Cytotoxic Effect of Low-Density Lipoproteins on Intact, Ischemic, and Reperfused Endothelial Cells

M. V. Bilenko, V. G. Ladygina, and S. V. Fedosova

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny,* Vol. 126, No. 9, pp. 302-306, September, 1998
Original article submitted January 14, 1998

The cytotoxic effect of low-density lipoproteins on cultured human umbilical vein endothelial cells increases with their concentration, degree of oxidation, and incubation time, being more pronounced in ischemia or ischemia+reperfusion than in aerobic conditions. Synergism of the cytotoxic effect of lipoproteins with the damaging effect of ischemia and reperfusion promotes the development of atherosclerotic lesions of the vascular wall at sites predisposed to the damage by the ischemia/reperfusion.

Key Words: *endotheliocytes; ischemia/reperfusion; cytotoxic effect of low-density lipoproteins; atherosclerosis*

High blood level of low-density lipoproteins (LDL) is a risk factor of atherosclerosis. The key role is played by oxidized LDL (oxLDL) which were detected in the blood of humans or animals with hypercholesterolemia [5,6]. Oxidized LDL exert a potent cytotoxic effect on endothelial cells (EC), activate monocytes and tissue macrophages to a greater extent than LDL, are taken up by them via the scavenger receptors, and are better degraded in these cells, which leads to accumulation of cholesterol and formation of foam cells [10,12]. The cytotoxicity of oxLDL causes EC damage, thus increasing the permeability of vascular wall for LDL [1] and facilitating migration of circulating monocytes into it [4].

EC are often exposed to ischemia/reperfusion damage, which develops in hypoxia, anemia, hypertension, stress, and locally at sites of vascular spasm [3,11]. Hypertension, cardio-, cerebrovascular, and limb spasms often anticipate the development of atherosclerosis in these vessels [7,9].

Group for Anti-Ischemic and Antiradical Protection, Institute of Biomedical Chemistry, Russian Academy of Medical Sciences, Moscow

We have verified the hypothesis that ischemic and reperfused EC are more sensitive to the cytotoxic effect of oxLDL than intact EC, which may explain the mechanism of selective deposition of atherosclerotic plaques at vascular sites more often subjected to ischemia/reperfusion.

MATERIALS AND METHODS

Experiments were carried out on confluent cultures of human umbilical vein EC of the second and third passages. EC were isolated from the vein of a fresh human umbilical cord using 0.1% collagenase solution (Sigma), incubated, and after the second and third passages reincubated for 7 days at 37°C in a CO_2 incubator (Assab) in the growth medium, pH 7.4, based on dry RPMI-1640 (Flow) containing (per 100 ml): Na bicarbonate (7.5 g), HEPES (15 ml), Na pyruvate (100 mM, 1 ml), L-glutamine (200 mM, 1 ml, Sigma), EC growth factor (1.5 mg), fetal calf serum (10 ml, Gamaleya Institute of Epidemiology and Microbiology), and gentamicin (30,000 U, Pharmachim). Disposable 24-well plates (Costar) were used. For experimental incubation (6 or 24 h), the growth medium was replaced by incubation

0007-4888/98/0009-0911$20.00

medium (RPMI-1640, half volume+sodium bicarbonate without substrate additives, EC growth factor, and fetal calf serum). Aerobic incubation was carried out in a CO_2 incubator (Assab) at 95% air+5% CO_2; incubation under conditions of ischemia was carried out in a chamber filled with gas mixture 95% N_2+5% CO_2 (O_2 content <0.001%), which together with a decreased content of substrate-deficient protein-free medium simulated a stop-flow model of *in vivo* ischemia [2]. Reperfusion was carried out by transferring the plate with ischemic EC into aerobic environment for 1 h. After incubation the medium was collected and centrifuged at 1500 rpm. EC were washed by phosphate saline buffer (Sigma), detached with 0.1% trypsin+0.02% EDTA (Sigma), and together with the precipitate left after centrifugation of the medium reinoculated into growth medium for further 24-h reincubation under aerobic conditions. The viability of EC in experimental wells was estimated from the number of cells newly attached to the bottom in the percentage of cells in control wells (Plating Efficiency Index) [6].

LDL (d=1.019-1.065 g/cm³) were isolated from donor plasma by preparative ultracentrifugation in the presence of 0.01% EDTA (Sigma) and stored at 2-4°C. One day before the experiment LDL were dialyzed for 18 h at 4°C against 6000 volumes of 10 mM phosphate buffer (pH 7.4), sterilized through 0.45 μ filters (Serva), and protein was measured by the Lowry method. Oxidized LDL were prepared by 24-h incubation of LDL at 37°C with $CuSO_4$, 5 μM $CuSO_4$ per 0.2 mg protein/ml. Oxidized modification of LDL was estimated from the content of products reacting with 2-thiobarbituric acid (TBA) [15] which were measured in a Beckman DU-7 spectrophotometer and its content was expressed as an equivalent content of malonic dialdehyde (MDA). LDL and oxLDL were added into wells with EC in concentrations 100-300 μg protein/ml, each concentration was tested in 3-4 wells, and the data were averaged. Results were processed using Student's test for small samples.

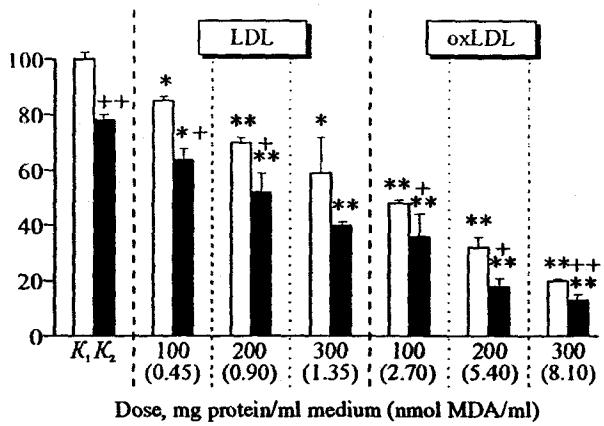

Fig. 1. Cytotoxic effect of nonoxidized and oxidized low-density lipoproteins (LDL and oxLDL) in human umbilical vein endotheliocytes incubated under aerobic conditions (light bars) and ischemia (dark bars) for 6 h (*M±m*). The content of TBA-reactive products in LDL and oxLDL before incubation was 4.5±0.9 and 27±0.3 nmol MDA/mg protein, respectively. Five series of experiments were carried out. Here and in Figs. 2 and 3: Ordinate: percent of viable endothelial cells. K_1 is the number of viable cells after aerobic incubation without LDL and K_2 is the number of viable cells after incubation under conditions of ischemia without LDL. All data are presented in percent of K_1 (100%). *p<0.01, **p<0.01 *vs.* respective controls; +p<0.05, ++p<0.01 *vs.* incubation under aerobic conditions.

RESULTS

LDL and oxLDL had a pronounced dose-dependent cytotoxic effect on intact human umbilical vein EC (Fig. 1). In experiments with LDL this effect could be explained by slight oxidation of LDL (Fig. 1), since it increased after double and triple increase of LDL concentration by protein, which led to an increase in the content of TBA-reactive products in LDL. The cytotoxic effect of oxLDL with initially 6 times higher degree of oxidation was stronger, and

TABLE 1. Cytotoxic Effect of LDL and oxLDL on EC during 6 or 24-h Incubation under Aerobic Conditions and Ischemia (*M±m*)

LDL dose, mg protein/ml medium		Conditions and duration of incubation, h			
		aerobic		ischemia	
		6	24	6	24
No LDL		100±2.0	100±3.0	78±0.7	64±1.0*
LDL	100 (0.45, 0.4)	85±2.0*	70±0.8***	64±4.0*	45±1.0***
	200 (0.9, 0.8)	70±3.0**	57±1.0***	52±6.0*	45±1.0**
	300 (1.35, 1.2)	59±11*	54±6.0**	40±0.3**	44±3.0**
oxLDL	100 (2.7, 3.3)	48±0.5**	50±2.0**	36±6.0**	41±2.0**

Note. *p<0.05, **p<0.01 *vs.* incubation under the same conditions without LDL, +p<0.05 vs. 6-h incubation. In brackets: nmol MDA/ml medium for 6- and 24-h incubation, respectively.

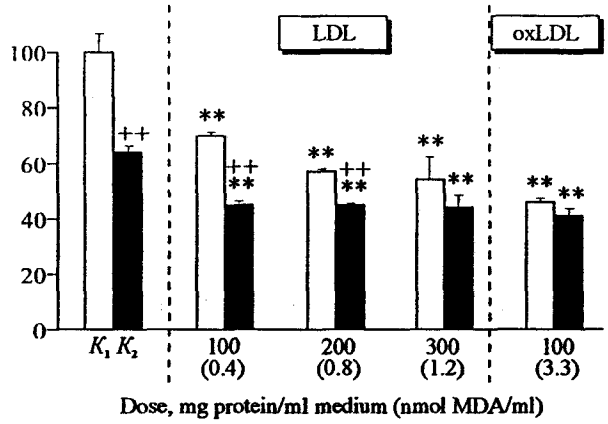

Fig. 2. Cytotoxic effect of nonoxidized and oxidized low-density lipoproteins (LDL and oxLDL) on endothelial cells of human umbilical vein incubated under aerobic conditions (light bars) and under conditions of ischemia (dark bars) for 24 h (*M±m*). The content of TBA-reactive products in LDL and oxLDL before incubation was 4±0.1 and 33±0.9 nmol MDA/mg protein, respectively. Five series of experiments were carried out.

at the highest concentration of oxLDL (300 μg protein/ml medium) the number of viable cells was only 20% of the aerobic control. During incubation of EC under conditions of ischemia, the cytotoxic effect of LDL and oxLDL was dose-dependent and significantly higher (and the number of viable cells significantly lower) than after incubation of intact EC with LDL and oxLDL in the same concentrations ($p<0.05$-0.01) and in control tests with ischemia ($p<0.05$-0.01). These data indicate that the effect of ischemia and LDL of different degrees of oxidation on EC is synergic.

The low content of TBA-reactive products in native LDL is probably be due to autooxidation of LDL during dialysis, because the addition of anti-

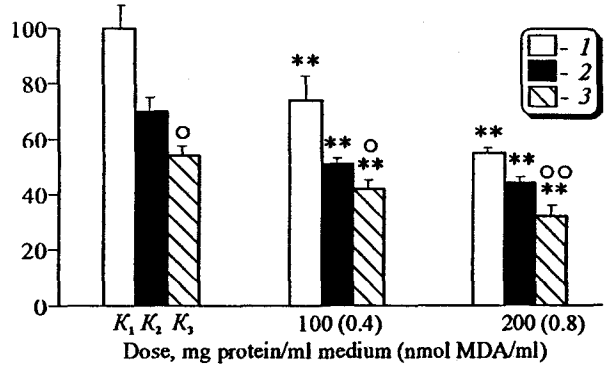

Fig. 3. Cytotoxic effect of nonoxidized low-density lipoproteins on human umbilical vein endotheliocytes incubated for 24 h under aerobic conditions (*1*), ischemia (*2*), and ischemia followed by reperfusion (*3*) (*M±m*). K_3 is the number of viable cells after 24-h incubation under conditions of ischemia+reperfusion (1 h) without LDL. °$p<0.05$, °°$p<0.01$ vs. incubation under conditions of ischemia. The content of TBA-reactive products in LDL before incubation was 4±0.1 nmol MDA/mg protein. Four series of experiments were carried out.

oxidants to the dialysate (EDTA, glutathione, ionol, vitamin E, superoxide dismutase, catalase) prevented the oxidation of LDL and their cytotoxic effect on EC [8]. The content of TBA-reactive products in LDL before incubation with EC in our experiments agrees with a previous report [10] as well as the data for oxLDL [10,14].

A 24-h incubation with LDL and oxLDL had a pronounced cytotoxic effect on intact and ischemic EC, but it was less dependent on the concentration of LDL in experiments with aerobic incubation than after 6-h incubation, while in incubation under conditions of ischemia it did not depend on the dose (Fig. 2). The number of live cells in experiments with LDL or oxLDL under conditions of ischemia was lower than in ischemic control at all concentrations ($p<0.01$), while with LDL concentrations of 100 and 200 μg protein/ml medium (0.4-0.8 nmol MDA/ml), the count of live cells was lower than at the same LDL concentrations in aerobic incubation ($p<0.01$). This indicates a synergic cytotoxic effect of ischemia and LDL on EC during 6 and 24 h incubation.

In control experiments on ischemia, prolongation of incubation decreased cell viability ($p<0.05$, Table 1). In experiments with LDL and oxLDL amplification of the cytotoxic effect due to prolongation of incubation under aerobic conditions was observed only with LDL containing no more than 0.8 nmol MDA/ml medium TBA-reactive products, while in incubation under conditions of ischemia the content of these products was no higher than 0.4 nmol MDA/ml medium. Thus, prolongation of EC incubation from 6 to 24 h abolished the dose- and time-dependent effects of all LDL except those with low or moderate content of TBA-reactive products. The cytotoxic effects of LDL with a high content of protein or high degree of oxidation (oxLDL) reached the maximum earlier (within the first 6 h of incubation). The cytotoxic effect of ischemia and LDL/oxLDL on EC was synergic up to 24 h. The prolonged cytotoxic effect of slightly oxidized LDL is of special interest because it may be due to their on-going oxidation under the effect of EC. EC modify LDL during prolonged co-incubation *in vitro* [13], but the ability of ischemic EC to oxidize LDL is unknown and will become the object of our further investigation.

Comparison of the cytotoxic effect of LDL on EC during a 24-h incubation under conditions of ischemia and ischemia followed by reperfusion showed that the number of viable EC in ischemia+reperfusion was smaller in the control and in experiments with LDL than after ischemia without reperfusion ($p<0.05$-0.01), and in experiments with

LDL it was significantly smaller than in the control ($p<0.05$-0.01). These data indicate that EC reperfusion after ischemia, as well as reperfusion of the whole organ, augments the ischemic injury and amplifies the cytotoxic effect of LDL. Prolongation of aerobic incubation of intact EC for 1 h, a period as long as the duration of reperfusion of ischemic EC, had no effect on the viability of EC (data not shown).

From our results it can be concluded that LDL of different degree of oxidation exert a cytotoxic effect on intact EC. During the first 6 h of incubation this effect is dose-dependent and depends on the content of TBA-reactive products in LDL. At moderate concentrations of LDL or their moderate oxidation this effect is time-dependent: it increases with prolongation of incubation from 6 to 24 h. Incubation of EC under conditions of ischemia decreased the percentage of viable cells to 78±0.7 and 64±1% after incubation for 6 and 24 h, respectively. LDL of different degrees of oxidation and ischemia exert a synergic cytotoxic effect on EC, which is more pronounced than that of ischemia or LDL alone. Reperfusion of EC subjected to long ischemia leads to a greater decrease in the percentage of viable cells than after ischemia without reperfusion, and the cytotoxic effect of LDL of different degree of oxidation on EC subjected to ischemia+ reperfusion is more pronounced than the effect of reperfusion or LDL alone or LDL+ischemia. In other words, reperfusion aggravates the injury to ischemic EC and potentiates the damaging effect of LDL on EC.

Thus, reperfusion potentiates the detrimental effect of LDL on vascular endothelium, which under conditions of hypercholesterolemia is an atherosclerosis risk factor.

The authors are grateful to Profs. P. Hochstein and A. Sevanian from the University of South California, Los Angeles, USA, for consultations on methods of culturing and evaluating the viability of EC.

The study was supported by the Russian Foundation for Basic Research (grant No. 97-04-48472).

REFERENCES

1. J. J. Alexander, D. J. Graham, and R. Miguel, *J. Surg. Res.*, **51**, No. 5, 361-367 (1991).
2. M. V. Bilenko, A. Sevanian, and P. Hochstein, *J. Mol. Cell. Cardiol.*, **25**, Suppl. 1, 85 (1993).
3. D. W. Crawford and D. M. Kramsch, *Exp. Mol. Pathol.*, **49**, 215-233 (1988).
4. A. Faggiotto, R. Ross, and L. Harker, *Arteriosclerosis*, **4**, 323-340 (1984).
5. G. Gazzolato, P. Avogaro, and G. Bittolo-Bom, *Free Radic. Biol. Med.*, **11**, 247-253 (1991).
6. H. N. Hodis, D. M. Kramsch, A. Sevanian, *et al.*, *J. Lipid Res.*, **35**, 669-677 (1994).
7. M. Marrilli and S. Goldstein, *Am. J. Cardiol.*, **47**, 1180-1181 (1987).
8. D. W. Morel, J. R. Hessler, and G. M. Chisolm, *Ibid.*, **24**, 1070-1076 (1983).
9. C. J. Nickerson, C. C. Haudenschild, and A. V. Chobanian, *Exp. Mol. Pathol.*, **56**, 173-185 (1992).
10. S. Parthasarathy, J. Barnett, and L. G. Fong, *Biochim. Biophys. Acta*, **1044**, 275-283 (1990).
11. S. L. Seidel and R. Strong, *Hypertension*, **8**, 103-108 (1986).
12. D. Steinberg, S. Parthasarathy, T. F. Carew, *et al.*, *N. Engl. J. Med.*, **320**, 915-924 (1989).
13. U. P. Steinbrecher, S. Parthasarathy, D. S. Leake, *et al.*, *Proc. Natl. Acad. Sci. USA*, **81**, 3883-3887 (1984).
14. C. E. Thomas and R. I. Jackson, *J. Pharmacol. Exp. Ther.*, **256**, 1182-1188 (1991).
15. M. Ushiyama and M. Michara, *Anal. Biochem.*, **86**, 271-278 (1978).