Autoantibodies Against Modified Low Density Lipoprotein

Nonlipid Factor of Blood Plasma That Stimulates Foam Cell Formation

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The blood serum of patients with coronary atherosclerosis possesses an ability to induce the accumulation of cellular lipids in primary cultures of human aortic intimal cells. Factors responsible for this property of the atherosclerotic patients' sera are represented by modified (desialylated) low density lipoprotein (LDL) and a nonlipid factor interacting with LDL. It was assumed that the nonlipid factor was antibodies against LDL. Total immunoglobulin G (IgG) fraction was isolated from the sera of atherosclerotic patients, and IgGs interacting with LDL (anti-LDL) were then purified by affinity chromatography on a sorbent with immobilized LDL. From the sera of patients, a 30-fold greater amount of anti-LDL has been isolated than from the sera of healthy donors. The affinity constant of anti-LDL to the lipoprotein obtained from the blood of healthy donors was 2×10^7 M^{-1}. The affinity of anti-LDL to the lipoprotein from the blood of atherosclerotic patients, as well as to LDL desialylated in vitro with neuraminidase, was much higher. Anti-LDL increased the uptake of LDL by cultured aortic cells by approximately 2.5-fold and substantially increased intracellular lipid accumulation. The obtained data suggest that autoantibodies against LDL are an essential factor of blood plasma responsible for its atherogenic potential. (*Arteriosclerosis and Thrombosis* 1991;11:316–326)

Recently, we[1,2] have found that the blood serum of coronary heart disease (CHD) patients with angiographically documented coronary atherosclerosis, as well as patients' plasma and plasma-derived low density lipoprotein (LDL), causes atherosclerosis-related alterations in cultures of human aortic intimal cells, which are manifested by the accumulation of cellular lipid, mostly free and esterified cholesterol. The serum- or LDL-induced deposition of lipids is accompanied by enhanced proliferation as well as increased synthesis of collagen, glycosaminoglycans, and total protein.[3,4] Serum and LDL of most healthy donors were unable to stimulate atherosclerosis-related processes in cultured cells.[1–6] We[7] have established that the patients' LDL causing intracellular lipid accumulation differs from native LDL of healthy donors by a low sialic acid content. On the other hand, desialylation of native LDL derived from healthy donors by neuraminidase treatment led to lipid accumulation in cultured human aortic cells.[7,8] These data suggest that it is desialylation that determines the capacity of the patients' LDL to accumulate cellular lipids.

We[1] have recently shown that desialylated LDL is not the sole factor in patients' blood plasma responsible for its ability to cause atherosclerosis-related manifestations at the arterial cell level. From the blood of atherosclerotic patients, we[9] have isolated immunoglobulin Gs (IgGs) having a higher affinity for LDL desialylated in vitro and for LDL obtained from patients' blood than for native LDL derived from the blood of healthy donors. These IgGs substantially increased the ability of patients' LDL to induce the accumulation of cellular cholesterol.[9] We assumed that Igs isolated from patients' blood samples are represented by autoantibodies against modified LDL. This study was undertaken to reveal the characteristics of these IgGs and to investigate their effect on the LDL-mediated accumulation of lipids in cultured subendothelial intimal cells of the human aorta.

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Methods

Reagents

The reagents were purchased from Sigma Chemical Co., St. Louis, Mo., unless stated otherwise.

Serum

After an overnight fast, blood was drawn from the ulnar vein into plastic tubes and incubated for 1 hour at 37°C. Sera were obtained by repeated centrifugation at 3,000 rpm for 15 minutes. We selected the blood sera of 15 CHD patients with angiographically documented coronary atherosclerosis and the sera of 15 healthy donors who were chosen on the basis of a single criterion, that is, the presence or absence, in this particular serum, of the ability to induce the accumulation of cholesterol in cultured cells. All the patients' sera selected were capable of inducing cholesterol accumulation, while all the sera of healthy donors were not capable of doing so. The characteristics of CHD patients and healthy donors have been described in detail elsewhere.[1,2] Characteristics such as age and sex in the groups of healthy donors and patients were similar. None of the donors had diabetes mellitus. The mean cholesterol level in both types of sera was the same: 186±4 mg/dl for patients and 176±6 mg/dl for healthy subjects. Healthy donors had no signs of heart disease as determined by epidemiological criteria (12-lead rest electrocardiogram ST test; Rose questionnaire; arterial pressure; and anthropometry). To isolate LDL and IgG from the blood plasma and serum of patients and healthy donors, the respective type of plasma or serum was pooled.

The lipoprotein-deficient fraction was obtained by centrifuging the serum at 300,000*g* (d=1.250 g/cm^3) for 48 hours at 4°C according to Lindgren[10] and dialyzed as described below. The obtained sera were sterilized by filtration (pore size, 0.45 μm).

Lipoproteins

LDL (d=1.030–1.050 g/cm^3) was isolated from the plasma obtained from patients and healthy donors according to the conventional method of ultracentrifugation in a stepwise gradient of NaBr[10] as described elsewhere.[1,2] Lipoprotein(a) (Lp[a]) was isolated by immunoaffinity chromatography involving an anti-apo(a)–Sepharose affinity column according to Gaubatz et al.[11] LDLs obtained from healthy donors were modified by acetylation,[12] glycosylation,[13] oxidation in the presence of Cu^{2+},[14] and malondialdehyde (MDA) alteration.[15] Desialylation of LDL was performed by agarose-bound neuraminidase (catalog No. N-4883) treatment for 3 hours at 37°C according to Camejo et al.[16] as described earlier.[7,8] As a result of this procedure, LDL lost 70% of sialic acid (determined according to Warren[17]). As a result of glycosylation, 6% of the apo B lysine residues were modified (determined according to Witztum et al[18]). MDA treatment resulted in the modification of 54% lysine residues (determined according to Yagi[19]). The MDA content of the LDL preparations obtained from healthy donors was 1.4±0.2 nmol/mg apo B, LDL from atherosclerotic patients was 1.6±0.2 nmol/mg apo B, and oxidized lipoprotein preparations were 16.2±0.5 nmol/mg apo B (as determined according to Yagi[19]). Lipoprotein preparations and the lipoprotein-deficient serum were dialyzed for 24 hours against 2,000 volumes of phosphate-buffered saline (PBS), sterilized by filtration, and stored at 4°C. Lipoproteins were used within 1–4 days after preparation. All lipoprotein preparations were filtered (pore size, 0.45 μm) immediately before addition to the culture. To investigate the cellular metabolism of LDL, lipoprotein was iodinated by the iodine monochloride method.[20] More than 98% of ^{125}I in the labeled lipoprotein preparation was precipitated in 10% trichloroacetic acid (TCA). Cells were incubated with labeled lipoprotein for 6 hours at 37°C. After incubation, an aliquot of the culture medium was taken to determine LDL degradation by the presence of the TCA-soluble (noniodide) ^{125}I.[21] To determine the uptake, cells were rinsed three times with PBS containing 0.2% albumin and seven times with PBS, after which they were dissolved in 0.1N NaOH to measure the radioactivity.

Cell Cultures

Cells were obtained from fresh segments of thoracic aortas taken at autopsy from 40- to 60-year-old men. Subendothelial cells were isolated from a grossly normal intima by dispersion with collagenase and were cultured as described elsewhere.[22] A suspension of the isolated cells was resuspended in the growth medium containing Medium 199, 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone (all reagents from GIBCO, Grand Island, N.Y.) and seeded into Linbro 24-well tissue culture plates (Flow Laboratories Ltd., Irvine, U.K.) with a density of 2 to 4×10^4 cells/cm^2 of the growth area. The cells were cultured at 37°C in an atmosphere of 95% air/5% CO_2 in a humidified CO_2 incubator. The medium was changed every day. All additions were diluted to the final concentration with Medium 199. The primary cultures contained a mixed cell population made up primarily of cells of smooth muscle origin.[22] It has been shown that by formal criteria (staining with antibodies against smooth muscle myosin, ultrastructural characteristics), these cells can be classified into typical and so-called modified smooth muscle cells.[22–24] However, these cells in culture accumulate LDL-derived lipids just as effectively as cultured macrophages do.[25,26] Thus, cultured intimal subendothelial cells of human aortas have properties similar to those of both smooth muscle cells and macrophages. Macrophages of monocytic origin accounted for less than 3% of cells in our cultures as determined by light and target electron microscopy[23,24] and less than 6–7% as determined by immunoperoxidase reaction with monoclonal antibodies HHF35 (muscle actin–specific monoclonal antibody) and HAM56 (antimacrophage monoclonal antibody).

Because the real origin of intimal cells is still obscure, we prefer to call them subendothelial intimal cells rather than smooth muscle cells.

Lipids

Before lipid determination, cells were rinsed three times with PBS, treated with 0.025% trypsin–EDTA for 5 minutes, and washed with PBS five times (all reagents were from GIBCO). The cells were removed from the substrate with 0.25% trypsin–EDTA and washed twice by centrifugation (200*g*, 10 minutes). Lipids were extracted from cells with a chloroform/methanol mixture (1:2, vol/vol) according to Bligh and Dyer.[27] The total cholesterol content in the lipid extracts was determined using the Boehringer Mannheim Monotest, cholesterol CHOD-PAP method (Boehringer Mannheim GmbH, Mannheim, F.R.G.). Cellular phospholipids, triglycerides, free cholesterol, and cholesteryl esters were separated by thin-layer chromatography and measured by scanning densitometry as described earlier.[22] Using light and electron microscopy,[25,26] we have demonstrated that an increase of cellular lipids is associated with the appearance of intracellular lipid inclusions.

Electrophoresis and Immunoblotting

Electrophoretic fractionation of serum proteins was performed according to Laemmli[28] with a 3% concentrating polyacrylamide gel and a separating polyacrylamide gel gradient (3–15%). Two microliters serum was diluted in 50 μl 40 mM Tris HCl, 10 mM EDTA, 4% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 20% glycerol (pH 7.0). After electrophoretic separation for 14–16 hours, proteins were transferred onto nitrocellulose (Bio-Rad Laboratories, Richmond, Calif.) at a constant current (0.5 A/3 hours).[29] Nitrocellulose transfers were blocked with 50 mM Tris HCl (pH 7.5) containing 0.5 M NaCl, 0.05% Tween-20, 2% bovine serum albumin (BSA), and 0.3% gelatin. Nitrocellulose strips were incubated for 3 hours with sera from healthy subjects and atherosclerotic patients diluted 30-fold with a blocking buffer as well as with the isolated human IgGs, which have an affinity for LDL (see below), or murine monoclonal antibody against human apo B obtained as described elsewhere.[26] After they were washed in 50 mM Tris HCl (pH 7.5) with 0.5 M NaCl and 0.05% Tween-20, peroxidase-labeled anti-human and anti-mouse goat antibodies (Amersham International plc, Amersham, U.K.) were incubated with respective replicas for 1 hour. This was followed by washing and an incubation in 0.05% 4-chloro-1-naphthol and 0.03% H_2O_2 in 50 mM Tris HCl, pH 7.5.

Immunoglobulin G Isolation

IgGs were isolated from the pooled blood sera of healthy donors and CHD patients by precipitation with 33% saturated ammonium sulfate and subsequent ion-exchange chromatography on diethylaminoethyl cellulose DE-52 (Whatman Ltd., Maidstone, U.K.).[30] The Ig content was determined immunochemically using Orion kits (Orion Diagnostica, Espoo, Finland).

Anti–Low Density Lipoprotein Purification

IgGs having an affinity for LDL (anti-LDL) were purified by affinity chromatography on a column with immobilized LDL. LDL immobilization on BrCN-activated Sepharose CL 4B was performed as described earlier.[1] Twenty to 30 milligrams of the total IgG fraction was applied to a column containing 10 ml LDL–Sepharose. The column was rinsed with 200 ml PBS and 50 ml 0.5 M NaCl. The IgGs bound to the column were eluted with 0.15 M glycine buffer, pH 2.5. The eluate was dialyzed against 2,000 volumes of PBS overnight and concentrated by reverse dialysis in Ficoll type 400 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden).

Binding Studies

The analysis of IgG interaction with LDL was performed using a micro enzyme-linked immunosorbent assay (ELISA) technique. The plates (Costar, Cambridge, Mass.) were coated with LDL (1 μg/well) at 20°C overnight. Delipidation of LDL was performed with a hexane/isopropanol mixture (3:2, vol/vol) for 1 hour at 20°C.[31] The plates were blocked for 1 hour with PBS containing 1% BSA, 0.05% Tween-20 (BSA–Tween–PBS). After a fourfold rinsing with PBS, the series of IgG dilutions in BSA–Tween–PBS were placed into the wells. Plates were incubated for 6 hours, washed with BSA–Tween–PBS, and combined with anti-human IgG antibodies conjugated with peroxidase and incubated for 1 hour. Then, plates were rinsed, and 100 μl substrate (0.1 mg/ml *o*-phenylenediamine and 0.003% H_2O_2 in 50 mM citrate buffer, pH 4.7) was added into the wells. The reaction was stopped by addition of 20 μl 50% sulfuric acid. The optical density was measured at 492 nm using a Titertek Multiskan automatic microphotometer from Eflab Oy, Helsinki, Finland.

Affinity Constant

The affinity constant of IgG to LDL was measured using ^{125}I-LDL of known specific activity (200–1,000 cpm/ng apo B). Serial dilutions of LDL in PBS containing 1% BSA and 0.05% Tween-20 (0.01–100 μg apo B/ml) were combined with 1 μg/ml of affinity-purified IgG and incubated for 8 hours at 20°C. The incubation mixture was transferred to plates precoated with goat anti-human IgG antibodies (Organon Teknika Corp., West Chester, Pa.). The same LDL dilutions incubated in the absence of affinity-purified IgG were used as controls. To immobilize the immune complex of IgG with LDL formed after an overnight incubation, the plates were rinsed with BSA–Tween–PBS with subsequent determination of radioactivity in each well. The identical procedures were performed in the presence of 0.001–1 mg/ml unlabeled LDL. The affinity constant (K_a) was calculated according to Müller,[32] proceeding from the known amounts of the radioactive and non-

radioactive LDL added as well as the amount of ^{125}I-LDL bound by IgG.

Immunoglobulin G Isoelectrofocusing and Native Blot

Isoelectrofocusing of IgG was performed according to Reinhart and Malamund.[33] Seventy micrograms IgG was focused in 5% polyacrylamide gel containing 4.5 M urea, 2.5% Nonidet P-40, and 2% (wt/vol) Pharmalyte, pH 3–10 (Pharmacia). The transfer of IgG separated by the charge from gel to the LDL-coated and the Tween-, BSA-, and gelatin-blocking nitrocellulose occurred via passive diffusion in a rising flow of PBS containing 0.3% BSA and 0.05% Tween-20 over 4 hours. After the termination of transfer, the replica was rinsed many times with large volumes of 50 mM Tris HCl, pH 7.5, containing 0.5 M NaCl, 0.3% BSA, and 0.05% Tween-20. The replica was treated with the antibodies against human IgG conjugated with peroxidase and revealed with 4-chloro-1-naphthol with H_2O_2 as described above.

F(ab')$_2$ Fragments

F(ab')$_2$ fragments of IgG were obtained by pepsin degradation with subsequent purification using Sephadex G-150.[35,36] F(ab')$_2$ fragments of IgG derived from healthy donors and CHD patients were labeled with ^{125}I[36] and added to the LDL-coated plates (10 μg apo B/well) in the concentration of 0.01–10 μg/ml. After a 6-hour incubation, plates were rinsed with BSA–Tween–PBS, and the radioactivity was measured.

C1q Component of Complement

LDL (30 μg/ml) was incubated for 6 hours with 30 μg/ml of the total IgG fraction or 30 μg/ml of anti-LDL obtained from the blood of patients. After that, 30 μg/ml ^{125}I-C1q[36] was added, and incubation was continued for another 4 hours. After the incubation, the samples were supplemented with an equal volume of 5% polyethyleneglycol 6000 and incubated for 18 hours. Samples were centrifuged for 15 minutes at 4,000 rpm, and the sediment was rinsed with 2.5% polyethyleneglycol 6000 with subsequent determination of its radioactivity.

Statistical Analysis

The significance of differences was evaluated by dispersion analysis methods using a BMDP statistical program package.[37]

Results

Isolation and Characteristics of Immunoglobulin Gs Interacting With Low Density Lipoprotein

First, we have attempted to demonstrate the presence of IgGs interacting with LDL in the serum of CHD patients and the serum of healthy subjects. Using immunoblotting, it has been confirmed that IgGs interacting with apo B, apoproteins of LDL, are present in the serum of CHD patients (Figure 1A). The reaction of apo B with autologous IgG of healthy donor serum was very weak (Figure 1B). Since the sera of patients and healthy subjects were characterized by approximately equal apo B contents, this weak reaction may be explained by a lower content of antibodies against lipoprotein in the serum of healthy donors.

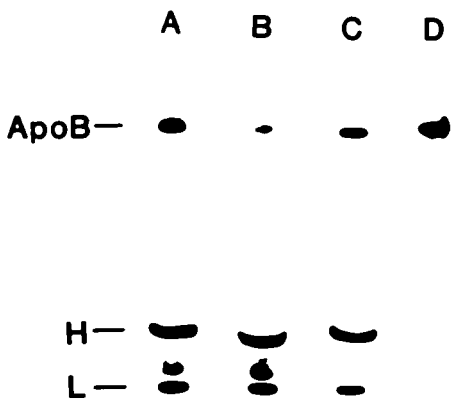

FIGURE 1. *Photograph of immunoblots after electrophoretic separation of proteins of blood serum from coronary heart disease patients (lane A) and from healthy subjects (lane B) and reactions with the same sera, affinity-purified immunoglobulin G (IgG) (lane C), or mouse monoclonal antibody against apolipoprotein (apo) B (lane D). Electrophoretic separation of serum samples and immunoblotting were performed as described in "Methods." Serum proteins were separated electrophoretically and transferred to nitrocellulose strips. Then, after the blocking step, nitrocellulose replicas were incubated with the same sera. If a serum contains antibodies against low density lipoprotein (LDL), the latter should bind to the antigen, i.e., LDL. Lanes A and B show immunoblots obtained after separation of serum proteins and incubation with initial serum, revealing human IgG with a peroxidase-labeled second antibody as described in "Methods." Four bands with anti-human Igs are revealed: light and heavy chains of IgG (L and H) as well as one band with a high molecular weight and another band with ~30-kd molecular weight. On the basis of electrophoretic mobility and positive reaction with monoclonal antibodies against apo B (lane D), the high molecular weight band was identified as apo B. The low molecular weight band was not identified. Patients' sera as well as healthy donors' sera contain anti–30 kd and anti–apo B as major autoantibodies (lanes A and B).*

To isolate the antibodies against LDL (anti-LDL) from the serum, at the first stage total IgG fractions were obtained. The yield of IgG resulting from the two isolation procedures, that is, precipitation with ammonium sulfate and ion-exchange chromatography, was about 50% (Table 1). Anti-LDL IgGs were purified from the total IgG fractions by affinity chromatography on a column with immobilized LDL. The amount of anti-LDL IgG isolated from the sera of patients accounted for 0.14% of the total IgG content in the serum. On the other hand, affinity chromatography of the total IgG fraction obtained from sera of healthy donors allowed us to isolate a 30-fold lower amount of IgG (<0.005% of the total IgG content in the serum).

TABLE 1. Immunoglobulin G Content at Different Purification Stages

Fraction	IgG content (μg/ml initial serum)	
	Healthy donors	CHD patients
Initial serum	12,900	15,600
$(NH_4)_2SO_4$ fraction	12,500	15,100
Total IgG fraction	6,520	6,940
Affinity-purified IgG	0.6	21

IgG, immunoglobulin G; CHD, coronary heart disease.

Figure 2 shows separation of the total IgG fraction by isoelectrofocusing. Subsequent immunoblotting with LDL revealed that in the total IgG fraction of CHD patients, only the Igs with an isoelectric point (pI) of about 8.5 (8.1–9.0) intensively bind LDL (Figures 2 and 3). IgG with a pI of approximately 8.5 obtained from the sera of healthy subjects bound the lipoprotein much less effectively (Figures 2 and 4). Anti-LDL IgGs purified from the total IgG fraction of patients' sera by affinity chromatography are represented by several bands with pIs of about 8.5 (Figure 3).

To test the specificity of anti-LDL IgG isolated on LDL–Sepharose, we performed immunoblotting with electrophoretically separated serum proteins. Affinity-purified LDL bound only to the apo B band but to no other serum proteins (Figure 1C).

The $F(ab')_2$ fragments obtained from affinity-purified anti-LDL IgG had an ability to bind LDL (Figure 4). $F(ab')_2$ fragments from the total IgG fraction of healthy donors were devoid of this ability (Figure 4).

Figure 5 shows the data on the binding of the complement C1q component by an LDL–anti-LDL–IgG complex. On the other hand, in the absence of anti-LDL IgG, lipoprotein failed to bind C1q. Incubation of LDL with the total IgG fraction obtained

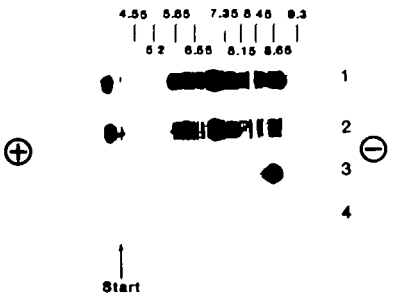

FIGURE 2. *Photograph showing separation by isoelectrofocusing (lanes 1 and 2) and immunoblotting (lanes 3 and 4) of the total immunoglobulin G (IgG) fraction derived from the blood of healthy subjects (lanes 2 and 4) and coronary heart disease patients (lanes 1 and 3). Isoelectrofocusing and transfer onto nitrocellulose coated with low density lipoprotein were performed as described in "Methods." Replica was treated with peroxidase-labeled antibodies against human IgG and revealed with 4-chloro-1-naphthol with H_2O_2. Gels 1 and 2 were stained with Coomassie Brilliant Blue G-250.*

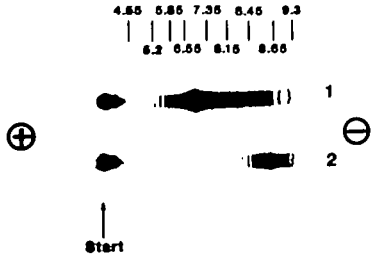

FIGURE 3. *Photograph showing separation of the total immunoglobulin G (IgG) fraction of coronary heart disease (CHD) patients (lane 1) and affinity-purified IgG (lane 2) by isoelectrofocusing. Affinity-purified IgGs were isolated from the total IgG fraction of the CHD patients' sera on a column with immobilized low density lipoprotein. Gels were stained with Coomassie Brilliant Blue G-250.*

from the blood of healthy donors did not lead to marked C1q binding either.

Thus, affinity-purified anti-LDL IgGs bind to LDL apo, but not to other blood serum proteins. $F(ab')_2$ fragments of these IgGs also possess an ability to bind to LDL. Finally, the LDL–IgG complex binds the C1q component of complement. All these data indicate that the anti-LDL IgG affinity purified on LDL–Sepharose from the total IgG fraction of patients' sera are antibodies against LDL (anti-LDL).

No significant differences in the interaction of antibodies with native LDL and lipoprotein delipidated by hexane/isopropanol were found, suggesting that isolated anti-LDLs are antibodies against the protein moiety of the lipoprotein particle (Figure 6).

Table 2 shows the affinity constants of purified anti-LDL to lipoproteins. The affinity constants for native LDL and Lp(a) obtained from the blood of a

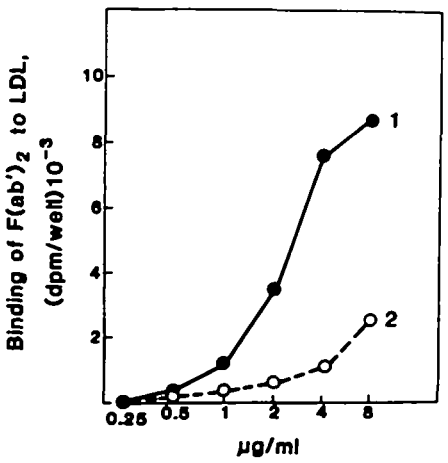

FIGURE 4. *Line plot of low density lipoprotein (LDL) binding of ^{125}I-$F(ab')_2$ fragments (dpm/well [10^{-3}]) obtained from affinity-purified anti-LDL immunoglobulin G (IgG, μg/ml) (1) and the total IgG fraction obtained from the blood of healthy subjects (2). ^{125}I-$F(ab')_2$ fragments obtained by pepsin treatment were added to LDL-coated wells and incubated over 6 hours. After incubation, wells were washed, and the radioactivity was measured.*

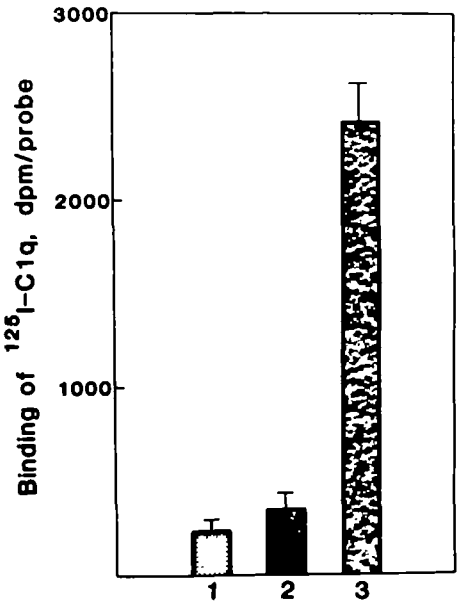

FIGURE 5. *Bar graph showing binding of the ^{125}I-C1q complement component (dpm/probe) by low density lipoprotein–immunoglobulin G (LDL-IgG) complex. ^{125}I-C1q complement component was incubated over 4 hours in the presence of LDL (1), LDL+total IgG fraction from the blood of healthy subjects (2), and LDL+affinity-purified IgG (3). Immune complexes were precipitated by polyethylene glycol 6000, after which the radioactivity of the precipitate was measured. Amounts of C1q, LDL, and IgG are indicated in "Methods." Data are represented as mean±SEM of three independent experiments.*

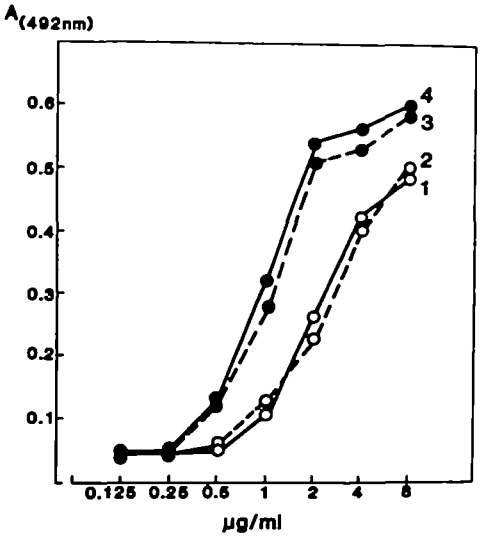

FIGURE 6. *Line plot showing binding of antibodies with native (1 and 3) and delipidated (2 and 4) low density lipoprotein (LDL, μg/ml) measured as absorbance (A) at 492 nm from the blood of healthy subjects (1 and 2, ○) and coronary heart disease patients (3 and 4, ●). The plate wells were coated with LDL (1 μg protein/well). Delipidation of LDL was performed with a hexane/isopropanol mixture (3:2, vol/vol). The interaction of anti-LDL with native and delipidated LDL was performed by enzyme-linked immunosorbent assay as described in "Methods."*

healthy donor as well as for glycosylated LDL, acetylated LDL, and LDL oxidized by Cu^{2+} were similar. The antibodies show a higher affinity for LDL isolated from the blood of CHD patients as well as MDA-modified LDL. The LDL desialylated in vitro by neuraminidase had the highest affinity constant among the modified lipoproteins.

Effect of Anti–Low Density Lipoprotein on the Low Density Lipoprotein–Induced Accumulation of Cellular Lipids

Patients' blood sera caused a more than twofold increase in total cholesterol in cultured cells of human aortic intima, while the blood sera of healthy donors proved to be ineffective in this respect (Table 3).

LDL isolated from plasma of healthy donors failed to stimulate the deposition of intracellular cholesterol, although in combination with the total IgG fraction derived from the patients' serum, LDL caused a twofold increase in cholesterol content in cultured cells. The total IgG fraction of healthy donor plasma had no effect on LDL-mediated cholesterol accumulation (Table 3).

Addition of affinity-purified anti-LDL to the cells cultured with the LDL of healthy donors substantially increased the cellular cholesterol content starting with the concentration of 25 μg/ml (Table 4). Anti-LDL brought about an increase in triglycerides and free and esterified cholesterol, while phospholipid levels remained unchanged (Table 5). Native LDL of healthy donors taken without anti-LDL did not induce lipid accumulation in cultured cells. LDL isolated from patients' sera was capable of inducing lipid accumulation in cultured cells; however, anti-

TABLE 2. Affinity Constants of Lipoprotein–Anti-Low Density Lipoprotein Interaction

Lipoprotein	Affinity constant ($\times 10^{-7}$ M^{-1})	LDL-induced cholesterol increment (% over control)
HD LDL	2.4	2±10
Glycosylated LDL	2.6	105±14*
Acetylated LDL	2.8	163±15*
Cu^{2+}-oxidized LDL	3.5	250±18*
Lp(a)	3.6	102±7*
MDA LDL	10.9	305±21*
Pt LDL	11.3	246±31*
Desialylated LDL	89.4	290±32*

Data from one of three representative experiments are presented. The affinity constant of anti-LDL was determined using native and modified ^{125}I-LDL of known specific activity (200–1,000 cpm/ng apolipoprotein B). Known amounts of added radioactive and nonradioactive LDL and the amount of ^{125}I-LDL bound to anti-LDL were used to calculate the affinity constant.

LDL, low density lipoprotein; HD LDL, healthy donors' LDL; Lp(a), lipoprotein(a); MDA, malondialdehyde; Pt LDL, coronary heart disease patients' LDL.

*Significant difference from HD LDL ($p<0.05$).

Table 3. Effect of Total Immunoglobulin G Fraction on Cellular Cholesterol Accumulation Induced by Low Density Lipoprotein

Addition	Cellular cholesterol (μg/ml cell protein)
Control (10% LDS)	50±4
Total HD serum, 40%	55±9
Total Pt serum, 40%	104±8*
HD LDL, 100 μg/ml	53±4
HD LDL+HD IgG, 100 μg/ml	64±8
HD LDL+Pt IgG, 100 μg/ml	96±7*

LDS, lipoprotein-deficient serum of healthy donors (HD); Pt, patients; LDL, low density lipoprotein; IgG, immunoglobulin G.
Values listed are mean of four to six determinations±SEM. Cells were isolated by collagenase dispersion from subendothelial part of human aortic intima. Sera, LDL, and IgG preparations were added to cultured cells on the 7th day in primary culture. Twenty-four hours later, cells were rinsed and extracted by chloroform/methanol, and cellular cholesterol was determined using a commercial kit as described in "Methods."
*Significant difference from control ($p<0.05$).

LDL enhanced this property of patients' LDL starting with a concentration of 10 μg/ml (Table 4). The effects of anti-LDL were dose dependent and reached a maximum at a concentration of 50 μg/ml. Thus, anti-LDLs alter initially native LDLs of healthy donors, imparting to them an ability to induce the accumulation of cellular lipids and, on the other hand, enhance this property of LDL of atherosclerotic patients. The IgG of patients that do not interact with LDL (not adsorbed on LDL–Sepharose) failed to increase cholesterol accumulation in cultured cells (Table 4).

The effect of anti-LDL on the cellular metabolism of lipoprotein was assessed by the uptake (surface-bound and internalized LDL) and degradation of ^{125}I-LDL by cultured intimal cells. The uptake of CHD patients' LDL was nearly twofold higher than that of healthy donors' LDL; at the same time, degradation of patients' LDL was significantly lower as compared with the degradation of healthy donors' LDL (Table 6). Addition of anti-LDL to cultured cells increased nearly 2.5-fold the uptake of both healthy donors' LDL and patients' LDL. Anti-LDL also increased the lipoprotein degradation, although to a lesser degree (Table 6).

We have investigated the effect of certain components of circulating immune complexes on the accumulation of cellular cholesterol. The C1q component of complement, added together with anti-LDL, caused a nearly threefold LDL-mediated increase in intracellular cholesterol, whereas anti-LDL in the absence of C1q caused a less than twofold increase (Table 7). An even greater rise of cholesterol level was observed when the culture containing LDL was supplemented with fibronectin in addition to anti-LDL and C1q. It should be pointed out that neither fibronectin nor C1q in the absence of anti-LDL stimulated the accumulation of cellular cholesterol (Tables 7 and 8). Combined addition of anti-LDL, C1q, and fibronectin to culture increased the uptake and degradation of LDL (Table 8). Addition of C1q and fibronectin to cell culture in the absence of anti-LDL had no effect on lipoprotein metabolism (Table 8).

Discussion

From the blood of CHD patients, we have isolated IgGs having affinity to the protein moiety of the LDL particle. The content of such IgGs in the blood of healthy donors was much lower than in the patients' blood. A high specificity of LDL to apo, a high affinity constant to LDL, the ability of $F(ab')_2$ fragments to bind to lipoprotein, and binding of the C1q component of complement by IgG–LDL complexes allows us to conclude that the isolated IgGs are autoantibodies against LDL.

We are not the first investigators to indicate the presence of antibodies against LDL in human blood. Earlier, the antibodies against lipoproteins or LDL-binding factors were found in the patients suffering from different diseases as well as in healthy sub-

Table 4. Effect of Anti–Low Density Lipoprotein Immunoglobulin G on Cellular Cholesterol Accumulation Induced by Low Density Lipoprotein

Addition	Cellular cholesterol (μg/mg cell protein)	
	Healthy donors	Patients
Control (10% LDS)	62±4	
LDL, 100 μg/ml	67±4	119±13
LDL+anti-LDL		
2 μg/ml	61±5	138±18
5 μg/ml	68±6	142±14
10 μg/ml	86±9	187±18†
25 μg/ml	129±14†	249±18†
50 μg/ml	150±14†	306±29†
100 μg/ml	143±14†	290±24†
LDL+residual IgG, 100 μg/ml*	63±5	133±10

LDS, lipoprotein-deficient serum; LDL, low density lipoprotein; IgG, immunoglobulin G.
LDL of healthy donors was used. Other details are the same as in Table 3.
*Residual IgGs are immunoglobulins of patients that do not interact with LDL (not adsorbed on LDL–Sepharose).
†Significant difference from LDL ($p<0.05$).

TABLE 5. Lipid Content of Cells Cultured in the Presence of Low Density Lipoprotein and Anti–Low Density Lipoprotein

Addition	Lipid content (μg/mg cell protein)			
	Phospholipids	Triglycerides	Free cholesterol	Cholesteryl esters
Control (10% LDS)	115±12	15±1	20±2	17±1
HD LDL	122±9	14±1	22±2	18±2
Pt LDL	117±8	24±2*	27±2*	38±4*
HD LDL+anti-LDL	123±10	27±3*	42±3*	42±4*
Pt LDL+anti-LDL	127±14	32±3*	45±4*	61±5*

LDS, lipoprotein-deficient serum; HD, healthy donors; LDL, low density lipoprotein; Pt, patients. LDL, 100 μg/ml; anti-LDL, 50 μg/ml. Other details are the same as in Table 3.
*Significant difference from control ($p<0.05$).

jects.[38–43] In 1965, Beaumont[44] described a situation in which hyperlipidemia, xanthomatosis, and atherosclerosis were apparently associated with anti-β-lipoprotein antibodies. Subsequently, the presence of antibodies against LDL in the blood of patients with coronary atherosclerosis and its clinical manifestations was confirmed by other studies.[39–43] It was demonstrated that Igs are major LDL binding proteins in human plasma.[45]

The antibodies isolated in this study had a higher affinity to the LDL of CHD patients that to the LDL of healthy donors. This fact corroborates our recent finding that patients' LDL differs from native LDL of healthy donors. Recently, we[7,8] have shown that LDL obtained from the plasma of atherosclerotic patients and causing the accumulation of cellular lipids has a much lower content of sialic acid as compared with native LDL of healthy donors and that desialylation of native lipoprotein leads to the accumulation of cellular lipids. This fact well agrees with the finding that in this study, the antibodies isolated from the blood of patients had the highest affinity to LDL desialylated with neuraminidase. Apparently, in atherosclerotic patients autoantibodies against LDL are produced in response to the emergence of desialylated lipoproteins in the bloodstream. The detection of antibodies with a high affinity to desialylated LDL in patients' blood serum is a strong argument in favor of the presence of desialylated LDL in patients' blood.

It cannot be ruled out that not only desialylation but other types of LDL modification might be responsible for the formation of autoantibodies against LDL. Immunization of animals with homologous LDL modified in different ways leads to the emergence of specific antibodies to methylated, ethylated, acetylated, carbamylated, and glycosylated lipoproteins.[46–48] Autoantibodies against homologous LDL modified by MDA were found in the blood of animals with experimentally induced atherosclerosis and in the blood of atherosclerotic patients, while antibodies against glycosylated LDL were detected in the blood of patients suffering from diabetes mellitus.[49–51] In the present study, we have found that anti-LDLs isolated from the blood of atherosclerotic patients have a higher affinity to MDA-LDL than to native LDL. It should also be pointed out that the antibodies isolated in this study represent a mixture of several IgG subfractions with variable charge. One may assume that the antibodies with a different charge and possibly other variable characteristics have a different affinity to different sites of both native and modified lipoproteins. It is also possible that the amount and the ratio of certain antibody subfractions vary in different CHD patients.

Igs and other components of immune complexes are accumulated in the vessel wall, and this deposition is most prominent at the site of an atherosclerotic plaque.[52–57] Certain evidence indicates that Igs

TABLE 6. Effect of Anti–Low Density Lipoprotein on ^{125}I-Low Density Lipoprotein Uptake and Degradation

Addition	Uptake (ng/mg cell protein/6 hr)	Degradation (ng/mg cell protein/6 hr)
HD LDL, 100 μg/ml	670±52	2,486±170
HD LDL+anti-LDL, 50 μg/ml	1,654±184*	3,458±308*
Pt LDL, 100 μg/ml	1,222±104*	1,814±188*
Pt LDL+anti-LDL, 50 μg/ml	2,896±272*†	3,912±270*†

Data of one of seven separate experiments are presented. Uptake includes both surface-bound and internalized LDL.
HD, healthy donors; LDL, low density lipoprotein; Pt, patients.
*Significant difference from HD LDL without anti-LDL ($p<0.05$).
†Significant difference from Pt LDL without anti-LDL ($p<0.05$). Other details are the same as in Table 3.

TABLE 7. Effect of Immune Complexes on Cellular Cholesterol Accumulation Induced by Low Density Lipoprotein

Addition	Cholesterol content (μg/mg cell protein)
Control (10% LDS)	38±3
LDL, 100 μg/ml	41±3
LDL+anti-LDL, 50 μg/ml	68±5*
LDL+C1q, 100 μg/ml	48±5
LDL+Fn, 100 μg/ml	42±4
LDL+anti-LDL+C1q	105±9*
LDL+anti-LDL+C1q+Fn	169±13*

LDS, lipoprotein-deficient serum; LDL, low density lipoprotein; C1q, complement component; Fn, fibronectin.
LDL of healthy donors was used. Other details are the same as in Table 3.
*Significant difference from control ($p<0.05$).

TABLE 8. Effect of Immune Complexes on ^{125}I-Low Density Lipoprotein Metabolism and Cellular Cholesterol Accumulation

	Uptake (ng/mg cell protein/6 hr)	Degradation (ng/mg cell protein/6 hr)	Cholesterol content (% of control)
LDL, 25 μg/ml (control)	242±18	839±75	98±8
LDL+anti-LDL, 25 μg/ml	428±29*	1,248±III*	137±9*
LDL+C1q, 50 μg/ml	296±34	796±69	105±9
LDL+Fn, 100 μg/ml	229±36	805±93	103±10
LDL+anti-LDL+C1q	617±39*	1,448±154*	176±12*
LDL+anti-LDL+C1q+Fn	629±62*	2,448±305*	253±14*

LDL, low density lipoprotein; C1q, complement component; Fn, fibronectin.
Details are the same as in Table 7.
*Significant difference from control ($p<0.05$).

and other immune proteins in atherosclerotic plaques and lipid-laden foam cells are localized together with lipoprotein apos.[55–58] This allows us to assume that the immune complexes containing LDL are either transported from the bloodstream into the vessel wall or are formed in the intima from the Igs and lipoproteins that have penetrated there separately. What is a possible role of immune complexes containing LDL, once they are in the vessel wall?

Antibodies against LDL can alter the metabolism of lipoproteins; LDLs that have penetrated the cell within an immune complex increase the synthesis of cholesteryl esters and cause their accumulation inside the cell.[26,43,59] The present study demonstrates that autoantibodies against modified LDL circulating in the blood of atherosclerotic patients substantially increase the ability of this lipoprotein to induce the deposition of intracellular fat. In addition, by interacting with initially native LDL of healthy donors, these antibodies make LDLs capable of inducing the accumulation of intracellular cholesterol. We assume that immune complexes containing LDL penetrate into intimal cells past specific receptors for LDL. Recently, we[8] have shown that the aggregation of modified LDL occurring under cell culture conditions is a necessary prerequisite for the accumulation of intracellular lipids caused by modified LDL. Furthermore, according to our data, LDL insolubilization brought about by the formation of an insoluble LDL associate with different agents, including immune complexes, leads to the accumulation of intracellular lipids.[26] We assume that the LDL-containing immune complex that stimulates the accumulation of lipids is transported inside the cell via phagocytosis. Earlier, we[25,26] have shown that cultured intimal cells are no less phagocytic than peritoneal macrophages. Within the immune complex, LDLs are taken up and degraded much more effectively than free lipoprotein. Capacity of the LDL–IgG complex to cause intracellular lipid accumulation is considerably increased after addition of other components of circulating immune complexes, that is, C1q and fibronectin, which are present in the plasma and in the vessel.[54–57, 60]

The relation between immunological mechanisms and atherosclerosis has been attracting the attention of many researchers for a long time.[38,61–64] Among various aspects of this relation, a special role in the pathogenesis of atherosclerosis was attributed to antibodies against LDL, giving rise to the autoimmune hypothesis of atherogenesis.[38,62–64] The purpose of this study was not to confirm or to refute the existing hypotheses, although our data suggest that antibodies against LDL might play the role of a major factor of blood plasma responsible for foam cell formation. Our results give reasons to assume that the primary factor present in the blood plasma of atherosclerotic patients is modified LDL (desialylated or, possibly, glycosylated or subjected to other types of modification). The second plasma factor is represented by autoantibodies produced in response to the emergence of modified LDL.

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KEY WORDS • antibodies • atherosclerosis • cell cultures • coronary arteriosclerosis • desialylation • foam cell formation • human aorta • immunoglobulin G • lipoprotein modification • low density lipoproteins

Autoantibodies against modified low density lipoprotein. Nonlipid factor of blood plasma that stimulates foam cell formation.

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