

Detection and Quantification of Lipoprotein(a) in the Arterial Wall of 107 Coronary Bypass Patients

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The aim of this study was to determine the extent of accumulation of lipoprotein(a) [Lp(a)] in human arterial wall and to define its potential role in atherogenesis. Biopsies routinely taken from the ascending aorta of 107 patients undergoing aortocoronary bypass surgery were analyzed for lipid and lipoprotein parameters, which were then correlated to serum values. A significant positive correlation was established between serum Lp(a) and arterial wall apolipoprotein (apo)(a) by enzyme-linked immunosorbent assay. High serum Lp(a) also led to a significant increase of apo B in the arterial wall. No significant correlation was found between apo B in serum and aortic tissue. Apo B was found to be partially linked to apo(a) in the aortic extract. Furthermore, apo(a) was found to be intact, as determined by its molecular weight in sodium dodecyl sulfate electrophoresis. This technique also revealed that the apo(a) isoform pattern of aortic homogenate was comparable to the individual serum pattern. Immunohistochemical methods demonstrated a striking colocalization of apo(a) and apo B in the arterial wall, predominantly located extracellularly. Both proteins were increased in atherosclerotic plaques. With density gradient ultracentrifugation, Lp(a)-like particles could be isolated from plaque tissue. This initial study showed that Lp(a) accumulates in the arterial wall, partly in the form of lipoprotein-like particles, therefore contributing to plaque formation and coronary heart disease. (Arteriosclerosis 9:579–592, September/October 1989)

Lipoprotein(a) [Lp(a)] is a lipoprotein similar to low density lipoprotein (LDL) in its lipid composition and the presence of apoprotein (apo) B-100. In contrast to LDL, Lp(a) contains an additional glycoprotein, designated (a), which is linked to apo B by disulfide bridges. The diameter of the particle is 250 Å, and it floats in a density range of 1.05 to 1.12 g/ml. The Lp(a) particle contains a high amount of neuraminic acid due to the highly glycosylated (a),^{1–4} and only a few areas of helical structure could be demonstrated in the glycoprotein(a).⁵ In spite of this low lipid-binding capacity, glycoprotein(a) is part of a lipoprotein and, therefore, it was generally agreed that it should be called apo(a).

Apo(a) is a high molecular weight protein with an apparent molecular weight of more than 500 kD in sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis (SDS-PAGE).⁴ A genetically determined heterogeneity in the form of several bands in SDS-PAGE has been described.^{6,7} The apparent molecular weight difference cannot be explained by the sialic acid moiety.⁶ In fresh human serum, 95% of apo(a) is lipoprotein associated.⁸

Recently, a striking homology between the human apo(a) and plasminogen was demonstrated in both amino

acid^{9,10} and DNA sequences.¹¹ Serum and liver samples from various species were analyzed for the presence of Lp(a), but only humans, primates,¹² and hedgehogs¹³ were found to express apo(a).

Lp(a) was first demonstrated in human plasma by Blumberg et al.¹⁴ and later by Berg and his associates.¹⁵ These and later studies provided evidence that Lp(a) is a qualitative and quantitative genetic trait.^{16,17,18} Around 70% of the normal population have serum Lp(a) levels below 25 mg/dl.¹⁹ Utermann et al.⁶ have postulated that there is a highly significant association between Lp(a) concentration in serum and the different apo(a) phenotypes in SDS-PAGE.

In a series of epidemiological studies, a positive correlation of high serum Lp(a) levels with coronary heart disease (CHD) has been demonstrated.^{20–23} In immunohistochemical studies, Walton et al.²⁴ detected apo(a) in the arterial wall; however, Lp(a) was not considered to participate in atherogenesis. There are several other studies, the first as early as 1958,²⁵ which extensively analyzed human arterial wall tissue for its lipoprotein content. None of these studies included Lp(a), and they mainly concentrated on LDL.^{26–32} Smith and Slater²⁶ observed a relationship between serum lipid levels and LDL in the aortic intima of 21 post-mortem samples, and "mobile and immobilized LDL" were described in atherosclerotic lesions by Smith et al.²⁷ To relate the apolipoprotein accumulation in the arterial wall to the development of arteriosclerosis, Hoff et al.²⁸ examined normal intima and plaques and quantified apo B in human arterial fatty streaks. The most recent work, published by a

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Finnish group,³³ demonstrated apo B- and apo E-containing lipoproteins in lesion-free human aortic intima.

In studies on the possible interaction between LDL and arterial wall, it has been demonstrated that the binding of Lp(a) to glycosaminoglycans is stronger than the binding of LDL.³⁴ Similar to modified LDL, dextran sulfate-modified Lp(a) caused an increase of cholesterol ester accumulation in macrophages.³⁵

The aim of this study was to investigate a possible accumulation of apo(a) in the arterial wall depending on serum Lp(a) concentrations and to compare these data to the relation between serum and arterial wall apo B. We did that by quantifying apo(a), apo B, and lipids in fresh arterial wall tissue. With these experiments, we wanted to determine whether Lp(a) is an independent risk factor for CHD.

Methods

Patients, Serum, and Tissue Samples

Preoperative fasting serum was collected from 306 patients (250 men, 56 women; mean age, 57 years) who were undergoing aortocoronary bypass surgery in the Department of Cardiovascular Surgery, Hamburg University Clinic. The blood was drawn upon admission to the hospital 48 hours before the operation. Of the patients, 20% were taking lipid-lowering medicines, but only 3% had reached normal lipid values with treatment. The control group was 72 factory workers from a local pharmaceutical company, who were fasting and were matched for sex and age.

Tissue samples were obtained from 107 of the coronary bypass patients (mean age, 59 years). We used the biopsies routinely taken during an aortocoronary bypass operation where the vein graft is attached to the ascending aorta. By histological screening, the biopsies showed different grades of intimal thickening compared with control tissue of newborns. No biopsies of severe plaque areas or complicated lesions were examined. Venous samples were taken from the vena saphena magna, which served as the bypass graft. The project was approved by the Physicians' Ethical Commission of Hamburg.

Post-mortem Blood and Tissue

Post-mortem tissue was obtained from autopsy cases within 24 to 48 hours after death. Samples from 11 different individuals were taken from the ascending aorta and the main stem of the left coronary artery and exhibited different degrees of atherosclerotic lesions. For immunohistochemistry, samples were taken from the left descending coronary artery (LAD). To gain a representative picture of the arterial wall, areas with or without plaques were used. Other samples were taken as indicated in the text below. No post-mortem serum was systematically evaluated because of questionable values due to hemolysis and dilution with other body fluids. For the lipoprotein particle study, we used the patients' pre-mortem blood obtained from the Department of Clinical Chemistry. These samples were stored as plasma for not more than 24 hours at room temperature before testing.

Lipoproteins, Lipids, and Apoprotein Determination

Cholesterol was estimated by the use of "Monotest" (CHOD-PAP method) from Boehringer Mannheim. For triglyceride determination, "Peridichrom" (GPO-PAP) from Boehringer Mannheim was used. High density lipoprotein (HDL) cholesterol was quantitated after precipitation of apo B-containing lipoproteins by phosphotungstic acid/Mg (Boehringer Mannheim, Mannheim, FRG).

The density gradient centrifugation of serum was carried out according to the method of Redgrave et al.³⁶ The stepwise gradients were layered as follows: 3 ml of serum adjusted to density $d=1.21$ g/ml with KBr; 3 ml of 0.9% NaCl, pH 7, adjusted to $d=1.063$ g/ml with KBr; 3 ml of the same solution adjusted to $d=1.019$ g/ml; and 1 ml of H₂O. The spin was carried out in a TH-641 Sorvall Dupont (Wilmington, DE) rotor from for 21 hours, 200 000 g (40 000 rpm) at 4°C. After the spin, 0.5-ml fractions were taken from the bottom of the tube (Beckmann fractionation-set up).

The Lp(a) in the 306 bypass patients (Table 1) was measured using radial immunodiffusion (Immuno, Heidelberg, FRG). The Lp(a) standard from Immuno Heidelberg was used for this assay and was adjusted to determine the protein portion of the particle. This means that total protein was measured in the isolated Lp(a) and then diluted in lipoprotein-free serum. The data in this paper, therefore, describe the apo(a) and apo B [apo B-(a)-complex] in the samples. The tissue apo(a) was calculated on the assumption that around 45% of the protein in the Lp(a) was apo B (Ewald Molinari, Immuno GmbH, Vienna, Austria, personal communication).

In the 107 patients with tissue samples, apo B and apo(a) in plasma and tissue homogenates were quantified with enzyme-linked immunosorbent assay (ELISA) (see below). In plasma, both parameters were also determined by radioimmunoassay (RIA) from Pharmacia (Uppsala, Sweden). A standard serum was supplied by Pharmacia to determine the total protein content of the particle, apo(a), and apo B. This additional assay was used to confirm the ELISA values by another commercially available method. The correlations between RIA and ELISA were $r=0.9$ for apo(a) and $r=0.6$ for apo B ($p<0.001$). The internal standards in our laboratory were in a 10% range. All data given in this paper for serum and tissue of the 107 coronary bypass patients were based on the ELISA system.

Antibodies

Production of Polyclonal Anti-apo B

Apo B was prepared from human serum LDL. After density gradient, the LDL fraction was reduced with 10% (vol/vol) mercaptoethanol; then solid SDS was added up to 5%, and the sample was applied to a Sepharose CL 2B column (94×2.6 cm) for apo B purification. Antibodies were raised in rabbits against this purified apo B. The antiserum was purified by an LDL affinity column where apo(a)-free LDL was coupled to CNBr-activated Sepharose. The purified antibodies were conjugated to peroxidase³⁷ and were used in the ELISA.

Polyclonal anti-apo B from Immuno was used for the apo B ELISA. This antibody was checked by immunoblotting for its specificity; it recognized only apo B-100, apo B-48, and the other known fragments of apo B-100. Monoclonal antibodies against apo B were kindly provided by Yves Marcel (Montreal, Canada). They were used only to control the validity of the results obtained with our polyclonal antibodies.

Antibodies against Apo(a)

To establish the ELISA system, monoclonal antibodies, KO 7 and KO 9 (peroxidase conjugated) against apo(a), were kindly provided by Jean-Charles Fruchard (Lille, France).³⁸ For this antibody, we showed by Western blotting and ELISA that the cross-reactivity with plasminogen was less than 5%. Polyclonal anti-apo(a) was produced in our laboratory against the purified apo(a) (see below) in rabbits.

In parallel, we produced monoclonal antibodies against the isolated apo(a). The purification was performed by recentrifugation of the density fraction $d=1.08$ to 1.15 g/ml in a density gradient after reduction with dithiothreitol.³⁹ Two apo(a) isoforms, a_3 and a_5 , were used for immunization (see below). The antigen was injected into Balb C mice.

The mouse lymphocytes were fused with NSO-1 myeloma cells by using PEG 1500 (Boehringer) and the protocol of Köhler and Milstein.⁴⁰ Modifications will be described elsewhere (Beisiegel et al., unpublished observation). The hybridoma supernatants were screened on 96-well plates coated with apo(a) or with plasminogen. Ninety percent of the apo(a)-positive supernatants showed cross-reactivity with plasminogen. The hybrids that were specific for apo(a) were subcloned. In this paper, we used the subclone, 8D3.

All monoclonal and polyclonal antibodies were tested on immunoblots, and all of them recognized the same banding patterns in 20 patients. The banding patterns included seven bands: one faster than apo B, one in the apo B position, and five above the apo B band. We designated them a_1 to a_7 , from low to high molecular weight.

Homogenization of Tissue

The freshly taken biopsies were rinsed in physiological NaCl several times. The adventitia was dissected and discarded. The biopsies were blotted dry and stored in liquid nitrogen until used. The biopsies were cut into small pieces and homogenized in a Potter glass homogenizer for 3 minutes with 25 μ l buffer (10 mM Tris/HCl, pH 8.0, containing 154 mM NaCl, 1 mM EDTA, and 1% Tween 20) per milligram wet weight (WW). The homogenate was then centrifuged at 56 000 rpm for 10 minutes in a Beckmann TL 100 ultracentrifuge with a TLA-100.2 rotor. The pellet was discarded, and the supernatant was analyzed for total Tween-soluble cell protein, total cholesterol, triglycerides, and the Tween-soluble apo(a) and apo B content. The protein was determined according to the method of Lowry et al.⁴¹ by using bovine serum albumin as the standard. Apo B and apo(a) were measured with ELISA as described below. The post-mortem tissue samples were prepared in the same way. For the study of

lipoprotein particles, a slightly different extraction method was applied to the post-mortem arterial wall. The tissue was cut into small pieces and was gently shaken overnight at 4°C in 2 ml/g WW of the buffer described above, but without detergent. After centrifugation, the supernatant was assayed as described above.

ELISA Techniques

Tissue Determinations

For quantification of apo B and apo(a) in the arterial wall homogenates, we used a sandwich ELISA technique, a modification of the ELISA system described by Vu-Dac et al.³⁸ For the apo(a) ELISA, 96-well plates were coated with monoclonal anti-apo(a) (KO7) in a concentration of 25 μ g/ml overnight at room temperature. Washing, blocking, and dilutions were performed in 0.1 M phosphate-buffered saline (PBS), pH 7.4, containing 1% bovine serum albumin (Sigma, fraction V) and 1% Tween 20. After washing and blocking, the homogenized samples were applied in quadruplicate or duplicate. At the end of the incubation (2 hours at 37°C), the homogenates from parallel wells (duplicates and quadruples) were taken out and pooled, and 50 μ l of the pool was transferred onto a second plate coated with anti-apo B (see below). The original plate coated with anti-apo(a) was washed, and then half of each set of quadruples or duplicates was treated with monoclonal anti-apo(a) [KO 9 (peroxidase conjugate) diluted 1:4000]. The other half was treated with our polyclonal anti-apo B (peroxidase conjugated, diluted 1:100) to determine the amount of apo B associated with the apo(a) [apo B-(a) complex]. For the quantification of the apo B, we used the apo B standard on an anti-apo B coat as described for the apo B ELISA. The incubations with the detection antibodies were performed at 37°C for 2 hours. After washing, the KO 9 peroxidase antibody was visualized with 100 μ l of *o*-phenyldiamine in 0.1 M citrate buffer (pH 5.0). The reaction was stopped after 10 minutes by the addition of 100 μ l 1 N HCl. The anti-apo B peroxidase was treated as described below.

To evaluate our assay systems, we used post-mortem tissue. Performing triplicates, the intra- and interassay variances were below 10% for both apoproteins. This was particularly necessary, since in 45 cases, we had only extremely small sample volumes (less than 500 μ l, from which all measurements had to be made), and we were not able to do all ELISA measurements in quadruplicate on the first plate. Therefore, we did not have duplicates for all final apo(a) and apo B measurements of all tissue samples.

The maximum capacity in the apo(a) ELISA was 35 ng/well, and if we stayed in this limit (by using the adequate dilutions), less than 5% of apo(a) could be measured in the transferred homogenates in a subsequent ELISA. The same kinds of transfer assay were performed with serum and lipoprotein fractions and were thereby proven to be dependable. This control was necessary to ensure that only apo B-containing particles were transferred and not residual Lp(a), which might not have been bound in the first incubation.

The apo B ELISA was used to determine the total apo B in fresh homogenates as well as to measure apo B particles that did not contain any apo(a) in the homogenates transferred from the apo(a) ELISA. The plates were coated with polyclonal anti-apo B antibodies (Immuno) in a 1:1000 dilution. The incubations were done under the same conditions as described for the apo(a) ELISA. The apo B was detected by using our peroxidase-conjugated polyclonal anti-apo B antibody. As substrate, 1,2-phenylenediaminedihydrochloride (Boehringer Ingelheim, Garching, FRG) was used, and, after 10 minutes, 8 N H₂SO₄ was added to stop the reaction.

With the combination of these two ELISAs, we could differentiate between the apo B linked to the apo(a) [apo B-(a) complex] and apo B not associated with apo(a). In 32 cases, the sum of these two apo B-containing subfractions was comparable to the total apo B as determined by the same ELISA (Table 3).

Plasma Determinations

For the quantification of apo(a) in the plasma samples, the same ELISAs were used; however, no Tween was added to the buffers. Apo B quantification in plasma included 0.05% Tween 20 in all buffers.

Standards

As a principle, all standards were treated exactly the same way as the samples. For the determination of apo(a) in the tissue samples, we therefore added 1% Tween 20 into the standard, since that was the Tween concentration we used in the homogenates and in all ELISA buffers.

In the apo(a) ELISA, the Lp(a) standard serum supplied by Immuno was used. We compared the Immuno standard with Lp(a) and apo(a) isolated from a serum pool. The isolated samples were examined for their protein content with the method of Lowry et al.⁴¹ The Lp(a) was found to be in good correspondence with the standard with a deviation of less than 10%, and the apo(a) was overestimated by around 50% as expected from using the "apo B-(a)" standard. Thus, for the calculation of the arterial wall apo(a), we might have overestimated the apo(a) content, depending on the ratio of free apo(a) in the tissue.

In the apo B ELISA, the apo B standard from Immuno was used when plasma samples were measured. For the tissue homogenates, we prepared our own pure apo B standard in 1% Tween 20. Apo B was isolated with column chromatography and was delipidated and solubilized in SDS. For the ELISA, apo B was dialyzed extensively against 1% Tween 20 to minimize the residual SDS content and to reach the same Tween content as the tissue samples. The protein content was determined by the method of Lowry et al.⁴¹

SDS-PAGE and Immunoblotting

Serum and tissue homogenates were delipidated in acetone/ethanol 1:1 (vol/vol). Samples were prepared in 0.02 M ethylmorpholine containing 5% SDS, were reduced with 10% mercaptoethanol at 100°C, and were applied to an 8% PAGE with 0.1% cross-linker according to the method of Neville.⁴² Subsequent immunoblotting was performed as

Table 1. Lipid and Lipoprotein Values in Coronary Bypass Patients and Controls

Parameters	Controls (n=72)	CHD patients (n=306)
Mean age (years)	52±5	57±11
Total cholesterol	237±48	256±58*
Triglycerides	130±66	189±125‡
VLDL-C	26±13	38±25†
LDL-C	161±45	175±51
HDL-C	51±15	44±12‡
Lp(a) >25 mg/dl	16%	40%‡
Lp(a) mean	14	25

Concentrations are given in mg/dl and are means±SD.

* $p<0.01$, † $p<0.001$, ‡ $p<0.001$.

CHD=coronary heart disease, VLDL=very low density lipoprotein, LDL=low density lipoprotein, HDL=high density lipoprotein, Lp(a)=lipoprotein(a).

previously described.⁴³ The legends to the figures indicate which antibodies were used for incubation of the blots.

Morphologic Methods

For immunohistochemistry, the tissue was rinsed in PBS and was fixed immediately in 3.7% PBS-buffered formalin. Following standard procedures, the tissue was paraffin-embedded, cut, and mounted on coated glass slides. Sections of autopsy material were histologically stained with hematoxylin-eosin and elastica-van Gieson. Lesions were classified into fatty streak, fibrous plaque, or complicated lesions according to common histological criteria. Immunohistochemical localization of apo B and apo(a) was performed by means of the avidin-biotin-peroxidase (ABC) method.^{44,45} The above-mentioned polyclonal rabbit anti-apo B and rabbit anti-apo(a), which were produced in our laboratory, as well as monoclonal anti-apo(a) antibodies (8D3), were applied to the sections. Controls consisted of replacement of the first antibody with a nonimmune serum of the same species.

Statistical Methods

For statistical evaluation, all parameters with a skewed distribution, such as serum Lp(a), tissue apo(a), and apo B, were transformed logarithmically for correlations. The statistical significance for these values was calculated with the Mann-Whitney *U* test.

Results

The serum lipid parameters from 306 patients who underwent aortocoronary bypass surgery and had angiographically determined CHD were measured and compared with an age-matched control group of normal factory workers (Table 1). The comparison of these groups showed significant differences in serum triglyceride ($p<0.0001$) and HDL cholesterol ($p<0.0001$). Total serum cholesterol was higher in the CHD group, even though it was not highly significant ($p<0.01$). In addition to the common lipoprotein parameters, serum Lp(a) was measured. In Table 1, the percentage of subjects with serum Lp(a) levels greater than 25 mg/dl is shown (16% in

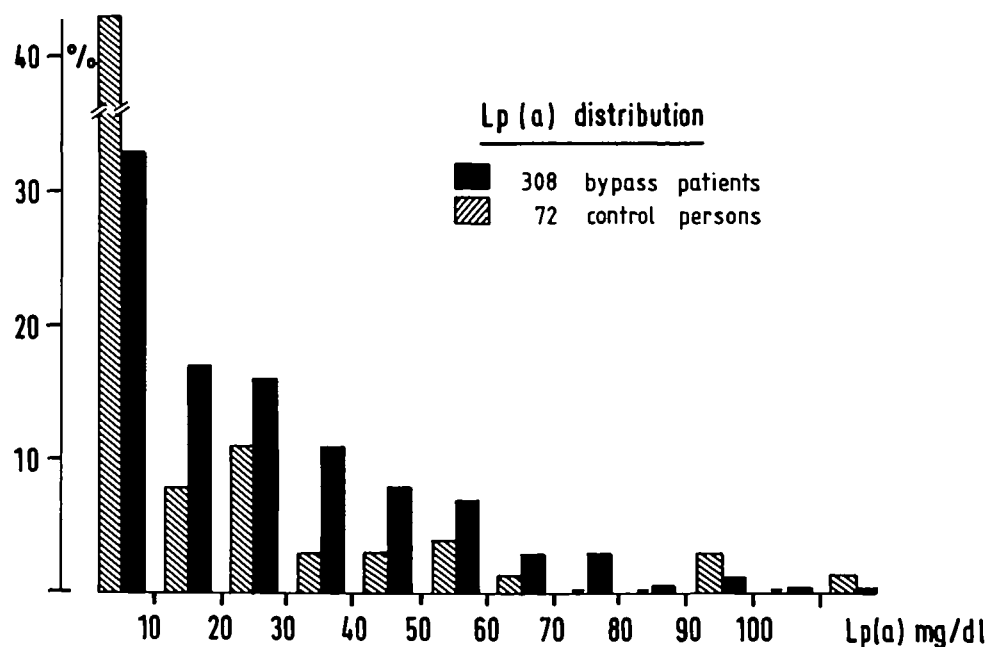


Figure 1. Distribution of lipoprotein(a) [Lp(a)] in 72 controls and 308 bypass patients. Percent of individuals per Lp(a) concentration is given in 10-mg/dl steps.

Table 2. Chemical Composition of Aortic Biopsies of 107 Patients

Parameters	All patients (n=107)	Patients with serum Lp(a) values	
		<25 mg/dl (n=61)	>25 mg/dl (n=46)
Total cholesterol (mg/g WW)	4.0±2.2	3.9±1.8	4.2±2.7
Triglycerides (mg/g WW)	3.3±4.4	2.7±2.7	4.1±5.9
Total protein (mg/g WW)	19.0±6.9	18.5±6.4	19.7±7.4
Apo B not linked to apo(a) (μg/g WW)	40.0±38.9	38.7±39.5	41.6±38.5
Apo B apo(a)-linked (μg/g WW)	27.1±43.7	13.1±22.5	45.7±56.5*
Apo(a) (μg/g WW)	21.8±25.4	13.8±16.5	32.6±30.8†

Values are means±SD.

* $p<0.001$, † $p<0.005$.

Lp(a)=lipoprotein (a), WW=wet weight, apo=apolipoprotein.

controls and 40% in the CHD group, $p<0.0001$). Lp(a) values were expressed as mg/dl apo B-(a) complex measured with immunological techniques. Mean values are also given in Table 1, but note that Lp(a) is not normally distributed, as shown in Figure 1. The difference between the groups is clearly confirmed by the mean values: 14 mg/dl in the controls and 25 mg/dl in the bypass patients.

From 107 of the 306 bypass patients, we obtained arterial wall tissue samples from the ascending aorta at the time of surgery. The tissue samples were used to demonstrate a possible correlation between lipoprotein levels in serum and arterial wall tissue. No biopsies of severe plaque areas were used, although the degree of intimal thickening varied.

Our main interest was to study apo B- and apo(a)-containing particles in the tissue. A special ELISA technique (described under Methods) allowed us to determine the concentrations of apo(a), apo(a)-linked apo B, and apo B not linked to apo(a) in the same sample. The lipoprotein parameters analyzed in the tissue samples are given in Table 2. Note that the standard deviation in Table 2 is very high, reflecting the fact that the lipid and protein content in the biopsy samples varied considerably, due to the degree of intimal thickening.

In patients with high serum Lp(a) levels, the apo(a) content in aortic biopsies were, as well as the apo(a)-linked apo B, significantly higher than in patients with low serum Lp(a). This led to an increase of total apo B in the arterial wall of patients with Lp(a) greater than 25 mg/dl.

Table 3. Chemical Composition of Aortic Biopsies and Samples of Saphenous Veins from 32 Patients

Parameters	Aortic biopsies	Venous samples
Total cholesterol (mg/g WW)	4.3±2.4	1.4±1.2
Triglycerides (mg/g WW)	2.6±1.5	1.9±2.0
Total protein (mg/g WW)	18.6±6.7	17.9±5.6
Apo B not linked to apo(a) (μg/g WW)	7.9±9.4	—
Apo B apo(a)-linked (μg/g WW)	35.0±35.4	—
Total apo B (μg/g WW)	42.0±37.4	23.2±30.2
Apo(a) (μg/g WW)	11.1±8.2	2.4±2.7

Values are means±SD.

WW=wet weight, apo=apolipoprotein.

Table 4. Correlation of Lipoprotein Values between Serum and Arterial Wall Homogenate in 107 Coronary Bypass Patients

Arterial wall homogenate	Serum			
	Total C	TG	Lp(a)	Apo B
Total C	0.1515	0.1186	0.0498	0.1601
TG	0.1026	-0.0772	0.1431	0.1341
Total protein	-0.1018	0.0427	0.0758	0.1549
Apo B not linked	0.0849	0.1482	0.0198	0.1566
Apo B (a)-linked	0.1280	-0.0644	0.4408*	0.0783
Apo(a)	0.0970	-0.0242	0.3759*	-0.0160

* $p < 0.001$.

C=cholesterol, TG=triglyceride, Lp(a)=lipoprotein(a), apo=apolipoprotein.

All other parameters in Table 2 were not significantly influenced by the Lp(a) serum level.

Table 3 compares the lipoprotein content of aortic biopsies to venous samples ($n=32$). The mean values for total apo B and apo(a) in the veins were significantly lower, 55% and 27%, respectively, of the aortic biopsy values. Total cholesterol and triglyceride levels were also much lower in veins (33% and 73% of the aortic values). Table 3 also includes the measurement for apo B linked to apo(a) and not linked to apo(a) (see Methods) for the aortic biopsies. The calculated sum of these two values (42.9 μg/mg WW) is in good agreement with the measured total apo B (42 μg/mg WW). In this subgroup of patients, 83% of the apo B was apo(a) linked. It seems, however, that the amount of apo B-(a) complex varied considerably (see Table 2).

The tissue lipoprotein parameters were correlated to the serum lipoprotein values in Table 4. The results shown in this table are confirmed in Figure 2A, where the correlation ($r=0.556$, $p<0.001$) between serum and arterial wall apo(a) is shown in more detail. In contrast, no significant correlation could be found between serum apo B and arterial wall apo B, as shown in Figure 2B ($r=0.0999$, $p=NS$).

The next question we raised was whether apo(a) could be detected in the arterial wall as an intact protein or whether it might be already partially degraded. In 8% SDS-PAGE and Western blotting, intact apo(a) with its normal high molecular weight was seen (Figure 3). In addition, the majority of immunodetectable apo B was found to be still intact as a 500-kD protein band (data not shown). We demonstrated that the apo(a) isoform pattern in the arterial wall corresponded to the serum pattern (Figures 3, 4, and 5). Moreover, in 10 arterial wall samples, we separated the three main layers of the thoroughly washed arterial wall and showed the following distribution: most of the apo(a) was present in the intima, there were traces in the media, and none was detected in the well-washed adventitia. These data were confirmed in 100 immunohistochemical preparations, where apo(a) and apo B were mainly detected in the intima.

Post-mortem arterial wall samples with different areas of intimal surface covered with atherosclerotic lesions were analyzed to determine the amount of lipids, apo(a), and apo B in relation to the percentage of plaque area (Table 5). We divided the samples into two groups according to the macroscopically visible plaque area (<50% or >50%). Included were 21 arterial wall tissue samples of the aorta and the left coronary artery from 11 patients. While triglycerides and protein did not differ between the two groups, cholesterol, apo(a), and apo B were greater in the group with >50% plaque area.

As in the bypass samples, we wanted to determine whether apo(a) and apo B were still intact as high molecular weight proteins in the post-mortem tissue. Figure 4 shows that most of the apo(a) was intact in its high molecular weight position on 8% SDS-PAGE. Figure 4 also shows that the apo(a) pattern was comparable between serum, aorta, and coronary artery. Moreover, in the post-mortem tissue, we studied the apo(a) content of the ascending aorta at the typical location where the biopsies were taken during bypass surgery, and we compared this to the content of the stem region of the right ascending (RA) and left ascending (LA) coronary artery, as well as to the

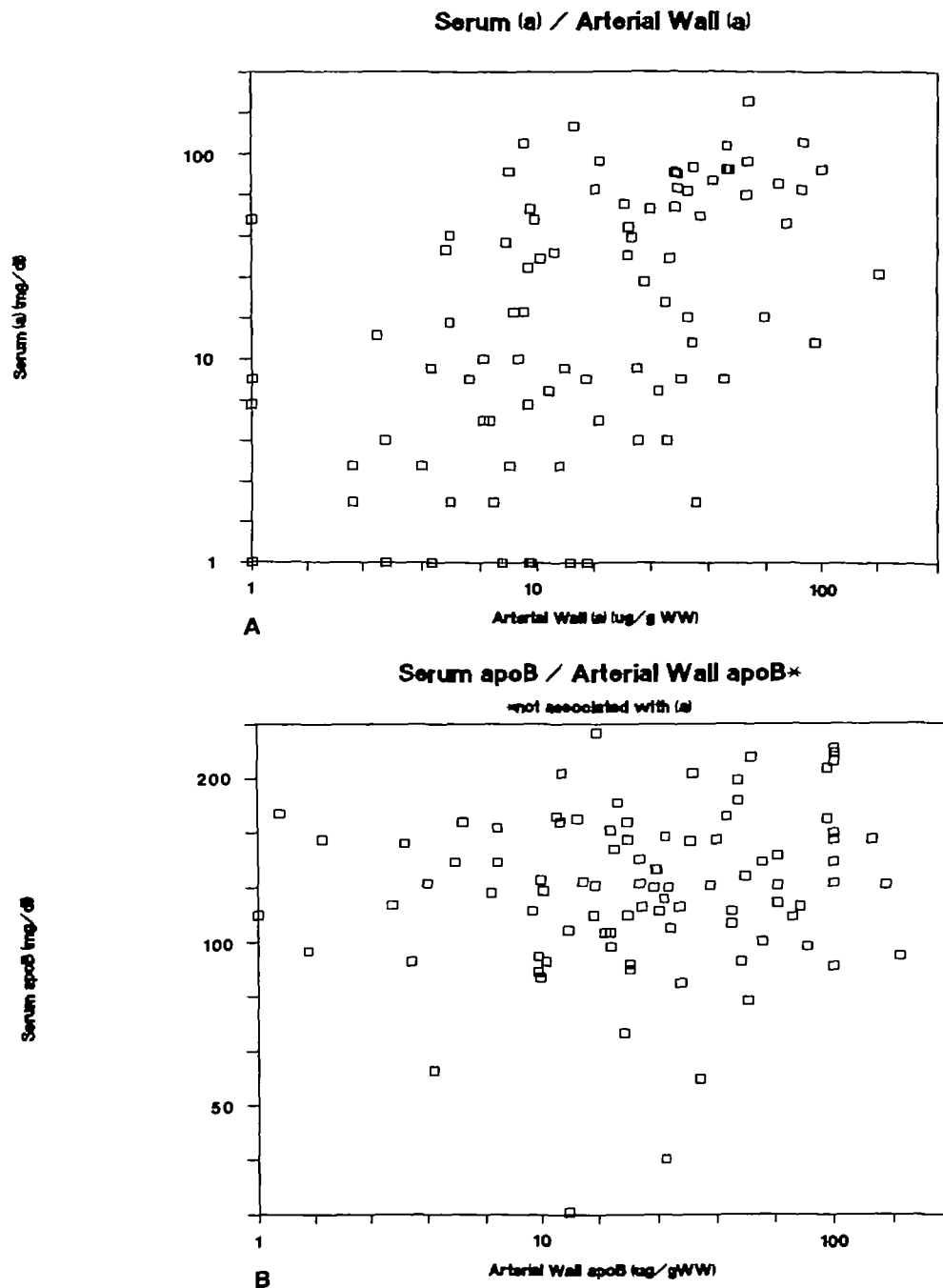


Figure 2. Correlation between serum and arterial wall values. **A.** Serum Lp(a) versus arterial wall apo(a) ($r=0.556$, $p<0.001$). **B.** Serum apo B versus arterial wall apo B, not linked to apo(a) ($r=0.099$, $p=NS$).

branching region of the LAD coronary artery and the circumflex coronary artery (CX). The different arterial wall sections contained comparable amounts of apo(a) and expressed the same isoform pattern (Figure 5). Only the branching regions of the coronary arteries (fatty streak) contained slightly more apo(a). This figure shows again the absence of apo(a) in the media (compare Figure 3).

Immunohistochemistry of the post-mortem arterial wall was performed parallel to the biochemical analysis. Three controls showed a complete lack of any staining (Figure 6A)

while the polyclonal and monoclonal apo(a) antibodies revealed comparable results (data not shown). Apo B and apo(a) were found to be exclusively located in the intima. Atheromatous lesions were significantly more severely affected than regular segments (Figure 6B).

Comparing the distribution of apo B and apo(a), a tight association of both was observed (Figures 6C and 6D). Patterns of apo B and apo(a) deposits were either almost congruent with a slightly stronger staining for apo B, or matched at least in major parts. Apo B and apo(a) were

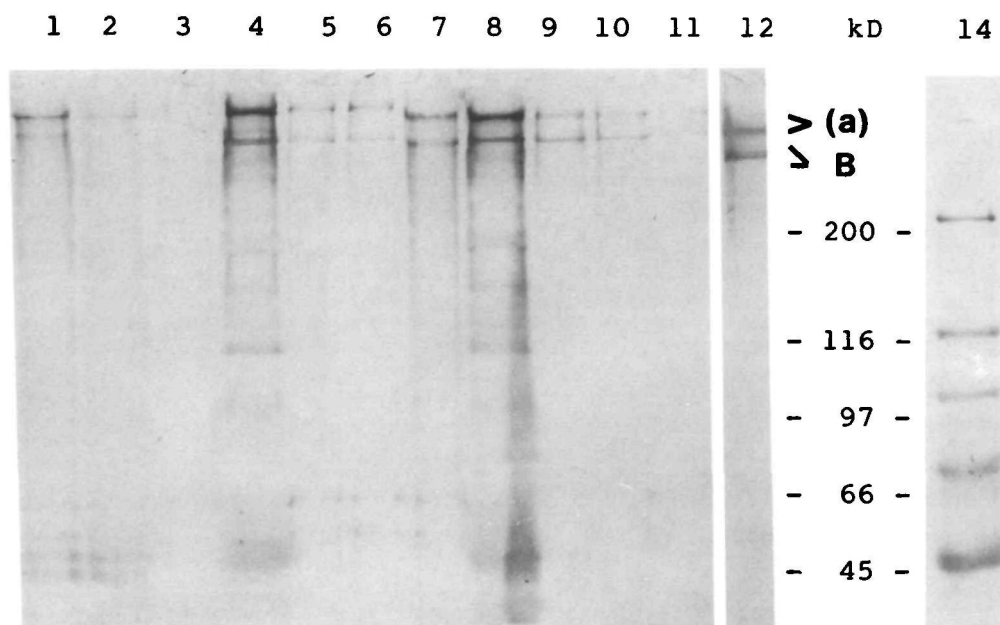


Figure 3. Immunoblotting with polyclonal anti-apo(a) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis from serum and aortic biopsies; 2 μ l of serum or density gradient fractions and arterial wall homogenate from 10 mg (wet weight) were applied. **Lanes 1 to 6.** Male patient, age 76, serum Lp(a) of 37 mg/dl. **Lane 1.** Intima of aortic biopsy. **Lane 2.** Media. **Lane 3.** Adventitia. **Lane 4.** Total serum. **Lane 5.** Lp(a) serum fraction after density gradient (diluted three times compared with lanes 4 and 6). **Lane 6.** Very low density lipoprotein (VLDL) fraction. **Lanes 7 to 12.** Female patient, age 49, serum Lp(a) 56 mg/dl. **Lane 7.** Aortic biopsy (intima, media). **Lane 8.** Total serum. **Lane 9.** Lp(a) serum fraction (diluted). **Lanes 10 and 11.** Intermediate density lipoprotein fractions. **Lane 12.** VLDL fraction. Lanes 1 to 12 were incubated with polyclonal anti-apo(a). Lane 12 was re-incubated with monoclonal anti-apo B (Yves Marcel). **Lane 14.** Amido black-stained molecular weight standards.

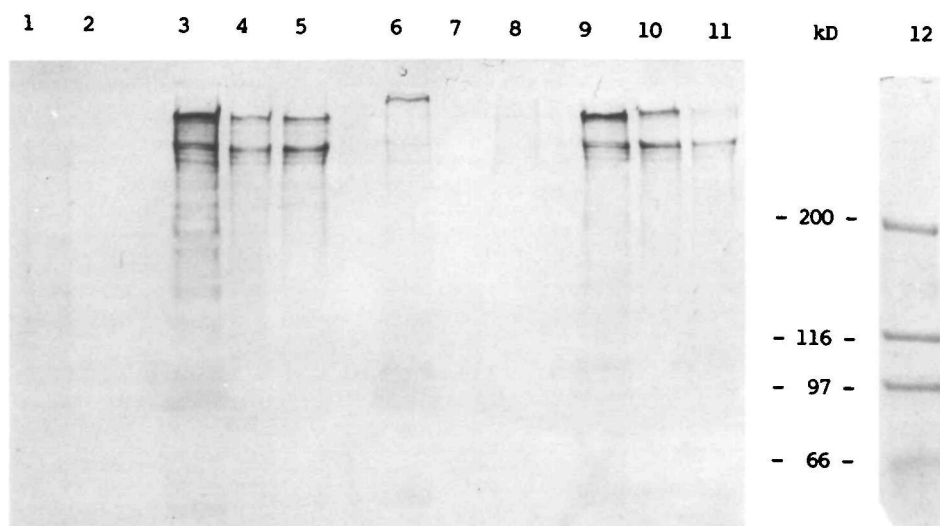


Figure 4. Immunoblotting with monoclonal anti-apo(a) (8D3) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis from post-mortem (pm) serum and from aortic or coronary artery tissue with various degrees of atherosclerotic lesions (grossly normal=gn, fatty streak=fs, complicated lesion=cl). Two microliters of serum and arterial wall homogenate from 10 mg (wet weight) were applied. **Lanes 1 and 2.** Woman, age 37, cause of death: leukemia, pm serum Lp(a)=1 mg/dl. **Lane 1.** Serum. **Lane 2.** Aorta (gn). **Lanes 3 to 5.** Woman, age 38, cause of death: myocardial infarction, pm serum Lp(a)=95 mg/dl. **Lane 3.** Serum. **Lane 4.** Aorta (fs). **Lane 5.** Coronary artery (fs). **Lanes 6 to 8.** Woman, age 39, cause of death: bronchial asthma, pm serum Lp(a)=17 mg/dl. **Lane 6.** Serum. **Lane 7.** Aorta (gn). **Lane 8.** Coronary artery (gn). **Lanes 9 to 11.** Man, age 47, cause of death: cerebral dysregulation, pm serum Lp(a)=50 mg/dl. **Lane 9.** Serum. **Lane 10.** Aorta (cl). **Lane 11.** Coronary artery (cl). **Lane 12.** Amido black-stained molecular weight standard.

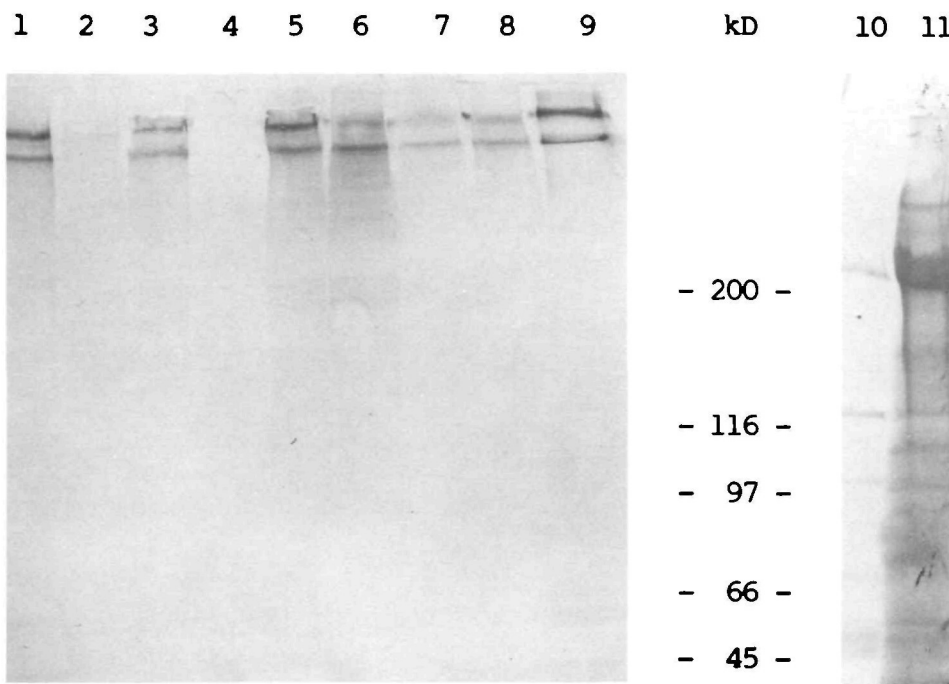


Figure 5. Immunoblotting with monoclonal anti-apo(a) (8D3) after sodium dodecyl sulfate polyacrylamide gel electrophoresis from homogenate of aortic regions (Intimal thickening) and coronary artery samples (fatty streaks) of a 28-year-old man. Cause of death: polytrauma, pm serum Lp(a)=13 mg/dl. Homogenate corresponding to 20 mg wet weight were applied. **Lanes 1 and 2.** Ascending aorta. **Lane 1.** Intima. **Lane 2.** Media. **Lanes 3 and 4.** Abdominal aorta. **Lane 3.** Intima. **Lane 4.** Media. **Lanes 5, 6, and 7.** Coronary arteries (Intima and media). **Lane 5.** Left ascending (stem region). **Lane 6.** Left anterior descending (branching region). **Lane 7.** Circumflex (branching region). **Lane 8.** Right ascending (branching region). **Lane 9.** Serum (2 µl). **Lane 10.** Amido black-stained molecular weight standard. **Lane 11.** Amido black-stained tissue sample.

Table 5. Chemical Composition of Post-mortem Aorta in Relation to Plaque Area

Parameters	Plaque area of total intimal surface	
	<50% (n=16)	>50% (n=5)
Total cholesterol (mg/g WW)	4.0±3.5	5.1±3.6
Triglycerides (mg/g WW)	2.4±1.5	2.6±1.5
Total protein (mg/g WW)	9.8±2.9	10.2±2.3
Total apo B (µg/g WW)	45.2±48.3	61.2±24.4
Apo(a) (µg/g WW)	38.0±17.0	43.8±21.2

Values are means±SD.

n=number of tissue samples.

WW=wet weight, apo=apolipoprotein.

found to be primarily associated with extracellular structures in a bundle-like staining pattern (Figures 6C and 6D). However, in fatty streaks, some foam cells could be identified as bearing the apo B and/or apo(a) intracellularly (Niendorf et al., unpublished observations).

To learn more about the form in which apo B and apo(a) is associated in the arterial wall, we analyzed post-mortem tissue extracted in NaCl without detergent in a KBr density gradient. We measured the total and free cholesterol, triglycerides, phospholipids, apo B, and apo(a) in the extract and in

the different density fractions. Figure 7 shows the distribution of cholesterol, apo B, and apo(a) in the density gradient of aortic extracts from two different samples (see legend). While no apo B was detected in the bottom fraction, 30% to 40% of apo(a) was found in this lipid-free form. There was apo B and apo(a) in the density range of Lp(a) and LDL, where most of the cholesterol was measured. In other experiments, we also demonstrated that the distribution of triglycerides and phospholipids was mainly associated with the LDL and Lp(a) regions (data not shown).

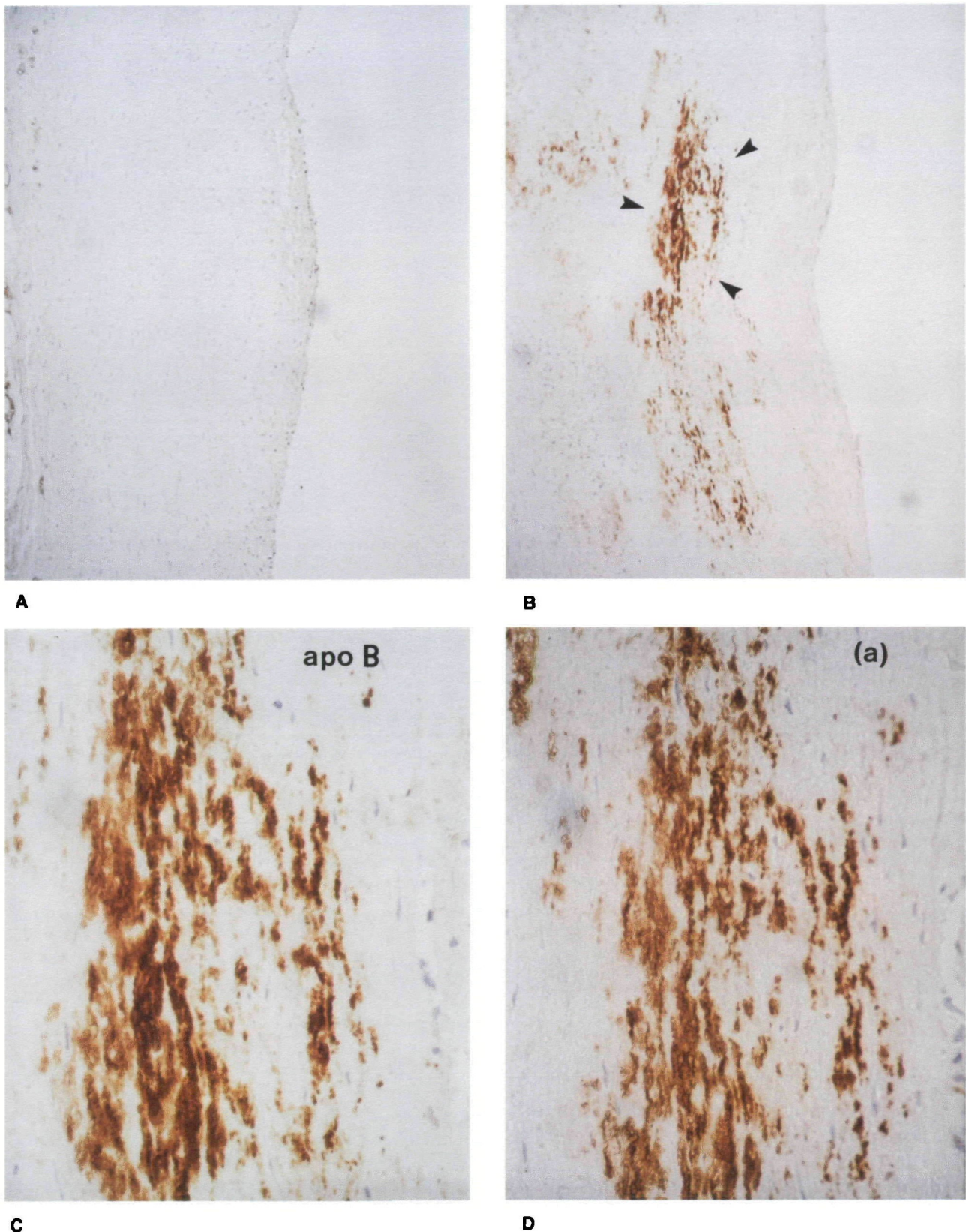


Figure 6. Immunohistochemical demonstration of apolipoprotein (apo) B and apo(a) in the main branch of the left coronary artery. **A.** Coronary artery of a 32-year-old patient without detectable serum Lp(a). No staining for apo(a) (this section) or apo B (data not shown) was observed. $\times 67$ **B.** Overview of the intima of a coronary artery with brown staining for apo B. A part of the fibrous cap and the core region of the complicated lesion are stained. No staining in the media. **C.** Higher magnification of the same section as B, marked there with an arrow. The staining is in a bundle-like pattern and is located mainly extracellularly. $\times 400$ **D.** Staining for apo(a) in a serial section of the same tissue shown in B and C. Compare C and D for a striking congruency in the corresponding areas.

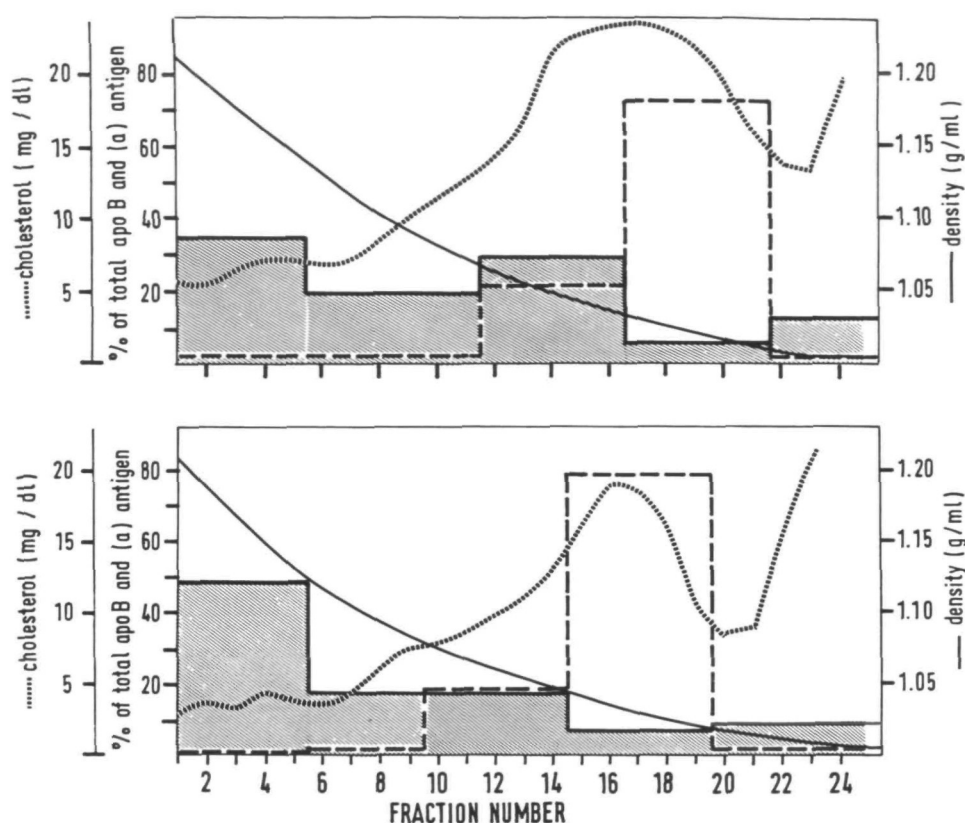


Figure 7. Density gradient centrifugation of post-mortem arterial wall extract from two individuals. **A.** Fatty streak region of a 60-year-old man who died of chronic renal failure. **B.** Aortic plaque area (mainly complicated lesion) of a 70-year-old man who died of cardiac failure. Extraction was accomplished without detergents. A density gradient was run as described in the Methods section. The fractions were analyzed for total cholesterol content and were pooled according to the lipoprotein density range. The total extract and the pooled fractions were analyzed for apolipoprotein (apo)(a) and apo B. The relative amount of apo(a) (shaded area and continuous line) and apo B (area under dashed line) are plotted. **A.** Total apo(a) content, 32 $\mu\text{g/g}$ wet weight (WW); total apo B, 14 $\mu\text{g/g}$ WW. **B.** Total apo(a), 5.5 $\mu\text{g/g}$ WW; apo B, 5.1 $\mu\text{g/g}$ WW. The density of the fraction is also indicated. Note the increased appearance of Lp(a)-like particles in fractions 12 to 16 (**A**).

Discussion

As background for our study, we analyzed lipoproteins in 306 CHD patients who underwent coronary bypass surgery. In this cohort, we confirmed earlier studies on lipoproteins and CHD. The importance of hyperlipidemia as a risk factor for CHD has recently been confirmed in a study of 40 bypass patients who underwent a second operation.⁴⁶ In addition to higher serum and LDL cholesterol levels, this study determined that CHD patients have significantly higher triglyceride values^{47,48,49} in most of the cases associated with lower high density lipoprotein cholesterol.⁵⁰

The most striking difference between normal controls and bypass patients in our study was the serum Lp(a) level ($p < 0.0001$). Forty percent of the patients had Lp(a) levels greater than 25 mg/dl, while only 16% of the controls surpassed this level. This supports the epidemiological evidence from others and our own group that Lp(a) has to be considered as an atherosclerotic particle.⁵¹ However, to date, the pathophysiological background for the atherogenicity of Lp(a) has not been studied exten-

sively. While Walton et al.²⁴ detected apo(a) in the arterial wall, they did not think that it participates in atherogenesis.

The level of apo(a) in the arterial wall tissue samples of 107 bypass patients compared with their serum Lp(a) showed a strong correlation. No significant correlation could be found between serum and tissue cholesterol or serum and tissue apo B. The apo(a)-linked apo B showed a correlation with Lp(a) in serum. These data support the role of Lp(a) as a risk factor for the development of arteriosclerosis independent from LDL.

The described results differ from earlier studies,²⁶ which found a relationship between high serum lipid levels and LDL in aortic intima. Based on our data, we speculate that the lack of correlation between serum and tissue apo B is due to intracellular degradation of LDL in the arterial wall.

If tissue lipoprotein values are analyzed (Table 2) according to the serum Lp(a) of the patients (under or above 25 mg/dl), two striking facts are seen: there is a high correlation between high serum Lp(a) and the concentration of apo(a) in the arterial wall, and there is a considerable amount of apo B linked to apo(a) (40% to 83%) in the aortic biopsies. Thus, high serum Lp(a) levels

can contribute significantly to the deposition of apo B in the arterial wall. These data were confirmed by immunohistochemistry; in post-mortem arterial wall tissue, most of the apo B was co-localized with apo(a). The fact that apo(a), as well as apo B, are immunologically detectable suggests that major parts of Lp(a) accumulate extracellularly instead of being digested in the cells.

To prove the suggestion from the immunohistochemistry that apo B and apo(a) might still be intact proteins, we performed SDS-PAGE. Apo B was found at 513 kD and apo(a), in the molecular weight range around and above apo B; these weights correspond to their normal total molecular weight. Moreover, the isoforms described for apo(a) in serum can be demonstrated in the comparable pattern in the arterial wall. Whether this is a general observation needs to be evaluated. Intact apo(a) was found in the biopsies as well as in the post-mortem samples, a particularly surprising fact.

For the quantification experiments, we compared different detergents for extraction of the tissue samples and found that in 1% Tween 20, the measurable amounts of apo B were comparable to those in 3% Triton (TX100), which was used by Hoff et al.²⁸ Both detergents left some SDS-soluble apo B in the pellet, but Tween buffer was slightly superior to Triton for the detection of apo(a).

Apo B has been measured in several studies with rather different results. The amount varied between 2 and 15 mg/g dry tissue^{29,31} and 5 to 50 $\mu\text{g/g}$ ⁵² or up to 600 $\mu\text{g/g}$ dry tissue.^{53,54} The 2 to 15 mg/g were described for human material, while the values in micrograms per gram were from swine⁵⁴ and monkey studies.^{52,53} We detected apo B in a mean range from 40 to 70 $\mu\text{g/g}$ wet tissue in our detergent-extracted tissue samples with the ELISA technique. Our data actually corresponded much better to the animal studies and did not reach values in the range of milligrams per gram as described by Hoff et al.^{29,31} for human tissue. However, we consider our results more reasonable since it is unlikely that apo B content is of the same order of magnitude as total protein in detergent-solubilized tissue (18 mg/g). We believe that 0.2% for aortic material and 0.12% for venous samples are more realistic for the percentage of apo B from total protein.

No determination of apo(a) in the arterial wall has been published so far. Ylä-Hertuala et al.³³ failed to detect apo(a) in the arterial wall by radial immunodiffusion (RID). On the basis of our results, we conclude that the RID method is not sensitive enough to allow a quantification of apo(a) in arterial wall tissue.

When the apo(a) and apo B content of the aortic biopsies were compared with the venous samples, significantly lower values were found in the venous tissue. Under physiological conditions, there seemed to be no comparable accumulation of lipoproteins in the veins. Hoff et al.,⁵¹ however, showed that serum Lp(a) levels were significantly associated with the degree of stenosis in saphenous vein grafts.

In 10 biopsies, as well as in post-mortem tissue samples, we dissected the different tissue layers and analyzed the intima separate from the media and adventitia. With biochemical and immunohistochemical methods, we showed that apo B and apo(a) are mainly localized in the

intima; there were only traces in the media and nothing was seen in the washed adventitia. Therefore, the lipoproteins must have entered the arterial wall via the endothelium rather than being contaminations from the vasa vasorum. With the post-mortem tissues, we had the chance to study different vessel areas and detected apo(a) with no differences in the individual isoform pattern. We have not yet quantitatively differentiated the various areas.

The next question was whether Lp(a) can be extracted from the tissue as lipoprotein particles. In preliminary studies with density gradient ultracentrifugation, we demonstrated that 50% to 65% of apo(a) was lipid-associated in the density range of 1.05 to 1.1 g/ml, and we found 20% of the apo B in this density fraction. We detected 70% to 80% of apo B in the density range of 1.02 to 1.05 g/ml. No apo B was found in the lipid-free bottom. We propose that these LDL-like particles are at least partly derived from Lp(a) that lost the apo(a) glycoprotein. The dissociated apo(a) was detected in the bottom fraction of the gradient. The stripping of apo(a) from the particle may be due to post-mortem changes in the tissue, because a similar dissociation was observed in serum samples that had been stored at room temperature for 24 hours. This hypothesis would also explain the strict co-localization of apo B and apo(a) in the immunohistochemistry. We were not able to obtain enough biopsy material to measure the free apo(a) content in fresh arterial wall tissue; therefore, we cannot fully verify the apo(a) values, which were calculated on the assumption that most of the apo(a) was in the apo B-(a) complex.

In addition to the already discussed set of data, we describe in this paper some preliminary results obtained by comparing different vessel wall sections in relation to the plaque area. The wall pieces with more than 50% visible plaque showed considerably higher values for apo(a), apo B, and cholesterol as compared with the samples with less than 50% plaque area. The protein content and triglycerides were comparable in both tissue samples. Hoff et al.²⁸ found also higher amounts of apo B in normal aorta than plaques using Triton for extraction. The exact definition of plaque grades will play an important role in the final answer to this question as well as the methods of extraction and quantification.

In conclusion, this is the first study showing a positive correlation of Lp(a) serum levels with apo(a) and apo B accumulation in the arterial wall. Also, the presence of intact apo(a) and Lp(a)-like particles in human arterial wall was demonstrated for the first time. We assume that in earlier studies on LDL-like particles in the arterial wall, the apo(a) might have been missed and, at least partly, Lp(a)-like particles might have been isolated. Our studies imply that Lp(a) enters the arterial wall and accumulates extracellularly, where apo(a) and apo B can be co-localized with immunohistochemistry. Further studies are necessary to define the way by which Lp(a) enters the vessel wall.

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Index Terms: lipoprotein(a) • arterial wall • coronary heart disease • apo B • arteriosclerosis

Arteriosclerosis, Thrombosis, and Vascular Biology



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