Volume 305, number 2, 155–159 FEBS 11213 June 1992
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Oxidized low density lipoproteins elicit DNA fragmentation of cultured lymphoblastoid cells

Isabelle Escargueil, Anne Nègre-Salvayre, Marie-Thérèse Pieraggi and Robert Salvayre

Department of Biochemistry, Metabolic Disease Laboratory, Faculty of Medicine in Rangueil, University Paul Sabatier, Toulouse, France

Received 16 April 1992; revised version received 8 May 1992

Lymphoblastoid cell lines continuously pulsed with mildly oxidized low density lipoproteins, exhibited a significant increase of DNA fragmentation induced by oxidized LDL internalized by cells. DNA fragmentation was associated with an increasing number of morphologically characteristic apoptotic cells simultaneously with the increase of cytotoxicity indexes, and the activation of the poly(ADP-ribose) polymerase, a nuclear enzyme stimulated by DNA strand breaks. The potential involvement of these biochemical and morphological changes in atherogenesis is discussed.

DNA fragmentation; Apoptosis; Oxidized LDL; Lymphoblastoid cell; Calcium; Cytotoxity

1. INTRODUCTION

Oxidized low density lipoproteins (LDL) are thought to play a major role in atherogenesis [1–4] by several mechanisms: (i) extensive oxidative alterations of apoB result in the metabolic deviation of oxidized LDL towards the scavenger–receptor pathway of macrophagic cells which accumulate cholesteryl esters and are transformed into 'foam' cells (characteristic of the early lesions of atheroma) [3,6]; (ii) lipid peroxidation of LDL [1,3–5] results in a cytotoxic effect towards cultured cells [7–11]. We have recently demonstrated that oxidized LDL elicit a delayed and sustained rise of cytosolic calcium $[Ca^{2+}]_i$ [12] which is involved in the cellular mechanism of the cytotoxicity [13]. A sustained $[Ca^{2+}]_i$ rise has been shown to be critically involved in the cytotoxicity by stimulating calcium-dependent injurious processes, for instance activation of degradative enzymes, resulting in damage of cellular components leading finally to cell death [13,14]. Supporting this view, several experimental studies have shown that a sustained $[Ca^{2+}]_i$ rise can stimulate calcium-dependent enzymes such as phospholipases and proteases which are probably involved in cytoskeletal alterations and blebbing of the plasma membrane (see review in [13]). Moreover, a sustained $[Ca^{2+}]_i$ rise has been shown to mediate DNA fragmentation [13,15–17], possibly potentialized by PKC inhibition [18]. This DNA fragmentation is one of the characteristic events of apoptosis, i.e. programmed cell death, and seems to be linked to activation of an endogenous nuclear endonuclease that clives chromatin into oligonucleosomes [19,20].

The actual mechanism by which cells exposed to oxidized LDL become damaged is still completely unknown. We report in the present work that the sustained $[Ca^{2+}]_i$ rise induced in lymphoblastoid cells by mildly oxidized LDL is followed by a significant increase of DNA fragmentation and of apoptotic cells number which could in part explain the mechanism of their cytotoxic effect.

Abbreviations: EBV, Epstein-Barr virus; LDL, low density lipoproteins.

Correspondence address: R. Salvayre, Laboratoire de Biochimie 'Maladies Métaboliques', CHU Rangueil, 1 Avenue J. Poulhès, 31054 Toulouse Cedex, France. Fax: (33) (61) 32 29 53.

2. MATERIALS AND METHODS

2.1. *Chemicals*

The calcium probe Quin-2/AM was purchased from Molecular Probes (Eugene, OR, USA), [^{3}H]thymidine (5 Ci/mmol) and [^{14}C]NAD (nicotinamide- [^{4}C]adenine dinucleotide, 273 mCi/mmol) from Amersham (Paris, France). Trypan blue, bovine serum albumin, sodium dodecyl sulfate, ethidium bromide and agarose from Sigma (St. Louis, MO, USA), RPMI 1640, fetal calf serum, glutamine, penicilline and streptomycin from Gibco (Cergy-Pontoise, France) and Ultroser HY from IBF (Villeneuve-la-Garenne, France), λ-DNA *Hin*dIII, proteinase K and ribonuclease A from Boehringer-Mannheim (Meylan, France). Other reagents and chemicals were obtained from Merck (Darmstadt, FRG) or Prolabo (Paris, France).

2.2. *Lymphoblastoid cell lines*

Lymphoblastoid cell lines were established by Epstein-Barr virus (B95/8 strain) transformation of blood B lymphocytes of healthy donors and were grown in RPMI 1640 medium containing 10% fetal calf serum, penicillin, streptomycin and glutamine [21]. 48 h before LDL incorporation, this medium was removed and replaced by RPMI 1640 containing 2% Ultroser HY (a serum substitute devoid of lipoprotein), as previously used [10,12].

2.3. *LDL isolation and oxidation*

LDL were isolated from human pooled fresh sera by ultracentrifu-

gation according to Havel [22], dialyzed, sterilized by filtration and their purity controlled as previously indicated [23]. Purified LDL were stored at 4°C under nitrogen (up to 2 weeks). For each experiment, 2 mg (as apoB) of LDL were exposed to UV-C radiations under the previously used standard conditions (254 nm, 0.5 mW/cm², for 2 h) [9,23] and immediately incorporated (at the concentrations indicated in the text) in the culture medium. Lipid peroxidation was evaluated by determining the content of thiobarbituric acid reactive substances (TBARS) according to the method of Yagi [24] as previously used [23].

2.4. *Determination of* $[Ca^{2+}]_i$

The concentration of $[Ca^{2+}]_i$ (free cytosolic calcium concentration) was determined by using Quin-2/AM according to Arslan et al. [25].

2.5. *Determination of cytotoxicity*

The cytotoxicity was determined by the Trypan blue test according to Morel et al. [26] and under the previously used conditions [9,10].

2.6. *Determination of DNA fragmentation and counting of apoptotic cells*

DNA fragmentation assays were essentially derived from the procedures previously used by Wyllie [15,19] and Orrenius' group [16–18]. Briefly, lymphoblastoid cells (approximately 10^6 incubated with oxidized LDL for variable times), were isolated by centrifugation for 10 min at 500 × *g*, allowed to lyse for 15 min in 1 ml lysis buffer (0.5% v/v Triton X-100 and 20 mM EDTA, 5 mM Tris, pH 8.0), then centrifuged for 20 min at 27,000 × *g* to separate the chromatin pellet from cleavage products. The pellet (re-suspended in 1 ml of 1 mM EDTA in 10 mM Tris-HCl, pH 8.0 buffer) and the supernatant were assayed for DNA determination by the fluorometric DAPI procedure according to Kapuscinski et al. [27] and Bumma et al. [28]. Similar results were obtained with the diphenylamine method [29], but using 5–10 × 10^5 cells per determination because of the slight sensitivity of this method.

Another procedure derivating from Zheng et al. [30] was alternatively used: lymphoblastoid cells (10^6/ml) were grown in a culture medium containing [^{3}H]thymidine (0.2 μCi/ml for 48 h) and carefully washed 3 times with phosphate-buffered saline. These pre-labelled cells were incubated with oxidized LDL for variable periods of time, then were lysed and centrifuged at 27,000 × *g* under the conditions indicated above and the relative DNA content was evaluated by radioactive liquid scintillation counting of supernatant and pellet fractions.

DNA electrophoresis was performed according to Trauth et al. [31] with the following modifications: briefly, 2 × 10^6 cells were pelleted (10 min at 600 × *g*), washed once with cold phosphate-buffered saline, re-suspended in NTE buffer, pH 8.0 (100 mM NaCl, 10 mM Tris, 1 mM EDTA) containing 1% SDS and 0.1 mg/ml proteinase K. After 12 h incubation at 37°C, the DNA was extracted with phenol, phenol/chloroform and chloroform (v/v) and precipitated in ethanol/Na-acetate (0.3 M) for at least 18 h at −20°C. After 5 min centrifugation at 13,000 × *g*, the DNA pellet was suspended in 20 μl of sterile water, digested with ribonuclease (1 mg/ml) and run on 1.5% agarose gel in TBE buffer (2 mM EDTA, 90 mM boric acid, 90 mM Tris, pH 8.4) in the presence of 0.05% Bromophenol blue. λ-DNA-*Hin*dIII digest was treated and used simultaneously as a size marker.

The percentage of apoptotic cells was determined by counting the lymphoblastoid cells microscopically after cytocentrifugation and staining by May Grünwald-Giemsa.

2.7. *Determination of poly(ADP-ribose) polymerase activity*

The poly(ADP-ribose) polymerase activity was determined exactly under the conditions described by Althaus et al. [32] and Schraufstatter et al. [33] on lymphoblastoid cells (3 × 10^6 cells/ml) previously incubated for variable periods of time with oxidized LDL, using [^{14}C]NAD as substrate: briefly, at the required time lymphoblastoid cells were pelleted by centrifugation (10 min at 500 × g), washed once with the incubation HEPES buffer (56 μM HEPES buffer, pH 7.4, containing 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$). The cell pellet was resuspended in 500 μl of HEPES buffer containing 0.01% digitonin and 250 μM NAD and 0.5 μCi/ml [^{14}C]NAD, the permeabilized cells were incubated for 5 min at 37°C and the proteins radiolabelled by ribosylation from [^{14}C]NAD were precipitated with 200 μl 50% TCA. The protein pellet was washed twice with TCA, solubilized in 2% SDS in 0.1 M NaOH (incubation overnight at 37°C) and its radioactivity determined by liquid scintillation counting (Packard Tricarb 4530).

Protein concentrations were determined using the procedure of Lowry et al. [34].

3. RESULTS

In agreement with our previous results [11,12], mildly oxidized LDL (LDL oxidized by UV-C irradiation under the standard conditions indicated above contained around 3 nmol TBARS/mg apoB) elicited a delayed and sustained $[Ca^{2+}]_i$ rise in lymphoblastoid cells, whereas the same dose of non-oxidized LDL did not induce any similar $[Ca^{2+}]_i$ peak (Fig. 1A). Under the standard conditions used here, UV-C induced a mild lipid peroxidation of LDL and no major structural or functional modification of apoB [10,23]; consequently, UV-oxidized LDL are internalized by lymphoblastoid cells through the apoB/E receptor pathway like normal LDL, as previously discussed [10]. When lymphoblastoid cells were continuously pulsed with 200 μg apoB/ml UV-oxidized LDL, the maximum of the $[Ca^{2+}]_i$ peak was observed around 12–14 h after the beginning of the pulse (Fig. 1A). This concentration of UV-oxidized LDL induces a delayed and progressive loss of cell viability (Fig. 1D) which has been shown to be, at least in part, calcium-dependent [12]. In contrast, the same concentration of non-oxidized LDL induced no $[Ca^{2+}]_i$ rise nor loss of cell viability.

We report here that the $[Ca^{2+}]_i$ rise was followed by a significant increase of DNA fragmentation level and apoptotic cell number (Fig. 1B and C). In EBV-transformed immortalized lymphoblastoid cell population, under the culture conditions used here (serum-free medium supplemented by 2% Ultroser HY) and during the exponential growth phase, we observed a basal level of cell death (around 22 ± 4% on the basis of Trypan blue staining) which is associated to a basal level of DNA fragmentation (around 10 ± 4%) and of morphologically apoptotic cells (4 ± 2%). When cells were pulsed with cytotoxic concentrations of UV-oxidized LDL (200 μg apoB/ml), the maximal level of DNA fragmentation was observed between 24 and 48 h after the beginning of the pulse. Then the level of fragmentation was apparently decreasing, perhaps because of the loss of DNA fragments (which might result from the loss of cell membrane integrity of dead cells: under the conditions used, around 70% cells were stained by Trypan blue after 48 h pulse). A good correlation was observed between the fluorometric (DAPI) and radiometric ([^{3}H]thymidine) methods for the quantitative determination of the DNA fragments. These results were con-

firmed by counting the number of apoptotic cells (Fig. 1C) (a microphotography of a morphologically typical apoptotic lymphoblastoid cell near normal cell is shown in Fig. 3) and by the presence of smears on the DNA electrophoresis (Fig. 2). DNA fragmentation induced by oxidized LDL was increased significantly and nearly parallel with the cytotoxicity index: the level of DNA fragmentation and of apoptotic cells represented an almost constant (but relatively low; around 20–25%) proportion of the cytotoxicity index (number of Trypan blue stained cells, i.e. cells with membrane damages).

Poly(ADP-ribose) polymerase activity was determined in lymphoblastoid cells grown for variable periods of time in the presence of oxidized LDL (under the conditions of Fig. 1, i.e. continuous pulse with 200 μg apoB/ml UV-oxidized LDL) and at the time of the determination, cells washed with the HEPES buffer, permeabilized with digitonin and incubated with [^{14}]NAD for radiolabelling of ribosylated proteins. A significant increase of poly(ADP-ribose) polymerase activity was observed with a maximum between 16 and 24 h after the beginning of the pulse (Fig. 4), thus concomitantly with the rise of DNA fragmentation indexes.

4. DISCUSSION

The data reported in this paper lead to several new conclusions on the cellular effects of oxidized LDL. The basal levels of DNA fragmentation reported here are consistent with the studies of Gregory et al. [35] which demonstrated that EBV-transformed lymphoblastoid cell lines are relatively protected from apoptosis by the expression of latent proteins (EBNAs and LMPs) induced by transforming strains of EBV. We used here these cells relatively resistant to apoptosis (particularly to serum deprivation-induced apoptosis), because we have to use serum-free culture medium for pulsing the cells with oxidized LDL in order to exclude non-oxidized LDL and protective molecules contained in the serum [7,8,36]. We can exclude that the DNA fragmentation reported here could be artifactual or non-specific since: (i) the same technique was used for all the samples (controls and cells treated by oxidized LDL); (ii) the three tests used here for detecting DNA fragmentation (DAPI fluorometric and [^{3}H]htymidine radiometric procedures and DNA electrophoresis) gave quite con-

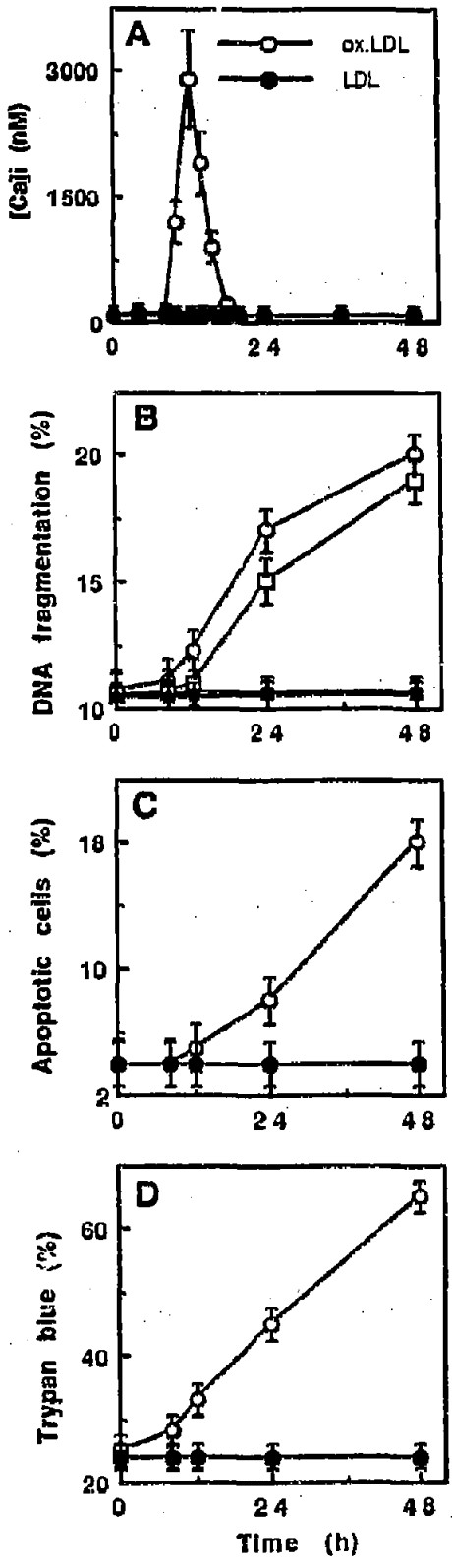

Fig. 1. Time course of [Ca^{2+}]$_i$ (A), DNA fragmentation (evaluated on 27,000 × g supernatant of gently lysed cells) by the fluorometric DAPI method (squares) or by the radiometric determination of [^{3}H]thymidine radiolabelled DNA fragments (circles) (B), apoptotic cells (microscopic morphological count) (C) and cytotoxicity indexes determined by counting Trypan blue stained cells (D). Cells were continuously pulsed with 200 μg of apoB/ml UV-oxidized LDL (open symbols) or non-oxidized LDL (filled symbols). Oxidation of LDL was performed under the standard conditions indicated in Materials and Methods; TBARS content in UV-oxidized LDL was 4 ± 1 nmol TBARS/mg apoB. The results are a mean of 5 separate experiments.

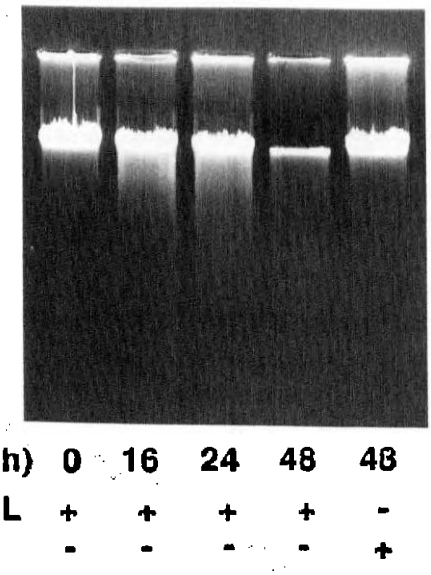

Fig. 2. Electrophoresis of DNA of cells grown in serum-free medium containing 2% Ultroser HY and continuously pulsed either by non-oxidized LDL or by oxidized LDL. All the procedures were performed as indicated in Materials and Methods. This experiment was done in quadruplicate and gave similar results.

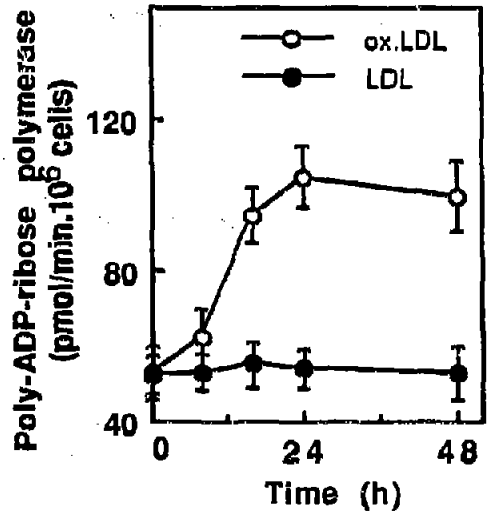

Fig. 4. Time course of poly(ADP-ribose) polymerase activity of lymphoblastoid cells pulsed with a fixed concentration of oxidized LDL (under the conditions used in Fig. 1). The enzyme activity was determined under the conditions indicated in Materials and Methods. Determinations were done in quadruplicate.

sistent results; (iii) the activation of poly(ADP-ribose) polymerase observed in our experiments strongly support the idea that DNA fragmentation occurred in the intact cell, since activation of this nuclear enzyme is associated to DNA strand break formation [32,33].

The difference between DNA fragmentation and cytotoxicity levels could be explained by different hypotheses. A first hypothesis is that oxidized LDL could kill cells by 2 separate mechanisms: the first one (predominating in a part of the cell population) could lead to DNA fragmentation and apoptosis, the second one (predominating in the other part of the cell population) could lead to damages of the plasma membrane and to the rise of cytotoxicity indexes. Yet, we do not know exactly if both events are subsequent to the $[Ca^{2+}]_i$ rise or not (the role of the sustained $[Ca^{2+}]_i$ rise being currently under investigation in our laboratory). A second hypothesis (less probable) is that cell death induced by oxidized LDL is mediated by a single mechanism (possibly the $[Ca^{2+}]_i$ rise) inducing DNA fragmentation and membrane damage as well, but DNA fragmentation is not complete or DNA fragments are lost in the culture medium subsequently to the loss of membrane integrity. An under-estimation of DNA fragmentation (resulting from a loss of DNA fragments) can probably be rejected since we did not detect any appreciable amount of DNA fragments in the culture medium (at times 24 and 48 h) and since the loss of total DNA was proportional to the cell loss when the cell population declined. It is more probable that DNA fragmentation is not complete in cells affected by the cytotoxicity: if all the cells affected by the cytotoxicity (i.e. Trypan blue stained, but not completely desintegrated) were also affected by DNA fragmentation, DNA fragmentation would be evaluated at around 20% of the total cellular DNA. However, it is to note that the number of apoptotic cells is largely lower than that of Trypan blue stained cells (around 18% vs. 65%, at time 48 h) and if the DNA fragmentation affected only apoptotic cells, the DNA of these cells would be almost completely fragmented. The mechanisms of apoptosis and of DNA fragmentation are only poorly understood [20] and are completely unknown in the case of oxidized LDL (since the present paper reports the phenomenon for the first time, to our knowl-

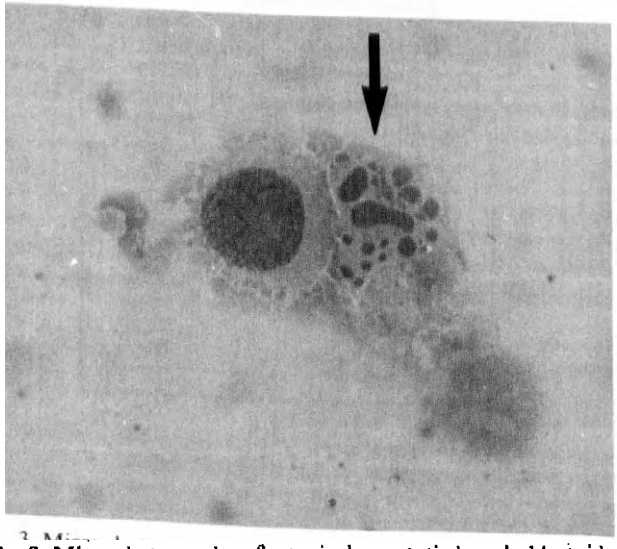

Fig. 3. Microphotography of a typical apoptotic lymphoblastoid cell (arrow) near a morphologically normal lymphoblastoid cell. Cells were grown for 24 h in the presence of UV-oxidized LDL under the conditions of Fig. 1 (May Grünwald-Giemsa staining, and magnification × 400).

edge), therefore we cannot today distinguish between the various hypotheses.

Acknowledgements: The authors wish to thank Drs. N. Paillous (IMRCP-CNRS) for helpful discussions. This work was supported by grants from INSERM (CRE 901004), Région Midi-Pyrénées (Contrats 8900699 and 907815), Université Paul Sabatier-Toulouse III (JE DRED 91), Fournier-Dijon Laboratories ('Aides à la Recherche') and ARCOL.

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