Chapter 1

Circulating Atherogenic Multiple-Modified Low-Density Lipoprotein: Pathophysiology and Clinical Applications

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Additional information is available at the end of the chapter

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Abstract

Low-density lipoprotein (LDL) circulating in human bloodstream is the source of lipids that accumulate in arterial intimal cells in atherosclerosis. *In-vitro*–modified LDL (acetylated, exposed to malondialdehyde, oxidized with transition metal ions, etc.) is atherogenic, that is, it causes accumulation of lipids in cultured cells. We have found that LDL circulating in the atherosclerosis patients' blood is atherogenic, while LDL from healthy donors is not. Atherogenic LDL was found to be desialylated. Moreover, only the desialylated subfraction of human LDL was atherogenic. Desialylated LDL is generally denser, smaller, and more electronegative than native LDL. Consequently, these LDL types are multiply modified, and according to our observations, desialylation is probably the principal and foremost cause of lipoprotein atherogenicity. It was found that desialylated LDL of coronary atherosclerosis patients was also oxidized. Complex formation further increases LDL atherogenicity, with LDL associates, immune complexes with antibodies recognizing modified LDL and complexes with extracellular matrix components being most atherogenic. We hypothesized that a nonlipid factor might be extracted from the blood serum using a column with immobilized LDL. This treatment not only allowed revealing the nonlipid factor of blood atherogenicity but also opened the prospect for reducing atherogenicity in patients.

Keywords: atherosclerosis, multiple-modified LDL, desialylated LDL, atherogenicity, circulating immune complexes, therapeutic approach

1. Introduction

Early stages of atherosclerosis development are characterized by abnormally high lipid accumulation in the arterial intima [1]. Formation of foam cells filled with lipids may be considered as the onset of the disease [2]. Low-density lipoprotein (LDL) circulating in human bloodstream is the origin of lipids that accumulate in the arterial intima cells [3]. However, intracellular cholesteryl ester accumulation could not be induced *in vitro* by native LDL [4]. On the other hand, *in vitro* –modified LDLs (acetylated, exposed to malondialdehyde, oxidized with transition metal ions, etc.) were demonstrated to cause lipid accumulation in cultured cells [5, 6]. Moreover, the question whether the modified LDL forms obtained *in vitro* fully correspond to the profile of modified LDL existing *in vivo* remains controversial. Therefore, the research community faces here a paradox: on one hand, a well-grounded opinion indicates LDL as the main source of lipid accumulation in the arterial wall, and on the other hand, native LDL failed to induce intracellular lipid accumulation in cultured cells. At the same time, *in vitro*–modified LDL was found to be atherogenic. However, detection of modified LDL in the bloodstream appeared to be challenging: acetylated LDL could not be found in the bloodstream, and the existence of oxidized LDL *in vivo* could not be demonstrated directly. Auto-antibodies against LDL modified by malondialdehyde, which is considered as a model of oxidized LDL, have been found in circulation [7]. It has to be kept in mind, however, that LDL conjugated with malondialdehyde (MDA-LDL) is a purely artificial modification, which cannot form in a living organism. Despite the fact that oxidized LDL has not been found in the bloodstream, the occurrence of antibodies against MDA-LDL is usually regarded as evidence of the existence of oxidized LDL *in vivo* [7].

2. Discovery of desialylated LDL in blood

In order to study modified LDL in atherosclerosis, we isolated LDL fraction from the blood of healthy subjects and atherosclerotic patients. We aimed to demonstrate that LDL from atherosclerotic patients can induce lipid accumulation in cultured cells. As a model, we used smooth muscle α-actin-positive cells, isolated from the intima of human aorta. These cells have been demonstrated to deposit lipids in atherosclerotic lesions *in situ* [8]. The method for isolation and cultivation of these cells has been previously established by our group [8]. After being cultured for 7 days, smooth muscle α-actin-positive cells (SMA(+) cells) originating from uninvolved intima of human aorta were subjected to a 24-hour incubation in Medium 199 supplemented with 10% lipoproteindepleted serum from a normal subject, as well as with LDL fraction with concentration 5–500 μg of apolipoprotein B (apo B)/ml. In the majority of experiments with the LDL samples isolated from normal subjects, there was no significant intracellular accumulation of phospholipids and neutral lipids [9]. By contrast, in the majority of experiments with LDL obtained from the plasma of coronary atherosclerosis patients, the intracellular levels of free cholesterol and triglycerides increased by 1.5 times and of the level of cholesteryl esters increased 1.5 to 5 times. Higher concentrations of LDL had no added effect on the intracellular lipid level. The results of the described experiments have

demonstrated that LDL fractions isolated from the blood of patients with atherosclerosis, but not from normal subjects, induced deposition of lipids in human vascular cells. This feature of LDL was referred to as atherogenicity [10].

What is the possible explanation of LDL atherogenicity? We focused on comparing the properties of atherogenic LDL circulating in the patients' blood and nonatherogenic LDL from healthy donors. One of the major observations we made was the significantly (2 to 3 times) decreased sialic acid (N-acetylneuraminic acid) in LDL isolated from patients with coronary atherosclerosis [11]. Sialic acid is a terminal residue of asparagine-bound biantennary carbohydrate chains in LDL glycoconjugate moiety. In case of its removal, galactose becomes the terminal exposed residue. It is therefore possible to use *Ricinus communis* agglutinin (RCA120), which has a strong affinity to the terminal galactose to specifically isolate desialylated LDL [12]. We applied the total LDL preparation on a column containing CNBr-activated agarose-bound RCA120. LDL with sialylated carbohydrate chains passed freely through the column, while desialylated LDL bound to the lectin sorbent and could later be eluted with 5–50 mM galactose. This method allowed us extracting subfractions of both sialylated and desialylated LDL from the total LDL preparation isolated from the blood of patients. Desialylated LDL was found to be only a fraction of the total LDL pool circulating in patients' blood. Using the lectin affinity columns and lectinsorbent assay, we demonstrated that the ratio of desialylated LDL in blood of patients with coronary atherosclerosis was 20–60% of the total LDL level, while for normolipidemic subjects, desialylated LDL accounted for 5–15% [89]. The sialic acid content in desialylated LDL subfraction isolated by lectin chromatography was 2–3 times lower than that of sialylated LDL [12].

We next studied the atherogenic properties of desialylated LDL. Cultured SMA(+) cells, derived from the intima of human aorta and incubated with sialylated LDL subfraction, had unaltered intracellular contents of phospholipids and neutral lipids [12]. By contrast, cells incubated with desialylated LDL demonstrated a 1.5- to 2-fold increase in the contents of lipids and nonesterified cholesterol, as well as a 2- to 7-fold surge in the cholesteryl esters content. Therefore, only the desialylated subfraction of human LDL was found to be atherogenic. Normally, sialylated LDL had no atherogenic effect and could be regarded as native unmodified LDL. In summary, we have isolated a subfraction of naturally occurring desialylated LDL that was able to induce lipid deposition in human arterial subendothelial cells.

3. Trans-sialydase: the unknown LDL-modifying enzyme

More than 98% of the sialic acid cleaved from LDL is not present in the free form in the blood but is transferred to various protein acceptors [13]. Therefore, the enzyme responsible for desialylation of LDL works as a trans-sialidase.

We found that, apart from LDL, other lipoproteins, glycoproteins, and gangliosides are also affected by the trans-sialidase activity. Free sialic acid can be transferred to glycoproteins and sphingolipids of human serum. It can also be transferred to a protein or a lipid moiety of lipoprotein particles. Both lipoprotein fraction of human blood serum and lipoprotein-deficient

serum had sialidase activity, as demonstrated by gel-filtration chromatography. Trans-sialidase activity was shown to be present in lipoproteins, as well as in a free form. The mechanism of trans-sialidase interaction with lipoproteins remains to be elucidated.

Using affinity chromatography, we succeeded in extracting a 65-kDa protein from lipoprotein deficient serum, which was a likely candidate to be the trans-sialidase [13]. The isolated enzyme was present in quantities from 20 to 200 μg/ml of human serum. The enzyme had three pH optima: 3.0, 5.0, and 7.0. The optimal pH spectrum indicated that the trans-sialidase would be active both in blood and in cellular organelles with low pH. Calcium and magnesium ions at millimolar concentrations could influence the enzyme activity *in vitro*. Thiol groups were found to be essential for normal enzyme functioning. Various blood proteins could serve as substrates for trans-sialidase activity. The enzyme successfully cleaved sialic acids from HDL, LDL, IDL, and VLDL particles. Trans-sialidase could also cleave sialic acid residues from glycoconjugates found in plasma, proteins (fetuin and transferrin), and gangliosides (GM3, GD3, GM1, GD1a, and GD1b). The rate of sialic acid transfer from these glycoconjugates was, however, much slower as compared to LDL. Among the sialylated LDL, VLDL, IDL, and HDL, the former has the highest affinity to the trans-sialidase. The mechanism trans-sialidase preference for LDL is unclear. It is possible that trans-sialidase activity is affected by the particle volume.

Importantly, isolated naturally occurring trans-sialidase was able to desialylate native LDL, which resulted in formation of desialylated LDL, which could induce cholesteryl ester accumulation in SMA(+) human aortic intimal cells [13]. This underscores the possible role of the enzyme in foam cell formation.

The role of plasma trans-sialidase remains to be established. Possible functions of trans-sialidase may include regulation of plasma proteins activities, cell-to-cell interactions, lifespan of glycoproteins, lipoproteins, and cells, etc [14].

Given its role in the formation of modified LDL, trans-sialidase activity may be an important component in the onset and progression of atherosclerosis. Trans-sialidase can also affect the interaction of lipoproteins with the arterial wall. Lipid accumulation induced by lipoproteins processed by trans-sialidase can be associated with the induction of proliferation and extracellular matrix synthesis. In conclusion, trans-sialidase may participate in all currently known cellular manifestations of atherosclerosis.

4. Physical properties of desialylated LDL

LDL is defined as a lipoprotein fraction with densities spanning from 1.019 to 1.063 g/l. Using ultracentrifugation and gradient gel electrophoresis, LDL particles can be segregated into four subfractions, including large, intermediate, small, and very small LDL [15].

4.1. Size and density

We first separated LDL particles based on their flotation rate using analytical ultracentrifugation [16].

We have determined densities of both native and desialylated LDL using gradient density ultracentrifugation [17]. Desialylated LDL fraction tended to have higher density than native LDL. Increased density was caused by lower amounts of phospholipids, free and esterified cholesteryl, and triglycerides.

Another method of LDL analysis, gradient gel electrophoresis, allows for separation of LDL subfractions by their electrophoretic mobility, which depends on the particle size and shape [18]. Gradient gel electrophoresis separation allows distinguishing 4 subclasses: large, intermediate, small, and very small LDL [19]. Correlation of LDL particle size and density is highly significant, as shown using ultracentrifugation, as well as gradient gel electrophoresis. However, these parameters are not always equal. Another method to analyze the weight and size of LDL particles is capillary gel electrophoresis is also used [20].

Another relatively new method for analysis of LDL is nuclear magnetic resonance (NMR). It is sometimes used for analyzing LDL subfractions in blood plasma, although the results obtained using this method cannot be compared directly with those obtained by ultracentrifugation of gel electrophoresis [21]. Other available methods of LDL analysis include high-performance liquid chromatography [22], dynamic light scattering [23], ion mobility analysis [24], and homogenous assay analysis [25].

Desialylated and native LDL particle size was estimated by our group using quasi-elastic laser scattering in a lipoprotein suspension followed by electrophoresis in polyacrylamide gel and scanning densitometry [26]. Native LDL particles from healthy subjects and atherosclerotic patients had sizes of 26.5 and 26.8 nm, respectively. Desialylated LDL of healthy subjects and atherosclerotic patients were 24.8 and 24.5 nm, respectively. The results of polyacrylamide gel electrophoresis were similar, with average diameters of native LDL being 26.3 and 26.2 nm for controls and for patients and those of desialylated LDL being 23.5 and 22.9 nm for controls and patients, respectively [17]. These results demonstrated that desialylated LDL had a reduced particle size in comparison with native LDL.

The origins of LDL subfractions remain unclear. According to Berneis, two types of precursors are secreted by the liver: triglyceride-poor apoB and triglyceride-rich apoB [27]. Triglyceride-poor lipoprotein gives rise to the large LDL subfraction while triglyceride-rich lipoprotein is a precursor for small dense LDL. This hypothesis explains the formation of small dense LDL from liver-secreted precursors and is supported by clinical results [27].

Genome-wide association studies have been used to search the factors affecting small dense LDL production. The available results indicate that small dense LDL metabolism is connected to genetic factors that may be considered as potential therapeutic targets for treatment of atherosclerosis [24].

Small dense LDL has a higher lifetime than large LDL, which is retrieved from the bloodstream through the LDL receptor pathway [28]. Small dense LDL tends to have lower levels of vitamins and antioxidatants than normal LDL. This means that small dense LDL is more oxidation prone than the larger forms of LDL [29].

It has been demonstrated that incubation of native LDL particles with atherosclerosis patients' blood plasma results in a significant decrease of the sialic acid contents [13]. Small dense

LDL particles have been shown to contain less sialic acid than larger LDL particles [30]. The increased ability of small dense LDL to form complexes with proteoglycans leads to the prolonged residence time of these particles in the subendothelial space or the arterial wall, where LDL may contribute to the development of atherosclerotic lesion [31]. In summary, LDL particle density reversely correlates with the particle size and sialic acid contents and directly correlates with atherogenicity.

4.2. Electronegativity

Desialylated LDL has been demonstrated to have a 1.2- to 1.4-times increased electrophoretic motility in comparison to native LDL [17]. Therefore, desialylated LDL has a lower charge than native LDL, that is, is more electronegative.

Agarose gel electrophoresis allows for specific isolation of electronegative LDL (LDL(−)). Isotachophoresis or ion exchange chromatography can be used as well [32]. The group of Avogaro was the first to discover and isolate atherogenic LDL(−) fraction [32] using ion-exchange chromatography.

More recent studies have revealed heterogeneity of LDL(−) particles, defining as many as five subclasses of LDL(−) [33]. The majority of electronegative subfractions correlated with cardiovascular (CV) risks, including, but not limited to hypercholesterolemia, smoking, myocardial infarction, and diabetes mellitus type II [34].

Several methods have been developed for isolation and analysis of LDL(−). Capillary isotachophoresis is another method used for LDL(−) extraction and analysis [35]. This technique allows for separation of LDL(−) from other LDL particles by its migration rate. Heparin precipitate LDL(−) was analyzed using capillary isotachophoresis [36]. Monoclonal antibodies allow for distinguishing LDL(−) by specific epitopes [37]. LDL(−) ELISA method is based on this technique and may prove to be useful in clinical practice [38].

It has been demonstrated that LDL(−) particles tend to aggregate [32], and the LDL particle aggregates have been shown to be atherogenic [39]. Gnarled structure of the lipoprotein was shown to be the principal cause of LDL(−) association [40]. The secondary structure of apoB in LDL(−) appears to be disturbed [70], with tryptophan residues abnormally exposed to the aqueous environment [41] and lysine residues having an altered ionization state [42]. Lipid moieties of LDL(−) particles also affect their surface tension/fluidity, rendering the particles more-aggregation prone [43].

Moreover, improper folding of apolipoprotein of LDL(−) particles affects its affinity to LDL receptors, which in turn leads to extended blood circulation times of LDL(−) [44]. On the other hand, the most electronegative fraction of LDL(−) is able to bind to lectin-like oxidized LDL receptor 1 (LOX-1) [45]. That subfraction of LDL(−) when added to cultured endothelial cells is able to increase the production of reactive oxygen species and to upregulate C-reactive protein levels via LOX-1 signaling pathway [46].

Therefore, LDL(−) is able to provoke pro-inflammatory and immune responses that contribute to the progression of atherogenesis. LDL(−) forms complexes with proteoglycans in the subendothelial intima where it resides for extended amounts of time. Subendothelial cells

take up LDL(−) via scavenger receptors, which leads to saturation of their cytoplasm with lipid deposits and results in foam cell formation. Autoantibodies to LDL(−) can contribute to the development of atherosclerosis as well [47]. LDL(−) is cytotoxic to endothelial cells, inducing apoptosis and provoking production of inflammatory molecules such as IL-8, VCAM-1, and MCP-1 [47]. Therefore, LDL(−) was demonstrated to be pro-atherogenic and pro-inflammatory.

4.3. Similarity of desialylated LDL with small dense LDL and LDL(–)

As discussed above, small dense LDL and LDL(−) are the forms of modified LDL that have been detected in human blood plasma [48]. Our group has performed a series of experiments comparing the properties of LDL particles modified *in vivo*. In a study conducted in collaboration with the group of Avogaro (Italy), we have demonstrated that the more electronegative LDL corresponds by its properties to desialylated LDL [49]. Desialylated LDL subfraction also turned out to be more electronegative [50]. Therefore, it is likely that desialylated LDL and electronegative LDL subfractions are similar if not identical. We have found desialylated LDL to be smaller and denser as compared to native LDL. Simultaneously, LaBelle and co-authors have shown that sialic acid centent was reduced in small dense LDL [51]. Therefore, converging evidence demonstrates that all modified LDL subfractions isolated by different methods may be the same subfraction that underwent multiple modifications.

4.4. Which of the LDL modifications conveys atherogenicity?

Atherogenic LDL naturally present in the blood was found to be small, dense, and highly electronegative. Atherogenic LDL is also characterized by altered protein, lipid, and carbohydrate compositions. Consequently, these LDL particles can be referred to as multiply modified. To understand which modifications convey LDL atherogenicity, we have investigated the relationship between changes in chemical and physical parameters of LDL and its ability to induce lipid accumulation in SMA(+) cells of human aortic intima. A significant reverse correlation ($r = -0.66$, $p < 0.05$) between LDL atherogenicity and the sialic acid content was observed. By contrast, no correlation was observed between atherogenicity and the LDL particle size and charge, as well as with the levels of phospholipids and neutral lipids. Levels of lipophilic antioxidants, lipid peroxidation products, free lysine amino groups, and susceptibility of LDL to oxidation did not correlate with atherogenicity significantly [50]. It is therefore likely that desialylation is the principal cause of lipoprotein atherogenicity.

5. Is oxidized LDL just a myth?

According to the mainstream current opinion, oxidized LDL is the main trigger of atherosclerosis [52]. However, oxidized LDL had never been found in blood. One of the possible explanations that has been proposed to explain this discrepancy was that LDL oxidation takes place not in the bloodstream but in the arterial wall. The oxidized LDL theory is based on the following observations:

- Antibodies against LDL-oxidized *in vitro* were able to bind to substrates originating from atherosclerotic lesions, where LDL was found to be co-localized with oxidation products [53].
- Part of LDL isolated from atherosclerotic plaques corresponds by its properties to oxidized LDL [54].
- Autoantibodies recognizing malondialdehyde-LDL have been found in the blood [7].

Worth noting here is the fact that circulating autoantibodies have affinity not to the oxidized LDL but rather to MDA-LDL, a model of oxidized LDL. Interestingly, antibodies recognizing MDA-LDL were demonstrated to have an even higher affinity to desialylated LDL [55]. It is therefore possible that anti-LDL autoantibodies that primarily react with desialylated LDL also show cross-reactivity with MDA-LDL. This observation, together with other facts, challenges the concept of the oxidative modification of LDL being the principal *in vivo* modification that causes the onset and progression of atherosclerotic lesions. Other modified LDL species that have been found in the blood probably deserve more attention from the scientific community.

5.1. Circulating atherogenic desialylated LDL is oxidized

The degree of lipoprotein oxidation is estimated by measuring the contents of hydroperoxides or thiobarbituric acid-reactive substances (TBARS). These compounds are usually formed in course of lipid peroxidation. However, chemical instability and hydrophilic nature of these substances may cause their loss from LDL particles during lipoprotein isolation and purification stages. We have established a new technique to evaluate the degree of LDL oxidation based on the assumption that chemically active lipid derivatives formed in the process of peroxidation are able to covalently bind to apoprotein B and thus may serve as a marker of lipoperoxidation occurring *in vivo* in lipoprotein particle [56]. We have discovered sterol and phosphates covalently bound to apoB in delipidated preparations of LDL oxidized by copper ions, azo-initiators, sodium hypochlorite, or cultured cells. Newly extracted and isolated LDL from healthy individuals contained no apoB-lipid adducts. It has been revealed that contrary to other parameters used to estimate the degree of lipid peroxidation in LDL, the level of cholesterol covalently bound to apoB of copper-oxidized LDL rised monotonously during incubation [50]. Therefore, the level of apoB-bound cholesterol is a parameter that reflects the degree of LDL oxidation.

Native LDL and desialylated LDL isolated from healthy subjects had apoB-bound cholesterol levels of 0.25 ± 0.08 and 0.28 ± 0.05 mol/mol apoB, respectively. ApoB-bound cholesterol level in native LDL of atherosclerotic patients did not differ significantly from its level in native LDL of healthy individuals. The content of apoB-bound cholesterol in desialylated LDL of patients was 7 times higher than in native LDL. Therefore, we have shown that desialylated LDL of coronary atherosclerosis patients is oxidized.

5.2. Desialylated LDL is oxidation prone

Desialylated LDL contains 2- to 4-fold more oxysterols compared to native LDL [17], which indicates the increased susceptibility of desialylated LDL to oxidation.

In addition to a high degree of *in vivo* oxidation, desialylated LDL possesses a higher susceptibility to *in vitro* oxidation, which was evaluated using the duration of lag-phase upon oxidation by copper ions [57]. Average duration of lag-phase of native LDL isolated from atherosclerotic patients did not differ from that of native LDL taken from healthy individuals. The lag-phase of desialylated LDL of healthy subjects and patients was significantly shorter (3- and 6-fold, respectively) than that of native LDL, indicating a higher *in vitro* proneness to oxidation of desialylated LDL. It should be noted that proneness to oxidation of total LDL preparations from healthy subjects and patients positively correlates with the proportion of desialylated LDL in the lipoprotein preparation.

In an attempt to find out the causes of increased degrees of *in vivo* oxidation and proneness to oxidation of desialylated LDL, we estimated the contents of major fat-soluble antioxidants in lipoprotein particles, analyzed dependences among the levels of tocopherols and carotenoids, coenzyme-Q10, and the concentration of cholesterol bound to apoB and duration of lag-phase.

The levels of all major antioxidants, including coenzymeQ10, lycopene, α-and y-tocopherols, and β-carotene, were 1.5 to 2 times lower in desialylated LDL than in native LDL. The amount of cholesterol bound to apoB in desialylated LDL positively correlated with the amount of ubiquinone and showed a negative correlation with ubiquinol and β-carotene concentrations. At the same time, a positive correlation was found between the amount of cholesterol bound to apoB and the ubiquinol level in native LDL. The length of lag-phase for desialylated LDL was positively associated with α-tocopherol and β-carotene amounts and negatively associated with the ubiquinone content. On the other hand, proneness to oxidation of native LDL positively correlated with ubiquinone level.

Based on these observations, we hypothesized that a) the levels of examined lipophilic antioxidants in desialylated LDL are lower than in native lipoproteins, which leads to the high proneness of desialylated LDL to oxidation; b) coenzyme-Q10 might play a pro-oxidational role in native LDL; c) *in vivo* lipid peroxidation in desialylated LDL is enhanced by the increased proportion of oxidized form of coenzyme-Q10; and d) the severity of *in vivo* oxidation in desialylated LDL is associated with oxidation degree of ubiquinoI and the amount of carotenoids loss.

6. Mechanisms increasing LDL atherogenicity

Based on the known rates of LDL uptake and degradation by the arterial wall cells, we estimate the time necessary for a normal intimal cell to become a foam cell, give 130 years . This estimation implies that there occurs no cholesterol efflux from the cell. The same estimation for desialylated LDL brings the result reduced to 15 years. However, according to angiographic and ultrasonographic data, atherosclerotic plaque can reduce the carotid artery lumen by one half within several weeks or months. Therefore, the actual rate of foam cell formation should be much higher than the estimated one, indicative of some processes that enhance the atherogenicity of desialylated LDL.

6.1. Self-association of LDL

It has been demonstrated that modified LDL particles are susceptible of self-association [58]. A positive correlation between atherogenicity of modified LDL and the degree of LDL association was demonstrated [59]. A dramatic increase of lipid accumulation rate by SMA(+) cells cultured from human aortic intima was observed upon incubation with lipoprotein associates. LDL associates removal from the incubation medium by filtration through filters with pore diameter 0.1 μm, completely eliminated any intracellular lipid accumulation. Therefore, association enhances LDL atherogenicity.

The absorption rate of associated LDL was 5–20 times higher than that of nonassociated LDL particles [39]. Latex beads (competiting phagocytic cargo) and cytochalasin B (inhibitor of phagocytosis) both inhibited the uptake of LDL associates [39]. It is therefore likely that LDL associates are absorbed via phagocytosis. The intracellular degradation rate of associated modified LDL apoB was 2–5 times slower than the rate of degradation of apoB of nonassociated particles. Therefore, high atherogenicity of lipoprotein associates is a result of enhanced absorption via phagocytosis and slow intracellular degradation rate.

6.2. LDL complexes with extracellular matrix

We have also demonstrated that LDL can form complexes with collagen, elastin, and proteoglycans of human aortic intima, as well as with cellular debris [60]. These complexes, once added cell culture, stimulated intracellular accumulation of lipids. Experiments with iodinated LDL have shown an increased absorption and diminished intracellular degradation rate of lipoprotein complexes, as compared to individual lipoprotein particles.

6.3. LDL-immune complexes

Multiply-modified lipoproteins are likely to be immunogenic. We succeeded in isolating circulating complexes containing LDL and anti-LDL autoantibodies from the blood of the majority of patients with coronary atherosclerosis [61].

We have observed a positive correlation between the levels of LDL-containing immune complexes in blood serum and the severity of coronary and extra-coronary atherosclerosis [62].

We have extracted LDL from circulating immune complexes by affinity chromatography on agarose with immobilized goat polyclonal antibodies against human LDL [63]. LDL from circulating immune complexes appeared to be desialylated, small, dense, more electronegative and with decreased contents of neutral lipids and phospholipids, as well as neutral saccharides. ApoB tertiary structure was also altered. Therefore, the LDL particles isolated from circulating immune complexes were similar if not identical to the desialylated LDL characterized previously.

We isolated antibodies to modified LDL from blood plasma of patients with coronary atherosclerosis [55]. These autoantibodies were identified as immunoglobulin G with an isoelectric point of about 8.5 (8.1–9.0), capable of interacting with the protein but not the lipid moiety of LDL. These autoantibodies were able to interact with native, glycosylated, acetylated, and

oxidized LDL, showing the highest affinity for malondialdehyde-treated LDL, desialylated LDL, and LDL isolated from patients with coronary atherosclerosis.

Autoantibodies bound to native LDL forming complexes that could induce lipid aggregation in SMA(+) cells cultured from uninvolved intima of human aorta. Moreover, autoantibodies enhanced the atherogenic properties of desialylated LDL via complex formation [55]. It was found that C1q complement component and fibronectin could bind to the LDL-antibody complexes leading to a more pronounced lipid aggregation in SMA(+) human aortic intimal cells. C1q complement component is produced by dendritic cells in the spleen, where C1q is binds to immune complexes [64]. Antigen-presenting dendritic cells are also present in atherosclerotic plaques [65]. Moreover, dendritic cells expressing C1q have been found in atherosclerotic plaques [66]. C1q was also expressed in macrophages, foam cells, and in neovascular endothelial cells [66]. Thus, C1q expression might be an important feature of cells located in the vessel wall of atherosclerotic lesions, causing them to capture and retain immune complexes [66].

In vitro interaction of mouse peritoneal and human pericardial macrophages with immune complexes isolated from blood serum of ischemic heart disease patients led to the transformation of macrophages into foam cells [67]. Macrophages incubated with immune complexes for 3 days acquired cytoplasmic lipid vacuoles, and the cisterns of endoplasmic reticulum (ER) in these cells were dramatically enlarged and filled with lipids. The accumulation of lipids within ER cisterns in macrophages may be accompanied by ER stress, which also plays a role in the development of atherosclerosis [68].

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7. Revealing a nonlipid factor of blood atherogenicity

We hypothesized that a nonlipid factor could be extracted from the serum using a column with immobilized LDL. We applied atherogenic serum, which has previously been shown to induce a nearly 5-fold increase of cholesterol content in cultured cells, to a column with LDL covalently bound to agarose. We found that the eluted serum lost its atherogenicity, i.e. it failed to induce a statistically significant lipid accumulation in cultured cells (**Figure 1**, from Ref. [69]). The substances retained on the column were eluted with glycine buffer and mixed with the sera samples that were previously treated by passing though the column, which resulted in the recovery of serum atherogenicity up to the initial level (**Figure 1**). It is therefore likely that patients' blood serum contains unknown atherogenic factors that can be absorbed on immobilized LDL.

We next used this method was to reduce atherogenicity of the blood of patients by extracorporeal perfusion. Four male volunteers aged 46–59 years with CHD, normal cholesterol levels,

functional class II–III angina pectoris, and angiographically documented stenosis of 2 to 3 coronary arteries have agreed to take part in the study [69]. Three individuals were smokers, and one had mild arterial hypertension. A pronounced decrease of plasma atherogenicity was registered after a 2-hour extracorporeal perfusion through a column with autologous LDL (**Figure 2(a)**, from Ref. [69]).

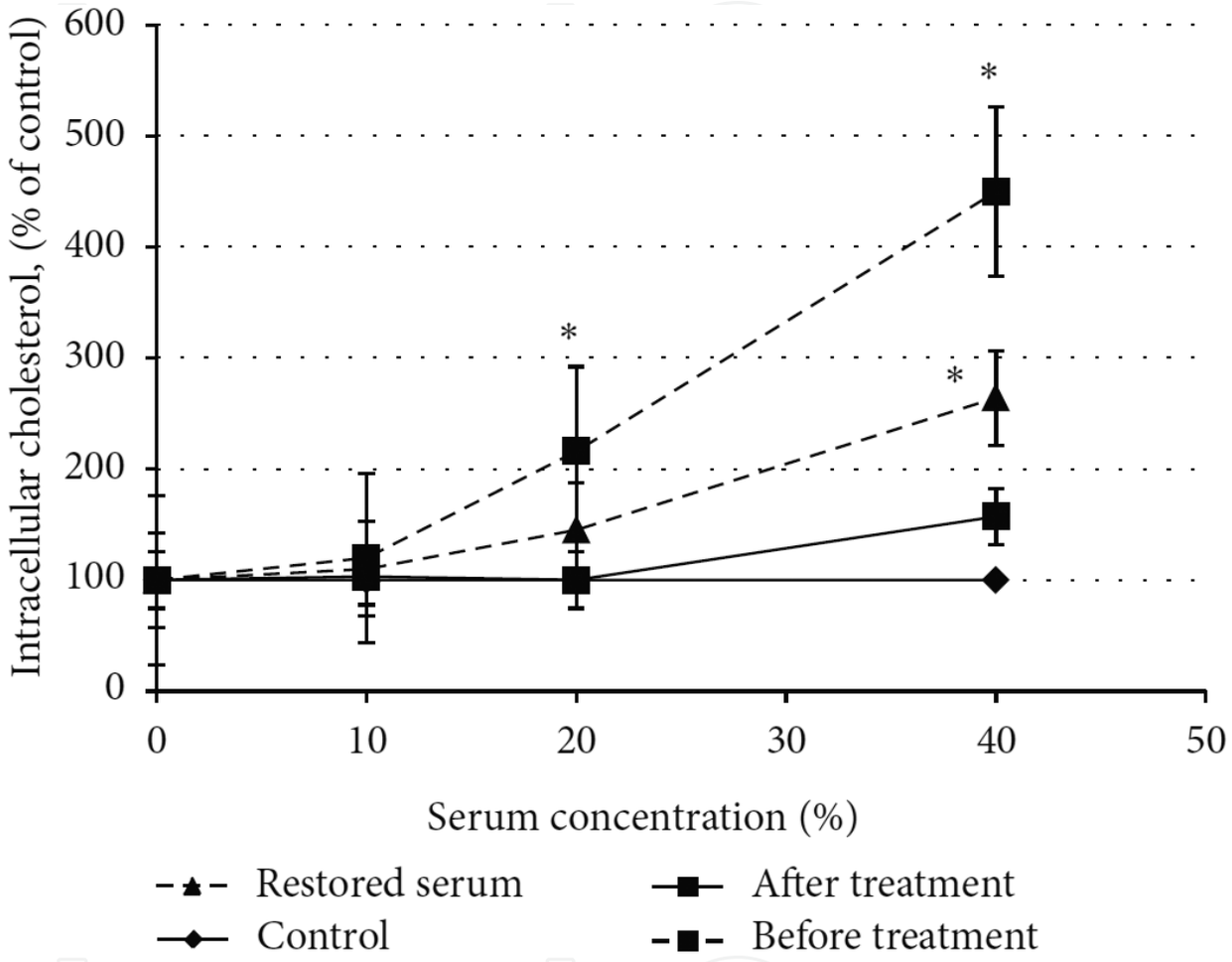

Figure 1. Elimination of serum atherogenicity with LDL-agarose column. Five milliliters of the serum were passed through the LDL sepharose column at a flow rate of 1 ml/min for 30 min. The sorbent was then eluted with 2-ml glycine buffer (pH 2.7), and the eluate was dialyzed against a 2000-fold excessive volume of medium 199 for 24 hours at 4°C. The cells were cultured in the presence of the initial or treated serum and with the proper volume of the dialyzed eluate.

The analysis of serum atherogenicity demonstrated that, in all four cases, it was reduced to a near-zero level 24 hours after the procedure and then gradually reappeared, reaching a significant level within 1 week. Repeated procedure resulted in a pronounced decrease of serum atheorgenicity, with the 2nd and the 3rd procedures reducing it for prolonged periods sufficient for reducing the frequency of the treatment. When applied once every 2 to 3 weeks, the procedure provided low levels of plasma atherogenicity for long periods (**Figure 2(b)**, from Ref. [69]). The procedure has been applied twice a month in one patient for 9 months and in another patient for more than 7 months. Each patient was examined taking into account the general state of health, number of angina pectoris episodes, the amount of medicine (nitrates) taken, and

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capacity for exercise. Bicycle test, 24-hour Holter ECG monitoring, and control of hematological and biochemical parameters have been performed every 3 months. During this trial, the patients have felt better, moved from functional class III to II (according to Canadian classification), and endured greater physical loads in the bicycle test [69]. Arterial blood pressure of patient 1 stabilized and reached a nearly normal level. Both patients have noted a heightened sexual activity and have associated this with reduced angina pectoris [69]. The repeated angiograms have been assessed after 20–25 months of treatment. There were no new stenoses, 50% stenoses have progressed, 25% regressed, and 25% remained unchanged. These observations suggest an improved disease progression in comparison to the normal course of coronary atherosclerosis [70].

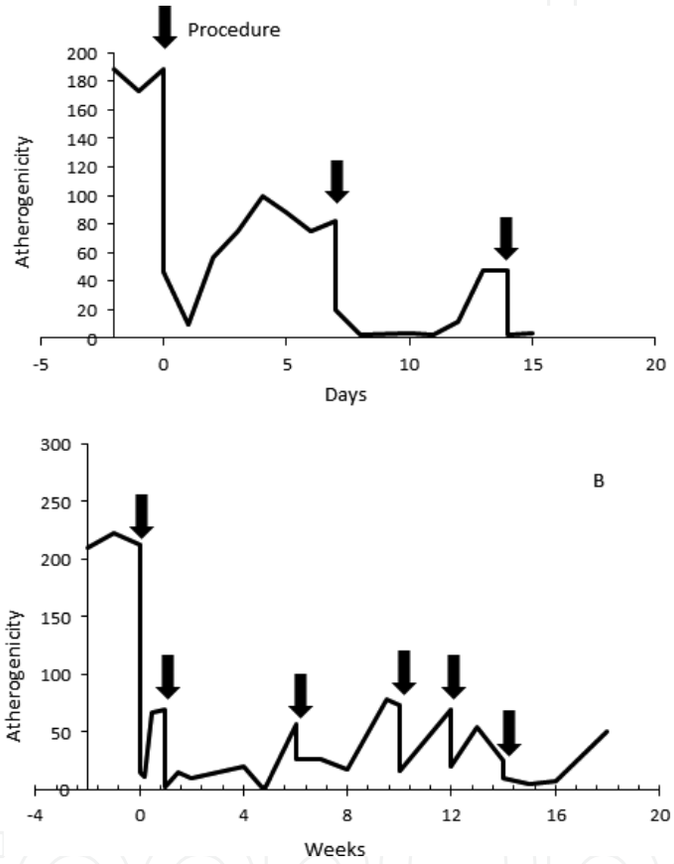

Figure 2. Monitoring of atherogenicity. The patient's plasma was subjected to 2-hour extracorporeal perfusion through a column with 200 mL of the sorbent; the flow rate was 30 ml/min. The total plasma volume of 2–3 liters was perfused through the column during the procedure. Blood serum atherogenicity after 3 procedures was assessed daily ((a), patient 3) and once or twice a week afterwards ((b), patient 1). Ordinate (atherogenicity), percent of cholesterol accumulation in the cells cultured in the presence of the serum from the CHD patient.

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8. Multiple-modified LDL: which type of LDL modification occurs early in the blood?

What are the mechanisms of multiple modifications of LDL? Do they take place in the blood plasma? A round the clock exposure of LDL to hepatocytes, intact endotheliocytes, smooth muscle cells, macrophages, or cell homogenates has not affected properties of native LDL [13].

After incubation for 24 hours at 37°C with whole blood or plasma taken from patients with coronary atherosclerosis, the sialic acid content of LDL became 2 times lower than that of LDL incubated with whole blood or plasma obtained from healthy individuals. Incubation with red and white blood cells had no effect on the sialic acid content. This points out that LDL modification takes place in the blood plasma [13].

A detailed analysis of LDL modification processes has been performed by our group [13]. Native LDL was extracted from the blood plasma using ultracentrifugation followed by lectin chromatography. Serum was cleared from apoB-containing lipoproteins by defibrination of the remaining LDL-deficient plasma. Afterwards, LDL and serum were reconstituted in the same proportion in the original plasma and incubated for different time points at 37°C.

After incubation, LDL was re-isolated by ultracentrifugation. The described method allowed for elimination of the effects of LDL originating from VLDL and IDL in the process of incubation. After 1 hour of incubation of native LDL with autologous plasma samples, a sharp decrease of sialic acid content was observed. At the same time, desialylated LDL concentration increased, as determined by lectin-sorbent chromatography (**Table 1**, from Ref. [13]). In parallel to the decrease of the sialic acid content, LDL acquired capability to induce a pronounced accumulation of cholesterol in SMA(+) cells cultured from unaffected human aortic intima. This could be registered as early as after 3 hours of incubation. After 6 hours of incubation with plasma, a steady decrease of phospholipid and neutral lipid contents, as well as LDL particle size could be observed.

1 h	3 h	6 h	12 h	24 h	36 h	48 h
↓Sialic acid ↑% of desialylated LDL	↑ Atherogenicity	↓Free cholesterol	↓Size ↓Cholesteryl esters ↓Phospholipids	↓Triglycerides	↑ Electronegativity	↑Apo B-bound cholesterol ↑Susceptibility to oxidation ↑Fluorescence ↓Vitamin E

Table 1. The outline of LDL modification.

After 36 hours of incubation, negative charge of lipoprotein particles became obvious. Longer incubation times (48 and 72 hours) led to a loss of α-tocopherol and to an increase of LDL

susceptibility to oxidation, as well as to aggregation of cholesterol covalently bound to apoB. Degradation of apoB was also registered at this point.

It can be concluded that desialylation is likely to be the primary and most important LDL modification that conveys its atherogenicity. Other known modifications may further increase the LDL atherogenicity.

9. Conclusion

We have obtained an LDL subfraction that was able to induce accumulation of lipids, primarily cholesteryl esters, in cultured SMA(+) cells. This helped to reconcile the facts that native LDL is not atherogenic and *in vitro*–modified LDL not present in circulation.

We have shown that atherogenic LDL is characterized by numerous alterations of carbohydrate, protein, and lipid moieties, and can therefore be termed multiple-modified LDL. Multiple modifications of LDL occur in human blood plasma. It was shown that circulating multiple-modified LDL loses the 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, as well as stimulation of re-esterification of free cholesterol, cause the intracellular accumulation of intracellular-esterified cholesterol.

The formation of LDL-containing large complexes (associates, immune complexes, and 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.

Therefore, we have been able to obtain and describe naturally occurring multiple-modified LDL capable of provoking all atherosclerotic manifestations at the cellular level.

Immune complexes, consisting of LDL and autoantibodies, have been discovered in the human blood stream circulation [71]. Amount of LDL-containing circulating immune complexes was directly correlated with the severity of atherosclerosis [71]. We hypothesize that anti-LDL autoantibodies and circulating immune complexes containing LDL can be the factors that convey blood atherogenicity. Although the anti-LDL cannot be proven the only atherogenic factor adsorbed on the column with immobilized LDL, the substances binding to LDL should be thoroughly studied. Columns with immobilized LDL allowed not only distinguishing and collect nonlipid factors of atherogenicity, but also opening a prospect for reducing atherogenicity in patients.

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