SUMMARY Low (LDL) and very low (VLDL) density lipoproteins retained in grossly normal and atherosclerotic human intracranial arteries have been quantitated using an electro-immunoassay directed against apolipoprotein B (apoB), the major protein of these two lipoprotein fractions. Buffer-homogenates of grossly normal arteries contained apoB amounts ranging from <0.04 to 1.58 μg/mg tissue dry weight, while those of atherosclerotic plaques gave values ranging from 0.80 to 3.9 μg per mg tissue dry weight. These results were consistent with immunofluorescence studies localizing apoB in these arteries. Plaques also contained a remaining fraction of tightly-bound apoB as evidenced by positive immunofluorescence in sections of pellets from buffer homogenates. This was in contrast to the negative results from grossly normal arteries. These results would suggest that retention of apoB by intracranial arteries correlates positively with vessel lesions. Arterial apoB is present in both grossly normal regions and plaques in a loosely-bound form, possibly representing intact lipoprotein. apoB is also present in a tightly-bound form in plaques.

THE PLASMA LOW DENSITY lipoprotein fraction (LDL), the major carrier of cholesterol in blood, has been linked to the atherosclerotic process. Elevations in plasma cholesterol have been shown to correlate positively with coronary heart disease resulting from atherosclerotic occlusion of coronary arteries. Elevations in plasma cholesterol have also been shown to correlate positively with stroke in man, resulting from atherosclerosis of extra- and intracranial arteries. In experimental animal models in which hypercholesterolemia has been induced by dietary manipulation, elevations in plasma cholesterol levels are accompanied by extensive atherosclerosis of the aorta and coronary arteries. A positive correlation has also been noted between elevations in plasma triglycerides, resulting from increases in plasma very low density lipoproteins (VLDL), and stroke in man.

Apolipoprotein B (apoB) is the major protein of both LDL and VLDL fractions. We have utilized the immunological reactivity of apoB as a marker for these lipoproteins in immunofluorescence studies to determine the apoB localization in both normal and atherosclerotic regions of aortic, coronary, as well as intracranial artery beds. Since immunofluorescence procedures are only qualitative, we have used an electro-immunoassay or "rocket" technique to quantitate apoB accumulation in intracranial arteries from both grossly normal regions and atherosclerotic plaques. Our aim has been to correlate the arterial apoB values with the degree of atherosclerosis in the vessel wall.

Methods

LDL was isolated from the plasma of normolipemic donors by sequential ultracentrifugation in KBr (d 1.020–1.050 g/ml). A 60 Ti rotor was utilized for the first ultracentrifugation run at 55,000 rpm for 18 hours, while an SW 50.1 rotor was used for the recentrifugation at a solvent density of 1.050 g/ml. ApoB was isolated by delipidation of LDL at 4°C with diethyl-ether:ethanol (3:1) and fractionation of the delipidated LDL on Sephadex G-150. Antiserum against apoB were raised in goats by immunization with apoB solubilized in 1 mM Na decyl sulfate. Globulin fractions of antisera against apoB were purified by affinity chromatography using LDL-Sepharose which functioned as a solid-phase immunoadsorbent. The purified anti-apoB gave precipitin lines of complete identity with LDL, VLDL and whole plasma, but failed to react with human serum albumin, high density lipoproteins (HDL) and its major apoproteins, apoA-I or apoA-II or the apoC proteins of VLDL either on double gel diffusion plates or in the electro-immunoassay to be described.

Samples of grossly normal and atherosclerotic-involved intracranial arteries (basilar, middle cerebral and communicating arteries) were obtained at autopsy within eight hours after death from 26 subjects between the ages of 17 and 90 years. Preliminary studies demonstrated no significant differences in apoB content in plaques from different arterial beds, such as basilar and middle cerebral. The number of lesions per case was usually small, and localized focally. Therefore, lesions or normal regions from different vessels of the same case were pooled. Cause of death in 14 cases was trauma, six cases died from coronary heart disease, while six cases died from miscellaneous causes.

Each arterial sample was divided into two parts. The central part was utilized for light microscopic assessment of the degree of pathology of the vessel wall and was further subdivided into two sections. One was immersed in formalin, paraffin embedded, sectioned, and stained with H&E and Movat’s pentachrome procedure which distinguishes between collagen, cell structure, elastin, glycosaminoglycans and fibrin. The other part was snap-frozen in isopentane-liquid nitrogen, cryostat sections cut and stained for neutral lipid with oil red O and for apoB using an immunofluorescence procedure described previously. Eriochrome black (Chroma CI 14645) was used to diminish tissue background fluorescence. A Leitz Orthoplan photomicroscope was used for bright field and fluorescence microscopy. For the latter an HBO 200 Mercury light source, a BG-12 primary filter, a K-530 secondary filter, and dark field condenser were employed. The major part of each sample was cut into a fine mince and homogenized for 15 seconds at 4°C in 0.13M Tris-HCl, 0.1% EDTA buffer, pH 7.4 with a Polytron homogenizer (Brinkmann, New York) equipped...
with a microprobe operated at full power. Following a 30 minute incubation at 4°C, the debris was sedimented by low speed centrifugation and the supernatant assayed for apoB in the electro-immunoassay to be described.

An electro-immunoassay (EIA) or Laurell "rocket" technique to measure apoB was employed using a Behring (Behring, New Jersey) water-cooled electrophoresis cell. In this technique antigens of known and unknown concentration are electrophoresed through an agarose slab gel containing homogeneously dispersed antibody directed against that antigen. At the equivalence point for that antigen-antibody system, a precipitin peak is formed, the height of which is proportional to the antigen concentration. A linear standard curve of peak height versus antigen concentration can be obtained for a given antibody concentration. In our study anti-apoB was added to 50°C molten agarose at a 1/300 dilution of the original antiserum. Sensitivity of the assay permitted us to measure apoB values down to 30 ng in a 5 μl sample. The apoB content in the LDL standard (d 1.020 to 1.050 g/ml) was determined by the Lowry procedure for protein determination. Further details concerning the EIA procedure can be found in an earlier paper. ApoB values were recorded as μg per mg tissue dry weight. The latter was determined by taking duplicate one ml aliquots of the homogenate, dialyzing to give salt-free tissue, lyophilizing, and measuring gravimetrically.

Results

It was conceivable that part of the immunological reactivity of endogenous apoB could be broken down by one or both of the following: (1) The mechanical disruption of the tissue during homogenization with a Polytron homogenizer, and (2) enzymatic digestion by hydrolytic enzymes released from lysosomes during homogenization. To determine if breakdown occurred due to homogenization, a known amount of LDL (36 μg apoB) was added to normal as well as plaque minces before and after homogenization. The extracts were then assayed by EIA. As seen in table 1, no appreciable differences were detected between the measured and expected values of apoB for either normal or plaque regions. To assess if breakdown occurred due to enzymatic digestion, LDL of varying amounts was added to 1 ml samples of either homogenates of grossly normal artery (1 to 94 μg apoB) or atherosclerotic plaques (0 to 50 ng apoB). Figure 1a and b illustrate the experimental values obtained by EIA as compared with the expected values. The ordinate shows the measured values of homogenate with exogenous LDL of varying amounts added to normal as well as plaque homogenates. Mean - 0.57 ± 0.57 S.D. using homogenates was 0.999 (p < 0.005). (b): LDL (0 to 50 ng apoB) added to 1 ml samples of minces of grossly normal artery which had previously been homogenized in 0.13M Tris-HCl-EDTA buffer pH 7.4 with a Polytron homogenizer. On the abscissa is recorded the amounts of exogenous apoB from LDL in 5 μl samples of supernatants of homogenates. Normal homogenate alone contained 45 ng of apoB in 5 μl. On the ordinate is recorded the recovered exogenous apoB calculated as the total apoB in a 5 μl sample minus the apoB content of the 5 μl sample of the homogenate alone. The 45° line signifies the position of the points if recoverability were 100%. Using linear regression analysis the correlation coefficient (r) for normal artery homogenates was 0.999 (p < 0.005). (b): LDL (0 to 50 ng apoB) was added to 1 ml samples of minces of atherosclerotic plaques previously homogenized as just described. 5 μl plaque homogenate alone contained 170 ng apoB. The correlation coefficient (r) was 0.994 (p < 0.005).

Grossly normal intracranial arteries are of the thin-walled muscular type with one very thick internal elastic membrane, usually lacking a thickened intima (fig. 2a). Occasionally focal thickening can be observed (fig. 2b), usually around branch points and in the basilar artery. ApoB was found only in such thickened areas, diffusely localized in the extracellular space usually together with neutral lipid. Table 2 summarizes the values of apoB found in pooled samples of grossly normal intracranial arteries obtained from the basilar, middle cerebral, and communicating artery beds. One-third of the samples failed to demonstrate measurable apoB from expected values was found in either normal arteries, (r = 0.999 p < 0.005) or in plaques (r = 0.994 p < 0.005).

Table 1

TABLE 1 Recovery of Exogenous LDL During Homogenization and Extraction of Intracranial Arteries

<table>
<thead>
<tr>
<th>Sample</th>
<th>LDL added before homogenisation</th>
<th>LDL added after homogenisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL + BSA</td>
<td>150 (180)*</td>
<td>180 (180)</td>
</tr>
<tr>
<td>normal + LDL tablets</td>
<td>195 (225)</td>
<td>225 (225)</td>
</tr>
<tr>
<td>plaque + LDL tablets</td>
<td>235 (255)</td>
<td>275 (255)</td>
</tr>
</tbody>
</table>

*Numbers in parenthesis represent theoretical value.

Table 2

TABLE 2 Buffer-Extracted ApoB from Grossly Normal Intracranial Arteries

<table>
<thead>
<tr>
<th>Age/sex</th>
<th>ApoB (μg/mg tissue dry weight)</th>
<th>ApoB</th>
<th>ApoB (μg/mg tissue dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17f</td>
<td>0.92</td>
<td>57m</td>
<td>1.04</td>
</tr>
<tr>
<td>42f</td>
<td>0.92</td>
<td>58m</td>
<td>1.46</td>
</tr>
<tr>
<td>43m</td>
<td>1.20</td>
<td>58m</td>
<td>1.17</td>
</tr>
<tr>
<td>44m</td>
<td>0.73</td>
<td>59m</td>
<td>0.34</td>
</tr>
<tr>
<td>47m</td>
<td>0.06</td>
<td>59m</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>48m</td>
<td>0.08</td>
<td>63m</td>
<td>1.58</td>
</tr>
<tr>
<td>50m</td>
<td>1.00</td>
<td>67m</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>50m</td>
<td>&lt;0.04</td>
<td>67m</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>53m</td>
<td>0.62</td>
<td>69f</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>54m</td>
<td>&lt;0.04</td>
<td>70f</td>
<td>1.30</td>
</tr>
<tr>
<td>55m</td>
<td>1.10</td>
<td>70m</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>56m</td>
<td>&lt;0.04</td>
<td>90m</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Mean = 0.57 ± 0.57 S.D.
FIGURE 2. Light micrographs of human intracranial arteries. The lumen (L) is to the top of all micrographs. (a): Grossly normal artery demonstrating no thickened intima, $\times 100$. (b): Grossly normal artery demonstrating some intimal thickening (I). The thick internal elastic membrane (e I) forms a sharp border between the intima and the underlining tunica media, $\times 100$. (c): Advanced atherosclerotic plaque illustrating the large fibromuscular cap (f) above a necrotic core (co) on the lumen side of the internal elastic membrane, $\times 125$. (d): Localization of neutral lipid in the necrotic core of an advanced atherosclerotic lesion (black areas). Oil red O stain, $\times 100$. (e): Localization of apoB in a plaque using the immunofluorescence procedure. ApoB is seen in the lipid core region and along bands of collagen in the fibrotic cap as white areas (except for the white autofluorescing internal elastic membrane), $\times 85$. (f): Immunofluorescence staining for apoB of a cryostat section from the pellet of a buffer homogenate from a normal artery. Note the almost complete lack of positive fluorescence depicting apoB, $\times 80$. (g): Same as fig. 2f but from a homogenate of an atherosclerotic plaque. Note the remaining positive fluorescence localized to granular material and bands of collagen fibers, $\times 80$.

quantities of apoB, that is, they had values below the sensitivity of the technique (0.04 $\mu$g/mg tissue dry weight). The remaining arteries contained apoB ranging from 0.06 to 1.58 $\mu$g/mg tissue dry weight.

Plaques in this study usually had a fibromuscular cap and necrotic core close to the internal elastic membrane (fig. 2c). Such necrotic cores were filled with neutral lipid (fig. 2d). ApoB (fig. 2e) was localized to some collagen fibers and to pools of extracellular lipid of the necrotic core (fig. 2d). Atherosclerotic lesions contained values of apoB ranging
from 0.60 to 3.91 μg per mg tissue dry weight. As can be seen in table 3, the apoB values in plaques were consistently higher than in grossly normal areas from the same individual case. The mean apoB content of the 11 plaques measured (2.01 μg/mg tissue dry weight) was statistically significantly higher (p < 0.05) than that of the mean of the normal regions (0.52 μg/mg tissue dry weight). No differences were detected between apoB values of either normal regions or plaques between arteries from younger or older individuals (tables 2 and 3).

To determine whether all apoB had been extracted with a standard buffer, pellets of buffer homogenates were snap-frozen, cryostat sectioned and subjected to immunofluorescence staining to localize any residual apoB. Although normal regions showed only negligible positive fluorescence (fig. 2f), plaques demonstrated significant positive fluorescence only slightly reduced from that of minces (fig. 2g). The apoB was localized primarily to some bands of collagen fibers and to pools of extracellular lipid.

**Discussion**

The buffer-extracted apoB content of both grossly normal and atherosclerotic-involving intracranial arteries was documented using an electro-immunoassay. In numerous cases the pooled uninvolved intracranial arteries gave buffer-extracted apoB values below the threshold of the immunoassay and were recorded as < 0.04 μg/mg dry weight. In other cases significant values were obtained. Immunofluorescence studies showed apoB localized only to those regions of grossly normal arteries demonstrating intimal thickening. This would suggest that these areas gave apoB values above the assay threshold. The values for buffer-extracted apoB in these normal arteries ranged from < 0.04 to 1.58 μg per mg tissue dry weight. They were appreciably lower than those found in the intimal lining of grossly normal human aortas. This is perhaps not surprising for a number of reasons: (1) only the intimal lining was used in the aorta in contrast to the total vessel in intracranial arteries. The tunica media has been shown in grossly normal arteries not to contain apoB, both by histochemical and quantitative techniques. (2) Most segments of normal intracranial arteries either showed no intimal thickening and therefore no apoB by immunofluorescence techniques, so that presumably only those areas with thickened intima contained apoB measurable by EIA.

Atherosclerotic plaques (table 3) contained a mean of four times more buffer-extracted apoB per tissue dry weight than normal arteries from the same case. This result is consistent with immunofluorescence procedures localizing apoB in normal areas and plaques. Total apoB in intracranial plaques as compared with normal regions may be even greater than that documented by buffer-extracted apoB alone. Sections of pellets of buffer homogenates from plaques demonstrated positive fluorescence for apoB while similar sections from normal areas showed no specific fluorescence. Preliminary studies utilizing detergents to extract this remaining fraction of apoB in plaques indicate that it is at least comparable in amount to the buffer-extracted fraction and appears to be bound to some collagen fibers and pools of extracellular lipid as evidenced by our immunofluorescence studies.

We have previously viewed saline extracts of intracranial plaques with the electron microscope following negative staining and have observed particles in the 250 to 800Å diameter range, similar to those of human plasma LDL and VLDL. These lipoproteins, it has been suggested, are retained in atherosclerotic lesions as complexes with tissue glycosaminoglycans (GAG). Such GAG-lipoprotein complexes have been isolated from saline extracts of atherosclerotic plaques and have been studied chemically. It is conceivable that the buffer-extracted apoB found in normal intracranial arteries, as documented in this study, and in the thickened intima of grossly normal human aortas, as studied recently, may represent sequestered intact LDL and VLDL that have complexed with intimal GAG. Differential ultracentrifugation of buffer extracts of grossly normal aortic intima, together with electro-immunoassay of apoB in each density fraction, have indicated that most apoB in such extracts is in the LDL range (d 1.006–1.063 g/ml) suggesting that only small amounts of VLDL, if any, are retained by the grossly normal aortic intima. It is possible that VLDL is broken down to LDL by a lipoprotein lipase on the endothelial surface as suggested by Zilversmit, allowing only LDL to enter the arterial wall. Such a lipase has recently been extracted from rabbit atherosclerotic lesions. The small amount of apoB-containing lipoprotein in the d > 1.063 fraction (5%) could represent LDL with some lipid removed, or a soluble complex of LDL with the denser GAG molecule.

Camejo et al. have isolated a glycoprotein from human aortic lesions that forms insoluble complexes with plasma LDL. Extracts of plaque elastin have also been shown to contain a glycoprotein. It has been suggested that this glycoprotein may be the cause of the interaction between plaque elastin preparations and cholesteryl esters from plasma LDL. Whether it is this glycoprotein, possibly adhering to connective tissues, or extracellular lipid that causes LDL retention in plaques is still unknown.

Although lipid is generally localized together with apoB in histochemical studies, the degree of lipodation of the tightly-bound apoB is uncertain. The question as to whether the protein or lipid portion of LDL is primarily responsible for the interaction with plaque components also remains to be answered. Studies presently in progress utilizing detergents to extract this tightly-bound apoB fraction may help clarify

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**Table 3 Buffer-Extracted ApoB from Normal and Atherosclerotic Intracranial Arteries**

<table>
<thead>
<tr>
<th>Age/sex</th>
<th>ApoB content (μg/mg tissue dry weight)</th>
<th>Normal apoB</th>
<th>Plaque apoB</th>
</tr>
</thead>
<tbody>
<tr>
<td>47m</td>
<td>0.08</td>
<td>0.06</td>
<td>2.04</td>
</tr>
<tr>
<td>48m</td>
<td>0.08</td>
<td>0.08</td>
<td>1.10</td>
</tr>
<tr>
<td>53m</td>
<td>0.62</td>
<td>0.62</td>
<td>5.39</td>
</tr>
<tr>
<td>56m</td>
<td>1.46</td>
<td>1.46</td>
<td>1.95</td>
</tr>
<tr>
<td>58m</td>
<td>&lt;0.04</td>
<td>0.04</td>
<td>0.80</td>
</tr>
<tr>
<td>62m</td>
<td>1.58</td>
<td>1.58</td>
<td>2.39</td>
</tr>
<tr>
<td>67m</td>
<td>&lt;0.04</td>
<td>0.04</td>
<td>3.91</td>
</tr>
<tr>
<td>69f</td>
<td>&lt;0.04</td>
<td>0.04</td>
<td>0.60</td>
</tr>
<tr>
<td>70f</td>
<td>0.51</td>
<td>0.51</td>
<td>0.93</td>
</tr>
<tr>
<td>70f</td>
<td>1.30</td>
<td>1.30</td>
<td>2.25</td>
</tr>
<tr>
<td>90m</td>
<td>0.15</td>
<td>0.15</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Mean = 0.32 and 2.01, S. D. ± 0.63 ± 1.49, p < 0.05.
these questions and enable us to correlate this apoB fraction with the degree of arterial pathology.

Acknowledgments

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Apolipoprotein B (apoB) retention in atherosclerotic intracranial arteries.
H F Hoff, C L Heideman and J W Gaubatz

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