Atherogenic Modified LDL in Diabetes

Igor A. Sobenin, Vladimir V. Tertov, and Alexander N. Orekhov

This paper summarizes the recent findings on LDL atherogenic modifications in diabetic patients. LDL from diabetic patients, unlike LDL from healthy subjects, caused a significant increase in cholesterol content of cells cultured from unaffected human aortic intima, i.e., produced a direct atherogenic effect. LDL was divided into two fractions (nonbound and bound) by affinity chromatography on *Ricinus communis* agglutinin-agarose. The amount of bound LDL was significantly higher in diabetic patients compared with healthy subjects. Bound LDL was characterized by significantly lowered sialic acid content and a significantly increased fructosyl lysine level compared with unbound LDL, i.e., was desialylated and nonenzymatically glycosylated lipoprotein. The bound (desialylated), but not unbound (sialylated), LDL subfraction induced cholesterol accumulation in cultured cells. Bound LDL was also characterized by a significantly lowered content of neutral lipids and demonstrated increased electrophoretic mobility on agarose gel electrophoresis. Bound and nonbound LDL differed significantly in hydrated density and particle size, as was determined by density gradient ultracentrifugation and native polyacrylamide gradient gel electrophoresis. The results of this study have shown that the in vivo modified atherogenic LDL subfraction in the blood of diabetic patients is represented by small, dense, more electronegative, desialylated, and glycated LDL. *Diabetes* 45 (Suppl. 3):S35–S39, 1996

Late complications of diabetes include a variety of clinical pictures, mainly related to the involvement of the arterial wall of both large vessels (macroangiopathy) and small vessels (microangiopathy) and of the peripheral nervous system (neuropathy). Within this division, macroangiopathy is generally thought to be of an atherosclerotic nature (1), although some pathomorphological changes specific for diabetes only are observed in arteries (2,3). Premature onset and rapid progression of atherosclerosis are characteristic features of diabetes, as has been definitely demonstrated in numerous epidemiological studies (4–7). Furthermore, cardiovascular events remain a leading cause of morbidity and mortality in patients with type I and type II diabetes (8–10).

However, the mechanisms of early atherogenesis in diabetic patients remain obscure. The deposition of intracellular lipids, mainly free and esterified cholesterol, in the vessel wall is a typical feature of early atherosclerotic lesion at the cellular level (11,12). LDL is believed to be the source of accumulating intracellular lipids, and LDL demands to be chemically modified in some way to provide an atherogenic effect (13). Evidence that several forms of LDL modification occur in vivo is rapidly accumulating, although their clinical relevance remains uncertain.

In this report we summarize recent findings concerning LDL atherogenic modifications in diabetic patients.

ATHEROGENICITY OF LDL IN DIABETIC PATIENTS

The experimental design of our studies was founded on the assumptions that *1*) atherosclerosis-related events occur in the intimal layer of human aorta and smooth muscle cells play a significant role in processes of intracellular lipid deposition (14–16), and *2*) the cells isolated from human aortic intima retain their properties in culture for a prolonged period of time (up to 7–14 days) (17,18). One of these properties was the ability to accumulate lipids in the presence of such additives as atherosclerotic patient's serum (19,20) or LDL (21,22). Taken together, these data confirmed the approach that cells cultured from human aortic intima proved to be a proper object for the studies of atherosclerosis-like intracellular changes.

Several years ago we developed a cell culture technique that allowed us to assess the so-called atherogenic potential of biological fluids. Further investigations have shown that not only atherosclerotic but also diabetic patients' sera possess atherogenic properties, with respect to intracellular cholesterol accumulation (23). On the other hand, the sera of healthy subjects, as a rule, failed to induce lipid deposition in cultured cells (19,20). As for patients with diabetes, in our study (180 patients) >55% of type I diabetic patients and nearly 90% of type II diabetic patients had atherogenic sera. On average, the sera from diabetic patients increased intracellular cholesterol content by 75% (24).

The question arose whether the atherogenic properties of the serum are associated with lipoproteins. For this purpose, LDL, VLDL, and HDL were isolated from atherogenic serum. Lipoprotein-deficient serum (deprived of all lipid-containing particles) lost its atherogenic potential entirely. Among all lipoprotein density classes, the LDL fraction (d = 1.019–1.063 g/ml) was the only lipoprotein possessing atherogenic properties. It was notable that LDL isolated from nonatherogenic diabetic sera did not affect cellular cholesterol level. By contrast, LDL isolated from atherogenic sera proved to be also atherogenic in 90% of cases. A strong positive correlation between the cholesterol-accumulating effects of sera and corresponding LDL was revealed (r = 0.89, P < 0.001) (24). So, it became possible to conclude that the atherogenic effect of serum in diabetes was mainly due to LDL. Moreover, these data seemed to be the substantial indication that diabetic patients' LDL may be modified, since native LDL of

From the Institute of Experimental Cardiology, Russian Cardiology Research Center, Moscow, Russia.
Address correspondence and reprint requests to Dr. I.A. Sobenin, Institute of Experimental Cardiology, 3rd Cherepkovskaya Str., 15-a, 121552 Moscow, Russia.
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apo, apolipoprotein.

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most healthy donors failed to induce intracellular accumulation of lipids.

LDL NONENZYMATIC GLYCOSYLATION

The nonenzymatic glycosylation of proteins is a complex chemical reaction resulting in the formation of a stable bond between a glucose molecule and a protein amino group. In diabetic patients, hyperglycemia may induce a large increase of glycated products. Kim and Kurup (25) were the first to demonstrate the elevated level of fructosyl lysine in diabetic patients' LDL. Nonenzymatic glycation of all classes of apolipoproteins (apos) including apoB was shown to occur in hyperglycemic subjects (26). Lopes Virella et al. (27) have shown that in vitro glycated LDLs are able to induce esterified cholesterol accumulation in cultured human monocyte-derived macrophages. Similarly, in vitro glycated normal LDL produced the same effect on cultured intimal human aortic cells (28). LDL isolated from the serum of type I diabetic patients stimulated the synthesis of cholesteryl esters in cultured monocyte-derived macrophages, thus leading to cholesterol accumulation within the cells, and this effect correlated well with the degree of LDL nonenzymatic glycation (29).

We have shown that the 2- to 2.5-fold increase of fructosyl lysine residue content during in vitro glycation resulted in an ability of initially nonatherogenic LDL to cause a moderate but significant cholesterol accumulation in cultured cells (up to 35% above the initial level) (I.A.S., V.V.T., A.N.O., unpublished data). LDL in vitro glycation was accompanied by a practically equimolar decrease in free amino group content (I.A.S., V.V.T., A.N.O., unpublished data), as glucose forms Shiff bases and further Amadori products presumably with ϵ-amino groups of lysine. Lysine residues are thought to play an important role in determination of the tertiary structure of apoB, and the decrease in their content may affect lipoprotein-to-cell interaction (30,31).

LDL from diabetic patients was characterized by increased nonenzymatic glycation, as can be judged by an average increase in fructosyl lysine content by ~25% compared with LDL of healthy subjects (24). It is notable that a significant positive correlation was revealed between fructosyl lysine content of diabetic LDLs and their atherogenic effect ($r = 0.57$, $P < 0.01$). So, nonenzymatic glycation per se seems to be nothing but in vivo atherogenic modification of LDL in diabetic patients.

However, the increase in glycated apoB levels in diabetic patients is not dramatically high or even is not observed (32), and the atherogenic effect of nonenzymatically glycosylated LDL is quite moderate. Taken together, these reasons do not allow us to ascribe to the glycated LDL a leading role in the initiation and progression of the atherosclerotic process. Numerous attempts have been made to explain the mission of glycosylated LDL in atherogenesis, and altered glycated apoB metabolism is suspected in diabetic patients (33,34). Metabolic abnormalities associated with the glycation of LDL include diminished recognition of LDL by the classic LDL receptor (35–37), retardation of the plasma clearance of LDL (38,39), enhanced uptake of LDL by macrophages, increased platelet aggregation (40), increased covalent binding of LDL in vessel walls (41), and generation of oxygen free radicals, possibly resulting in oxidative damage to both lipid and protein moieties of LDL (42). However, the real status and role of nonenzymatically glycosylated LDL in atherosclerosis development is far from being understood.

SIALIC ACID–POOR LDL IN DIABETES

Desialylated LDL is another definitely known type of modified lipoprotein occurring in vivo. Previously it was demonstrated that LDL treatment with neuraminidase in vitro leading to a significant loss of lipoprotein-bound sialic acid results in an ability of LDL to induce massive lipid accumulation in cultured monocyte-macrophages (43) and smooth muscle intimal cells (44). Further clinical studies have shown that LDLs from atherosclerotic patients are characterized by a lowered content of sialic acid; the latter correlates negatively with LDL atherogenic effects revealed in cell culture (45). However, the reduced sialic acid level turned out to be a particular feature of atherosclerotic patients' lipoprotein, but was also observed in diabetic patients. In our studies, up to 90% of patients had a low sialic acid content in LDL that was decreased by 30% on average compared with native LDL from healthy subjects (24). Interestingly, a significant negative correlation was observed between LDL sialic acid level and LDL atherogenicity revealed in cell culture ($r = -0.51$, $P < 0.001$) (24). These data allowed us to suppose that desialylation (or any process leading to a formation of sialic acid–poor LDL) is an additional, if not the dominant way of LDL modification in diabetes. The mechanisms of desialylation are not understood yet. No significant elevation in neuraminidase activity was found in the blood of atherosclerotic or diabetic patients, but an elevated level of free sialic acid in plasma was observed under various pathological conditions, including atherosclerosis (46–48). On the other hand, desialylated LDL may appear in the circulation as a result of impaired posttranslational glycosylation of apoB by hepatocytes, but this possible mechanism is not investigated at all and may be regarded only as a speculation. The preliminary data obtained in the LDL turnover study using labeled precursors showed that desialylated LDL may be a senescent lipoprotein (49), and in this case desialylation may happen as damage that occurs due to longer circulation time or sequestration of LDL in tissues.

LDL PHYSICOCHEMICAL AND FUNCTIONAL HETEROGENEITY IN DIABETES

Recently, a lectin affinity chromatography technique was developed for the isolation of sialic acid–poor LDL from human blood (50). This method was based on the proposition that if apoB is deprived of sialic acid, it begins to express galactose as a terminal sugar in its polysaccharide moiety. *Ricinus communis* agglutinin (RCA_{120}), a lectin isolated from castor beans, is able to bind glycoproteins that bear terminal galactose residues in polysaccharide chains. Being covalently bound to agarose matrix, it provides a convenient tool for affinity isolation of asialoglycoproteins, including desialylated LDL. The fraction of desialylated LDL found in the blood of atherosclerotic patients was characterized in detail (51–55). Similarly, LDL from diabetic patients could be easily subdivided into two fractions, sialic acid–rich and sialic acid–poor LDL. It turned out that the proportion of desialylated LDL is significantly higher in diabetic patients compared with healthy subjects (35 vs. 12%, on average) (24,56).

Sialic acid–rich LDL that was unable to bind to affinity gel seemed to be nearly identical to native LDL from healthy persons and was characterized by practically normal levels

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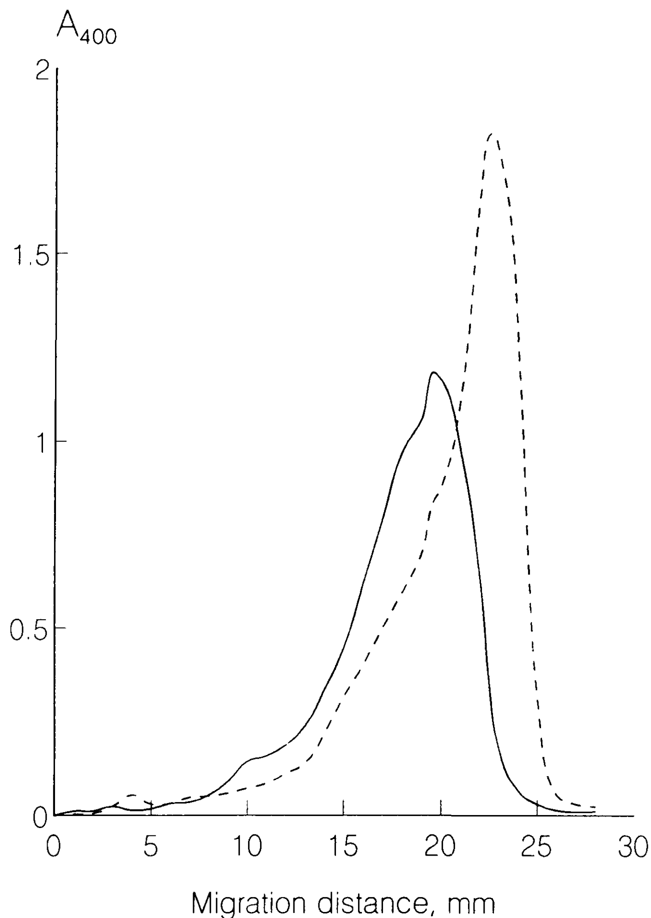

FIG. 1. Densitometry profiles of sialylated (solid line) and desialylated (dashed line) LDL after native gradient electrophoresis on 3–10% polyacrylamide gel and silver staining. LDL particle sizes were further determined using calibration curve of globular proteins with known molecular diameters.

of sialic acid. As for other physicochemical parameters, there were no or insignificant differences from native LDL, with respect to nonenzymatic glycosylation, free and esterified cholesterol content, electrophoresis mobility, and particle density and size. This LDL was unable to cause any significant accumulation of cholesterol in cultured human intimal cells, in spite of the marked atherogenicity of the total LDL preparation (24,56).

The picture changed dramatically for bound (sialic acid–poor or desialylated) LDL. The diminished content of sialic acid (by 25–60%, compared with nonbound LDL) appeared to be not the only characteristic of this LDL subfraction. This desialylated LDL fraction was also characterized by a lowered content of neutral lipids (especially esterified cholesterol) and elevated level of lysophospholipids (24,56). Another important finding was that desialylated LDL appeared to be significantly more glycated than nonbound (sialic acid–rich) LDL: the amounts of fructosyl lysine residues were 1.3- to 1.7-fold higher. Recently, we demonstrated that simultaneous in vitro modification of native LDL in two ways (i.e., nonenzymatic glycation and desialylation) results in an unexpectedly extensive rise in LDL atherogenicity, compared with LDL modified in a singular manner (57). In other words, desialylation and glycation produce a synergetic effect on LDL atherogenicity. It could be expected that the bound (sialic acid–poor) LDL fraction, being glycated as well, is responsible for the high atherogenic potency of diabetic LDL. Indeed, bound LDL induced a 2.5- to 4.7-fold increase in intracellular cholesterol content, whereas total LDL induced only a 1.4- to 2.3-fold cholesterol accumulation (24,56). The lower atherogenicity of the whole LDL preparation may be explained by the presence of a substantial amount of unmodified LDL.

The most recent data yield the additional characteristics of the modified LDL fraction in diabetes. It has been demonstrated by polyacrylamide gradient gel electrophoresis performed under nondenaturing conditions that the size of bound LDL particles is smaller by 0.5–2.3 nm compared with nonbound LDL (Fig. 1) (58). Bound LDL is also characterized by increased hydrated density, as it was shown by gradient ultracentrifugation (Fig. 2) (58). These findings correlate well with the data on the diminished lipid content of this LDL fraction. It is notable that the ability of bound LDL to accumulate cholesterol in cultured cells increased substantially according to the increase in density. The presence of small dense LDL in human circulation is claimed to be strongly associated with atherosclerosis development (59–61). Taking into account the high atherogenic effect of bound LDL revealed in cell culture, we may assume that the smaller size and increased density of these particles is a typical attribute of modified LDL.

The bound LDL was also characterized by increased electronegative net surface charge, as was judged by higher

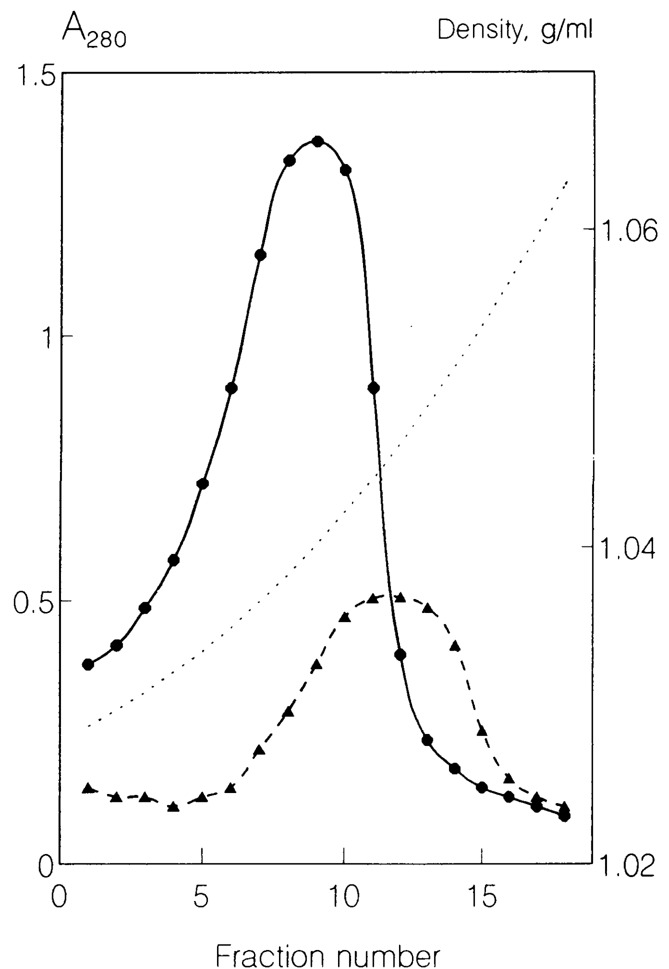

FIG. 2. Density profiles of sialylated (———) and desialylated (- - - -) LDL after density discontinuous gradient (· · · · ·) ultracentrifugation.

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electrophoretic mobility (58). The more electronegative LDL fraction was isolated recently from the blood of atherosclerotic subjects and characterized as a modified atherogenic LDL (62–64). It is necessary to note that numerous techniques of in vitro LDL modification (e.g., acetylation, methylation, maleylation, acetoacetylation, metal-dependent oxidation, malonic dialdehyde treatment, etc.) lead to the formation of anionic LDL, thus rendering them atherogenic (13). It is possible that the LDL surface charge plays a significant role in the processes of lipoprotein-to-cell interaction, and charge alterations may substantially modify LDL cellular metabolism, thus resulting in lipid deposition.

CONCLUSIONS

The results of our studies allow us to conclude that there is a fraction of modified LDL along with native LDL in the blood of diabetic patients. This naturally occurring modified LDL is characterized by various alterations in its physicochemical properties: *1*) it is a desialylated and nonenzymatically glycosylated lipoprotein; *2*) it is a small dense LDL; *3*) it is more electronegative than native LDL; and *4*) it is atherogenic in terms of intracellular cholesterol accumulation. So, the discovered atherogenic lipoprotein can be regarded as multiply-modified LDL that may be ascribed a significant role in early atherogenesis in diabetes. However, the origin and metabolic fate of this in vivo modified LDL remain to be studied.

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