meylan, France). M199, 4.5 and 1 g/l glucose DMEM were from BioWhittaker (Fontenay sous Bois, France). Ham's F10 medium with and without phenol red, cysteine-free Ham's F10 and cysteine free and phenol red-free Ham's F10 medium were from Gibco BRL (Cergy Pontoise, France). Horse-heart cytochrome c was from Sigma (St. Louis, MO, USA). J774 murine macrophage-like cells were from the American Type Culture Collection (Rockville, MD, USA). EA.hy 926, a permanent human endothelial cell line, was kindly provided by Dr Cora Jean S. Edgell (University of North Carolina, Department of Pathology, 7525 Chapel Hill, NC 27599, USA). Na-^{125}I (13–17 Ci/mg) was from Amersham (Buckinghamshire, UK). The kit used to determine TBARS was from SOBIODA (Grenoble, France).

LDL preparation. LDL ($1.019 < d < 1.063$) was prepared from normal human plasma as previously de-

scribed[16]. After isolation, it was dialyzed against 0.01 mol/l Tris/HCl buffer, pH 7.4, containing 1 mmol/l EDTA, passed through a 0.2 μm filter, collected in a sterile tube and stored at 4 °C in the dark. LDL was dialyzed against 0.02 mol/l phosphate buffer, pH 7.4, containing 0.15 mol/l NaCl, to remove EDTA before incubation with endothelial cells.

Cell culture. HUVEC were obtained from umbilical cords, from normotensive, nondiabetic, infection-free and nonsmoking women, using dispase instead of collagenase in the method of Jaffe et al.[17]. They were cultured in M199 medium supplemented with 10% FCS, antibiotics, HEPES and glutamine. When confluent, cells were trypsinized and resuspended in Ham's F10 medium supplemented with 10% FCS, antibiotics, HEPES and glutamine at an initial density of 0.15-0.20 × 106/ml. LDL oxidation experiments were performed on confluent cells. EA.hy 926 cells were maintained in DMEM containing 4.5 g/l glucose, 10% FCS, antibiotics, glutamine and HAT. For the study of LDL oxidation, EA.hy 926 cells were trypsinized in the same way as HUVEC. The J774 macrophage cell line was maintained in suspension in RPMI 1640 supplemented with 10% heat-inactivated FCS and antibiotics. For the determination of LDL degradation, cells were seeded in 35 mm dishes at a density of 1.5×10^6/dish. All experiments were performed on confluent cultures (about 3×10^6 cells/dish).

LDL oxidation by cells. EA.hy 926 and HUVEC were grown in 4-well NUNC plates at an initial cell density of $0.15–0.20 \times 10^6$/well. When confluent, they were rinsed twice with FCS-free Ham's F10. LDL was diluted to 0.2 mg/ml in FCS-free Ham's F10 supplemented with antibiotics, HEPES and glutamine. Ham's F10 medium contains 25 mg/l cysteine, which autoxidizes to cystine during storage[18]. A growth control for each cell line, and cell-free controls, were run in each experiment. After 48 h of incubation, the medium was removed and oxidation was stopped by the addition of 1 mmol/l EDTA and 0.02 mmol/l butyl-hydroxy-toluene. Part of the supernatant was dialyzed against 0.01 mol/l Tris/HCl buffer, pH 7.4, containing 1 mmol/l EDTA, then filtered and injected into the FPLC system as previously described[16]. Determinations were done in duplicate. Lipid peroxidation products were measured fluorometrically (at excitation wavelength 532 nm and emission wavelength 553 nm) by using the SOBIODA kit based on a modified version of Yagi's test[19], also in duplicate. This assay is based on the reaction between two molecules of thiobarbituric acid (TBA) and one molecule of the three-carbon compound, malondialdehyde (MDA), during heating at 95 °C and at acid pH, to produce a fluorescent complex. Results are expressed in nmol equivalent malondialdehyde/mg LDL protein, with MDA as standard. To minimize day-to-day fluctuations in TBARS determination[20], a TBARS index was calculated as follows:

$$\text{TBARS index} = [\text{TBARS}]\text{sample}/[\text{TBARS}]\text{cell-free control.}$$

TBARS index = 1 for cell-free control, TBARS index > 1, when LDL oxidation in the sample is higher than in the cell-free control.

Changes in the net negative charge of LDL were assessed by FPLC and agarose gel electrophoresis at pH 8.6, using the Ciba Corning system (Le Vézinet, France). Apo B fragmentation was assessed after delipidation by SDS-PAGE using the Pharmacia Phast system with Phastgel gradient 4–15%[16]. We used the separation technique and silver staining method recommended by the manufacturer.

LDL degradation. ^{125}I-labelling of LDL was performed as described by Bilheimer et al.[21]. Specific radioactivity was 157 cpm/ng LDL protein, and the free iodine content was 0.7%. Oxidation of [^{125}I]-LDL by cells was first carried out with 0.2 mg/ml LDL as previously described[16]. The medium was then transferred to J774 cells for the study of degradation. Degradation of native LDL, cell-free controls, HUVEC- and EA.hy 926-modified LDL were studied according to Goldstein and Brown[22]. The results are expressed in ng LDL per mg cell protein.

Superoxide anion production. Cells were incubated for 10, 20, 40 and 60 min in phenol red-free Ham's F10 medium in the presence of 20 μM cytochrome c according to the modified method of Steinbrecher et al.[23]. Superoxide anion release was calculated from the difference in absorbance at 550 nm in the absence and presence of superoxide dismutase (50 U/ml), using a molar extinction coefficient of 20 $\text{mmol}^{-1}\ \text{cm}^{-1}$. Each experiment was performed at least twice in duplicate. Superoxide anion production is expressed as nmol/min/mg cell protein. The same experiment was performed in cysteine free and phenol red-free Ham's F10.

Protein assay. Total protein was measured by using Peterson's method[24] with bovine serum albumin as standard.

Statistical analysis. Results are expressed as means ± SEM, and data were compared using Student's unpaired t-test.

Results

Assessment of LDL modifications. Oxidative modifications of LDL were assessed by TBARS measurement, chromatographic separation (FPLC), agarose electrophoresis and SDS-PAGE. We determined the TBARS index on supernatants of cell-free controls (n = 10), HUVEC (n = 11) and EA.hy 926 (n = 11). The TBARS index (1 for cell-free controls) was 2.64 ± 0.18 (m ± SE) and 3.12 ± 0.24 for HUVEC and EA.hy 926, respectively. LDL were significantly more

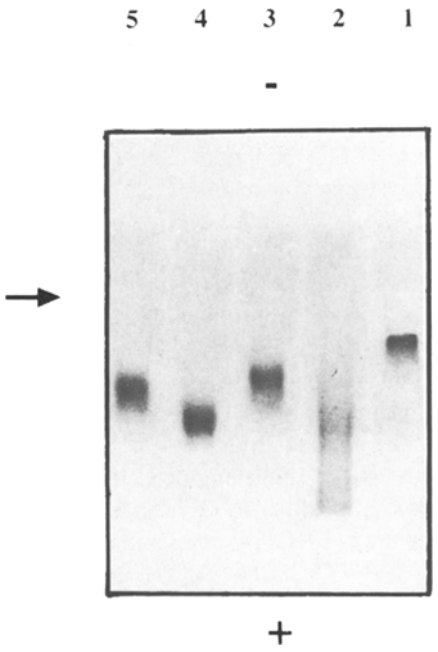

Figure 1. Oxidation of LDL by EA.hy 926 cells and HUVEC, as assessed by agarose gel electrophoresis. LDL (0.2 mg/ml) was incubated for 48h in 1.9 cm^2 wells in the absence or presence of endothelial cells. Oxidation was stopped as described in 'Materials and methods'. LDL were concentrated by ultracentrifugation. The sample size was 1.8 µg LDL protein. The arrow indicates the origin. Lane 1 = native LDL; lane 2 = LDL oxidized by 5 µM copper; lane 3 = cell-free control; lane 4 = LDL oxidized by EA.hy 926; lane 5 = LDL oxidized by HUVEC.

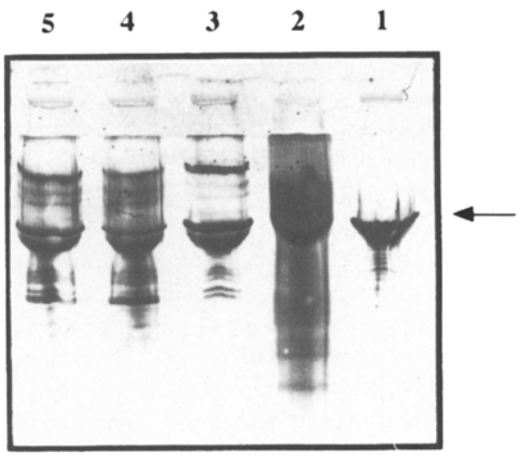

Figure 2. Cell-induced modifications of LDL assessed by SDS-PAGE. LDL was prepared as described in the legend of figure 1 and delipidated[16]. Native LDL was run in lane 1; copper-oxidized LDL in lane 2; cell-free control in lane 3; EA.hy 926-modified LDL in lane 4 and HUVEC-modified LDL in lane 5. The arrow indicates the position of native apo B100.

markedly modified by both cell types ($p < 0.001$) than in cell-free medium; no significant difference was found between HUVEC- and EA.hy 926-modified LDL. We have previously described an ion-exchange chromatographic method based on FPLC[16]. This methodology allowed us to separate native, cell-free and endothelial cell-modified LDL into three populations A, B and C on the basis of the particle charge and to quantify the percentage of the different fractions. The fraction A corresponded to native LDL and fractions B and C corresponded to modified LDL. Fraction C was the most electronegative, therefore the most modified fraction, and was obtained only in the presence of cells. No fraction C was detected in cell-free controls. Indeed, we have assessed the modifications of LDL by FPLC and expressed them as the percentage of fraction C[16]. Fraction C generation by HUVEC ($n = 34$) and EA.hy 926 ($n = 10$) represented $14 \pm 3\%$ and $22 \pm 13\%$, respectively (not significant). The electrophoretic mobility of LDL particles is presented in figure 1. Compared to native LDL (lane 1), copper-oxidized LDL (lane 2), LDL incubated in control cell-free medium (lane 3), EA.hy 926-modified LDL (lane 4) and HUVEC-modified LDL (lane 5), all showed increased mobility. SDS-PAGE (fig. 2) showed that after 48 h of incubation of LDL in cell-free medium (lane 3) or in the presence of EA.hy 926 (lane 4) and HUVEC (lane 5), the native apo B100 band (indicated by the arrow and observed in native LDL (lane 1), disappeared in parallel to the appearance of both lower and higher molecular weight fragments. After incubation with endothelial cells (lanes 4 and 5), low molecular weight fragments were seen in larger amounts than in the cell-free control (lane 3). Aggregates (stopped at the end of the stacking gel) were present in all lines except native LDL (lane 1). Compared to native LDL (lane 1), control copper-oxidized LDL (lane 2) contained less apo B100 and more aggregates.

Superoxide anion production. As endothelial cells produce superoxide anion, which could be involved in oxidative modifications of LDL[23], we also determined superoxide anion production by ten different HUVEC lines and EA.hy 926 cell line (11 determinations). Values (determined in duplicate) were 0.12 ± 0.04 ($m \pm SE$) and 0.07 ± 0.01 nmol/min/mg cell protein for HUVEC and EA.hy 926, respectively (difference was not significant). Superoxide anion production was undetectable in cysteine-free and phenol red-free Ham's F10 (data not shown).

Role of cysteine/cystine in cell-mediated modifications of LDL. As thiols (cysteine/cystine in particular) play an important role in cell-mediated modifications of LDL[18, 25–28], we also investigated LDL oxidation by HUVEC and EA.hy 926 in cysteine-free Ham's F10 medium. As with HUVEC, the presence of cysteine in the culture medium was necessary for EA.hy 926-mediated oxidation of LDL assessed by the TBARS index (fig. 3). In the presence of L-cysteine, the mean TBARS index was 1 for cell-free controls, 1.61 ± 0.14, ($m \pm SE$, $n = 5$), for HUVEC; and 1.81 ± 0.16 ($n = 5$) for EA.hy

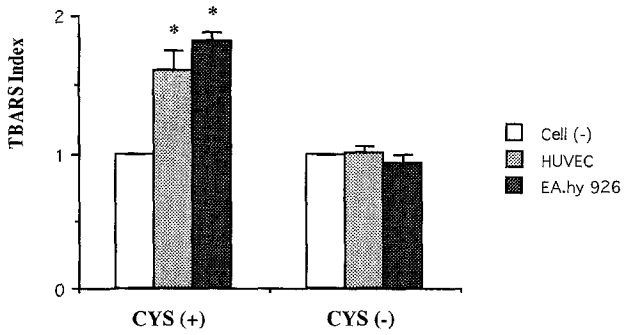

Figure 3. Oxidation of LDL in cell-free medium, and by HUVEC and EA.hy 926 cells, as assessed by the TBARS index in the presence and absence of L-cysteine. Each experiment was performed in duplicate. *$p < 0.001$ versus cell-free controls (Student's unpaired t-test).

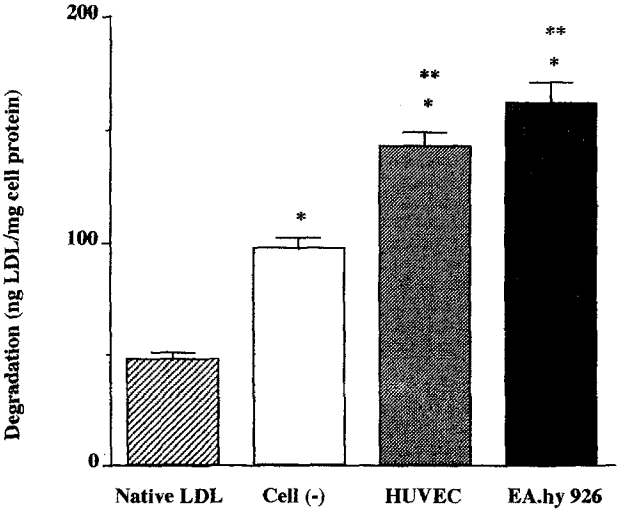

Figure 4. Degradation of LDL modified by HUVEC and EA.hy 926 cells. ^{125}I-labelled LDL (0.2 mg/ml) was incubated for 48h in 1.9 cm^2 wells in Ham's F10 medium in the presence or absence of 200,000 cells. The supernatant was collected and incubated for 4 h at 37 °C with J774 macrophages. Native radiolabeled-LDL (0.2 mg/ml) were incubated with J774 macrophages under the same experimental conditions in order to control the degradation prior to any modifications with or without cells. The rate of degradation of ^{125}I-labelled LDL was estimated in the supernatant by measuring radioactive non-iodide tricloroacetic acid-soluble degradation products. Values are the means ± SE of 7 wells with the same LDL preparation. *$p < 0.001$ versus native LDL and **$p < 0.001$ versus cell-free control [cell(-)] (Student's unpaired t-test).

926 cells; endothelial cells modified significantly more LDL than cell-free controls ($p < 0.001$) and there was no significant difference between the two cell types. In the absence of cysteine, the TBARS index (1 for cell-free controls; $n = 5$) was 1.004 ± 0.06 ($n = 5$) for HUVEC and 0.932 ± 0.01 ($n = 5$) for EA.hy 926 cells. The latter values were not significantly different.

Degradation of cell-modified LDL by J774 macrophages. As oxidized LDL are rapidly taken up and degraded by macrophages, endothelial cell-induced modifications were also assessed in terms of degradation of LDL by J774 macrophages (fig. 4). Native radiolabeled-LDL (0.2 mg/ml) were incubated with J774 macrophages under the same experimental conditions in order to control the degradation prior to any modifications with or without cells. Degradation of cell-free-, HUVEC- and EA.hy 926-modified LDL increased by 100, 200 and 240%, respectively, compared with native LDL ($p < 0.001$). Moreover, the degradation of HUVEC- and EA.hy 926-modified LDL by J774 macrophages increased by 50 and 70%, respectively, compared with cell-free controls ($p < 0.001$). No significant difference was found between the two cell types.

Discussion

One of the main problems in endothelial cell biology has been the lack of cell lines for use as models in in vitro studies. HUVEC are difficult to culture and can only survive through a limited number of passages. The frequent generation of new primary cultures is time-consuming and leads to problems such as batch-to-batch and individual variability. The availability of immortalized but well-differentiated endothelial cells would overcome the dependence on umbilical cords, save time and money, and be more applicable to longitudinal studies. A permanent HUVEC cell line was established by Edgell et al.[15] in 1983. The EA.hy 926 cell line[15] was derived from HUVEC by fusion with the relatively undifferentiated A549/8 line[29]. The differentiated properties of EA.hy 926 include the presence of von Willebrand factor[15, 30], Weibel-Palade bodies[31], prostacyclin[32], tissue plasminogen activator and plasminogen activator inhibitor type 1[33], platelet activating factor[34], thrombomodulin[35, 36], GMP-140[37], vitronectin receptor[38], CD-31[39], CD-34[40], CD-59[41], and endothelin-1[42], and the uptake of modified LDL[36]. This cell line can be loaded with cationized LDL, but not with acetylated LDL[43]. It is used as a model to study cholesterol efflux rates[44, 45] and possesses saturable HDL3 binding sites[46]. Moreover, like HUVEC, it can express heat shock protein 70 (which has been detected in atherosclerotic lesions) after exposure to oxidized LDL[47]. Rieber et al.[48] have shown that the EA.hy 926 cell line sustained transcription of many genes that are differentially expressed in endothelium, accounting for about 10% of its mRNA. Another advantage of this cell line is its human specificity, whereas many authors use a permanent rabbit aortic endothelial cell line (REC B4)[18, 23, 49, 50].

When endothelial cells modify LDL in vitro, the LDL particle undergoes a number of changes, including an increased negative charge, increased apo B100 fragmentation and altered receptor recognition. This requires the presence of micromolar concentrations of copper or iron. Lipid peroxidation is involved in this mechanism, as cell-modified LDL contain TBARS. In this study, we found that EA.hy 926 oxidized LDL in the same way as HUVEC, as shown by the increase in the TBARS index, the increase in electronegativity (FPLC and agarose gel

electrophoresis), fragmentation of apo B100, increased degradation by J774 macrophages, and altered fibroblastic receptor apo B/E recognition. Moreover, EA.hy 926, like HUVEC, produced superoxide anion. The mechanism by which EA.hy 926 oxidizes LDL was explored by using cysteine-free Ham F10 medium. Indeed, thiols autoxidize in the presence of trace amounts of metal ions, forming reduced metal ions, thyil radicals and superoxide anion, all of which promote lipid peroxidation. Heinecke et al.[26] showed that LDL oxidation and superoxide anion production were blocked in medium lacking L-cystine, and that the addition of L-cystine to the medium restored both phenomena in a concentration-dependent manner. More recently, Sparrow and Olszewski[18] showed the importance of L-cysteine in endothelial cell-mediated LDL oxidation. As L-cysteine in the culture medium rapidly autoxidizes into L-cystine[18], it has been suggested that cells could take up L-cystine via a membrane transport system (such as the Xc-system described by Bannai et al.[51] in fibroblasts and by Miura et al.[52] on HUVEC), reduce the disulfide to a thiol inside the cell and export the thiol into the extracellular medium where it could act by promoting lipid peroxidation[18,53]. This is supported by LDL oxidation by thiols in a cell-free model[25,28]. We checked that the presence of L-cysteine in the culture medium was necessary for HUVEC- and EA.hy 926-mediated LDL oxidation. The mechanism of LDL oxidation and the oxidative properties of the hybrid endothelial cell line EA.hy 926 are thus very similar to those of HUVEC. The vigorous growth, unlimited replication potential and clonal purity of the human endothelial cell-derived EA.hy 926 line could facilitate studies of LDL oxidation pathways.

Acknowledgements. We thank Beatrice Chappey for LDL labelling and Martine Auclair for her technical assistance in binding and degradation studies.

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