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LIPOPROTEIN AGGREGATION AS AN ESSENTIAL CONDITION OF INTRACELLULAR LIPID ACCUMULATION CAUSED BY MODIFIED LOW DENSITY LIPOPROTEINS

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SUMMARY: We have tested a hypothesis that aggregates of modified low density lipoproteins (LDL) play the key role in the accumulation of lipids by cells of unaffected aortic intima. It was demonstrated using analysis of relative dispersion of light transmission fluctuations as well as gel filtration on Sepharose CL-2B that LDL modified by oxidation, glycosylation, desialylation and malondialdehyde treatment form aggregates under the conditions of culture. Native LDL failed to aggregate under the same conditions. It was demonstrated that modified LDL, unlike native LDL, bring about a 2- to 3-fold rise in cholesteryl ester levels of cultured cells. Moreover, direct and strong correlation (r=0.86) was observed between the degree of lipoprotein aggregation and the amount of cholesteryl esters accumulated. Removal of modified LDL aggregates by filtration through a 0.1 μm filter or gel filtration completely prevented the intracellular accumulation of cholesteryl esters. These findings indicate that LDL aggregates play an essential, if not the decisive, role in the intracellular accumulation of lipids in vitro.

The accumulation of intracellular lipids is a characteristic feature of human atherosclerosis. Presently, it is considered to be an established fact that the source of these lipids is low density lipoprotein (LDL) circulating in the blood (1-3). However, the mechanism of the LDL-mediated accumulation of intracellular fat remains obscure. Several authors demonstrated that native LDL isolated from the blood of healthy subjects fail to induce elevation of lipid levels in cultured macrophages and smooth muscle cells (4-7). The accumulation was observed only in the presence of LDL chemically modified by acetylation (4), acetoacetylation (8), carbamylation (9), maleylation (4), malondialdehyde or glutaraldehyde treatment (10), oxidation (11), desialylation (12), etc. The most intriguing thing about this is that such a broad range of chemical LDL modifications cause the same response at the cellular level, i.e. the accumulation of lipids. This observation led us to

Abbreviations: LDL, low density lipoprotein; MDA-LDL, malondialdehyde-treated low density lipoprotein.

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the assumption that in all the cases described we deal with one and the same process, i.e. lipoprotein aggregation, underlying the increase in intracellular lipid levels. This hypothesis may be indirectly corroborated by the data indicating that the associates of LDL with connective tissue matrix components [proteoglycans (13), collagen (14), elastin (15), fibronectin (14) as well as large particles of artificial origin (15)] are able to increase the intracellular lipid content. The ability of LDL to bind to lipoprotein(a) and other apolipoprotein B-containing lipoproteins was demonstrated by Ye et al. (16). Avogaro et al. (17) reported that a tendency to aggregate is seen in LDL with an increased negative charge. Khoo et al. (18) found that the aggregates prepared by vortexing LDL facilitate the intracellular deposition of lipids in monocyte-macrophages. In this study, we have attempted to provide experimental evidence in favour of the hypothesis suggesting the crucial role of spontaneously formed modified LDL aggregates in the process of intracellular lipid accumulation inside the cells of human aortic intima.

MATERIALS AND METHODS

Low density lipoprotein (1.019-1.063 g/ml) was isolated by ultracentrifugation in KBr density gradient from pooled blood of healthy subjects (19). LDL was glycosylated in vitro for 2 weeks in the presence of 50 mM glucose (20). Desialylation of LDL was carried out by agarose-bound neuraminidase (SIGMA Chemical Co., St.Louis, MO; cat. no. N-4883) treatment for 2 h at 37°C. As a result of this procedure, LDL lost 70% of sialic acid of which the content was measured according to Gaubatz et al. (21). Oxidation of lipoprotein was performed in the presence of 10 μM Cu^{2+} according Steinbrecher et al. (11). Malondialdehyde-modified LDL (MDA-LDL) was obtained using the method of Fogelman et al. (10). Gel filtration of native and modified lipoproteins was performed on a column of Sepharose CL-2B (25x0.6 cm) at the rate of 0.15 ml/min. Subsequently, 0.30 ml fractions were collected to measure the total cholesterol content.

The degree of lipoprotein aggregation was evaluated by the method based on the analysis of light transmission fluctuations in LDL suspensions (22). As is known, relative dispersion of the optical density fluctuations (D) caused by random changes in the number of particles in the optical channel reflects alterations in their average size, i.e. the degree of aggregation. To measure light transmission fluctuations, a semiconductor laser with collimating optics was used (wavelength - 860 nm) (22).

Smooth muscle cells of unaffected aortic intima were isolated by collagenase and cultured as described earlier (15). The autopsy material for cell isolation was taken from males who suddenly died of myocardial infarction. On the 7th day in culture, cells were incubated with medium 199 containing a 5% lipoprotein-deficient serum of a healthy donor obtained by ultracentrifugation (d>1.250 g/ml) (19) as well as 100 μg/ml native or modified LDL. The medium with lipoprotein preparations was filtered (0.22 μm) immediately before it was added to culture. After incubation, cells were rinsed and lipids were extracted with hexane-isopropanol mixture (3:2, v/v) (23). To determine the content of cholesteryl esters, the amount of free and esterified cholesterol was measured according to Seidel (24) using Boehringer Mannheim Kits (Mannheim, FRG). Cell protein was measured according to the method of Lowry et al. (25). Significance of differences was evaluated by multiple t-test of one-way analyses of variance using BMDPIV program from BMDP77 package (26).

RESULTS AND DISCUSSION

At the first stage, we attempted to reveal LDL aggregation under the conditions of culture. To analyze the degree of LDL aggregation we have used a recently developed approach which allows, at a fixed volume concentration of

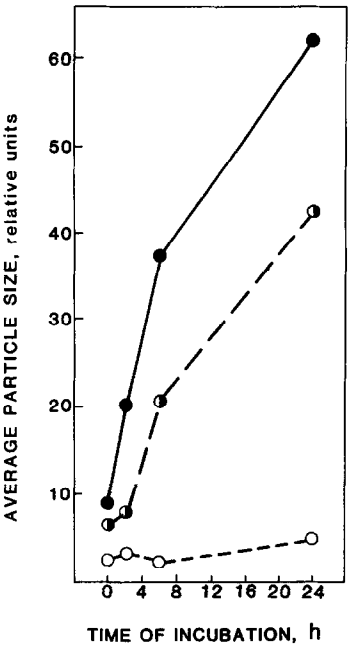

Fig.1. Kinetics of the increase in the average radius of the particles during LDL incubation with cells. 100 μg/ml native lipoproteins (○), oxidized LDL (◐) as well as MDA-LDL (●) were incubated with cells in medium 199 containing 5% lipoprotein-deficient serum for 6 hours. After incubation, the medium was withdrawn and centrifuged for 10 min at 4000 rpm with subsequent determination of average particle size.

particles in suspension, to assess alterations in their average size (22). This method has certain advantages over conventional optical techniques. Firstly, unlike optical density, relative dispersion of light transmission fluctuations is a monotonous function of particle size. Secondly, there is no sensitivity threshold at the registration of aggregates containing a small number of particles.

Native and modified lipoproteins were incubated with cells in medium 199 containing 5% lipoprotein-deficient serum at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Fig. 1 shows the kinetics of aggregation for native LDL, oxidized LDL as well as MDA-LDL. One can see that native LDL practically do not aggregate under the conditions of culture for as long as 6 h of incubation. Within the same period, we observed intensive aggregation of oxidized LDL and MDA-LDL. Aggregate formation was also seen in the preparations of glycosylated LDL (TABLE I).

In addition to the modified LDL mentioned above, we have used desialylated lipoproteins. Earlier, we have demonstrated (12) their presence in the blood of patients with coronary heart disease. Besides, it was shown that LDL treated with neuraminidase bring about the accumulation of lipids in the cells of unaffected human aortic intima. Desialylated LDL just as other types of modified lipoproteins formed aggregates during cultivation with cells (TABLE I).

TABLE I. Aggregation of native and modified LDL and their effect on the cholesteryl ester content of human aortic intimal cells

	Cholesteryl ester accumulation % over control	Average particle size, relative units Incubation	
		before	after
Native LDL	17±6	6.3	6.8
Glycosylated LDL	117±11*	7.5	30.0
Desialylated LDL	184±14*	7.8	39.5
Oxidized LDL	186±17*	8.9	45.2
MDA-LDL	217±28*	7.3	52.3

Freshly filtered (0.22 μm) LDL preparations (100 μg/ml) were incubated with human aortic intimal cells in medium 199 containing 5% lipoprotein-deficient serum for 6 h. After incubation in the medium, they were used to study LDL aggregation. Following cell rinsing, their cholesteryl ester content was measured. Control cells were incubated in medium 199 containing 5% lipoprotein-deficient serum. The cholesteryl ester content of control cells was 27.5±1.9 μg/mg protein. Values listed are means of 3 determinations ± SEM. *, significant difference from the control, $p<0.01$.

It should be pointed out that all the above mentioned lipoproteins showed a similar degree of aggregation when cultured under the same conditions, but in the absence of cells (data not shown).

The formation of modified LDL aggregates was also demonstrated using gel filtration on Sepharose CL-2B. Fig. 2 shows the elution profiles of native and oxidized LDL incubated with cells for 6 h. It can be clearly seen that oxidized LDL gave a larger peak as compared to native LDL. It should be pointed out that the aggregates account for 5 to 7% of lipoprotein cholesterol. Similar results were obtained for desialylated and glycosylated LDL (data not shown).

Subsequently, we have studied the ability of lipoproteins to induce the accumulation of cholesteryl esters in the cells of unaffected aortic intima.

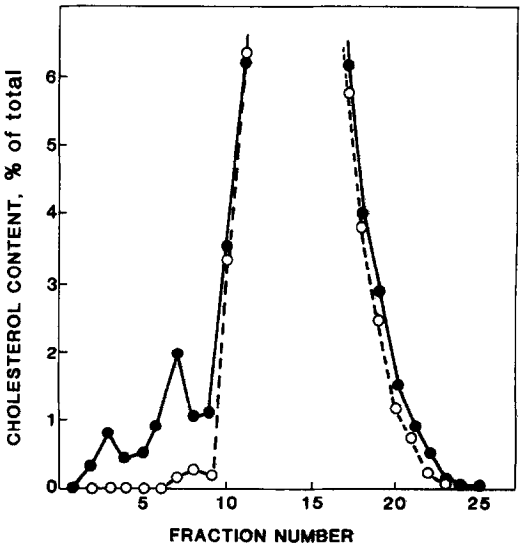

Fig.2. Chromatography of native (○) and oxidized (●) LDL on Sepharose CL-2B. Freshly filtered LDL preparations were incubated with cells for 6 h after which gel filtration was carried out.

Native LDL failed to cause a significant increase in the intracellular content of cholesteryl esters (TABLE I). At the same time, elevation of cholesteryl ester levels was seen in the cells incubated with modified LDL (TABLE I), a direct and strong correlation being revealed between the degree of LDL aggregation and cholesteryl ester accumulation in the cells of unaffected human intima ($r=0.86$, $p<0.001$, $n=21$).

Then, we have attempted to obtain additional evidence proving that LDL aggregates induce the intracellular accumulation of cholesteryl esters. For this purpose, freshly isolated and filtered (0.22 μm) preparations of modified (desialylated and oxidized) LDL were incubated under the conditions of culture and subjected to repeated filtration through filters of different pore size. These LDL were added to culture of intimal cells and incubated for 6 more hours with subsequent determination of the intracellular cholesteryl ester content. The data listed in TABLE II indicate that preincubated oxidized and desialylated LDL brought about a 2.5- to 3-fold accumulation of cholesteryl ester. The filtered preparations caused a lesser increase in cholesteryl esters. Moreover, the preparations filtered through an 0.1 μm filter failed to induce a significant rise in the intracellular cholesteryl ester level within 6 h of incubation.

Briefly summarizing these findings, one can conclude that aggregation of modified LDL occurs in vitro under the conditions of culture. It is exactly modified, but not native LDL, that can cause the accumulation of cholesteryl esters in the cells of human aortic intima, a very close correlation being revealed between the degree of aggregation and the amount of accumulated cholesteryl esters. Finally, removal of aggregates from the medium prevents the accumulation of esterified cholesterol inside the cells. All these data

TABLE II. Effect of oxidized and desialylated LDL filtration on their ability to induce the accumulation of cholesteryl esters in the cells of unaffected human aortic intima

	Pore size, μm	Cholesteryl ester content, μg/mg protein
Control		37±2
Native LDL		43±2
Oxidized LDL		87±7*
	0.45	56±4*
	0.22	49±3
	0.10	39±4
Desialylated LDL		108±8*
	0.45	73±6*
	0.22	62±3*
	0.10	41±2

Preparations of freshly modified LDL were filtered through 0.22 μm filters and preincubated for 6 h in medium 199 containing 5% lipoprotein-deficient serum (0.5 mg apolipoprotein B/ml). After preincubation, LDL preparations were carefully filtered through 0.1 μm, 0.22 μm and 0.45 μm filters and added to cells in the concentration of 0.1 mg/ml. Following 6 h of incubation, cells were rinsed and their cholesteryl ester content was measured. Values listed are means of 3 determinations ± SEM. *, significant difference from the control, $p<0.01$.

indicate that LDL aggregation plays an essential, if not the decisive, role in the processes of lipid accumulation by cells in vitro.

Naturally, the question arises whether aggregation occurs in vivo and brings about the intracellular deposition of lipids during atherosclerosis. As yet we have no answer. Further investigations will be required to prove the existence of aggregate formation in vivo and to elucidate the mechanisms of the LDL aggregate-mediated accumulation of lipids.

REFERENCES

1. Smith, E.B. (1974) Adv. Lipid Res. 12, 1-49.
2. Schonfeld, G. (1979) Artery 5, 305-329.
3. Nicoll, A., Duffied, R. and Lewis, B. (1981) Atherosclerosis 39, 229-242.
4. Goldstein, J.L., Ho, Y.K., Basu, S.K. and Brown, M.S. (1979) Proc. Natl. Acad. Sci. USA 76, 333-337.
5. Traber, M.G. and Kayden, H.J. (1980) Proc. Natl. Acad. Sci. USA 77, 5466-5470.
6. Shechter, I., Fogelman, A.M., Haberland, M.E., Seager, J., Hokom, M. and Edwards, P.A. (1981) J. Lipid Res. 22, 63-71.
7. Leak, D.S. and Peters, T.J. (1982) Atherosclerosis 44, 275-291.
8. Mahley, R.W., Innerarity, T.L., Weisgraber, K.H. and Oh, S.Y. (1979) J. Clin. Invest. 64, 743-750.
9. Weisgraber, K.H., Innerarity, T.L. and Mahley, R.W. (1978) J. Biol. Chem. 253, 9053-9062.
10. Fogelman, A.M., Shechter, I., Seager, J., Hokom, M., Child, J.S. and Edwards, P.A. (1980) Proc. Natl. Acad. Sci. USA 77, 2214-2218.
11. Steinbrecher, U.P., Parthsarathy, S., Leake, D.S., Witztum, J.L. (1985) Proc. Natl. Acad. Sci. USA 81, 3883-3887.
12. Orekhov, A.N., Tertov, V.V., Mukhin, D.N. and Mikhailenko, I.A. (1989) Biochem. Biophys. Res. Commun. (accepted for publication).
13. Vijayagopal, P., Srinivasan, S.R., Jones, K.M., Radhakrishnamurthy, B. and Berenson, G.S. (1985) Biochim. Biophys. Acta 837, 251-261.
14. Falcone, D.L., Mated, N., Shio, H., Minick, C.R. and Fowler, S.D. (1984) J. Cell Biol. 99, 1266-1274.
15. Orekhov, A.N., Tertov, V.V., Mukhin, D.N., Koteliansky, V.E., Glukhova, M.A., Khashimov, Kh.A. and Smirnov, V.N. (1987) Biochim. Biophys. Acta 928, 251-258.
16. Ye, S.Q., Trieu, V.N., Stiers, D.L. and McConathy, W.J. (1988) J. Biol. Chem. 263, 6337-6343.
17. Avogaro, P., Bittolo Bon, G. and Cazzolato, G. (1988) Arteriosclerosis 8, 79-87.
18. Khoo, P. and Stainberg, D. (1988) Arteriosclerosis 7, 35-45.
19. Lindgren, F.T. (1975) in Analysis of Lipis and Lipoproteins (Perkins, E.G., ed.), pp. 205-224, American Oil Chemical Society, New York.
20. Kim, H.-J. and Kurup, I.V. (1982) Metabolism 31, 348-353.
21. Gaubatz, J.W., Cushing, G.L. and Morrisett, J.D. (1986) Methods Enzymol. 129, 167-186.
22. Gabbasov, Z.A., Popov, E.G., Gavrilov, I.Yu. and Pozin, E.Ya. (1989) Thrombosis Res. (accepted for publication).
23. Hara, A. and Randin, N.S. (1978) Anal. Biochem. 90, 420-426.
24. Seidel, J., Hagele, E.O., Ziegenhorn, J. and Wahlefeld, A.W. (1983) Clin. Chem. 29, 1075-1080.
25. Lowry, O.H., Rosenbrough, N.I., Farr, A.L. and Randall, L.Y. (1951) J. Biol. Chem 193, 265-275.
26. Dixon, W.J. and Brown, M.B. (1977) Biomedical Computer Programs. P-Series, pp. 185-198, University of California Press, Berkeley.