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MODIFICATION OF LOW DENSITY LIPOPROTEIN BY DESIALYLATION CAUSES LIPID ACCUMULATION IN CULTURED CELLS:

DISCOVERY OF DESIALYLATED LIPOPROTEIN WITH ALTERED CELLULAR METABOLISM IN THE BLOOD OF ATHEROSCLEROTIC PATIENTS

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SUMMARY: Low density lipoprotein (LDL) isolated from the blood of healthy donors was partially desialylated by incubating the lipoprotein with sialidase (neuraminidase). The addition of LDL treated with neuraminidase to cultured human aortic intimal cells of smooth muscle origin caused a substantial increase in intracellular cholesteryl esters, free cholesterol and triglycerides. Cultured cells took up and degraded desialylated LDL much more effectively than untreated (native) LDL. LDL were also isolated from an atherogenic blood plasma of patients with coronary artery disease, i.e. the plasma capable of inducing the accumulation of lipids in cultured cells. Patients' LDL, similarly to the mother plasma, were atherogenic, i.e. stimulated the accumulation of intracellular lipids. LDL isolated from nonatherogenic plasma of healthy donors proved to be nonatherogenic. Atherogenic patients' LDL had a 2- to 5-fold lower level of sialic acid as compared with nonatherogenic LDL of healthy donors. The uptake and degradation of atherogenic patients' LDL were much more effective than in the case of nonatherogenic LDL of healthy donors. We assume that atherogenic properties of LDL obtained from patients' blood plasma are explained exactly by a low sialic acid content.

The major manifestation of atherosclerosis at the cellular level is the deposition of lipid within vascular cells which is apparently induced by low density liporotein (LDL) transported into the vessel wall from the blood steam (1). However, native LDL failed to induce the intracellular cholesterol accumulation (2). On the other hand, LDL chemically modified in vitro are able to cause massive accumulation of cholesterol in cultured cells (2). Chemical modification in vitro may include acetylation (3), acetoacetylation (4,5), carbamylation (5), methylation (5), maleylation (5), succinylation (4), malondialdehyde or glutaraldehyde derivatization (3-5), oxidative LDL modification (6), etc.

Sialic acid is a component of native LDL (7) which plays an important role in lipoprotein function (8-11). It has been demonstrated that desialylation of LDL with neuraminidase leads to increased binding and uptake of LDL by cultured fibroblasts and smooth muscle cells (9,10). We decided to

Abbreviations: CAD, coronary artery disease; LDL, low density lipoprotein.

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find out whether LDL desialylation leads to the accumulation of lipids in cultured cells. Furthermore, we assessed the content of sialic acid in the LDL circulating in the blood of atherosclerotic patients which, as had been shown earlier, can induce the accumulation of intracellular lipids (12,13).

MATERIALS AND METHODS

Subendothelial cells for culture were isolated from grossly normal intima by dispersion of human aortic tissue with 0.15% collagenase (14), suspended in the growth medium containing Medium 199, 10% fetal calf serum, 2 mM L-glutamine, and antibiotics, seeded into 24-well tissue culture plates with a density of $2\text{-}4x10^4$ cells per 1 cm^2 of the growth area and cultured as described earlier(14). The primary cultures contained a mixed cell population made up primarily by typical and modified smooth muscle cells as defined by the ultrastructural and immunofluorescent features (14). The medium was changed every day. Starting from the 7th day in primary culture, cells were incubated for 24 hrs in the media containing 100 μg protein/ml LDL and 10% lipoprotein-deficient serum of a healthy donor. On the 8th day the cell were rinsed and cellular lipids were determined.

Lipids were extracted from cells with a chloroform-methanol mixture (1:2, v/v) according to Bligh and Dyer (15) as described earlier (14). The total cholesterol content in the lipid extracts was determined using Boehringer Mannheim Monotest, Cholesterol CHOD-PAP Method (Cat. no. 236691, Boehringer Mannheim GmbH, Mannheim, FRG). Cellular phospholipids, triglycerides, free cholesterol and cholesteryl esters were separated by thin-layer chromatography and measured by scanning densitometry as described earlier (14). Each determination of lipids was done in triplicate.

Blood for LDL isolation was drawn in the morning before meals. The group of healthy donors included males aged 30-49 yrs without signs of ischemic heart disease as judged by the absence of complaints and signs of myocardial ischemia on bicycle exercise test. In the group of coronary artery disease (CAD)-patients, there were males aged 32-49 yrs with effort angina pectoris. The degree of stenosis of 1-3 coronary arteries assessed by selective coronarography was 75% or higher. The groups of patients and healthy donors were comparable with respect to such parameters as sex and age. The total cholesterol blood content varied from 190 to 220 mg/dl (CAD-patients) and from 150 to 220 mg/dl (healthy subjects). None of the donors had diabetes mellitus or arterial hypertension. Selection of CAD-patients and healthy donors was carried out on the basis of a single criterion - the presence or absence in the blood plasma of the ability to induce the accumulation of cholesterol in cultured cells (atherogenic and nonatherogenic plasma, respectively). "Atherogenicity" of LDL or plasma manifested in cell culture was evaluated as described elsewhere (12,13,16,17) and expressed as the increment of total cholesterol in cultured cells. LDL or plasma sample was considered atherogenic if its addition to primary culture of human intimal aortic cells (LDL concentration 100 μg/ml, plasma concentration 40%) increased the intracellular cholesterol level by 2- to 3-fold within a 24 hr incubation. All the plasma samples of selected patients were atherogenic while all the plasma samples of selected healthy subjects were not.

LDL ($d=1.030\text{-}1.050$ g/cm^3) was isolated by ultracentrifugation in a NaBr density gradient according to Lindgren (18) as described earlier (12,13). The LDL sialic acid content was measured according to Warren (19). The determination of sialic acid content in each sample was done in duplicate, and the difference between the obtained values did not exceed 5%.The total protein content was determined according to Lowry et al (20).

LDL was desialylated by treatment with agarose-linked Clostridium perfringens neuraminidase according to Daniels et al. (21) and Camejo et al. (22). Control LDL was incubated with agarose-linked bovine serum albumin. Sialic acid removal from control LDL was not detectable.

The uptake and degradation of LDL labeled with [^{125}I] according to Bilheimer et al. (23) by intimal cells was measured as described by Goldstein et al. (3).

The significance of differences was evaluated by dispersion analysis methods using a BMDP statistical program package (24). Significance of the correlation coefficient difference from zero was estimated using Fisher z-transformation (25).

RESULTS

LDL isolated from the blood of healthy donors was partially desialylated by incubating the lipoprotein with sialidase (neuraminidase). LDL

TABLE 1. Lipid Accumulation in Cultured Cells Caused by LDL Desialylation

Period of neuraminidase treatment, hr	Sialic acid content, μg/mg protein	Intracellular lipid content, μg/mg cell protein			
		Cholesteryl esters	Free cholesterol	Triglycerides	Phospholipids
0 (control)	23.9	28±1	25±2	18±1	157±10
0.5	10.7	57±7*	45±3*	25±2	146±13
1	7.3	67±10*	54±6*	33±2*	163±17
2	6.6	67±10*	68±2*	35±3*	168±12
4	7.0	74±8*	69±7*	34±3*	149±16
6	6.8	72±6*	71±3*	36±2*	170±14
8	7.0	69±7*	69±14*	34±3*	166±19

*, Significant difference from the control ($p<0.05$). Control and desialylated LDL taken in the concentration of 100 μg protein/ml were incubated with cultured cells for 24 hrs.

desialylation resulting from neuraminidase treatment was most prominent within the first 1 hr (Table 1). Incubation of cultured cells with neuraminidase-treated LDL caused a nearly 2-fold increase of triglycerides and a more than 2-fold increase of intracellular cholesteryl esters and free cholesterol; phospholipid level was unchanged (Table 1). A more prolonged incubation with neuraminidase slightly increased LDL atherogenicity, i.e. its ability to cause the accumulation of intracellular lipids. The correlation coefficient between the degree of LDL desialylation and lipid accumulation was 0.98 for cholesteryl esters, 0.91 for free cholesterol and 0.93 for triglycerides ($p<0.05$).

LDL desialylated by neuraminidase were taken up by cultured cells much more effectively than untreated native LDL (Table 2). Degradation of desialylated LDL by cultured cells was also higher than that of native LDL (Table 2).

LDL derived from atherogenic blood plasma of CAD-patients (atherogenic LDL) displayed atherogenic properties in culture causing over a 3-fold rise of intracellular cholesteryl esters and increasing triglycerides (Table 3). LDL obtained from nonatherogenic blood plasma of healthy subjects failed to manifest atherogenicity in culture. The sialic acid content in the LDL obtained from patients' blood was 4-fold lower than that of healthy donors' LDL (Table 3).

TABLE 2. Uptake and Degradation of Desialylated LDL by Cultured Cells

Lipoprotein		Uptake	Degradation
		ng/mg cell protein/12 hrs	
Native LDL	25 μg/ml	131±9	93±7
	100 μg/ml	415±27	547±29
Desialylated LDL	25 μg/ml	546±73*	405±39*
	100 μg/ml	1043±115*	1185±187*

*, Significant difference from native LDL. Desialylated LDL were obtained by treating native LDL with neuraminidase for 3 hrs at 37°C. Values listed are means±SE of 4 determinations.

TABLE 3. Lipid Accumulation in Cultured Cells Caused by Atherogenic LDL of CAD-Patients

Lipoprotein	LDL sialic acid content, μg/mg protein	Intracellular lipid content, μg/mg cell protein			
		Cholesteryl esters	Free cholesterol	Triglycerides	Phospholipids
None (control)	-	19±1	25±2	15±1	187±14
Nonatherogenic LDL	31.3	20±1	26±1	15±2	204±20
Atherogenic LDL	7.8	62±6*	30±4	21±2*	176±14

*, Significant difference from the control and nonatherogenic LDL. Cells were cultured with 10% lipoprotein-deficient serum of healthy donor. Nonatherogenic LDL (100 μg protein/ml) taken from nonatherogenic blood plasma of CAD-patients were incubated with cultured cells for 24 hrs.

We compared several LDL preparations obtained from an atherogenic plasma of CAD-patients and nonatherogenic plasma of healthy donors. The preparations isolated from an atherogenic plasma were atherogenic while all preparations derived from healthy donors proved to be nonatherogenic (Table 4). The highest sialic acid content among the patients' LDL preparations was more than 2-fold lower than the lowest sialic acid content registered among the LDL preparations of healthy donors (Table 4).

Cultured intimal cells took up and degraded atherogenic patients' LDL much more effectively than nonatherogenic LDL of healthy donors (Table 5).

DISCUSSION

This study has confirmed the data of Filipovic et al. (9,10) indicating that partial LDL desialylation by neuraminidase treatment alters the cellular metabolism of lipoproteins. Besides, we have shown that, unlike native LDL, the LDL treated with neuraminidase induce the accumulation of lipids in intimal cells of human aorta. Apparently, this finding is explained by the fact that intimal cells take up desialylated LDL more effectively than native LDL. Thus, LDL desialylation is an atherogenic modification of lipoproteins

TABLE 4. Sialic Acid Content in Atherogenic LDL of CAD-Patients

Original blood plasma	Number of subjects in the pool	Atherogenicity (cholesterol increment)	Sialic acid content, μg/mg protein
Nonatherogenic, pool I	10	8±11	27.4
Nonatherogenic, pool II	8	11±17	33.9
Nonatherogenic, pool III	4	9±13	29.8
Nonatherogenic, pool IV	12	5±9	32.0
Atherogenic, pool V	10	103±19*	11.5
Atherogenic, pool VI	7	131±13*	8.3
Atherogenic, pool VII	12	117±10*	9.2
Atherogenic, pool VIII	10	145±15*	6.5

LDL atherogenicity was determined as described in Materials and Methods and expressed as total cholesterol increment in the cells cultured with the lipoprotein tested. Cholesterol increment is expressed as the percentage of control value (initial cholestrerol content, 63±5 μg/mg cell protein).
*, Significant difference from the control ($p<0.05$).

TABLE 5. Uptake and Degradation of Atherogenic LDL Obtained from CAD-patients

Lipoprotein	Uptake	Degradation
	ng/mg cell protein/18 hrs	
Nonatherogenic LDL	564±18	1555±215
Atherogenic LDL	1438±152*	2280±260*

Nonatherogenic and atherogenic LDL were the same as in Table 3. *, Significant difference from nonatherogenic LDL.

leading to the accumulation of intracellular fat.

We have found that LDL isolated from an atherogenic plasma of CAD-patients have a lower sialic acid content than the LDL obtained from non-atherogenic plasma of healthy donors and, unlike the latter, can stimulate the deposition of intracellular lipids. Evidently, atherogenic properties of patients' LDL are explained exactly by a low sialic acid content since desialylation of originally nonatherogenic LDL of healthy donors makes them atherogenic.

As was noted above, there are many ways of atherogenic LDL modification in vitro leading to the LDL-mediated accumulation of intracellular lipids (2-6). It is postulated that the presence of modified LDL in the circulation or the vessel wall is a prerequisite for the initiation of intracellular lipid accumulation occurring in atherosclerosis. As yet, however, very little is known about the nature of atherogenic LDL modification in vivo and modified LDL have not yet been found in the blood of atherosclerotic patients.

Recently, Avogaro et al. have reported the existence of modified LDL in healthy donors which are capable of inducing the accumulation of cellular lipids in humans (26). This LDL differs from a native lipoprotein by the tendency to aggregate as well as a higher negative charge, low phospholipid levels, elevated content of free cholesterol and protein. However, the authors did not measure the sialic acid content in this LDL subfraction, therefore it is difficult to say whether the modified LDL seen by Avogaro et al. and those found by ourselves are related or not.

We assume that at least one type of atherogenic LDL modification in vivo consists exactly in the lipoprotein desialylation. Elucidation of the mechanism(s) of atherogenic LDL modification in vivo would considerably increase our understanding of the pathogenesis of atherosclerosis.

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