Circulating desialylated low density lipoprotein

In memory of Vladimir Tertov

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ABSTRACT

Accumulation of lipids is the central event in the development of atherosclerotic lesion. Circulating low density lipoprotein (LDL) particles are known to be the major source of cholesterol and other lipids stored in atherosclerotic plaque. However, not all LDL particles possess atherogenic properties. In order to induce lipid accumulation in arterial cells, LDL particles have to undergo modifications. Although among such modifications the oxidation remains the most studied one, other atherogenic LDL modifications have been described. According to a series of studies conducted with blood serum and LDL from atherosclerotic patients, desialylation is one of the earliest if not the first atherogenic modification of LDL. Desialylation occurs in the bloodstream and is followed by a cascade of other modifications, including the reduction of LDL particle size and increase of its density, acquisition of negative electrical charge, oxidation and formation of highly atherogenic complexes. In this mini-review we will discuss the concept of multiple atherogenic modification of LDL leading to initiation and progression of atherosclerosis.

Keywords:
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Introduction

Extra- and intracellular deposition of lipids, predominantly of cholesteryl esters, in the arterial intima is one of the earliest manifestations of atherosclerosis [1–4]. The formation of lipid-laden foam cells is recognized as the triggering factor in the pathogenesis of atherosclerosis [5,6]. By the end of 1970s, it was found that low density lipoprotein (LDL) circulating in human blood is the source of lipid accumulation in vascular cells [7,8]. However, only modified LDL, and not native lipoprotein, cause intracellular lipid accumulation.

Currently, PubMed lists 8692 articles indexed under “oxidized LDL” and 4108 under “oxidized LDL and atherosclerosis”. The hundreds of reviews on this topic remove the need to emphasize the oxidative modification of LDL in detail. It is generally accepted that oxidized LDL causes foam cell formation and triggers atherogenesis [9–11]. However, other atherogenic modifications of LDL have been detected in the bloodstream of atherosclerosis patients, which have attracted much less attention until now.

Small dense LDL is regarded as atherogenic lipoprotein subfraction circulating in the blood. It has been described in a number of excellent reviews [12,13]. Another form of circulating modified LDL is electronegative LDL (LDL(−)), which was detected using methods, sensitive to the particle’s electric charge, such as agarose gel electrophoresis, isotachophoresis or ion exchange chromatography [14,15]. The atherogenic LDL(−) fraction was first isolated by Avogaro and co-authors using ion-exchange chromatography [15]. The authors dedicate this review to the memory of their colleague, Vladimir Tertov, a recognized leader in the research on modified lipoproteins, who died fifteen years ago. However, the research that he initiated continues. In this review, we provide an overview of the results obtained in course of Dr. Tertov’s research, as well as of more recent studies published after his death as a tribute to the memory of our colleague.

Desialylated LDL

The search for atherogenic LDL circulating in human blood resulted in the discovery, isolation and characterization of desialylated LDL followed by the studies of the mechanisms of atherogenic modification. As a first step, LDL was isolated from the blood of healthy subjects and cardiovascular patients with angiographically proven coronary atherosclerosis. The ability of LDL to induce intracellular lipid accumulation (atherogenicity) was tested in a primary culture of human aortic intima smooth muscle -actin-positive cells (typical smooth muscle cells and pericyte-like cells), which correspond to the cell types accumulating fat in atherosclerotic lesions in situ [19]. In most cases, LDL samples obtained from healthy individuals induced no intracellular accumulation of phospholipids and neutral lipids [20,21], whereas most of the samples of LDL isolated from the plasma of patients with coronary atherosclerosis increased the intracellular content of triglycerides, free cholesterol and cholesteryl esters [21,22].

What is the reason for LDL atherogenicity? Comparison of atherogenic and non-atherogenic LDL properties demonstrated a significant difference in the sialic acid content of lipoprotein particles [23,24]. Sialic acid is a terminal monosaccharide of asparagine-bound biantennary carbohydrate chains within LDL glycoconjugate moiety. After removal of sialic acid, galactose becomes the terminal saccharide. This fact was used to isolate the subfraction of desialylated LDL from total LDL preparation using Ricinus communis agglutinin (RCA120), which possesses high affinity to the terminal galactose [25]. Incubation of cultured cells with normally sialylated LDL subfraction had no effect on the intracellular phospholipid and neutral lipid content [25,26]. By contrast, desialylated LDL subfraction induced a significant increase in the intracellular lipids.

Atherogenicity of desialylated LDL

Two approaches were used to elucidate the mechanisms of intracellular lipid accumulation caused by desialylated LDL: (1) evaluation of binding, uptake and degradation of LDL; and (2) determination of the rate of hydrolysis and esterification of lipids in LDL particles. The uptake of desialylated LDL was much higher than the uptake of native LDL, especially by cells that were cultured from atherosclerotic lesions [27]. Binding to the scavenger-receptor, asialoglycoprotein-receptor and proteoglycans may account for the enhanced cellular binding and uptake of desialylated LDL. On the other hand, degradation rate of internalized desialylated LDL was lower than that of native LDL [27]. The enhanced uptake and the low rate of intracellular degradation lead to the accumulation of desialylated LDL. Desialylated LDL stimulates intracellular esterification of free cholesterol [27]. This can explain the accumulation of cholesteryl esters in human arterial cells caused by desialylated LDL.

In addition to intracellular lipid accumulation, increased proliferative activity and enhanced synthesis of the extracellular matrix components by subendothelial cells are generally recognized as major manifestations of atherosclerosis at the cellular level [28]. Intracellular lipid accumulation induced by desialylated LDL was found to be accompanied by the enhanced proliferative activity and synthesis of the connective tissue matrix components [28,29]. Therefore, desialylated LDL can induce all known atherosclerotic manifestations at the cellular level.

Properties of desialylated LDL

Desialylated LDL differs considerably from native LDL by its carbohydrate and lipid composition [26]. Desialylated LDL particles are smaller and denser and more electronegative than native LDL particles [26]. In apoprotein B-100 (apoB-100) of desialylated LDL, the amino group domain is chemically modified, whereas another domain is masked due to the changes in the tertiary structure of apoB-100 [26]. To evaluate the degree of LDL oxidation, a new approach based on apoB-lipid adduct measurement was developed [30]. This approach allowed demonstrating
that desialylated LDL of coronary atherosclerosis patients is an oxidized lipoprotein. In addition to a high degree of in vivo oxidation, desialylated LDL exhibits a higher susceptibility to in vitro oxidation. To determine the causes of the increased degree of in vivo oxidation and oxidizability of desialylated LDL, the contents of the major fat-soluble antioxidants in lipoprotein particles were determined, and the correlations between the contents of coenzyme-Q10, tocopherols and carotenoids and the level of apoB-cholesterol adducts and the susceptibility of LDL to in vitro oxidation were analyzed [31]. It was demonstrated that the content of all investigated lipid-soluble antioxidants in desialylated LDL is lower than in native lipoproteins. This can explain the high oxidizability of desialylated LDL.

We isolated the total immunoglobulin G fraction from the sera of atherosclerotic patients and purified the immunoglobulins that interact with LDL (anti-LDL) using affinity chromatography [32]. Compared to healthy individuals, higher amounts of anti-LDL immunoglobulins were isolated from the sera of atherosclerotic patients. The affinity constant of anti-LDL to LDL obtained from healthy subjects was lower than that to modified LDL from atherosclerotic patients. The affinity constants for in vitro glycosylated LDL, acetylated LDL, and LDL oxidized by Cu2+ were similar to the affinity constant for LDL from atherosclerotic patients. The affinity constant of anti-LDL to LDL obtained from healthy subjects was lower than that to modified LDL from atherosclerotic patients. The affinity constants for in vitro glycosylated LDL, acetylated LDL, and LDL oxidized by Cu2+ were similar to the affinity constant for LDL from healthy subjects. The anti-LDL autoantibodies showed a higher affinity for LDL from atherosclerotic patients and malondialdehyde-modified LDL (MDA-LDL), chemical modification, which is considered to reflect natural oxidation of LDL. The LDL that was desialylated in vitro by neuraminidase possessed the highest affinity constant among all of the tested forms of modified LDL. Thus, autoantibodies against LDL are generated as a response to desialylated LDL. Cross-reaction with the MDA-LDL can be explained by a similar conformation of certain epitopes of desialylated LDL and MDA-LDL.

**Multiple modification of LDL in human blood**

Therefore, naturally occurring atherogenic LDL circulating in the blood appears to be small, dense, and more electronegative. Modified LDL particles possess altered lipid, protein and carbohydrate moieties compared to native LDL. Therefore, these lipoprotein particles can be regarded as multiple-modified LDL (Fig. 1). To determine the modifications that govern LDL atherogenic potential, the correlations between changes in different chemical and physical parameters of LDL and the ability of LDL to induce lipid accumulation in cultured subendothelial cells of human aortic intima were analyzed. This analysis revealed a significant negative correlation between LDL atherogenicity and the sialic acid content. Other parameters, such as size and charge of the LDL particles, the phospholipid and neutral lipid content, fat-soluble antioxidants and lipid peroxidation products, the amount of free lysine amino groups, and the degree of oxidation and oxidizability of LDL, were not significantly correlated with atherogenicity [26,33]. Thus, desialylation is most likely the most important modification that results in lipoprotein atherogenicity.
ed. After 36 h of incubation, the negative charge was increased. Further incubation (48 and 72 h) reduced α-tocopherol and increased LDL oxidizability and oxidation. In addition, the degradation of apoB-100 was observed. Thus, desialylation of LDL particles is the primary modification. Subsequent modifications enhance the atherogenicity of LDL. Multiple modifications of LDL in the blood occur through a cascade of successive changes: desialylation, decrease of lipid content, reduction of the particle size, increase of its density and negative charge and peroxidation of lipids [37].

These observations fully explain the detection of various forms of modified LDL in the blood, including desialylated, small dense, electronegative and oxidized. This sequence is likely to represent the chain of atherogenic modifications of LDL occurring in human blood plasma. It should be considered that, in contrast to the popular belief, oxidation may be just one of the atherogenic modifications of LDL, which occurs at later stages following several other modifications, and its importance for the increase of the atherogenic potential of modified LDL might be overestimated.

Because desialylation is one of the earliest, if not the first atherogenic modification of the LDL particle, elucidation of the mechanism of LDL desialylation in blood is extremely important. Trans-sialidase (approximately 65 kDa protein) was isolated from lipoprotein-deficient serum using affinity chromatography [38]. Treatment of native LDL with isolated trans-sialidase led to LDL desialylation and then induced cholesterol ester accumulation in human aortic intimal cells [38]. Thus, trans-sialidase may be responsible for LDL desialylation in the blood.

Enhancement of LDL atherogenicity

Microscopic studies of atherosclerotic plaque development revealed that foam cell formation occurs faster than expected. We hypothesized that modified LDL atherogenicity may be enhanced by a number of mechanism and identified three of them: association of LDL particles, formation of circulating immune complexes containing modified LDL, and formation of LDL complexes with the extracellular matrix.

LDL forms complexes with components isolated from human aortic intima, namely: cellular debris, collagen, elastin and proteoglycans [39–41]. Multiple-modified LDL as a part of these complexes has an increased ability to induce intracellular lipid accumulation. This enhancement is a result of increased uptake and decreased intracellular degradation of LDL in complexes compared to complex-free particles.

Tertov et al. [42–44] showed that multiple-modified LDL possesses a tendency to spontaneous self-association. Moreover, the degree of LDL association correlates with atherogenicity of modified LDL [43,44]. In cell culture, LDL self-associates isolated from human plasma induced much higher elevation of intracellular lipids as compared with initially non-associated particles [43]. Self-associates of multiply-modified LDL are formed spontaneously in cell culture conditions and cause intracellular accumulation of lipids [43]. At the same time, prevention of complex formation by continuous filtration of the culture medium resulted in a complete prevention of lipid accumulation in cells [44]. It has been found that the increased atherogenic potential of LDL associates is a result of increased uptake by phagocytosis and reduced degradation rate of LDL particles [44]. These observations allowed us to draw a conclusion that, without modification of LDL particles, formation of associates does not occur, and without LDL association accumulation of intracellular lipid does not take place, i.e. the atherogenic potential of LDL is not manifested. This implies that it is possible to prevent atherogenesis by suppressing LDL association. Further investigation of the mechanisms of LDL association revealed the factors capable to promote or prevent the association [45–50]. It turned out that the most promising are poloxamers [50–52]. We consider poloxamers and similar substances as candidates for antiatherosclerotic drug development.

Multiple modified LDL can be immunogenic. We have found circulating immune complexes (CIC) containing auto-anti-LDL and multiple-modified LDL in blood of most atherosclerotic patients [53–58]. Serum levels of LDL containing CIC correlated with coronary, femoral, and carotid atherosclerosis [57,58]. LDL isolated from CIC had properties similar to those of multiple-modified LDL, such as lower content of sialic acid, neutral lipids, phospholipids and neutral saccharides, smaller size, and increased density and electronegativity [56]. Immunoglobulin G interacting with the protein moiety of LDL was isolated from plasma of coronary atherosclerosis patients [53,57]. Isolated anti-LDL autoantibodies interact with different forms of chemically modified LDL, with the highest affinity demonstrated to desialylated LDL, and a lower affinity – to MDA-LDL and total LDL from atherosclerotic patients [53]. Anti-LDL in complex with native LDL stimulated lipid accumulation in cells cultured from uninvolved subendothelial human aortic intima. Autoantibodies also potentiated atherogenicity of modified LDL [53,57]. Further investigation of CIC containing LDL revealed the diagnostic and prognostic potential for atherosclerosis [54,55,58,59]. Clinical implications of CIC LDL measurement in the blood are described in the next section.

In conclusion, formation of large complexes containing multiple-modified LDL (self-associates, immune complexes and complexes with extracellular matrix) substantially increases atherogenic potential of multiple-modified lipoproteins.

Clinical implications

The accumulating knowledge generated over the many years of studying modified LDL was translated into the development of clinically relevant applications. In particular, as mentioned above, studies of LDL-containing immune complexes led to the development of a diagnostic test [54,55,58,59].

A method based on precipitation of CIC using polyethylene glycol followed by determination of cholesterol or apoB in the precipitate was described [58]. Because both
approaches to evaluation of LDL content in CIC yield similar results, a simpler and cheaper option based on the measurement of cholesterol (CIC-cholesterol) could be applied. Patients with atherosclerosis had significantly higher CIC-cholesterol levels than healthy individuals. The threshold level of CIC-cholesterol of 16 μg/mL was established. The diagnostic value of CIC-cholesterol was compared with other lipid and lipoprotein parameters used as clinical markers associated with atherosclerosis. CIC cholesterol, as well as the apo B/apo A-1 ratio allowed distinguishing individuals with and without atherosclerosis [58]. Total cholesterol, triglycerides, HDL cholesterol, apo B, Lp[a] and apo A1 were not reliable in that regard. CIC-cholesterol-based analysis had the highest sensitivity, specificity and accuracy of diagnosis as compared to other parameters. Two-year prospective study was performed in asymptomatic individuals with carotid atherosclerosis [55]. The increased levels of CIC-cholesterol, total cholesterol and LDL cholesterol had similar prognostic significance in respect to atherosclerosis progression. Normal level of CIC-cholesterol (below 16 μg/mL) was the only parameter that predicted the absence of atherosclerosis progression. Therefore, CIC-cholesterol level may be used as a marker of atherosclerosis and can have a prognostic value sufficient for clinical implication.

The diagnostic significance of the level of deglycosylated (desialylated) LDL in blood remained unexplored until a method for diagnostics of predisposition to atherosclerosis development was established by our group. This method is based on the comparison of simultaneous measurement of total and desialylated apoB-100 in serum and calculation of desialylated apoB-100 fraction size [60]. In brief, 130 serum samples from healthy persons and patients with carotid atherosclerosis were taken for measuring desialylated apoB-100 using ELISA-based technique previously developed by Tertov et al. [61]. Quantitative diagnosis of atherosclerosis in study participants was conducted by ultrasound examination of carotid arteries in high-resolution mode, followed by detection of atherosclerotic plaques and intima-media thickness measurement. In healthy individuals, mean value of desialylated apoB-100 fraction size was 12%, and in patients with carotid atherosclerosis – 27%. Desialylated apoB-100 level was not correlated with any of the traditionally considered risk factors for cardiovascular diseases. These data suggest that serum levels of desialylated LDL may serve as an independent discriminant for the diagnosis of atherosclerotic disease. The threshold value of desialylated apoB-100 fraction size accounted for 18%, thus allowing the best way to differentiate the participants in the study by the presence of atherosclerotic plaques in the carotid arteries as instrumental signs of atherosclerosis [60]. Additionally, we have studied the ability of the mixtures of native (non-atherogenic) and desialylated LDL to induce accumulation of cholesterol in vitro in primary culture of monocyte-macrophages obtained from the blood of healthy donors. Desialylated LDL was obtained by treatment of native LDL with neuraminidase. Incubation with mixtures containing 1–15% of desialylated LDL did not lead to cholesterol deposition in cultured cells, while incubation with LDL mixtures containing 20–50% desialylated LDL resulted in a significant increase in cholesterol in cells, and this effect was dose-dependent. The extrapolation of results established the same threshold value of desialylated apoB-100 fraction of 18%, in which the mixture of modified and native acquired the ability to induce cholesterol accumulation in cells in vitro [60]. These results, besides their practical utility, also support the hypothesis on the role of modified (desialylated) LDL in pathogenesis of atherosclerosis in humans.

The discovery of such atherogenic factors as autoantibodies against modified LDL and LDL-containing circulating immune complexes stimulated the development of approaches to eliminating these factors. We have developed a procedure for extracorporeal removal of autoantibodies against LDL from bloodstream. As a kind of prototype for this procedure, LDL-apheresis uses columns with immobilized anti-LDL antibodies involved in the extracorporeal perfusion system. During the plasma passage through such column, LDL is adsorbed by anti-LDL, which decreases the blood level of LDL dramatically. We used a similar principle for isolation of autoantibodies. Columns were filled with immobilized autologous LDL instead of anti-LDL [65]. Such a column effectively removed autoantibodies to LDL from plasma. The columns with immobilized LDL were used for extracorporeal perfusion. In a pilot study, this procedure was applied to four patients with coronary atherosclerosis twice a month for 7–9 months. As a result, there were no new coronary stenoses, 50% stenoses progressed, 25% regressed, and 25% remained unchanged [65]. This suggests a beneficial effect as compared to normal history of coronary atherosclerosis progression.

The search for therapeutic approaches to atherosclerosis treatment was a central topic of our research. To this end, we have created a cellular models based on the assessment of the degree of intracellular lipid accumulation caused by modified LDL [66–68]. Using these models, we have identified several effective anti-atherosclerotic pharmacological agents, including botanicals, and developed drugs based on these agents. These drugs have significantly reduced the atherogenic potential of the blood. We have conducted three clinical atherosclerosis regression studies and found that newly developed drugs prevent the development of carotid atherosclerosis and induce regression of already existing atherosclerotic lesions [69–72]. Thus, our basic studies revealed that lipid accumulation in arterial cells induced by modified LDL is the key trigger process in atherogenesis. Therefore, the results of the performed basic research were successfully translated into clinics, with clinically demonstrated efficacy of both novel approaches and novel drugs.

**Conclusions**

In conclusion, we have identified a subfraction of LDL that is capable of inducing accumulation of lipids, primarily cholesteryl esters, in subendothelial cells derived from uninvolved human aortic intima. We have shown that atherogenic LDL is characterized by numerous alterations of carbohydrate, protein and lipid moieties and can be regarded as multiple-modified LDL. Multiple modification of LDL occurs in human blood plasma. Cir-
culating multiple-modified LDL has a decreased affinity for the B,E-receptor and acquires the ability to interact with a number of other cellular membrane receptors and proteoglycans. The enhanced cellular uptake of desialylated LDL, low degradation rate of apolipoprotein and cholesteryl esters, and stimulation of re-esterification of free cholesterol cause the intracellular accumulation of esterified cholesterol.

The formation of LDL-containing large complexes (associates, immune complexes, complexes with the extracellular matrix components) can stimulate lipid accumulation in intimal smooth muscle cells. In addition to cholesteryl ester accumulation, desialylated LDL stimulates cell proliferation and synthesis of the connective tissue matrix. Thus, we have discovered and characterized naturally occurring multiple-modified LDL capable to induce all atherosclerotic manifestations at the cellular level. This discovery has found its application in clinical practice. Obtained knowledge allowed developing a diagnostic method based on measurement of modified LDL in circulating immune complexes. We have developed an approach to extracorporeal removal of autoantibodies to modified LDL from blood based on the use of immobilized LDL. In addition, cell models evaluating the degree of LDL atherogenicity were established to develop anti-atherosclerotic therapies.

Authors' contributions
AO designed and wrote the manuscript Sections 1–5 and 8, AM is the author of Section 6, IS is responsible for Section 7, EI drew the figure and performed the text correction and preparation for publication.

Conflict of interest
None declared.

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Ethical statement
Authors state that the research was conducted according to ethical standards.

References
[28] A.N. Orekhov, V.V. Tertov, S.A. Kudryashov, V.N. Smirnov, Triggerlike stimulation of cholesterol accumulation and DNA and extracellular matrix synthesis induced by atherogenic


[34] V.V. Tertov, G. Bittolo-Bon, I.A. Sobenin, et al., Naturally occurring modified low density lipoproteins are similar if not identical: more electronegative and desialylated lipoprotein subfractions, Experimental and Molecular Pathology 62 (1995) 166–172.


[38] V.V. Tertov, V.V. Kaplun, I.A. Sobenin, et al., Human plasma trans-sialidase causes atherogenic modification of low density lipoprotein, Atherosclerosis 159 (2001) 103–115.


