Original Article

Effects of Oxidized LDL on In Vitro Proliferation and Spontaneous Motility of Human Coronary Artery Endothelial Cells

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Objective

To investigate the effects of low concentrations of oxidized LDL (oxLDL) on the proliferation and spontaneous motility of human coronary artery endothelial cells (HCAEC) in culture.

Methods

Cultures of HCAEC were treated with low concentrations of native LDL (nLDL) isolated from human plasma and with LDL minimally oxidized through different chemical methods; the effects were compared.

Results

Native LDL had no deleterious effects on in vitro proliferation and motility of HCAEC; however, at its highest concentration and for a longer exposure, nLDL inhibited cell proliferation. The LDL chemically oxidized by spermine nonoate (SNO) and 3-morpholinylsydnonimine (SYN-1) had significant inhibiting effects on in vitro proliferation and motility of HCAEC, which were proportional to the greatest concentrations and degrees of oxidation of LDL.

Conclusion

OxLDL has a cytotoxic effect, inhibiting the proliferation and spontaneous motility of HCAEC in culture. This effect is proportional to the concentration and degree of oxidation of LDL; native LDL is relatively innocuous.

Key words

oxidized LDL, coronary artery endothelial cells, cardiovascular atherosclerotic disease

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High plasmatic levels of low-density lipoprotein (LDL) are considered one of the major risk factors for the development of cardiovascular atherosclerotic disease. The hypothesis of response to injury to explain atherosclerosis proposes that the first step in atherogenesis is endothelial dysfunction induced by the action of risk factors, especially the exposure of the vascular endothelium to oxidized LDL (oxLDL) [1].

Preliminary studies revealed that preparations of isolated human LDL have toxic effects on endothelial cells in culture [2-4]. Native LDL (nLDL) undergoes chemical alterations related to peroxidation of polyunsaturated fatty acids, components of the lipoprotein, which result in a great increase in its susceptibility to phagocytosis and break down by macrophages [5].

The major types of vascular wall cells, the endothelial cells, the smooth muscle cells, and the macrophages were shown in vitro to be able to oxidize native LDL[6]. Oxidized LDL inhibits endothelial cell migration, which is an essential mechanism in the processes of reestablishing vascular integrity after injury and angiogenesis, proportionally to the concentration and degree of oxidation of LDL, mediated by the formation of lipid hydroperoxides[7].

Recent studies have shown that the lipid components of oxLDL [8,9] can cause a paradoxical increase in the production of vascular endothelial growth factor (VEGF) by endothelial cells in culture, which may be a protective mechanism in face of cellular injury. OxLDL was observed to have a double effect on cell cycle: to induce cell proliferation at low concentrations (5 to 10 µg/mL), and apoptosis at concentrations above 50 µg/mL [10].

The endothelial cells constitute a heterogeneous population. Endothelial cells originating from different vascular beds express characteristic surface antigens, specific intracellular transporters, and regulation of different intracellular enzymes [11].

Considering the importance of endothelial cell heterogeneity and the contradictory data in the literature, and aiming at contributing to a better understanding of the basic processes involved in the interaction between oxLDL and endothelial cell, we carried out the first systematized study using human coronary artery endothelial cells (HCAEC) in culture, whose role in coronary atherogenesis is primordial.

This study aimed at investigating and describing the effects of low concentrations of nLDL and LDL chemically oxidized through different methods and oxidation times on in vitro cell proliferation and spontaneous motility.

Methods

This study was performed with the cell line of human coronary artery endothelial cells obtained through CLONETICS (BioWhittaker Inc., Walkersville, MD, USA). The culture medium used was MCDB-131 (GIBCO) with the addition of the following: EGF (INTERGEN), 10 μg/mL; hydrocortisone (SIGMA), 1.0 μg/mL; amphotericin B (GIBCO), 50 μg/mL; penicillin, 100 U/mL; streptomycin (GIBCO), 100 U/mL; and 10% fetal calf serum (GIBCO) with 1 μM L-glutamine (GIBCO). The experiments were performed in culture media with 5% fetal calf serum.

Cell count for assessing cell proliferation and migration was performed under direct microscopy (Nikon-TMS) with a 100X magnification in 3 different representative microscopic fields in each culture plate. The mean of the visualized cells was calculated.

The assay of cell migration was performed according to the model of in vitro healing established by Burk [12]. Briefly, the endothelial cells were cultivated in 60-mm culture plates (FALCON) with 5 mL of culture medium and 10% fetal calf serum. Two days after the culture became totally confluent, we created a lesion in the unicellular confluent layer with the aid of a razor blade pressed against the floor of the culture plate, cutting the cell layer and marking the plate. The razor blade was then carefully moved to the side, removing part of the unicellular layer. The cells were twice washed with PBS for removing the cell material pushed aside. Experimental culture medium (5% fetal calf serum) supplemented with variable concentrations of nLDL and oxLDL was added. Four culture plates were used as control or experimental, for each phase of the experiment.

After 24 hours of incubation, the number of cell nuclei that crossed the line demarcated in the culture plate was counted, in 3 distinct microscopic fields representative of each culture plate. The mean number of cells that surpassed the demarcated line was calculated.

nLDL was extracted from human plasma and purified according to the method reported by Sevanian et al [13]. nLDL was stored at a temperature lower than -70ºC until its use.

The LDL samples (0.5 μg/mL of protein) underwent oxidation with 1.0 mM of 3-morpholinylsydnonimine (SYN-1) or 1.0 μM of spermine nonoate (SNO) (SIGMA). The samples were incubated in a hot-water bath (37°C) under constant agitation at the following times: 1, 5, 10, 15, 30, and 60 minutes. The reaction was blocked with 100 mM of diethylene tetramine pentacetic acid, 100 mM of butylhydroxytoluene, 125 units/mL of superoxide dismutase, and 125 units/mL of catalase. All samples were maintained in the freezer at -20°C until the time of the experiments [14].

In statistical analysis, for comparing the concentrations, the Kruskal-Wallis nonparametric test was used, because it is considered the most efficient test for independent samples, and the Dunn test was used for multiple comparisons [15]. Nonparametric tests were used because the supposition of data normality was rejected. The significance level adopted for the tests was 5%.

Results

Figure 1 shows the effect of nLDL preparations on cell proliferation in partially confluent (60%) cultures of human coronary artery endothelial cells. After 72 hours of exposure to varied concentrations of lipoproteins, the cultures were washed twice with PBS (GIBCO) and stained according to the Giemsa method. Cell count was performed under microscopy (NIKON-TMS) with a 100X magnification in 3 different representative microscopic fields for each culture plate, and the mean number of cells was calculated.

An inhibiting effect of nLDL was observed on cell proliferation at the maximum concentration used, which may be attributed to the longest exposure and oxLDL by the endothelial cells in culture.

Figures 2 and 3 depict the effects of LDL oxidation by the SNO system for 1 and 10 minutes, respectively, on cell proliferation. An inhibitory effect proportional to the duration of oxidation is observed in both figures.

Figures 4 and 5 show the important inhibitory effects of oxLDL by the SYN-1 system for 1 and 10 minutes on cell proliferation.

Briefly, the results of the treatment of partially confluent endothelial cell cultures for 72 hours with nLDL and oxLDL by the SNO and SYN-1 chemical systems on cell proliferation were as follows: oxLDL by SNO and SYN-1 had an inhibitory effect on cell proliferation proportional to the duration of oxidation and to the concentration of oxLDL; LDL oxidized by SYN-1 had a more intense toxic effect than the other treatments did; nLDL had an inhibitory effect only at the greatest concentration studied.

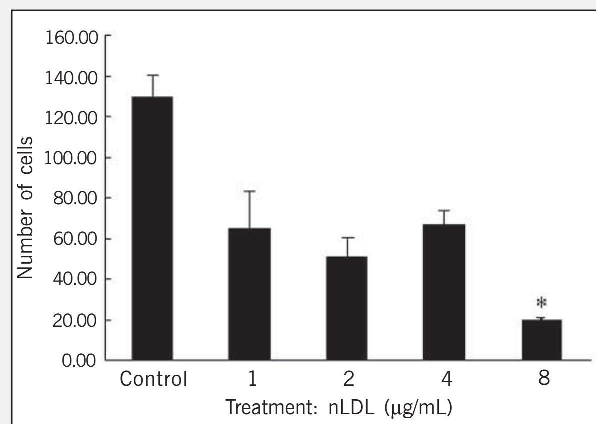

Fig. 1 - Effect of various concentrations of native LDL during 72 hours on the number of cells per microscopic field. Control differs from the treatment with 8 μg/mL *($P < 0.05$).

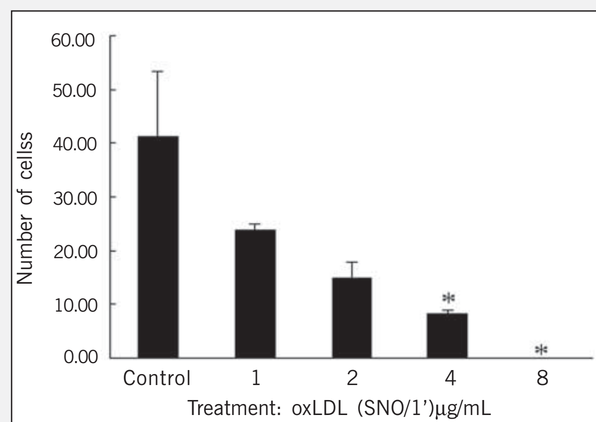

Fig. 2 - Effect of various concentrations of oxidized LDL (SNO/1') during 72 hours on the number of cells per microscopic field. Control differs from the treatments with 4 and 8 μg/mL *($P < 0.05$).

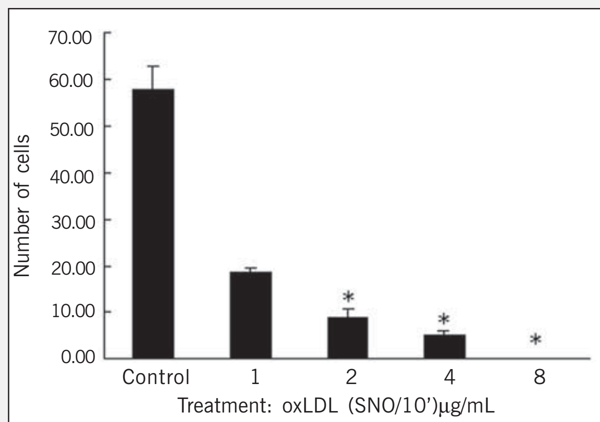

Fig. 3 - Effect of various concentrations of oxidized LDL (SNO/10') during 72 hours on the number of cells per microscopic field. Control differs from the treatments with 2, 4, and 8 µg/mL *(P < 0.05).

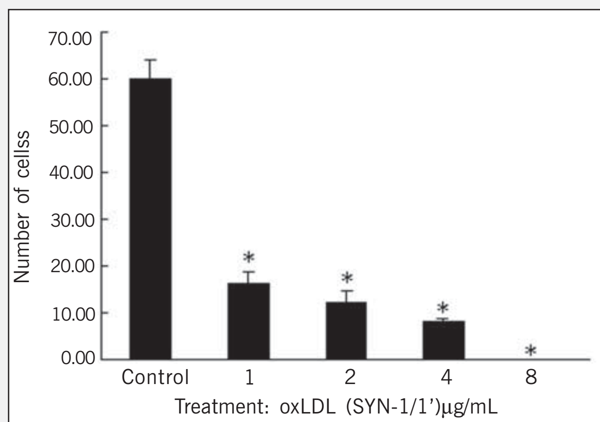

Fig. 4 - Effect of various concentrations of oxidized LDL (SYN-1/1') during 72 hours on the number of cells per microscopic field. Control differs from the treatments with 1, 2, 4, and 8 µg/mL *(P < 0.05).

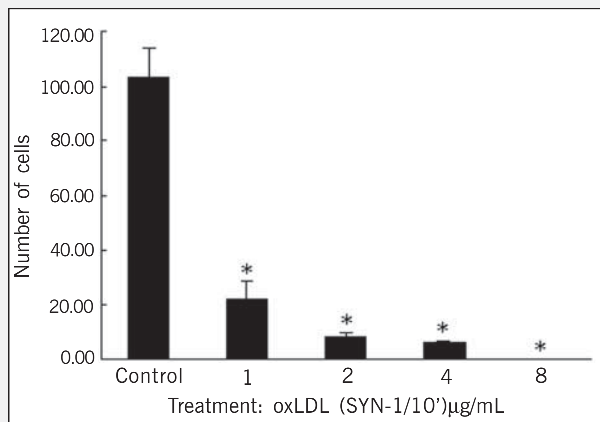

Fig. 5 - Effect of various concentrations of oxidized LDL (SYN-1/10') during 72 hours on the number of cells per microscopic field. Control differs from the treatments with 1, 2, 4, and 8 µg/mL *(P < 0.05).

Figure 6 shows the effect of the nLDL preparations on the spontaneous motility of human coronary artery endothelial cells according to the cell migration assay performed using the Burk technique. No inhibitory effect of nLDL on cell migration was observed.

Figures 7 and 8 show the effects of oxLDL by SYN-1 for 1 and 10 minutes, respectively. The effects were directly related to the concentration and duration of oxLDL.

Figure 9 is a microphotography representing the experiment of cell motility. A clearly smaller number of cell nuclei crossed the demarcated line in the treated field (B) as compared with that in the control field (A). This shows inhibition of cell migration.

Briefly, the effects of the treatment of endothelial cell cultures with native and SYN-1 system oxLDL for 24 hours on cell motility, according to the Burk technique, were as follows: nLDL had no inhibitory effect on cell migration; SYN-1 oxLDL inhibited cell migration; the effect observed was proportional to the duration of oxidation and the concentration of oxLDL.

Discussion

Recent studies have shown that the products of cholesterol oxidation generated by the oxidative modification of LDL, the cholesterol hydroperoxides, provide cytotoxicity to the particle, are relevant to the pathogenesis and progression of atherosclerosis, and have been identified in atheroma plaques and human plasma [16,17].

The molecular mechanisms that initiate in vivo oxidation of LDL are yet to be identified; however, some chemical substances, such as peroxynitrite, participate effectively in the process of LDL modification, the lipid peroxidation [18]. The method of LDL chemical oxidation through 2 systems, SYN-1 and SNO, was used. SYN-1 is known as a generator of peroxynitrite, and its use in the expe-

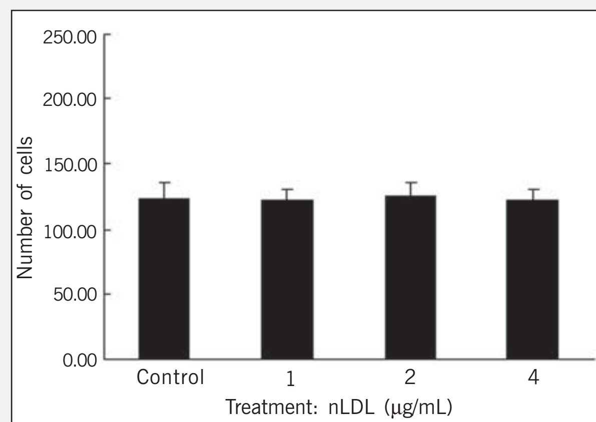

Fig. 6 - Effect of various concentrations of native LDL on the number of cells that migrated beyond the demarcated line. No significant difference was observed between the treatments (P=0.8920).

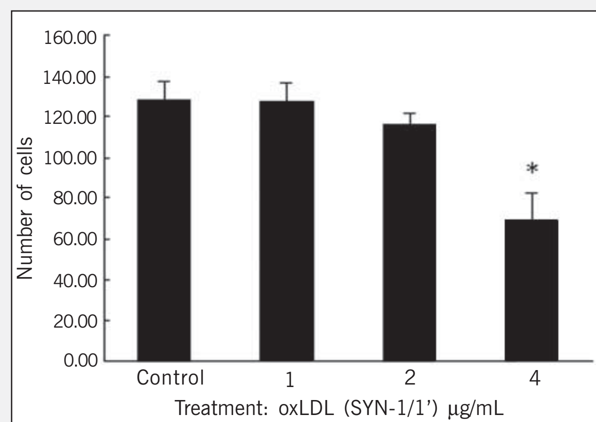

Fig. 7 - Effect of various concentrations of oxidized LDL (SYN-1/1') on the number of cells that migrated beyond the demarcated line. The treatment with 4 µg/mL differs from those with other concentrations and from control *(P < 0.05).

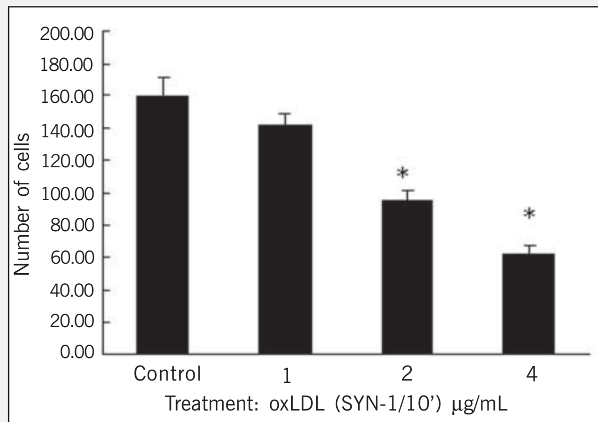

Fig. 8 - Effect of various concentrations of oxidized LDL (SYN-1/10') on the number of cells that migrated beyond the demarcated line. Control differs from the treatments with 2 and 4 µg/mL *($P < 0.05$).

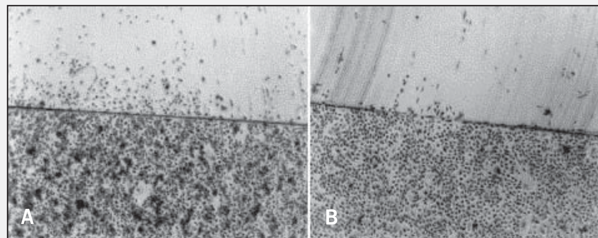

Fig. 9 - Microphotography of cell fields representing cell migration. A: control; B: treatment: OxLDL (SYN-1/ 10'); (40X magnification).

riments may be justified because the process is slow and constant, and, therefore, more similar to the path of peroxynitrite formation in physiological situations. SYN-1 has a high capacity of oxidation, because it oxidizes both the protein and lipid portions of LDL [19,20]. On the other hand, SNO, generator of nitric oxide, has a low oxidation capacity, because it oxidizes only the lipid portion of LDL. Therefore, the generation of products of oxidation by the 2 systems produces an LDL particle with different degrees of oxidation in different sites of the molecule [14].

The concentrations of oxLDL commonly used in the experiments (25 to 100 µg/mL) are considerably more elevated than the levels of oxLDL found in the human plasma (0.1 µg/mL) [21,22]. It is worth nothing, however, that, in atherosclerotic lesions, oxLDL is located and concentrated in the subendothelial space, because it is retained in the extracellular matrix during the formation of the atheroma plaque. Therefore, large quantities of oxLDL, in much higher concentrations than the plasmatic ones, accumulate in that region. The LDL retained in that microenvironment, ideal for oxidation, is more intensely oxidized and has characteristics similar to those of LDL oxidized by the different systems used in experimental studies [23].

In this study, we chose to use low concentrations (1 to 8 µg/mL) of LDL aiming at obtaining values closer to those of the real physiological situation, and, therefore, at investigating the biological effects of LDL on cell cultures.

Experimental data obtained with endothelial cells originating from a certain organ should not be automatically extrapolated to other systems, due to the heterogeneity of that cell line. A considerable part of the information in the literature about the complex mechanisms involved in human atherosclerosis and angiogenesis was obtained with endothelial cells of the umbilical cord, which would, therefore, require certain care in interpreting the results. The endothelium of the umbilical cord is submitted to extremely high concentrations of the steroid hormones and low pressure regimen, differently from the endothelial bed that participates in the coronary atherosclerotic process [11]. Thus, in our study, we chose to use the human coronary artery endothelial cell line.

Totally confluent endothelial cell cultures may serve as a model for in vitro studies of the vascular endothelium, because the basic properties of the endothelial cell are preserved. The cells in partially confluent culture (60%) are proliferating, and in that phase they are much more sensitive to adverse agents than in the state of plain integrity of total confluence.

In our experiments, oxLDL inhibited in vitro proliferation and motility of human coronary artery endothelial cells, effects directly related to the concentration and degree of oxidation of the lipoprotein. Our results may suggest the occurrence of similar effects of the oxLDL present in vascular walls, atherosclerotic plaques, and even circulating in the plasma, reinforcing the data in the literature that indicate the participation of oxLDL in cardiovascular events [24,25].

Recently, elevated oxLDL plasma levels were shown for the first time to directly relate to plaque instability in atherosclerotic lesions of human coronary arteries. OxLDL levels were measured in patients with acute myocardial infarction, unstable angina, stable angina, and controls, revealing a positive correlation with the severity of acute coronary syndrome. The serum levels of oxLDL were 4 times more elevated in patients with acute myocardial infarction when compared with those of controls, suggesting that circulating oxLDL may be a marker of severity in cardiovascular events [26].

Our results indicate that oxLDL concentrations similar to those found in the acute phase of coronary syndromes inhibit the *in vitro* proliferation and motility of human coronary artery endothelial cells. Considering that, one may suggest that elevated levels of oxLDL, due to its cytotoxicity, may negatively interfere not only with instability of the atherosclerotic plaque, but also with the reestablishment of the postinjury vascular integrity, worsening the prognosis of the patients.

In conclusion, the effects of oxLDL on the coronary endothelial cells in culture, inhibiting cell proliferation and motility, capital mechanisms in reendothelization of the injured areas of the wall and in the process of vascular angiogenesis, are proportional to the concentration and degree of oxidation of LDL. One could thus infer that clinical interventions to control and preserve the integrity of these variables should be pursued to minimize the deleterious effects and change the natural history of coronary artery disease.

Our data, however, should be interpreted considering the limitations inherent to in vitro experimentation, which is very valuable in clarifying pathophysiological mechanisms. Nevertheless, our results should not be directly extrapolated to the in vivo situation, upon which innumerous other noncontrolled factors act.

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