The interaction of human apoB-containing lipoproteins with mouse peritoneal macrophages: a comparison of Lp(a) with LDL

F. Krempler, G. M. Kostner, A. Roscher, K. Bolzano, and F. Sandhofer

First Department of Medicine, Landeskrankenanstalten, A-5020 Salzburg, Austria, Institute of Medical Biochemistry, University of Graz, A-8010 Graz, Austria, and Department of Pediatrics, University of Graz, A-8010 Graz, Austria

Abstract Cholesteryl ester accumulation in macrophages and foam cell formation is believed to play an important role in atherogenesis. The effect of Lp(a) on the incorporation of [^{14}C]oleate into cholesteryl esters was studied in mouse peritoneal macrophages. In view of the physico-chemical similarities between Lp(a) and LDL, the results were compared with those obtained with LDL. Native Lp(a) and LDL did not stimulate cholesteryl ester formation. Incubation of macrophages with Lp(a)- or LDL-dextran sulfate complexes caused a significant increase in cholesteryl ester formation. A similar effect was observed when Lp(a) or LDL were incubated with macrophages in the presence of antibodies directed against the specific Lp(a) apoprotein or against LpB. Treatment of Lp(a) with acetic anhydride or malondialdehyde (MDA) was followed by precipitation of most of the lipoprotein. Therefore, these modifications were not suitable to study the uptake of modified Lp(a) by macrophages. Studies with acetyl-LDL or MDA-treated LDL caused the well-known stimulation of [^{14}C]oleate incorporation into cholesteryl esters. Thus, the modification of Lp(a) by sulfated polysaccharides or by treatment with antibodies yields similar cholesteryl ester deposition in mouse peritoneal macrophages as observed with modified LDL. This might be one mechanism by which Lp(a) exerts its atherogenicity.—**Krempler, F., G. M. Kostner, A. Roscher, K. Bolzano, and F. Sandhofer.** The interaction of human apoB-containing lipoproteins with mouse peritoneal macrophages: a comparison of Lp(a) with LDL. *J. Lipid Res.* 1984. **25:** 283–287.

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Foam cells derived from macrophages and arterial smooth muscle cells, which are loaded with cholesteryl esters, seem to play an important role in atherogenesis (1–3). The mechanisms of cholesterol uptake by these cells have been extensively studied in human monocyte-derived macrophages (4–6) and in macrophages isolated from the peritoneal cavity of mice (7–9).

In contrast to human monocyte-derived macrophages, mouse peritoneal macrophages do not bind and internalize native low density lipoproteins (LDL) by specific high affinity receptors similar to the LDL receptors originally described by Brown and Goldstein (10).

It has been demonstrated that macrophages are unable to take up large amounts of native LDL, but massive cholesteryl ester deposition can be produced by LDL which has been modified by acetylation (7) or malondialdehyde (MDA) treatment (4), thus giving the LDL particles more negative charge.

For the uptake of modified lipoproteins, receptors have been demonstrated on human monocyte-derived macrophages and on mouse peritoneal macrophages which have been termed "scavenger receptors" (7, 11). These receptors exhibit saturable high affinity binding of modified LDL.

After binding of LDL to the classic LDL receptor or to the scavenger receptor, LDL is internalized and degraded, and the cholesteryl esters are hydrolyzed (9, 12). Free cholesterol is then reesterified with oleic acid. From numerous studies it has been suggested that not the classic LDL receptor but the scavenger receptor is responsible for the massive accumulation of cholesteryl ester in macrophages and their transformation to foam cells (7, 8).

Lipoprotein (a) (Lp(a)) is a lipoprotein which has many physical and chemical properties in common with LDL. The lipid composition of both lipoproteins is almost identical and the main apoprotein is apoprotein B. In contrast to LDL, Lp(a) has an additional apoprotein, the specific Lp(a) apoprotein. Lp(a) has a higher content of carbohydrate, especially of sialic acid, and therefore more negative charge than LDL (13). Increased serum concentrations of Lp(a) are considered to be an independent risk factor for premature cardiovascular disease (14). Recently it has been demonstrated that Lp(a) is bound and de-

Abbreviations: LDL, low density lipoprotein; Lp(a), lipoprotein (a); DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

graded by high affinity receptors of cultured human fibroblasts with similar affinity as LDL (15). The uptake of Lp(a) by macrophages has not been investigated until now.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Bio-Cult, Glasgow, Scotland. Fetal calf serum (FCS) was obtained from Seromed, München, West Germany. Tissue culture dishes were purchased from Falcon, Oxnard, CA. Malonaldehyde bis(dimethyl acetal) and acetic anhydride were obtained from Merck, Darmstadt, West Germany. Neuraminidase was purchased from Sigma Chemical Co., St. Louis, MO. DEAE cellulose was from Whatman, London, England, [^{14}C]oleic acid from the Radiochemical Centre, Amersham, England, and dextran sulfate from Pharmacia, Uppsala, Sweden.

Mouse peritoneal macrophages

Mouse peritoneal macrophages were collected from unstimulated mice in phosphate-buffered saline (PBS) containing 20 units heparin/ml. Approximately 3×10^6 cells were harvested per mouse. Macrophages from 150 mice were pooled and collected by low speed centrifugation at 1000 *g* for 5 min at room temperature. The cells were then resuspended in DMEM containing 20% (v/v) FCS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (medium A). During the centrifugation steps approximately 40% of the cells was lost due to adherence on the silicone-coated glass surface and incomplete sedimentation. Cells ($3–4 \times 10^5$ per cm^2) were dispensed into multitray dishes and allowed to adhere for 2 hr at 37°C in a humidified 5% CO_2/95% air atmosphere. Then the cells were washed twice with PBS to remove nonadherent cells. Sixty to 80% of the total mononuclear cells dispensed into the dishes were found to be adhering macrophages. After addition of 2 ml of fresh medium A, the cells were further incubated for 24 hr. At the end of this incubation each dish contained approximately 50 μg of cell protein (16).

Lipoproteins

Lp(a) and LDL were isolated as previously described (15, 17). During all isolation procedures, NaN_3 and Na_2EDTA were present in a concentration of 1 mg/ml. Lp(a) and LDL were isolated from sera of individuals with Lp(a) concentrations above 20 mg/dl. Sequential isopycnic ultracentrifugation was performed to isolate density fractions ranging from 1.006 to 1.055 g/ml and from 1.055 to 1.110 g/ml. Both density fractions were applied to agarose columns (Biogel A 5m, Bio-Rad, Richmond, CA) for purification of LDL and Lp(a). The elution profiles have been published elsewhere (17). The isolation procedure took 4 days.

The preparations of Lp(a) and LDL were investigated on PAGE (18). Electrophoresis was carried out in 3.5% polyacrylamide gels; the gels were stained with Coomassie blue R 250.

Modification of lipoproteins

Lp(a) and LDL were acetylated as described by Basu et al. (19). Prior to use, lipoproteins were dialyzed exhaustively against 0.15 M NaCl, 0.02 M sodium-phosphate, pH 7.5. Malondialdehyde-treated Lp(a) and LDL were prepared as described by Fogelman et al. (4). Lipoprotein-dextran sulfate complexes were prepared by addition of 0.1 μg of dextran sulfate (mol wt 500,000) to 1 μg of LDL- or Lp(a)-protein.

Preparation of antibodies

Antisera against LpB and Lp(a) obtained by immunization of rabbits with highly purified antigens were the same as described earlier (20). Anti-Lp(a) was monospecific and gave no cross reaction with LDL (LpB). The gamma globulin fraction from these antisera was prepared by DEAE-cellulose column chromatography (21). The optimal Lp(a) and LDL/antibody ratio that resulted in maximal precipitation of the antigen was determined in previous experiments. Then the amount of antibody added was decreased stepwise to a point where no precipitin formation could be observed after incubation for 1 hr at room temperature (approximately $^1/_5$–$^1/_{10}$ of the optimal antibody concentration). This lipoprotein/antibody ratio was used for the further experiments.

Experimental procedures

After preincubation for 24 hr, medium A was removed from the macrophage monolayers and substituted with 1 ml of medium B (medium A containing 20 μCi of [^{14}C]oleic acid/ml) plus various amounts of Lp(a) or LDL.

Incubation studies. Macrophage monolayers were incubated with Lp(a), LDL, or acetyl-LDL at a concentration of 100 μg of cholesterol/ml medium B for 4, 8, 16, and 24 hr. The medium was then removed and the monolayers were washed four times with PBS. Extraction of cholesteryl ester and calculation of incorporation of [^{14}C]oleic acid into the cholesteryl ester fraction of the macrophages was performed according to Brown, Ho, and Goldstein (9).

In further experiments, macrophage monolayers were incubated with medium B or with medium B containing different concentrations of native or modified lipoproteins up to 500 μg cholesterol of each lipoprotein. Incubation was performed at 37°C for 24 hr. Incorporation of [^{14}C]oleic acid into cholesteryl ester was also measured by incubation of macrophages with Lp(a) and LDL in the presence of antibodies against LpB or Lp(a), and in the presence of dextran sulfate.

RESULTS

When macrophages were incubated with native Lp(a) or native LDL, both in concentrations up to 500 μg of cholesterol/ml of medium, no measurable incorporation of [^{14}C]oleate into the cholesteryl ester fraction could be observed within 24 hr. These results were obtained in five experiments using five different preparations of Lp(a) and LDL from various blood donors. Since Lp(a) is highly susceptible to proteolytic degradation, the lipoproteins were tested on PAGE (**Fig. 1**). With apoLp(a), two bands were obtained which are typical for apoLp(a) on SDS-PAGE: a minor band migrating in the position of apoB and a major band with higher molecular weight than apoB. In the presence of a disulfide reducing agent (mercaptoethanol), apoLp(a) migrates near apoB. No additional bands of lower molecular weight indicating proteolytic degradation could be detected. Using polyacrylamide gels of more than 3.5% monomer concentrations, all the proteins concentrated on top of the separating gels and no band was seen moving into the gels, indicating a very high molecular weight and excluding degradation of the protein moiety of Lp(a) or LDL by proteolysis. The incubation of macrophages with acetyl-LDL resulted in the well-known accumulation of [^{14}C]cholesteryl oleate (**Fig. 2**).

Fig. 1. Polyacrylamide gel electrophoresis of Lp(a) and LDL. Electrophoresis was carried out in 3.5% polyacrylamide gel in the presence of 0.1% SDS; 250 μg of protein was applied to each gel. A, ApoLp(a) solubilized in 5% SDS. B, ApoLp(a) solubilized in 5% SDS + 2% mercaptoethanol. C, ApoLDL solubilized in 5% SDS. D, ApoLDL solubilized in 5% SDS + 2% mercaptoethanol.

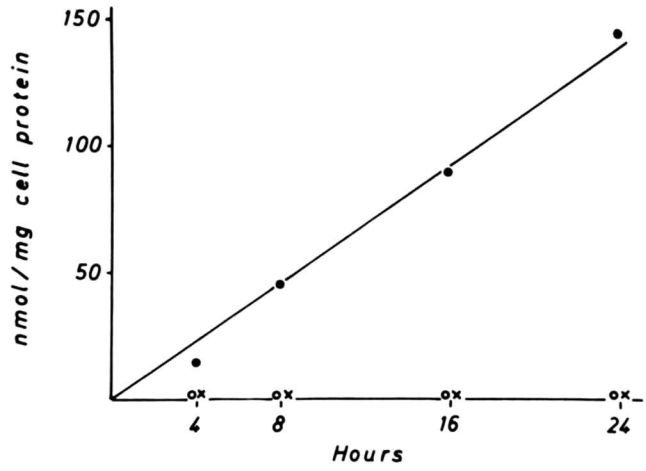

Fig. 2. Effect of native Lp(a) (×), native LDL (○), and acetyl-LDL (●) on the cholesteryl ester formation in mouse peritoneal macrophages as a function of incubation time.

Acetylation of Lp(a) or treatment of Lp(a) with malondialdehyde caused a massive precipitation of approximately 95% and 83% of the lipoprotein, respectively. The material remaining in solution exhibited little if any alteration in electrophoretic mobility as compared to native Lp(a) (**Fig. 3**). This led us to conclude that the modification of Lp(a) by these agents is impossible because of the instability of Lp(a). The small amount of material in the supernatant was considered as not, or only slightly, modified. When this material was incubated with macrophages, no incorporation of [^{14}C]oleate into cholesteryl ester could be observed. Acetylation of LDL or MDA treatment of LDL caused a marked modification of the surface charge and a pronounced anodical shift upon

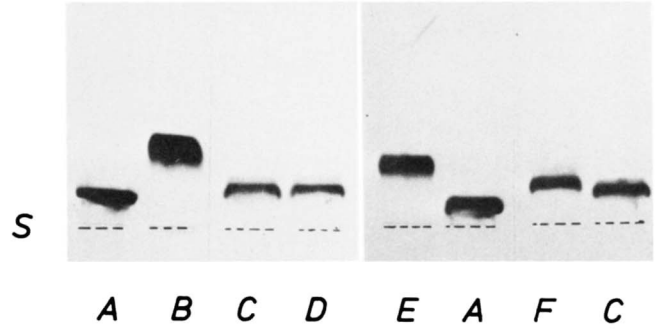

Fig. 3. Agarose gel electrophoresis of various lipoproteins before and after modification. A, Native LDL. B, Acetyl-LDL. C, Native Lp(a). D, Soluble Lp(a) after treatment with acetic anhydride. E, MDA-LDL. F, Soluble Lp(a) after treatment with MDA.

agarose gel electrophoresis (Fig. 3). These modified LDL preparations enhanced the incorporation of [^{14}C]oleate into cholesteryl esters when incubated with macrophages (**Fig. 4**) as described by others (4, 7).

When macrophages were incubated with Lp(a) in the presence of dextran sulfate or antibodies against LpB or Lp(a), a massive incorporation of [^{14}C]oleate into the cholesteryl ester fraction was observed (**Fig. 5**). In this respect, Lp(a) behaved very similar to LDL which caused a similar cholesteryl ester formation when dextran sulfate or antibodies against LpB were added (Fig. 5).

DISCUSSION

There are strong indications that an increased serum level of Lp(a) represents an independent risk factor for coronary vascular disease (14). One possible explanation for this atherogenicity of Lp(a) would be an enhanced interaction with macrophages as compared to native LDL. Our in vitro studies, however, indicate that this assumption is unlikely. The negative charge of native Lp(a) seems to be lower than that of acetyl-LDL or MDA-LDL, as can be delineated from the difference in their electrophoretic mobility. Thus the difference in the electric charge between Lp(a) and LDL might be not sufficient to cause an enhanced interaction of Lp(a) with macrophages. On the other hand, the well-known influence of the negative charge of lipoproteins on the uptake by macrophages was demonstrated with acetyl-LDL and MDA-LDL. On agarose gel electrophoresis, acetyl-LDL showed a greater mobility as compared to MDA-LDL, and thus should have a greater negative charge than the latter one.

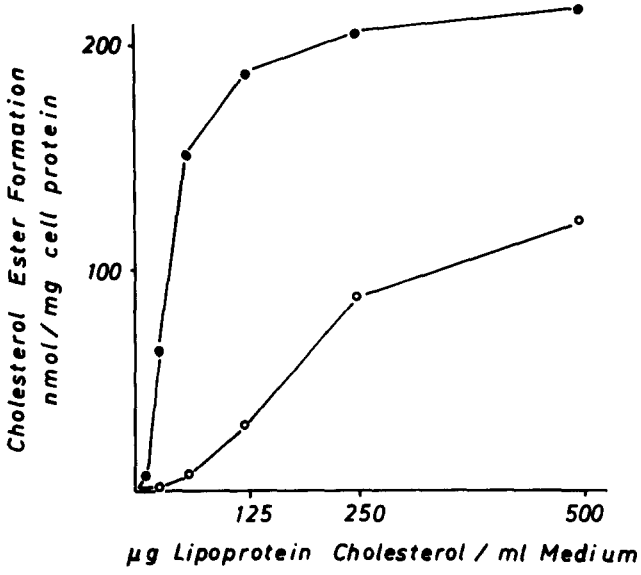

Fig. 4. Effect of acetyl-LDL (●) and MDA-treated LDL (○) on cholesteryl ester formation in mouse peritoneal macrophages as a function of lipoprotein cholesterol concentration in the incubation medium.

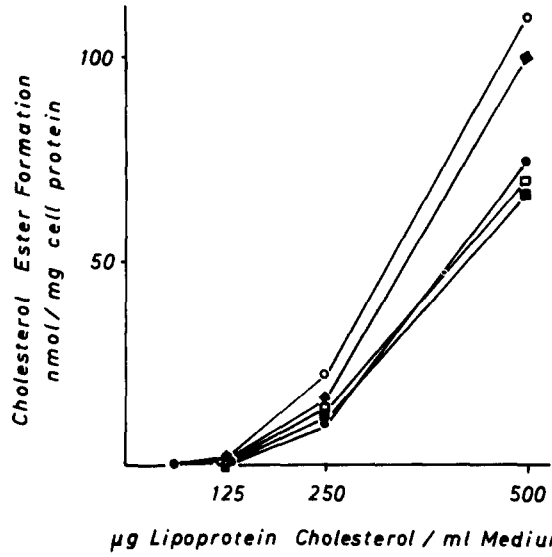

Fig. 5. Effect of Lp(a)-dextran sulfate complex (■), Lp(a)-anti-Lp(a) antibody complex (◆), Lp(a)-anti-LpB antibody complex (●), LDL-dextran sulfate complex (□), and LDL-anti-LpB antibody complex (○) on cholesteryl ester formation in mouse peritoneal macrophages as a function of lipoprotein cholesterol concentration in the incubation medium.

This difference in negative charge seems to correlate well with the observed cholesteryl ester incorporation, which was higher for acetyl-LDL than for MDA-LDL (Fig. 4).

In further experiments we tried to study the uptake of modified Lp(a) in analogy to modified LDL. Acetylation or MDA treatment of Lp(a), however, was followed by precipitation of the lipoprotein. Apparently, Lp(a) was less stable than LDL when chemial manipulations were applied. Therefore, these procedures are not suitable for studying the uptake of modified Lp(a) by scavenger receptors in vitro. There remains the possibility that the instability of Lp(a) and its tendency to aggregate could favor the uptake of Lp(a) by macrophages in vivo in a way similar to that shown in vitro for the Lp(a)-dextran sulfate complexes. That the size of the aggregates is important for the uptake and degradation has been discussed by Basu et al. (22), who found that only large aggregates formed by LDL with dextran sulfate of 500,000 daltons but not of 40,000 daltons were effective.

Our studies show that the Lp(a)-dextran sulfate complex stimulates the incorporation of [^{14}C]oleate into cholesteryl esters of macrophages to the same extent as the LDL-dextran sulfate complex. Dextran sulfate, a sulfated polysaccharide with a negative charge, is known to compete with acetyl-LDL for the binding sites of macrophages (7). Since dextran sulfate forms complexes with lipoproteins, it can transport these lipoproteins by a "piggy back" mechanism into the macrophages. Basu et al. (22) were able to demonstrate that the LDL-dextran sulfate complex is taken up with high affinity by mouse macrophages and that this complex markedly stimulates cholesteryl ester

deposition. Their data suggest that the effect of the LDL-dextran sulfate complex on cellular cholesterol metabolism is similar to that of acetyl-LDL (22). It has been speculated that in vivo large proteoglycan complexes could play a similar role as that of high molecular weight dextrans and promote the uptake of LDL by macrophages (22). The same could apply for Lp(a).

The incubation of Lp(a) or LDL with macrophages, in the presence of specific antibodies directed against their apoproteins, caused a marked stimulation of oleate incorporation into cholesteryl esters. It is well established that macrophages exhibit cell surface receptors that bind to the Fc moiety of antibodies (23). Our study shows that Lp(a), when incubated with specific antibodies, stimulates cholesteryl ester formation in macrophages to a similar extent as that already demonstrated for LDL. Whether or not a similar mechanism may play a role in vivo in the removal of Lp(a) or in atherogenesis remains to be established.

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