Apolipoprotein B Retention in the Grossly Normal and Atherosclerotic Human Aorta

HENRY F. HOFF, CAROL L. HEIDEMAN, ANTONIO M. GOTTU, JR., AND JOHN W. GAUBATZ

SUMMARY Apolipoprotein B (apoB) was measured in buffer-extracted homogenates of grossly normal and atherosclerotic human aortic intima by means of an electroimmunoassay procedure. The apoB values which were expressed as μg per mg tissue dry weight, varied widely, ranging from 0.34 to 18.45 in normal intima and from 0.8 to 12.5 in fatty fibrous plaques. No consistent differences in apoB content were found between normal intima from thoracic and abdominal aortic regions. There was a statistically significant positive correlation between the quantity of buffer-extractable apoB in normal regions and the plasma cholesterol and triglyceride concentration. Buffer-extractable apoB values were significantly higher in fatty fibrous plaques than in ulcerated lesions from the same vessel. However, fatty fibrous plaque apoB values were significantly lower than those from grossly normal regions from the same aorta, although the topographical distribution of apoB was more widespread in plaques than in normal regions, as shown by immunofluorescence studies. This apparent discrepancy reflected the incomplete extraction of apoB from plaques as contrasted to normal regions. The relatively loosely bound apoB, extractable by standard buffers, may represent intact low density lipoprotein (LDL) and/or very low density lipoprotein (VLDL), while the tightly bound fraction may represent insoluble complexes of intact lipoproteins within the plaque or delipidated apoB.

THE ENTRY and retention of plasma lipoproteins in the arterial wall is believed to be a significant factor in the pathophysiology of atherosclerosis. Many studies have shown a relationship between elevations of plasma lipids and lipoproteins and coronary heart disease. Cholesterol feeding studies in experimental animals have also added support to the sterol theory of atherogenesis. We have in the past used the immunological properties of the apolipoproteins in the different lipoprotein fractions to localize them in human arteries with atherosclerotic involvement. Recently we have employed an electroimmunoassay directed against apoB to quantify the amounts of low density lipoprotein (LDL), intermediate density lipoprotein (IDL), and very low density lipoprotein (VLDL) retained in the intimal lining of grossly normal human aortas. This report represents a continuation of the past study and documents the buffer-extractable apolipoprotein B (apoB) content of both grossly normal and atherosclerotic regions of the human aortic intima. Our aim has been to correlate the arterial apoB content with the pathology of the arterial wall and the plasma lipoprotein profile as estimated by concentrations of cholesterol and triglyceride.

Methods

PREPARATION OF ANTI-apoB

Plasma from normolipemic donors was subjected to sequential ultracentrifugation in KBr to isolate the LDL.

Support by the National Heart and Blood Vessel Research and Demonstration Center, Baylor College of Medicine (National Institutes of Health Grant HL 17259), National Institutes of Health Grant NS 09287, and a Grant from the Texas Affiliate, American Heart Association.

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Received December 29, 1976; accepted for publication June 17, 1977.

Electrophoresis was carried out at 4°C for 18 hours. Lipid-free LDL was prepared by delipidation at 4°C with diethylether-ethanol (3:1). The apoB was purified by fractionation of lipid-free LDL on Sephadex G-150. Goats were immunized by injecting apoB solubilized in 1 mm sodium decyl sulfate as described earlier. On Ouchterlony plates, the globulin fraction of antisera gave only single lines of complete identity with apoB, LDL, VLDL, and serum, but it did not react with human serum albumin, high density lipoprotein (HDL), its major apoproteins, apoA-I or apoA-II, or the apoC proteins from VLDL.

ELECTROIMMUNOASSAY (EIA)

A more detailed description of the EIA or "rocket" technique employed in this study can be found elsewhere. Briefly, 5-μl samples of supernatant fluids from tissue homogenates and dilutions of an LDL standard, isolated as described above, were dispensed into wells of the antibody gel. Precipitin peaks for each sample were formed after electrophoresis for 5.5 hours at 3.5 V/cm. Antibody dilutions (approximately 1/300) used in the gel were chosen to give peak heights between 0.5 and 4 cm with apoB values ranging between 30 and 300 ng for any 5-μl sample. As demonstrated in an earlier detailed report, apoB in the unknowns first was measured relative to the standard LDL and then was converted to micrograms of apoB per milligram of tissue, dry weight. Dry weights of arterial tissue were determined on duplicate samples of homogenates first by dialyzing to give a salt-free tissue sample and then by lyophilizing and measuring the sample gravimetrically. Sensitivity of the overall assay was 0.2 μg/mg tissue dry weight. The assessment of reproducibility and variability for these procedures has been described previously.
Tissue samples

The descending aorta from the arch to the iliac bifurcation was excised at autopsy within 12 hours after sudden death from 75 subjects between the ages of 12 and 90 years. Thirty-nine subjects were trauma victims, 22 died from ischemic heart disease, 3 from cerebral infarction, and 11 died from such miscellaneous causes as drug overdose, renal failure, or pulmonary embolism. The rules of the Baylor Institutional Review Board for Human Research and the U.S. Department of Health, Education and Welfare for studies on human subjects were observed. Samples of both grossly normal regions and grossly raised atherosclerotic plaques were removed and the intimal lining was stripped from the underlying media as described previously.10 Most of the grossly normal regions were taken from the descending thoracic aorta. Larger surface areas of the thin intima were pooled only from individuals under 25 years of age, since the sensitivity of the extraction procedure and the EIA necessitated obtaining at least 100 mg wet weight of intima. In six cases, samples of both grossly normal thoracic aorta (proximal to the arch) and abdominal aorta (proximal to the iliac bifurcation) were excised from the same vessel. In another set of six cases, but from aortas with confluent atherosclerosis, samples from the same aorta of fatty fibrous and ulcerated lesions were excised. The intimas of each sample were stripped from the underlying media and processed further as described below.

Extraction Procedure

Minces of aortic intima were homogenized in a standard buffer (0.13 μM Tris-HCl, 0.1% EDTA buffer, pH 7.4) using a Polytron homogenizer (Brinkmann Instruments) equipped with a microprobe and operated at full power for 30 seconds at 4°C.10 Following a 30-minute incubation at 4°C, the homogenate was centrifuged to remove all debris and the apoB content of the supernatant fluids was assayed by EIA.

Plasma lipid values

Whenever possible, postmortem blood samples were taken during autopsy from sudden death victims, and all elements were removed by centrifugation. Previous studies had shown that differences between antimortem and postmortem plasma cholesterol determinations are small.15 Total plasma cholesterol and triglyceride concentrations were determined on each plasma sample according to the procedures previously described.16,17

Statistical Methods

Correlation coefficients (r) were calculated for pairs of variables using a Wang model 720 calculator. The statistical significance of each correlation was determined either by t-test for groups under 30 or by z-test for groups over 30.18 The critical P value for significance was set at the 5% level. The least squares regression lines were calculated using apoB as the dependent variable. For paired observations of apoB in different regions of each vessel, the significance level was obtained using differences in the paired t-test.

Microscopy

Slices through the center of each aortic specimen were taken prior to homogenization for light microscopy. One piece was snap-frozen; cryostat sections were cut and were subjected to the immunofluorescence procedure5 to localize apoB. Eriochrome black (Chroma I 14645) was used to reduce tissue autofluorescence.19 Serial sections also were stained with oil red O-hematoxylin to localize neutral lipids and nuclei, respectively. Another segment of aorta was fixed in formalin prior to dehydration and embedding in paraffin. Sections of these blocks were stained with hematoxylin and eosin and Movat's pentachrome stain20 to assess the general morphology of the aortic specimen. Pellets of aortic homogenates as well as aortic minces also were snap-frozen. Cryostat sections were cut from these pellets and were stained to localize apoB by the immunofluorescence procedure.6 A Leitz Orthoplan photomicroscope was used for bright field and fluorescence microscopy. For the latter technique, an HBO 200 mercury light source, a BG-12 primary filter, a K-530 secondary filter, and a dark field condenser were employed.

Results

The following experiments were performed to determine whether a loss in immunological reactivity occurred due to mechanical disruption during homogenization or to incubation with tissue in which hydrolytic enzymes from lysosomes might have been released. Exogenous LDL was added to minces of aortic plaques before homogenization and to minces that already had been homogenized. A loss in reactivity of exogenous apoB would suggest that a breakdown of endogenous apoB might occur during the total homogenization procedure. A sample of LDL was added both before and after homogenization to separate samples of minces of aortic plaque. The apoB content in the supernatant fractions of this set of homogenates then was measured by EIA and compared to the value of apoB in supernatant fractions of homogenates without exogenous LDL. No appreciable loss of apoB from LDL occurred either during homogenization or during the combined homogenization procedure and incubation with tissue (Table 1). When varying amounts of LDL (5-50 μg/ml) were added to identical samples of

<table>
<thead>
<tr>
<th>Sample</th>
<th>LDL added before homogenization</th>
<th>LDL added after homogenization</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL + BSA</td>
<td>0.25</td>
<td>0.28</td>
</tr>
<tr>
<td>Plaque + LDL</td>
<td>0.58 (0.60)</td>
<td>0.63 (0.60)</td>
</tr>
</tbody>
</table>

LDL was added to both samples of minces of atherosclerotic intima, as well as previously homogenized samples, to give a final concentration of exogenous apoB of 54 μg/ml in each sample. Following homogenization for 30 seconds at 4°C, the samples were incubated for 30 minutes at 4°C, then centrifuged for 10 minutes at 5,000 rpm, and 5-μl samples of each supernatant fraction were added in duplicate to the gel of the electroimmunoassay. A sample of LDL (apoB, 54 μg/ml) in 1% bovine serum albumin (BSA) was also homogenized. Five-microliter samples of the homogenate of plaque alone contained 0.33 μg of apoB. The numbers in parenthesis represent theoretical values.
**Figure 1** Light micrographs of the human thoracic aorta. \( L = \) lumen. Magnification, \( \times 100 \).

Part a, Movat's pentachrome stain of a paraffin section of a grossly normal aorta from a 40-year-old man. Note that the tunica intima (I) is thickened. Smooth muscle cells, collagen fibers, and some fragmented elastic fibers can be discerned on the lumen side of the first intact elastic membrane that forms the border between tunica intima and media. \( \times 100 \).

Part b, immunofluorescence staining for apolipoprotein (apoB) in cryostat sections of a grossly normal aorta. Note the positive fluorescence localized diffusely within the tunica intima (I). Elastic fibers of the media display tissue autofluorescence. \( \times 100 \).

Part c, Movat's pentachrome stain of a paraffin section of a fatty fibrous aortic plaque from a 62-year-old male. Note the cellular fibromuscular surface layer above a fibrous layer (f) which in turn lies above a lipid core with cholesterol clefts (arrow). \( \times 50 \).

Part d, immunofluorescence staining of apoB in a cryostat section of a fatty fibrous plaque. Note the widespread positive fluorescence in the lipid core (co) in this section. Some positive fluorescence can also be seen on collagen fibers close to the lumen. \( \times 100 \).

Part e, ulcerated plaque in a human aorta. Note the presence of a thrombus filled with fibrin on the surface of a cellular cap covering a large core filled with extracellular lipid. \( \times 120 \).

Part f, cryostat section of a pellet of a buffer homogenate of a grossly normal aortic intima. After immunofluorescence staining for apoB, positive fluorescence is minimal, found only on isolated collagen fibers. \( \times 120 \).

Part g, cryostat section of a pellet of a buffer homogenate of an aortic fatty fibrous plaque stained by immunofluorescence procedure to localize apoB. Note the widespread localization of apoB, in particular along bands of collagen fibers. \( \times 120 \).
TABLE 2 Comparison of Buffer-Extractable apoB in Intima of Grossly Normal Thoracic and Abdominal Aorta

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Thoracic aorta (µg/mg tissue dry weight)</th>
<th>Abdominal aorta (µg/mg tissue dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>4.08</td>
<td>6.05</td>
</tr>
<tr>
<td>39</td>
<td>3.56</td>
<td>3.42</td>
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<tr>
<td>47</td>
<td>8.57</td>
<td>5.89</td>
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<tr>
<td>50</td>
<td>6.54</td>
<td>8.24</td>
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<td>52</td>
<td>6.13</td>
<td>7.03</td>
</tr>
<tr>
<td>56</td>
<td>9.6</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Mean 6.91 ± 2.71  P > 0.25

Table 2: Comparison of Buffer-Extractable apoB in Intima of Grossly Normal Thoracic and Abdominal Aorta

Buffer-extractable apoB was measured by EIA in grossly normal aortas from 61 subjects. The buffer-extractable apoB content of the normal intimas ranged from 0.34 to 18.45 µg/mg tissue dry weight with a mean of 7.18 ± 3.7 SD. These data are an expansion of the 25 cases reported earlier as preliminary data. The age range of the subjects was 12-80 years. No apoB could be measured in homogenates of unica media at the level of sensitivity of the assay (<0.2 µg/mg tissue dry weight). No consistent differences were found between the intimal apoB content of thoracic and abdominal aortas from the same vessels. The values for six cases between the ages of 17 and 56 years did not differ significantly (P > 0.25) (Table 2).

Strong correlations were found between the quantities of buffer-extractable apoB from normal intima and the concentration of cholesterol (Fig. 2a) and triglyceride (Fig. 3a) in postmortem plasma. When the same comparisons were made between the buffer-extractable apoB from plaques, the correlations with postmortem plasma concentration of cholesterol and triglyceride were weaker (Figs. 2b and 3b).

In 17 of 25 subjects between 37 and 77 years of age, the quantity of buffer-extractable intimal apoB was greater from grossly normal regions than from fatty fibrous plaques (Table 3). The mean value of apoB from normal regions was 7.13 ± 4.38 SD, while from plaques it was 4.72 ± 2.82 SD. This difference was statistically significant (P < 0.001). In contrast to the quantity of buffer-extractable apoB, fluorescence microscopy indicated that apoB was more widely distributed in the intimas from plaques than in the intimas from normal regions (Fig. 1b and d). The situation was clarified by the use of cryostat sections of minces and pellets of homogenates to stain for localization of apoB as viewed with the fluorescence microscope. Pellets of buffer homogenates of grossly normal aortic intima qualitatively showed only negligible specific fluorescence (Fig. 1b). Pellets of buffer homogenates of aortic fatty fibrous plaques, on the contrary, qualitatively demonstrated in-
tense positive fluorescence (Fig. 1g) localized to similar areas in the original plaque (Fig. 1d), to the lipid core, and to bands of collagen fibers. Lipid was also localized to the same regions as apoB.

In six such cases examined, the buffer-extractable apoB content of fatty fibrous plaques was consistently higher than that obtained from ulcerated lesions from the same artery (Table 4). The \( P \) value for the differences was <0.001.

**Table 3** Buffer-Extractable apoB in Intima of Human Aorta

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Grossly normal</th>
<th>Fatty fibrous plaque</th>
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</thead>
<tbody>
<tr>
<td>37</td>
<td>6.27</td>
<td>7.66</td>
</tr>
<tr>
<td>39</td>
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<td>67</td>
<td>6.52</td>
<td>3.97</td>
</tr>
<tr>
<td>70</td>
<td>1.07</td>
<td>1.10</td>
</tr>
<tr>
<td>75</td>
<td>2.66</td>
<td>2.11</td>
</tr>
<tr>
<td>77</td>
<td>2.82</td>
<td>2.04</td>
</tr>
</tbody>
</table>

**Mean**

7.13

**SD**

\( \pm 4.38 \)

\( P < 0.001 \)

**Table 4** Comparison of Buffer-Extractable apoB in Fatty Fibrous Plaques and Ulcerated Lesions

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Fatty fibrous</th>
<th>Ulcerated</th>
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</thead>
<tbody>
<tr>
<td>47</td>
<td>9.13</td>
<td>0.17</td>
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<td>1.61</td>
</tr>
<tr>
<td>58</td>
<td>3.42</td>
<td>2.95</td>
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<tr>
<td>59</td>
<td>3.79</td>
<td>1.69</td>
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<tr>
<td>60</td>
<td>6.82</td>
<td>1.92</td>
</tr>
<tr>
<td>75</td>
<td>2.81</td>
<td>0.41</td>
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**Mean**

5.26

**SD**

\( \pm 2.41 \)

\( P < 0.001 \)

**Discussion**

Several investigators previously have reported quantitation of apoB in the human aorta. One approach used has been first to extract minces of arteries with saline of high or low ionic strength and to follow with differential ultracentrifugation of the extract to isolate the VLDL (d < 1.006) and LDL (d 1.006-1.063) fractions. A number of technical problems are involved in these studies. First of all, extraction of the tissue minces is not a quantitative procedure as we have shown previously. Second, lipoproteins could be lost or degraded in the lengthy incubation and ultracentrifugation steps. Third, there is the possibility that lipid-protein complexes in the density range of VLDL or LDL could be formed during the mincing or extraction procedure.

Another approach to the measurement of lipoprotein in the arterial wall has been to squeeze fluid from the tissue with a specially designed press. The VLDL and LDL in the tissue fluid subsequently are precipitated with heparin-manganese. It is questionable whether this procedure is quantitative because lipoproteins trapped by arterial components might not be removed by this procedure.

A third approach, extensively used by Smith and co-workers and Onitiri et al., is the application of minces of arterial tissue directly to the wells of an EIA.
for apoB. Loosely bound lipoproteins migrate out of the whole tissue under the impetus of an electric field. However, the removal of apoB from whole tissue minces has proved to be less quantitative than the removal from homogenized tissues. We recently found that three times as much apoB can be removed from the intima of normal human aortas by homogenization than the amount recovered from whole minces. The quantity of apoB present in normal intima in our study was approximately twice that obtained by Smith and Slater. Smith et al. recently examined the lack of quantitation obtained with their previous methods. They have employed a further treatment of the mince with several different agents, including plasmin, collagenase, trypsin, and chondroitinase A, B, or C. A further fraction of lipoproteins was removed by these procedures. Hollander obtained similar results using salt extraction and enzymatic digestion followed by differential ultracentrifugation.

Another factor to be taken into account in measuring quantitatively the amount of lipoproteins in the arterial wall is the weight of tissue used for comparisons. For example, if the whole mince is used, then the dry weight determination of the mince following electrophoresis will be lower than the weight of the original mince by the quantity of material extracted by the electrophoresis procedure. The result would be a falsely high value of apoB when the concentration is expressed per unit of dry weight.

Because of this plethora of technical problems confounding the quantitation of lipoproteins, and specifically of apoB, from the arterial wall, we undertook a study to improve the recovery and measurement of apoB, using a combination of homogenization and EIA. In an earlier report with normal tissue, we found no loss of immunological reactivity of apoB during the procedure. In the present study, similar results were obtained for atherosclerotic plaques; that is, no immunologically detectable degradation of apoB occurred during the procedure. In the study with normal intima, the extraction procedure appeared quantitative, since no apoB could be detected in the pellets recovered after homogenization when we employed an immunofluorescence staining procedure for apoB. By contrast, in this paper we report the presence of significant positive fluorescence for apoB in the pellets of atherosclerotic plaques. This finding shows that the extraction of apoB by homogenization in the standard buffer was incomplete and accounts for the otherwise surprising finding that normal intima appears to contain more apoB per unit weight than do adjacent plaques.

The fraction of apoB extracted with a standard buffer from either a region of normal artery or from an atherosclerotic plaque appears to represent lipoprotein that is relatively loosely bound within the intima. Complexes of both VLDL and LDL with glycosaminoglycans (GAG) previously have been isolated from human aortic plaques by saline extraction. Both the present study and a previous one by Smith and Slater found a positive correlation between the concentration of plasma cholesterol and the quantity of apoB in the normal intima. Our report of a positive correlation between the concentration of plasma triglyceride and the quantity of apoB in the normal intima is a new finding. Possibly, both LDL and VLDL are in equilibrium with pools of these lipoproteins in the arterial intima. Whether VLDL can be degraded to LDL within the arterial intima by lipoprotein lipase is not known, although such activity has been found in the atherosclerotic plaque of cholesterol-fed rabbits. The fact that the correlations are much poorer between the concentrations of plasma cholesterol or triglyceride and the buffer-extracted apoB within atherosclerotic plaques could reflect the incomplete extraction of apoB from the lesions by our procedure.

That plaques contain apoB not extractable by standard buffers could be due to the formation of insoluble complexes with lipoprotein or to delipidation of the lipoprotein resulting in the accumulation of water-insoluble apoB. Formation of insoluble complexes of VLDL and LDL with GAG has been demonstrated in vitro. The finding that apoB and neutral lipids are superposed in both atherosclerotic lesions and pellets of buffer-extracted plaque homogenates suggest that not all apoB is in the lipid-free form. Topographically, apoB appears in plaques to be associated in part with the lipid core and in part with bands of collagen and elastic fibers. The removal of additional apoB by treatment of aortic minces with collagenase, chondroitinase ABC, plasmin, or elastase suggests that a portion of the lipoproteins is tightly bound to aortic connective tissue. It has not yet been established how much of the additionally extractable apoB is complexed to aortic tissue or how much represents lipoproteins physically trapped within the homogenate. There are a number of reports in the literature concerning the binding of lipoproteins to arterial elastin, glycoproteins, and collagen. The precise interaction of VLDL and LDL with substances in the lipid core of a plaque remains to be identified. It is of interest that we found that relatively low quantities of apoB could be extracted from ulcerated lesions as compared to fatty fibrous plaques. One interpretation of this finding is that more tightly bound apoB may be bound to the lipid core which generally makes up a larger fraction of the total volume of the ulcerated lesion than of most fatty fibrous plaques.

An investigation of the most efficient and practical way for extracting the remaining quantity of tightly bound apoB from atherosclerotic plaques is under way in our laboratory. In a preliminary study, we were successful in extracting a further portion of apoB with the detergent Triton X-100. The significance of the loosely bound vs. the more tightly associated fractions, or pools of apoB, is currently under study.

Acknowledgments

We acknowledge the help of the staff of the Harris County Medical Examiners who assisted in the procurement of arterial specimens. We further thank Dr. David Scott for evaluating our statistical analyses.

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Apolipoprotein B retention in the grossly normal and atherosclerotic human aorta.
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Circ Res. 1977;41:684-690
doi: 10.1161/01.RES.41.5.684

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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