EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Cytotoxic Effect of Low-Density Lipoproteins

V. A. Nagornev, A. N. Voskan'yants, A. G. Vinogradov, O. Yu. Zhdanova, and A. D. Denisenko

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 135, No. 1, pp. 107-109, January, 2003
Original article submitted October 11, 2002

The cytopathogenic effect of various modified lipoproteins on cultured peritoneal macrophages was studied *in vitro*. After minor peroxide modification lipoproteins induced apoptosis of macrophages. Immune complexes containing oxidized lipoproteins caused cell necrosis.

Key Words: *modified lipoproteins; peritoneal macrophages; apoptosis; necrosis*

Peroxidatively modified low-density lipoproteins (mLDL) enriched with cholesterol are constantly formed in humans and mammals. mLDL play a key role in atherogenesis. However, the mechanisms of their atherogenic activity are poorly understood. mLDL gain autoantigenic properties, which results in the formation of autoimmune complexes containing LDL and antibodies [3,10]. As differentiated from native LDL (nLDL), mLDL and immune complexes with these particles are engulfed by macrophages in the wall of arteries. This process is realized via scavenge receptors and leads to the appearance of cholesterol-loaded foam cells and formation of atherosclerotic damage to arteries.

All stages of atherogenesis are accompanied by cell death. Therefore, the vascular wall contains apoptotic cells. The mechanisms triggering cells death and role of mLDL in this process are poorly known. Studies performed in the past decade showed that during atherogenesis cell death in the vascular wall is realized via necrosis and apoptosis. The question arises: whether apoptosis is a result of lipid accumulation in cells or it is an integral component of atherogenesis, *i.e.* proceeds in parallel with the formation of mLDL and immune complexes including antigenic lipoproteins in the vascular wall [5-7]. Here we studied the role of mLDL and immune complexes containing these particles in the induction of cell death.

Research Institute of Experimental Medicine, Russian Academy of Medical Sciences, St. Petersburg. ***Address for correspondence:*** van@iem.spb.ru. Nagornev V. A.

MATERIALS AND METHODS

LDL (d=1.025-1.055 g/ml) were isolated from the donor' blood by consecutive centrifugation [8]. LDL were oxidized by incubation with $CuSO_4$ in doses of 10 and 1 μM at 37°C for 18 h to obtain mLDL and minimally modified LDL (mmLDL), respectively.

Autoantibodies to mLDL were isolated from the plasma of patients with coronary heart disease by affinity chromatography on a CNBr-Sepharose 4BCL column with immobilized human LDL modified with malonic dialdehyde. IgG were the major immunoglobulins in isolated autoantibodies. Immune complexes containing LDL and antibodies (molar ratio 4:1) were prepared by the addition of affinity isolated autoantibodies to mmLDL.

Peritoneal macrophages were isolated from male outbred albino mice [2]. These cells were cultured on cover glasses in Petri dishes using Eagle's medium. The medium contained 5 mg/ml proteins from lipoprotein-free human plasma and 50 μg/ml nLDL, mLDL, and mmLDL (free, in complexes with autoantibodies, or with 15 μg/ml free autoantibodies).

The cytotoxic effect of LDL was determined visually by the immunocytochemical method using FITC-labeled annexin B in combination with propidium iodide (PI). Apoptest-FITC Kit was used (Dako).

0007-4888/03/1351-093$25.00 © 2003 Plenum Publishing Corporation

The method is based on binding of annexin B to phosphatidylserine reoriented and localized on the membrane surface in apoptotic cells. Phosphatidylserine is localized on the surface of cell membranes starting from the early stage of apoptosis to the complete destruction of cells. Therefore, this localization of phosphatidylserine is typical of all stages of apoptosis. Annexin B is conjugated to fluorochrome, binds phosphatidylserine on the cell surface in the presence of Ca^{2+}, and serves as a marker for apoptosis. PI allows performing visual examination of cells in the late stage of apoptosis or necrosis.

After incubation the growth medium was removed from Petri dishes with peritoneal macrophage monolayer, and cells were washed with 0.1 M phosphate buffer. The cells (nonfixed cell culture) were incubated with immunocytochemical reagent containing 1 μl annexin B, 10 μl PI, and binding buffer (Annexin-V Binding Buffer) for 15 min in dark. After washout with the binding buffer cover glasses were mounted on slides.

After prefixation with 4% buffered paraformaldehyde macrophages from each sample were stained for lipids with Oil Red agent to study accumulation of lipids and transformation into foam cells. This procedure allowed us to estimate the quantitative ratio between normal macrophages and foam cells.

Apoptotic and necrotic cells were counted in 10 fields of view under a luminescence microscope (×400). Foam cells were counted in 10 fields of view under an Opton microscope (×400).

RESULTS

In our experiments 30 and 91.5% macrophages underwent transformation into foam cells after incubation with mLDL for 12 and 48 h, respectively. However, foam cells were not revealed after incubation of macrophages with nLDL. mmLDL induced transformation of 10% macrophages into foam cells. After addition of immune complexes containing mmLDL and antibodies, the ratio of foam cells was 30% of total cell count.

Our results are consistent with published data that mLDL induce accumulation of lipids in macrophages and appearance of foam cells. This process depends on modification of lipoprotein particles. Immune complexes containing LDL and antibodies are more potent than free mmLDL in inducing lipid accumulation in macrophages.

After 12-h incubation we observed death of only individual cells, which did not depend on the type of added lipoproteins (nLDL or mLDL).

The ratio of apoptotic cells increased most significantly with lengthening of macrophage incubation with mLDL. Incubation with mLDL for 48 h caused apoptosis in practically all cells. However, in experiments with nLDL only 26% cells underwent apoptosis, and 24% cells were labeled with PI.

Thus, oxidized LDL produce a strong cytotoxic effect on cells accompanied by apoptosis of macrophages transformed into foam cells and not containing lipids. The induction of apoptosis did not depend on the degree of LDL oxidation. It is not surprising that 50% macrophages underwent apoptosis or necrosis after 48-h incubation with nLDL. True nLDL are present only in the blood. These particles are oxidized during isolation and incubation with cells. The longer is the period of incubation, the greater is the degree of nLDL peroxidation. Therefore, arterial walls contain no nLDL during atherogenesis [1].

Our results agree with published data that oxidized LDL can induce apoptosis of cells [9]. It should be emphasized that mLDL produce a cytotoxic effect even without lipid accumulation. Therefore, this effect of mLDL is not associated with lipid accumulation in macrophages and their transformation into foam cells.

Of particular interest is the effect of immune complexes containing antigenic mmLDL on cell death. After 24-h incubation practically all cells underwent necrosis, while the percentage of foam cells did not surpass 30% of the total cell count. These results complement published data that in the presence of autoimmune complexes with mLDL in the vascular wall, atherogenesis runs a severe course and leads to the

TABLE 1. Count of Necrotic Cells (%) after Incubation of Mouse Peritoneal Macrophages with mmLDL and Immune Complexes Containing Antigenic mmLDL (*M*±*m*)

Cells	Incubation, h					
	mmLDL		mmLDL+IgG		IgG	
	24	48	24	48	24	48
Unlabeled	30.4±6.4	0	3.0±1.9	0	68.0±4.1	0
Labeled with FITC-annexin B	53.4±6.2	99.0±0.5	3.0±1.7	5.0±1.6	14.0±3.3	7.0±1.9
Labeled with PI	16.2±3.6	1.0±0.5	94.0±2.9	95.0±1.6	18.0±1.5	93.0±1.9

formation of giant phagolysosomes in macrophages, their destruction, and cell death [4].

The surprising result was obtained after incubation of peritoneal macrophages with autoantibodies to mLDL in the absence of LDL. The cytotoxic effect of autoantibodies on macrophages was similar to that produced by immune complexes containing mLDL. After 48-h incubation necrosis was observed in all cells. The question arises: which factors contribute to the development of immune inflammation in the wall of arteries during *in situ* accumulation or formation of autoimmune complexes with mLDL? Previously, mLDL were believed to play the major role in this process [3,4,10]. *In vitro* experiments demonstrated that antibodies to LDL can also gain cytotoxic properties.

Our results show that depending on conditions of LDL modification, their atherogenic effect is manifested in apoptosis and/or necrosis in cells. It should be emphasized that apoptosis of cells can proceed in the very early stages of LDL modification.

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