Quantitation and Localization of Apolipoproteins [a] and B in Coronary Artery Bypass Vein Grafts Resected at Re-operation

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Lp[a] is a lipoprotein whose plasma concentration is highly correlated with cardiovascular disease. Its protein moiety, apoLp[a], consists of two large polypeptides, apo[a] and apo B. The possible contribution of Lp[a] to atherosclerosis in saphenous vein aortocoronary bypass grafts was studied in a population of patients undergoing coronary re-bypass surgery. The vein graft tissue levels of apoLp[a] were compared with graft duration, gross and light microscopic pathology, as well as plasma levels of apoLp[a]. The localization pattern of apo[a] and apo B in vein graft tissue was determined. In addition, the plasma levels of cholesterol, triglycerides, apoproteins (apo) A-I, A-II, and E were measured. In a representative subpopulation of 17 patients with a mean age of 63 years from whom grafts with a mean duration of 112 months were resected, the mean total plasma cholesterol level was 221 mg/dl, the mean high density lipoprotein cholesterol level was 31 mg/dl, and the mean plasma triglyceride level was 228 mg/dl. In normal saphenous veins, the level of apoLp[a] was below measurable limits (<2 ng/mg), and the level of apo B was very low (3.3 ng/mg). In resected grafts, the mean tissue level of apoLp[a] was 32 ng/mg, and that of apo B was 70 ng/mg, demonstrating the net accumulation of these apoproteins in veins from the time of their grafting into the arterial bed. The apoLp[a]/apo B ratio was determined in 77 tissue segments from 59 grafts (28 patients) and was found to be 0.313. This tissue ratio was significantly higher (p=0.02) than the plasma apoLp[a]/apo B ratio from these patients, which was 0.132. Immunostaining showed co-localization of apo[a] and apo B in the neointima of grafts. The most abundant pathologic features observed in resected grafts were proliferated intima (43/52), thrombus (28/52), and atherosclerotic core regions (21/52). The level of tissue apo B correlated well with the abundance of core regions (r=0.501), whereas the level of tissue apoLp[a] did not correlate as well with this feature (r=0.233).

Although apo[a] and apo B are almost absent from normal saphenous vein, these apoproteins (and presumably the lipoproteins Lp[a] and low density lipoprotein) accumulate in bypass vein grafts. The data support the view that these lipoproteins play a significant role in vein graft atherosclerosis.

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Lipoprotein[a] (Lp[a]) is a lipoprotein whose plasma concentration has been highly correlated with cardiovascular disease. Several clinical studies have shown a connection between stenosis resulting from atherosclerosis of native coronary arteries and plasma levels of Lp[a]. This relationship has also been demonstrated for stenosis of carotid and cerebral arteries. A recent study has demonstrated a correlation between serum Lp[a] levels and stenosis of saphenous aortocoronary bypass vein grafts. Although the two major apoproteins of Lp[a], apolipoprotein [a] (apo[a]) and apo B, have been demonstrated by immunochemical localization to accumulate in these vessels, the extent of this accumulation has not been previously determined, nor has the abundance of either apoprotein in vascular tissue been correlated with the corresponding plasma levels of these apoproteins. Saphenous vein bypass grafts resected at re-operation afford an unusual opportunity to study these possible correlations. Normally, it is difficult to obtain sufficient amounts of fresh human arterial tissue to perform meaningful measurements of tissue apoproteins; usually, such measurements are made on autopsy material obtained at varying times after death. This problem is largely circumvented when fresh bypass graft tissue is obtained at the time of re-operation and is analyzed promptly thereafter. An additional advantage accrues from the knowledge of the graft duration, making it possible to assess the effect of particular plasma lipid and lipoprotein concentrations on vessel pathology. In the
The mean age of this group was 61.7 years and the mean duration of their initial grafts was 104.6 months (Table 1).

Patients from whom blood was drawn immediately before re-bypass surgery, and whose analytes could be corrected. Bloods drawn in the operating room frequently had lower lipid and apolipoprotein levels than bloods drawn >12 hours before surgery. For patients with cholesterol data at both times, apolipoprotein values were corrected by multiplying their apoprotein level at surgery by the cholesterol level at >12 hours before surgery and dividing by the cholesterol level at surgery. HDL-high density lipoprotein, ApoLp[a]-the protein moiety of Lp[a], which includes apo[a] and apo B. Apo-apolipoprotein. In this study, we have expressed amounts of Lp[a] in terms of its protein moiety, apoLp[a]. Hence, measurement of total protein mass in an Lp[a] primary standard has been related to its immunoreactivity with anti-apo[a]; whereas the former derives from the amount of apo[a] and apo B in Lp[a], the latter derives only from the amount of apo[a] in Lp[a]. It is not experimentally desirable to use purified apo[a] as the primary standard since its immunoreactivity in that form is much lower than when it is disulfide-linked to apo B in the intact lipoprotein (see reference 10).

present study, we report the tissue and plasma levels of apo[a] and apo B in a population of patients undergoing re-operation, and we demonstrate significant correlations with tissue pathology.

Methods

Patient Population

Seventy-five patients undergoing aortocoronary re-bypass surgery at The Methodist Hospital (Houston, TX) were studied from May 1985 to June 1987. A human research protocol for study of these patients was approved by the Review Boards from The Methodist Hospital and Baylor College of Medicine. Each patient signed a statement giving informed consent to the study. One representative subpopulation (Group A) consisted of 30 patients from whom blood was drawn immediately before re-bypass surgery but from whom we could not obtain sufficient vein graft tissue for quantitation of tissue apoproteins. In our experience, bloods drawn in the operating room frequently have had lower lipid and apolipoprotein levels than bloods drawn >12 hours before surgery. Fortunately, we were able to obtain from the patient files other plasma cholesterol data that allowed correction of apoprotein levels determined on bloods drawn at surgery. The mean age of this group was 61.7 years and the mean duration of their initial grafts was 104.6 months (Table 1).

A second subpopulation (Group B) consisted of 17 patients from whom blood was drawn immediately before surgery and from whom sufficient graft tissue could be obtained for quantitation of apoproteins. The mean age and graft duration of these patients were quite similar to those of patients in Group A (Table 2).

A third subpopulation (Group C) was comprised of patients from whom blood was drawn immediately before re-bypass surgery and from whom vein graft tissue was obtained. This population consisted of 28 patients from whom 59 grafts were resected; of these grafts, 77 tissue segments were analyzed.

Sample Acquisition and Processing

Diseased coronary artery bypass saphenous vein grafts were harvested from patients undergoing re-operation for significant, symptomatic coronary artery disease.11 Segments of normal autologous saphenous veins were harvested from these same patients and used for controls. Tissues were stored at 4°C in specimen cups containing sterile gauze soaked with 50 mM Tris, 150 mM NaCl, pH 7.4 (Tris-buffered saline, TBS), containing the proteolytic inhibitors aprotinin (Trasylo!, Mobay Chemical Corporation, 10 000 kallikrein inactivator units/ml, 1:100 dilution), and phenylmethylsulfonyl fluoride (10% in

Table 1. Characteristics of Group A

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mean±SD</th>
<th>N</th>
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<tbody>
<tr>
<td>Patient age (yrs)</td>
<td>61.7±7.5</td>
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</tr>
<tr>
<td>Graft duration (mo)</td>
<td>104.6±40.7</td>
<td>29</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>217.9±50.6</td>
<td>30</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>216.1±159.5</td>
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</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>31.5±7.0</td>
<td>29</td>
</tr>
<tr>
<td>Apo Lp[a] (µg/ml)</td>
<td>174.3±189.1</td>
<td>30</td>
</tr>
<tr>
<td>Apo B (µg/ml)</td>
<td>1334.9±367.0</td>
<td>30</td>
</tr>
<tr>
<td>Apo A-I (µg/ml)</td>
<td>1403.7±427.1</td>
<td>29</td>
</tr>
<tr>
<td>Apo A-II (µg/ml)</td>
<td>499.9±212.2</td>
<td>29</td>
</tr>
<tr>
<td>Apo E (µg/ml)</td>
<td>54.7±39.1</td>
<td>26</td>
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</tbody>
</table>

Plasma apoLp[a]/plasma apo B

Patients from whom blood was drawn >12 hours before re-bypass surgery and from whom vein graft tissue was obtained. See Table 1 for explanation of abbreviations.

Table 2. Characteristics of Group B

<table>
<thead>
<tr>
<th>Characteristics</th>
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<tbody>
<tr>
<td>Patient age (yrs)</td>
<td>63.1±6.8</td>
<td>17</td>
</tr>
<tr>
<td>Graft duration (mo)</td>
<td>111.6±34.4</td>
<td>17</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>221.0±55.0</td>
<td>17</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>228.0±182.0</td>
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<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>31.0±8.3</td>
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<tr>
<td>Apo A-I (µg/ml)</td>
<td>1370.0±444.8</td>
<td>16</td>
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<tr>
<td>Apo A-II (µg/ml)</td>
<td>455.8±254.6</td>
<td>17</td>
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<tr>
<td>Apo E (µg/ml)</td>
<td>62.2±46.6</td>
<td>13</td>
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<tr>
<td>Plasma apoLp[a] (µg/ml)</td>
<td>222.0±232.5</td>
<td>17</td>
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<tr>
<td>Plasma apo B (µg/ml)</td>
<td>1338.9±410.0</td>
<td>17</td>
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<tr>
<td>Plasma apoLp[a]/plasma apo B</td>
<td>0.156±0.146</td>
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<tr>
<td>Mean tissue apoLp[a] (ng/mg wet wt)</td>
<td>32.3±40.4</td>
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<td>Mean tissue apo B (ng/mg wet wt)</td>
<td>70.0±43.1</td>
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<tr>
<td>Tissue apoLp[a]/tissue apo B</td>
<td>0.396±0.425</td>
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<tr>
<td>Tissue apoLp[a]/tissue apo B</td>
<td>3.33±2.98</td>
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</tbody>
</table>

Patients from whom blood was drawn >12 hours before re-bypass surgery and from whom vein graft tissue was obtained. See Table 1 for explanation of abbreviations.

Table 3. Characteristics of Group C

<table>
<thead>
<tr>
<th>Characteristics</th>
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<tbody>
<tr>
<td>Plasma apoLp[a]/plasma apo B</td>
<td>0.132±0.132</td>
<td>28</td>
</tr>
<tr>
<td>Mean tissue apoLp[a] (ng/mg wet wt)</td>
<td>24.0±33.8</td>
<td>28</td>
</tr>
<tr>
<td>Mean tissue apo B (ng/mg wet wt)</td>
<td>69.1±47.1</td>
<td>28</td>
</tr>
<tr>
<td>Tissue apoLp[a]/tissue apo B</td>
<td>0.313±0.362</td>
<td>28</td>
</tr>
<tr>
<td>Tissue apoLp[a]/tissue apo B</td>
<td>3.55±3.67</td>
<td>28</td>
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</table>

Patients from whom blood was drawn immediately before re-bypass surgery and from whom vein graft tissue was obtained. This population consisted of 28 patients from whom 59 grafts were resected and 77 tissue segments were analyzed. See Table 1 for explanation of abbreviations.
methanol, 1:1000). Tissues were rinsed briefly in several changes of TBS solution with the same concentration of proteolytic inhibitors to remove any loosely adherent blood components. The resected graft pieces were examined in their intact, tubular form; the length and cross-sectional area of the lumen at each end were measured. Probable focal lesion areas were selected by inspection of the cut ends, by tactile hardness, by aneurysmal dilation, or at random intervals if there were no salient external morphologic features. During the latter part of the study, some lesion areas were identified by magnetic resonance imaging of fresh grafts. Two adjacent transverse segments, 2 to 3 mm in length, were cut so that the original lumen orientation was maintained. The first segment was formalin-fixed with hematoxylin-eosin (H&E) and Movat pentachrome stains for light microscopic pathological examination. The second segment was frozen for immunochemo-localization of apo[a] and apo B, and for demonstration of neutral lipids on serial sections stained with oil red O. The remaining adjacent segment from each lesion area was then opened longitudinally and, if necessary, rinsed to remove any loosely adhering blood components not previously accessible. If the 2 to 3 mm transverse segments were observed to be unrepresentative of the gross morphology of the remaining segment, additional segments were cut to achieve a one-to-one correspondence between segments to be used for microscopic pathological examination, for localization of apo[a] and apo B, and for apoLp[a] and apo B quantitation.

The adventitial layer was then stripped from tissues to be used for quantification by electroimmunoassay. After the surface area of the tissue segment was measured, the tissue was blotted to remove excess moisture, was weighed, then finally was minced with a scalpel blade and mixed to make as homogeneous a mixture as possible. Five to 10 mg of tissue was used for quantitation of apo B, and 25 to 40 mg for apoLp[a]. Replicate (two or more) samples were run whenever sufficient tissue was available. Since apoLp[a] and apo B were not electrophoresed from the very same piece of tissue, it is possible that the estimated apoLp[a]/apo B ratios do not exactly reflect the actual ratios in the same tissue piece.

To minimize the magnitude of this difference, we selected vessel regions that were grossly homogeneous and made multiple estimations that were used to calculate the mean of apoLp[a]/apo B ratios.

Autologous, 12-hour fasting blood samples were collected in Vacutainer tubes with ethylenediaminetetraacetic acid (EDTA) either 12 to 18 hours or immediately before surgery. Samples were run whenever sufficient tissue was available. The estimated values must be regarded as "apparent apoLp[a]" since the immunoreactivity of apo[a] in vein graft tissue relative to that of apo[a] in plasma has not yet been determined.

Samples were electrophoresed at 3.5 volts/cm for 18 hours, followed by 4.5 volts/cm for 4 hours. Tissues were then sliced from the gels, and the gels were gently agitated in 150 mM NaCl for 18 hours followed by deionized water for 4 hours. Electrophoresed gels were dried and stained with Coomassie blue R-250 (Bio-Rad, Richmond, CA). The areas under all rockets were measured by triangulation; tissue values of apoLp[a] and apo B were

**Electrophoresis**

Rabbit polyclonal antibodies directed against apo[a] and apo B were purified by immunofinity chromatography of anti-Lp[a] (which contained antibodies against both apoproteins) over low density lipoprotein (LDL) Sepharose. Anti-apo[a] passed through the column unretarded, and anti-apo B was bound until released by a high-pH buffer. Rabbit anti-human apo B antibody was added to 25 ml molten (55°C) 1% agarose (type C, Behring Diagnostics, La Jolla, CA) at a 1:100 dilution. Goat anti-human polyclonal apo[a] antibody was added to agarose (55°C) in a separate flask at a 1:200 dilution. These mixtures were then poured into the openings of separate 18 × 9.5 × 1.5 mm glass sandwich forms and allowed to solidify. Wells were then made in each gel with a 4 mm diameter punch used in conjunction with an electrophoresis template (Bio-Rad, Richmond, CA). Gels were then placed on cooled plates (10°C) of separate electrophoresis chambers (Behring Diagnostics, Sommerville, NJ) containing barbital buffer. Ultrawicks (LKB Instruments, Gaithersburg, MD) were used to complete contact between gels and the buffer chambers. Tissue segments were loaded into the larger wells, and molten agarose was used to fill the residual volume not occupied by the segment. Dilutions of plasma secondary standards containing 150 to 1500 ng apo B and 100 to 1000 ng apoLp[a] were added to the smaller wells. Two secondary standards were calibrated against purified Lp[a] and LDL whose protein content had been determined and whose immunoreactivity was measured by both electroimmunoassay and enzyme-linked immunosorbent assay. The two techniques showed excellent correlation (r = 0.99, p < 0.001). Representative apoLp[a] rockets are shown in Figure 1. The estimated values must be regarded as "apparent apoLp[a]" since the immunoreactivity of apo[a] in vein graft tissue relative to that of apo[a] in plasma has not yet been determined.
Figure 1. Electrommunoassay of apoLp[a]. Appropriate aliquots from a secondary standard of normolipidemic plasma containing 100 to 1000 ng of apoLp[a] was loaded in the left 10 wells of a 1% agarose gel containing anti-apo(a). Segments of minced vein graft tissue were loaded into pre-cut wells on the right side of the gels, and the remaining space was filled with molten agar. After electrophoresis, the gels were dried, stained, and measured as described in Methods.

estimated relative to the plasma standards. These values were then divided by the tissue wet weight to obtain the nanograms of apoprotein/milligrams of tissue.

Immunochemical Localization In Tissue

Flash-frozen segments of the vein grafts were sectioned at 10 μm with a cryostat (American Optical Histostat, Buffalo, NY). The sections were flash-dried onto alcohol-cleaned (1% acetic acid/95% ethanol), pollysine-coated (1 mg pollysine/ml distilled water) slides and allowed to dry. The sections were fixed in acetone, then delipidated in acetone. After delipidation, the sections were rinsed in phosphate-buffered saline (PBS), then pre-incubated in 1% normal nonimmune goat serum to block nonspecific binding of biotinylated goat anti-rabbit IgG. The serum was blotted from the tissue section, which was then treated with affinity-purified anti-apo[a] or anti-apo B. These primary antibodies were diluted in 50 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS) to a final protein concentration of about 4 μg/ml. Tissue sections were incubated in the primary antibody, then rinsed (gentle shaking) in PBS three times. Affinity-purified biotinylated goat anti-rabbit IgG was diluted in PBS to a final concentration of 34 μg/ml, then applied to the tissue sections. Avidin DH and biotinylated glucose oxidase (Vector Laboratories, Burlington, CA) were mixed (1 mol avidin/1 mol biotin) to generate the avidin-biotin-glucose oxidase complex. This complex was then incubated with the tissue sections. Sections were shaker-rinsed in PBS, followed by several washes in 50 mM Tris (pH 8.2). The final color was developed by using a VECTASTAIN substrate kit (Vector Laboratories), which contained glucose, phenazine methosulfate, and nitroblue tetrazolium. The tissue sections were incubated in this disclosing reagent in the dark for 30 minutes or until a definite blue-purple precipitate was observed. The slides were rinsed thoroughly with distilled water to terminate the reaction, then stored in the dark without coverslips until viewed and photographed. The following controls were performed to assess the extent of nonspecific staining: 1) omission of primary antibody, 2) omission of primary and secondary antibodies, 3) incubation with disclosing reagent only, 4) use of an unrelated antibody instead of anti-apo[a] or anti-apo B, and 5) use of an unrelated, biotinylated antibody instead of the goat anti-rabbit IgG antibody. The tissue sections were viewed, and photomicrographs were taken using a Leitz Dialux 22 photomicroscope equipped with a Wild camera assembly (Figure 2).

Morphological Evaluation of Tissue

H&E- and Movat-stained slides were examined by light microscopy to identify the morphologic features present in normal and diseased tissue. The fraction of the original luminal area occupied by various morphologic structures was estimated by visual inspection. The percentages of the original luminal area occupied by residual lumen, proliferated intima, thrombus, atherosclerotic core region, and nonintimal fibrotic material were visually estimated by two trained graders. In addition, the presence or absence of significant pathologic features were noted: fibrous proliferation alone; fibrous proliferation with lipid; and fibrous proliferation with blood elements, foam cells, cholesterol clefts, and calcification. Statistically significant correlations were sought between plasma lipid and apoprotein levels, tissue apoprotein levels, and pathologic features (Figure 3).

Data Analysis

Data storage, reduction, retrieval, statistical analysis, and graphic display were performed on the PROPHET Biomedical Computer System. To determine the significance of differing ratios of apoLp[a] and apo B concentrations in tissue versus plasma, the ratios of ratios were
Figure 2. Immunohistochemical localization of (A) apo[a] and (B) apo B in sections from aortocoronary bypass vein grafts. Vessel lumen is at the top, adventitial surface, at the bottom. Co-localization of the protein antigens within the neointima is evident. An intense band of staining is present approximately 250 μm below the luminal surface. This may represent an early core region, especially evident at left. The location of the original internal elastic lamina is uncertain in this specimen, but the relatively loose tissue structure and immunostaining of the upper third of the vessel wall describe, at least, a functional intimal layer. A double-antibody glucose oxidase technique was used for localization Bar=100 μm. ×75

subjected to arctangent transformation, and a t test was performed against the null hypothesis of a mean ratio of one (arctangent=1.548 radians). A nonparametric test of the same hypothesis was performed by counting ratios >1 and <1. The p value was obtained from the binomial expansion (two-tailed), assuming a 50% likelihood for each ratio <1.

Results

Plasma Lipid and Apolipoprotein Levels

Mean plasma lipid and apoprotein levels for patients (>90% male) in Group A (from whom bloods, but not graft tissue, were obtained) are shown in Table 1. The total cholesterol level (218 mg/dl) was quite similar to that of a normal, age-matched population (220 mg/dl).²¹ In contrast, the HDL cholesterol level (32 mg/dl) was significantly lower than that of a normal population (50 mg/dl). Plasma triglycerides (216 mg/dl) were significantly higher than a normal population matched for age (135 mg/dl). The distribution of plasma apoLp[a] levels is illustrated in Figure 4A. The mean level was 174 μg/ml, roughly equivalent to 57 mg Lp[a]/dl. This is significantly higher than the level (32 mg/dl) reported by Hoff et al.⁶ for a population of 135 patients with bypass graft stenosis. The distribution of plasma apo B levels in Group A is shown in Figure 4B. The mean (1335 μg/ml) was about 1.8 times higher than that of a randomly selected normolipidemic population of comparable age.²² The apo A-I and apo A-Il concentrations in Group A patients (1404 and 500 μg/ml) were about 20% and 50% above those in a normolipidemic group of 50 subjects.²³ Finally, apo E levels in Group A patients (55 μg/ml) were about 50% lower than those of a comparable normolipidemic population.²⁴

Light Microscopic Tissue Pathology

Although vein grafts were removed because of stenosis demonstrated by preoperative angiography, the most diseased segments may or may not have been included in the pathologic specimen depending upon what portion of the diseased vein graft could be dissected by the operating surgeon. Of the 52 graft sections retained in their intact tubular form, 21 were determined by light microscopy to be completely occluded.

Table 4 summarizes the prevalence of features that characterize the overall light microscopic appearance of the 65 graft segments studied. Five of the segments were occluded and markedly atrophic, probably indicating early closure of the graft soon after its placement into the arterial bed. Seven of the graft segments were characterized by fibromuscular (intimal) proliferation alone, without evidence of lipid or blood elements in the sections studied. A large majority (53/65) had intimal proliferation together with lipid and/or thrombus. Lipid was identified by the presence of foam cells or cholesterol clefts in paraffin sections, which correlated well with oil red O staining of frozen sections. Lipid and thrombus were commonly found together in a complex pattern, suggesting either rupture of atherosclerotic plaques with mixing of cholesterol crystals and blood elements, or alternatively, formation of cholesterol crystals within old thrombi or hematomas. Material that could be described macroscopically as atheromatous gruel had the microscopic appearance of blood elements and cholesterol clefts. Figure 3 shows these features.

The mean intima to media (I/M) area ratio was found to be 2.51 (±0.82). The number of graft segments containing various pathologic features and the percentage of the area within the internal elastic lamina occupied by these features is presented in Table 5. Thrombi comprised the largest percentage (34.5%). Thrombi and lipid-rich core regions appeared to be intimately related, since they were seen together in the same area about 50% of the time.
Figure 3. Hematoxylin-eosin–stained sections from vein graft segments of resected coronary artery bypass vein grafts. A. Segment of a bypass graft resected from a 66-year-old man, 11 years after implantation. Intimal thickening is evident around the full circumference of the lumen, which is otherwise highly patent and unaltered. ×36. B. Segment of a vein graft resected from a 64-year-old man, 12 years after implantation. This segment is nonfunctional and highly atrophic. ×57. C. Segment of a vein graft resected from a 67-year-old man, 11 years after initial implantation. The section illustrates intimal thickening and a large lipid-rich core region containing some calcification. ×36. D. Segment of vein graft resected from a 68-year-old man, 8.5 years after original implantation. At least two luminal channels and a prominent pink area containing degenerated red cells and cholesterol clefts are visible. These constitute evidence of old and new thrombus. ×27

Immunochemical Localization of Apoprotein[a] and Apolipoprotein B

Thirty lesions from six different patients were examined immunohistochemically for localization of apo[a] and apo B. These two apoproteins co-localized in each specimen examined, and their patterns of localization were associated with lipid deposition reflected by oil red O staining in the vast majority of specimens. However, in a few sections, there was apoprotein staining without associated lipid staining.

Atherosclerotic plaques exhibited intense apoprotein staining in the core regions, but moderate staining was also exhibited throughout the lesions and in the non-raised adjacent intima (Figure 2). The media stained only sparsely except in cases where adjacent involvement of intima was very intense. In areas where frank lesions were not present, staining was still found in the intimal layer adjacent to the lumen. Areas of new thrombus did not show staining, but old organized thrombus and areas of atherosclerotic gruel did show staining. Apoprotein staining was not seen within foam cells.

Control sections of diseased grafts that had been treated as described in the Methods section showed either no reaction product or very light background staining that was easily distinguished from the more intense staining seen after application of specific immunohistochemical reagents. Fresh, ungrafted saphenous veins showed little or no staining for apo[a] and apo B.

Tissue Apoprotein Levels

The estimated levels of apoLP[a] and apo B distributed in vein graft tissues from population C are summarized in Figure 5. The mean value for apoLP[a] was 24 ng/mg tissue and that for apo B was 69 ng/mg tissue. The level of apoLP[a] in ungrafted saphenous vein was below measurable limits (<2 ng/mg), and the level of apo B was very low (3.5 ng/mg tissue). Based on the arc tangent transformation, the apoLP[a]/apo B ratio was 56% higher in tissue than in plasma for the same 28 patients (Figure 6). A t test on the transformed data revealed that the apoLP[a]/apo B ratio was significantly higher in tissue than...
Table 4. Microscopic Characterization of Stained Paraffin Sections from Vein Graft Segments Adjacent to Those Used for Apol[a] and Apo B Estimation

Overall microscopic characterization N
Totally occluded and atrophic 5
Fibromuscular proliferation alone 7
Fibromuscular proliferation with lipid and/or blood elements 53
Total segments 65

Table 5. Microscopic Characteristics of 52 Graft Segments from 28 Patients

<table>
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<tr>
<th>Pathologic feature</th>
<th>Mean % of area within IEL</th>
<th>N</th>
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<tbody>
<tr>
<td>Proliferated intima</td>
<td>22.0</td>
<td>43/52</td>
</tr>
<tr>
<td>Atherosclerotic core region</td>
<td>12.2</td>
<td>21/52</td>
</tr>
<tr>
<td>Thrombus</td>
<td>34.5</td>
<td>28/52</td>
</tr>
<tr>
<td>Fibrotic material of indeterminant origin</td>
<td>2.0</td>
<td>2/52</td>
</tr>
<tr>
<td>Residual lumen</td>
<td>29.3</td>
<td>31/52</td>
</tr>
</tbody>
</table>

N = Number of graft segments showing this feature. IEL = internal elastic lamina.

Correlations of Tissue Data
Positive correlations were found between the measured plasma levels of apol[a] and apo B, and their respective estimated tissue levels ($r=0.590$, $p=0.001$ for apol[a]; $r=0.540$, $p=0.001$ for apo B) in plasma ($p=0.022$). Nonparametric testing was confirmatory ($p=0.014$).

Figure 4. Distribution of plasma apolipoprotein [a] (A) (apol[a]) and apo B levels (B) among patients undergoing aortocoronary bypass re-operation.

Figure 5. Distribution of graft tissue apol[a] and apo B levels among patients undergoing aortocoronary bypass re-operation.

Figure 6. Comparison of the apol[a]/apo B ratios in the plasma and graft tissues of patients undergoing bypass re-operation.

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tissue apoLp[a]/apo B ratio is higher than the corresponding plasma ratio, suggesting that Lp[a] has a particular propensity for deposition in vascular tissue. Since we have not yet demonstrated that the immunoreactivity of apoLp[a] or apo B in vein graft tissue is the same as that in purified Lp[a] (primary standard) or plasma (secondary standard), nor that all apoLp[a] has been electrophoresed from the tissue, our measurements must be regarded as

Discussion

In this study, we have demonstrated that the apoproteins apo[a] and apo B, and presumably the lipoproteins Lp[a] and LDL, accumulate in saphenous vein aortocoronary bypass grafts of long duration. We have shown that apo[a] and apo B co-localize in areas of atherosclerosis (Figure 2), and that the estimated tissue levels of these two apoproteins are positively correlated (Figure 8). The
estimates at this time. Clearly, further work is required to determine if our measurements accurately indicate the mass of apoprotein present in tissue.

The majority of the grafts in our study showed advanced atherosclerosis. This feature of late vein graft failure has been documented by many investigators. Atherosclerosis as a cause of graft failure is significantly different from thrombotic occlusion in the early postoperative period and from progressive fibrointimal proliferation, both of which have been described as causes of early graft failure. Where the demarcation between early and late graft failure occurs is unclear, but a case has been described in which evidence of atherosclerosis has been found in a vein graft of 6.5 months duration. We were not able to demonstrate localization of apo[a] in homologous saphenous veins harvested from their normal anatomic location, which is consistent with published studies indicating that atherosclerosis rarely occurs in veins.

Intimal proliferation of vein grafts begins soon after placement into the arterial circulation. It is one of the earliest pathologic changes and has been well described previously. It can progress to total graft occlusion, as did a few of the grafts examined in our study (e.g., Figure 3B), but occlusion on this basis is generally considered to be an uncommon occurrence, and moderate to marked intimal proliferation is felt to be compatible with long-term graft patency. We were unable to correlate tissue apoprotein levels with the amount of original luminal area occupied by intimal proliferation. We did localize focal accumulations of both apoproteins in early atherosclerotic lesions near the luminal surface of intimal proliferation, which appeared to be analogous to the fatty streaks seen in arterial disease. Walton et al. have reported localization of apo B in a weak, diffuse pattern in peripheral bypass grafts that were in place for a period of less than 3 months. This must be interpreted in light of our finding of small amounts of tissue apo B that were electrophoresed from intact normal saphenous veins, indicating that this apoprotein finds its way into the intima of intact vein tissue, at least in this population of patients. The findings of Hoff et al. agree with ours, in that control veins exhibit various degrees of intimal thickening but no localization of apo B.

Using immunofluorescence, Walton et al. demonstrated apoLp[a] in human arterial atherosclerosis in a pattern similar to that seen for apo LDL. The present study shows, for the first time, localization of apoLp[a] in bypass grafts. Apo[a] localizes in the areas where apo B is present. This is not surprising since both apoproteins are present in Lp[a], usually as a disulfide-linked complex.

The mean level of apo B estimated in vein graft tissue was 69 ng/mg wet tissue. This level is comparable with that estimated by the same methodology in the aortic arch of primates fed an atherogenic diet for 30 months. It was anticipated in this study that the tissue levels of apoproteins would depend upon their concentration gradients between the plasma and tissue as reported by other investigators for apo B. We observed a stronger correlation between the plasma and tissue levels of apoLp[a] than for apo B, but this is likely due to the greater variability of tissue and plasma levels of apoLp[a] than apo B. Most importantly, apoLp[a] appears to have a stronger tendency for tissue accumulation than apo B, based on the respective ratios of concentrations of these antigens in tissue and plasma. It is possible that intact Lp[a] and LDL enter the vein graft wall at the same rate, but that the efflux of Lp[a] from the tissue may be slower. One biochemical property that may support this model is the tendency for Lp[a] to aggregate in the presence of calcium ions. The significantly greater accumulation of apoLp[a] in vein graft lesions supports previous findings that Lp[a] is associated with coronary artery atherosclerosis despite its relatively modest contribution to total plasma cholesterol levels.

Our study has demonstrated positive correlations between estimated tissue concentrations of apoproteins and light microscopic features of atherosclerosis. The percent of the original luminal area occupied by lipid-rich core regions and characterization of the lesion as fibrous proliferation plus lipid both correlated positively with apo B; the presence of foam cells correlated positively with apoLp[a]. Further studies are needed to determine the ultrastructural localization and correlation with various cell types and extracellular components present in the lesions.

No positive correlations were found between vein graft duration and tissue apoprotein concentrations or between graft duration and the amount of atherosclerotic core region present. However, a strong positive correlation was found between vein graft duration and the amount of thrombus present (r = 0.673, p = 0.0001, n = 23). The reason for this correlation is unclear. Known causes of late graft failure include: 1) thrombus superimposed on an atherosclerotic plaque and 2) rupture of atherosclerotic plaques. A more likely natural history is the development of atherosclerosis to a point of vessel narrowing and superimposition of thrombus on, or rupture of, the necrotic core with mixing of blood and atheromatous debris, both of which were commonly seen in the grafts of the present study.

The association between elevated plasma total cholesterol and triglyceride levels and vein graft atherosclerosis has been reported by many investigators. More recently, two groups have shown an inverse relationship between HDL cholesterol levels and vein graft atherosclerosis. In addition, Campeau et al. have shown that the relationship between vein graft atherosclerosis is more highly correlated with the plasma apo B level than with either LDL or total cholesterol levels. Hoff et al. recently showed that serum Lp[a] levels retrospectively predict vein graft stenosis in patients with coronary artery bypass grafts.

Nevertheless, many workers in this field have observed atherosclerosis in vein grafts even in the presence of apparently normal lipid levels. The patients in our study had a mean total plasma cholesterol level (Tables 1 and 2) similar to that of a normal population of comparable age. Despite this observation, we found apo B levels elevated well above those of a normal population of comparable age. In addition, our patients had elevated plasma Lp[a] levels greater than 50% above those of a normal population of comparable age. Mean plasma HDL cholesterol levels in our patients were 25% below those of a normal
population,21 an observation consistent with that of Campeau et al.,28 who found an inverse relationship between HDL cholesterol and angiographic evidence for atherosclerosis in both vein grafts and native coronary arteries. Our study shows that the ratios of constituent apoproteins of HDL, apo A-I, and apo A-II were abnormal in these patients. The observed mean of the apo A-I/apo A-II ratios corresponded to an HDL2/HDL3 ratio of 1:9, contrasted to 1:3 for the same ratio in a normal male population of comparable age.46 This unfavorable HDL2/HDL3 ratio, together with elevated triglyceride levels in these same patients, suggests that they may suffer from a decreased rate of very low density lipoprotein \( \rightarrow \) LDL conversion, a process coupled to the conversion of HDL3 to HDL2.47

Yet another link between lipid levels and graft failure can be postulated in view of the findings of Simpson et al.46 that hypertriglyceridemia can lead to hypercoaguability.48 Virtually every study on vein graft atherosclerosis, including the present one, has documented hypertriglyceridemia in at least a portion of the patients. Allard et al.49 found that early vein graft occlusion was associated with either preoperative or postoperative hypertriglyceridemia compared with those patients with normal triglycerides. Although the majority of our patients’ grafts appeared to have survived the early postoperative period, it seems reasonable to expect that hypertriglyceridemia could lead to excessive coagulability at a later date, particularly in the setting of luminal narrowing by an atherosclerotic lesion.

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Quantitation and localization of apolipoproteins [a] and B in coronary artery bypass vein grafts resected at re-operation.

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