Increased Apolipoprotein Deposits in Early Atherosclerotic Lesions Distinguish Symptomatic From Asymptomatic Patients

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Objective—Apolipoprotein E (apoE) and apolipoprotein B100 (apoB) are both involved in receptor-mediated uptake of atherogenic lipoproteins by the liver. Inefficient hepatic clearance of these lipoproteins leads to symptomatic atherosclerosis. Using arterial tissue microarrays, we tested the hypothesis that apoE and apoB accumulation in the arterial wall discriminates between patients with symptomatic atherosclerosis and patients who never experienced cardiovascular events.

Methods and Results—In a postmortem study involving 49 patients (22 patients with symptomatic atherosclerosis), we quantified apolipoprotein deposits in arterial rings obtained from the left main coronary, the common carotid, the common iliac, and the renal artery applying tissue microarray technology and semiquantitative immunohistochemistry. In early atherosclerotic lesions, even before atheroma appeared, symptomatic patients had significantly more arterial apoE and apoB deposits than patients without cardiovascular events \((P < 0.001)\). Among the symptomatic patients, those without diabetes had more intense apolipoprotein deposits than diabetics. Large amounts of apoE and apoB were found in advanced atherosclerotic lesions, regardless of the activity of the disease.

Conclusions—Increased apolipoprotein deposits are an early sign of symptomatic atherosclerosis. They may reflect either enhanced retention of atherogenic lipoproteins or impaired local apolipoprotein degradation. The arterial lipoprotein turnover may be different in diabetic patients. (Arterioscler Thromb Vasc Biol. 2006;26:359-364.)

Key Words: arteriosclerosis ■ lipoproteins ■ apolipoproteins ■ diabetes mellitus ■ tissue microarray

Atherosclerosis is an inflammatory lipid storage disease affecting large and medium-sized arteries.\(^1\) Abnormal lipoprotein retention in the arterial wall has been described as one of the earliest steps in the development of atherosclerotic lesions.\(^4\) The apolipoproteins are the protein part of the lipoprotein particles and play an important role in the interaction of lipoproteins with cells and the extracellular matrix. Apolipoprotein E (apoE) and apolipoprotein B100 (apoB) are synthesized in the liver and other organs. Both apoE and apoB mediate low-density lipoprotein (LDL) receptor-mediated uptake of lipoproteins by the liver, the prerequisite of cholesterol elimination from the body.\(^7\) Mutations of the apoB and apoE gene, which affect LDL receptor binding, lead to familial hypercholesterolemia\(^a\) or dysbetalipoproteinemia.\(^b\) Both conditions are associated with premature atherosclerosis because of an impaired hepatic clearance of atherogenic lipoproteins. Intimal lipoprotein retention has been shown to accelerate atherosclerosis in an animal model of the disease,\(^5\) and extensive intimal lipid accumulation is a structural hallmark of the advanced and vulnerable atherosclerotic lesion.\(^10\)\(^11\) However, although intimal lipoprotein accumulation is known to precede plaque formation,\(^12\) little is known about the significance of early lipoprotein retention for the development of symptomatic atherosclerosis in humans. We have developed the human arterial tissue microarray technique to identify morphological signs of common pathways, which lead to unstable or vulnerable plaques.\(^13\) Forty percent to 50% of patients who are treated in our hospital have experienced cardiovascular events during their lifetime and fulfill the definition of symptomatic atherosclerosis.\(^1\)\(^2\) Therefore, the prospective inclusion of hospitalized patients in a nonselective manner assembles a cohort of patients that is highly representative for the population that this hospital is serving. We have successfully used the tissue microarray technique to systematically investigate the arterial wall at different lesional stages in patients with symptomatic atherosclerosis.\(^13\) We described previously the typical pattern of neovascularization and inflammation in vulnerable patients...
with symptomatic atherosclerosis, that is, in patients who experienced cardiovascular disease during their lifetime. In the present analysis, we used the tissue microarrays to investigate the pattern and extent of apolipoprotein deposits in the arterial wall of vulnerable patients and compared these findings with asymptomatic patients who did not develop cardiovascular events during their lifetime.

Methods

Arterial Tissue Microarrays

All of the investigations were approved by the institutional ethical review board. Between December 2002 and April 2003, 49 patients who died at our hospital (Department of Medicine, University Hospital Bruderholz, Bruderholz, autopsy rate 75%) were consecutively included in this study. Twenty two of the 49 patients experienced cardiovascular events (eg, myocardial infarction, stroke, or peripheral arterial occlusive disease) during their lifetime, that is, they had symptomatic atherosclerosis. The other 27 patients had no cardiovascular events, that is, they were asymptomatic with respect to atherosclerosis. The clinical characteristics of these patients are summarized in the Table. At autopsy, a 0.5-cm-long ring segment of the left common carotid, the left renal, and the left common iliac artery was removed, fixed immediately in 4% phosphate-buffered formalin (pH 7.4), and embedded in paraffin. When acute coronary heart disease was suspected, a postmortem coronary angiography was routinely performed. Therefore, the left main coronary artery segment, which was not treated equally for all patients, was obtained only from the 28 patients who did not undergo postmortem angiography. The arterial tissue microarrays from the iliac, the renal, and the carotid arteries have been described previously, and the microarrays from the coronary arteries were constructed in a similar way. In brief, the complete arterial ring sections were stained with Elastica-van Gieson and examined histologically for the presence of atherosclerotic lesions. From each arterial ring, the best preserved sections of coronary arteries were selected.

Immunohistochemistry

Six-micrometer-thick sections of the paraffin block were cut and transferred to glass slides. The sections were air dried (60°C, 45 minutes), and the slides were hydrated. Endogenous peroxidase activity was blocked by 2% H2O2. For antigen retrieval, slides were either incubated for 2 minutes in 10 mmol/L citrate buffer in a steam pressor chamber for apoE staining or treated with proteinase K (SIG-9740-02, Alexis Biochemicals, Lausen, Switzerland) for 20 minutes of incubation at room temperature. After 60 minutes of incubation at room temperature, the slides were washed twice and incubated with a peroxidase-conjugated rabbit anti-goat antibody (Z0233, DAKO, Glostrup, Denmark) for apoB staining. Slides were rinsed in PBS and incubated with the first antibody: polyclonal goat anti-human apoE (ab7616, Abcam, Cambridge, UK) or monoclonal mouse anti-human apoE (SIG-9740-02, Alexis Biochemicals, Lausen, Switzerland). After 60 minutes of incubation at room temperature, the slides were washed twice and incubated with a peroxidase-conjugated rabbit anti-goat antibody (Universal Quick kit, Novocastra, Newcastle-on-Tyne, United Kingdom) or goat anti-mouse antiserum (Envision system HRP mouse, DAKO). After 30 minutes of incubation, slides were washed twice and incubated with diaminobenzidine as a substrate. Hemalaun was used for counterstaining before the sections were dehydrated and embedded (Pertex, Medite, Nunningen, Switzerland).

Semiquantitative Assessment of Apolipoprotein Deposits in the Tissue Arrays

ApoE and apoB were detected in the extracellular matrix of the intima and, to a lesser extent, in the media layer of the arteries. In most of the microscopically healthy appearing arterial sectors, apolipoproteins were generally not detectable, and the amount of deposits was generally not detectable, and the amount of
Quantification of Human Plasma Apolipoproteins

Human EDTA plasma was obtained from 26 patients (10 without a history of cardiovascular events and 16 with symptomatic atherosclerosis) after written informed consent was given. The plasma was diluted 1:10 in PBS and 1:5 in reducing sample buffer. ApoE was separated on a 10% and apoB on a 6% SDS-PAGE, respectively. The proteins were transferred in transfer buffer (25 mmol/L Tris base; Merck), 192 mmol/L glycin (Fluka), and 20% pure methanol to separate on a 10% and apoB on a 6% SDS-PAGE, respectively. The proteins were transferred in transfer buffer (25 mmol/L Tris base; Merck), 192 mmol/L glycin (Fluka), and 20% pure methanol to cellulose nitrate (Protran BA83, Schleicher&Schüll). After washing the membrane in Tris-buffered saline, nonspecific protein binding sites were blocked for 60 minutes in 5% fat-free milk. The membrane was then incubated with the first antibody for 60 minutes at room temperature. After extensive washing in Tris-buffered saline, the membrane was transferred either to the peroxidase-conjugated secondary rabbit anti-goat antibody (P0449, DAKO) or to the peroxidase-conjugated goat anti-mouse antibody (115-035-071, Jackson Laboratories, Bar Harbor, Maine) and incubated for 60 minutes at room temperature. The membrane was washed again and incubated with chemiluminescence substrate (Supersignal West Pico, Pierce, Perbio Science). A photographic film ( Biomax, Eastman Kodak) was exposed to the membrane and developed using an automatic processor (Crurix 60, Agfa). A molecular weight standard [Color Marker (wide range), α-Fluka] was used to calibrate the electrophoretic separation of plasma proteins. Band intensity was measured with an image analysis software (ImageJ, please see online at http://rsb.info.nih.gov/ij/, National Institutes of Health) and expressed in relative units. ApoE and apoB concentrations were normalized to normal pool plasma (set to be 100%). ApoB concentrations in the normal pool plasma were quantified by immunonephelometry on the Beckman nephelometric Image 800 (Beckman-Coulter Inc) according to the manufacturer’s instructions. The band intensity of apoB as quantified by Western blotting correlated linearly with the amount of apoB measured nephelometrically ($R=0.93; P<0.001$).

Statistics

The statistical analyses were performed using SPSS 11.0 software (SPSS Inc). Plaque burden and apolipoprotein staining scores between asymptomatic and vulnerable patients and apoE and apoB staining scores at the different stages of the disease were compared by Mann-Whitney U test. The interobserver correlation was analyzed by the Spearman’s rank test. $P$ values $<0.05$ indicated significant differences. Unless otherwise stated, median values and the inter-quartile range are shown.

Results

Histological Analysis of Apolipoprotein Accumulation in the Arterial Wall

Fully developed and advanced atherosclerotic lesions (AHA type 4 to 6) showed extensive deposits of apoE and apoB covering most of the intima area (Figure 1D). The distribution of apoE and apoB in the atherosclerotic plaques was indistinguishable and, therefore, the same staining score was used to quantify both apolipoproteins. Both apoE and apoB are synthesized in the liver. The liver tissue, which was included as a control in each of the tissue microarray blocks, displayed hepatocellular staining for both proteins (data not shown). ApoE is also synthesized and secreted by macrophages. However, in the tonsillar tissue, which is included as a control in each of the tissue microarray blocks, displayed hepatocellular staining for both proteins (data not shown). ApoE is also synthesized and secreted by macrophages. ApoE and the anti-apoB antibody both detected a single protein band of the expected size (32 kDa and 500 kDa, respectively).

Interobserver Correlation of the Apolipoprotein Staining Score

The amount of apolipoproteins in the intima at early stages of the disease was assessed by a semiquantitative scoring system (Figure 1A through 1D). The interobserver correlation for this scoring system was high ($y=0.96x +0.2$, $r=0.87, P<0.001$ for apoE and $y=0.94x +0.3$, $r=0.85, P<0.001$ for apoB). In fully developed atherosclerotic lesions, apolipoproteins were found most abundantly in the center of the plaques. A cellular association with endothelial, fibromuscular, or inflammatory cells was not detected.

Arterial Apolipoprotein Deposits Are a Panarterial Sign of Atherosclerosis

The apolipoprotein deposits were quantified in asymptomatic patients at all 4 of the arterial sites, that is, the renal, the common carotid, the common iliac, and the left main coronary artery. The tissue arrays that were used for apolipoprotein staining contained a substantial number (247 of a total of 350 sectors) of arterial sectors with early atherosclerotic lesions (AHA type 1 to 3) and even 31 sectors without any pathologic changes of the arterial wall (Figure 2A; Table). Therefore, the gradual increase of apolipoprotein deposits in
The subendothelial area of the intima could be reliably analyzed (Figure 2B and 2C). At all 4 of the arterial sites, no or minimal apolipoprotein deposits were detectable in normal arterial sectors or in sectors with intimal thickening (AHA type 0 or 1), multilamellar apolipoprotein deposits at sites with fatty streaks, or preatheroma (AHA type 2 or 3) but uniformly extensive apolipoprotein deposits covering nearly 100% of the arterial intima in fully developed atheroma and advanced lesions (AHA type 4 to 6). Therefore, apoE and apoB retention are panarterial signs of atherosclerosis. The iliac and the coronary arteries both have a higher plaque burden than the renal and carotid artery (Figure 2A). However, the amount of apolipoproteins accumulating in these arteries is determined by the severity of atherosclerotic lesions at all 4 of the arterial sites (Figure 2B and 2C) and not by the plaque burden of the examined vascular bed.

**Arterial Apolipoprotein Deposits Are an Early Sign of Symptomatic Atherosclerosis**

We next analyzed whether apolipoprotein accumulation was different in symptomatic patients who experienced cardiovascular events and asymptomatic patients. Vulnerable patients had a significantly higher plaque burden [average AHA plaque type 2.7 (range, 2.1 to 3.3) versus 2.2 (range, 1.9 to 2.6); *P*=0.01] and significantly more apoE and apoB deposits [staining score 2.6 (range, 2.2 to 3.0) versus 2 (range, 1.25 to 2.1), *P*=0.002 for apoE and 3 (range, 2.7 to 3.3) versus 2 (range, 1.3 to 2.4), *P*<0.001 for apoB] compared with patients who never experienced cardiovascular events. When arterial sectors with similar plaque types were compared, vulnerable patients had significantly more apolipoproteins deposited at the earliest stages of the disease. In the microscopically healthy appearing arterial wall or in intimal thickening (AHA plaque type 1), symptomatic patients had a higher apoB and apoE staining score than asymptomatic patients [staining score 2 [range, 1 to 3] versus 1 [range, 0 to 1], *P*<0.001, for apoB and 1 [range, 1 to 2] versus 0 [range, 0 to 1], *P*<0.001, for apoE, respectively; Figure 3). At intermediate atherosclerotic lesions such as fatty streaks (AHA plaque type 2) or preatheroma (AHA plaque type 3), symptomatic patients had still higher apoB deposits than asymptomatic patients (staining score 3 [range, 3 to 3] versus 2 [range, 2 to 3], *P*<0.001; Figure 3), but apoE deposits were not significantly different. Elevated plasma apolipoprotein levels could explain the increased deposits in vulnerable patients. However, plasma apoE and apoB levels were not significantly different between the 2 groups of patients (Figure 4).

Diabetes mellitus is a known risk factor for atherosclerosis, and we tested whether, among the symptomatic patients, patients with diabetes mellitus would have increased apolipoprotein deposits. Unexpectedly, vulnerable patients with diabetes mellitus had significantly less apoB and apoE deposited in the arterial wall at early stages of atherosclerosis compared with patients without diabetes [staining score 1 (range, 0.9 to 2) versus 2.5 (range, 1.75 to 3), *P*=0.02, for...
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apoB and 1 (range, 0.5 to 1.1) versus 2 (range, 1 to 2.1), \( P=0.02 \), for apoE, respectively]. Nonenzymatic glycation could modify the antigenic structure of apolipoproteins, thereby reducing the avidity of the antibodies used for immunohistochemistry. To rule out that antibody binding was impaired by any posttranslational mechanism precipitated by diabetes mellitus, we performed Western blot analysis of plasma apoE and apoB in 3 diabetic patients with elevated HbA1c (>10%) and compared it with nondiabetic control patients. The staining intensity of the apoprotein bands was not influenced by posttranslational modification, such as nonenzymatic glycation (data not shown). Therefore, reduced antibody binding to modified apolipoproteins in diabetic patients with poor metabolic control cannot explain the modest staining of apolipoproteins in the arterial intima.

Discussion

In this tissue microarray analysis of arterial apolipoprotein deposits, we found that vulnerable patients, that is, patients who experienced symptomatic atherosclerosis during their lifetime, have increased apoE and apoB deposits at the earliest lesional stages, that is, during intimal thickening or even in microscopically healthy arteries. The histomorphological pattern of apolipoprotein distribution in the arterial wall was nearly identical for apoE and apoB. As an unexpected finding, patients with diabetes mellitus had minimal intimal apolipoprotein deposits in early atherosclerosis as detected by immunohistochemistry.

The net amount of apolipoproteins in the arterial wall is the sum of delivery or influx, local synthesis or retention, and degradation or efflux. Our finding that apolipoproteins first accumulate in the arterial intima, that media deposits are found only when extensive intimal deposits are present, and that apolipoproteins are virtually absent from the adventitia supports the concept of a centrifugal influx of apolipoprotein from the luminal side of the arterial blood vessel. We have observed a similar, centrifugal deposition of von Willebrand factor, another large molecule, in the extracellular matrix of large arteries (data not shown). ApoE is known to be synthesized by macrophages, particularly, activated lesional macrophages in atherosclerosis, and is supposed to mediate reverse cholesterol transport. However, we had no evidence that apoE would show cell-bound macrophage or foam cell staining, nor that it would be preferentially deposited around macrophage-rich areas of the atherosclerotic plaques. The proteoglycan binding site of apoE is located between amino acids 142 and 147. The mutational analysis of the proteoglycan binding sites of human apoB has demonstrated in several transgenic mouse lines that reduced matrix retention of LDL in the arterial wall was associated with diminished formation of atherosclerosis. Similarly, apoE is directly involved in the retention of lipoproteins to the arterial wall. ApoB and apoE are components of different lipoproteins (chylomicron remnants, LDL, very low–density lipoprotein, intermediate-density lipoprotein, and high-density lipoprotein), which exert proatherogenic and antiatherogenic effects. The similar pattern of apoB and apoE deposits and the close association with symptomatic atherosclerosis suggest that, in the arterial wall, both apolipoproteins are tracing the local delivery of proatherogenic lipoproteins. Finally, reduced cellular uptake and degradation by resident arterial cells, such as macrophages and smooth muscle cells, could also explain our observational data. We have shown previously that macrophages are present in early stages of atherosclerotic lesions and, therefore, cells would be locally available for the phagocytosis of lipoprotein particles. The extracellular pattern of apolipoprotein deposits found in the human arterial wall suggests that, with the progression of lesion formation, cellular uptake and degradation of apolipoproteins are increasingly ineffective to remove these proteins from the arterial intima.

The fact that patients with diabetes have less apolipoprotein deposits in the arterial wall is an intriguing finding of this study. Diabetes mellitus is a known risk factor for symptomatic atherosclerosis. In fact, 10 of 11 diabetic patients in our cohort are in the group with symptomatic disease. Endothelial dysfunction is a hallmark of diabetic macroangiopathy and microangiopathy. Therefore, diminished transendothelial influx may not explain the reduced level of apolipoprotein deposits in these patients. Poor metabolic control and hyperglycemia may facilitate nonenzymatic glycation of extracellular matrix proteins and, therefore, reduce avidity of apolipoproteins for proteoglycans. We showed by Western blotting that immune recognition of apoB and apoE by the antibodies used in this study is not affected by nonenzymatic glycation or other posttranslational modifications occurring in the blood. Diabetic patients have preferentially small LDL particles, and these atherogenic lipoproteins have been shown to increase binding and uptake by monococyte-derived macrophages. Our data could be explained as follows: in diabetic patients, scavenger receptor–mediated uptake of intimal lipoproteins is enhanced, and, therefore, intimal apolipoprotein deposits are reduced. Because LDL uptake and foam cell formation are c-Jun kinase 2–dependent processes, this signaling pathway may be activated during the pathogenesis of diabetic macroangiopathy. Selective uptake of cholesterol esters leaving back apolipoproteins extracellularly could be the favorite pathway of lipoprotein uptake in nondiabetic patients. Because we cannot definitely exclude that modifications of apolipoproteins in situ, by oxidation, or contain negatively charged glycosaminoglycan chains, which preferentially bind to basic amino acids.
by other mechanisms that are exclusively active in the arterial wall could explain the low immunohistochemical staining observed in diabetic patients, our findings need to be interpreted with caution.

The experimental approach described here is limited by the fact that, in this postmortem study, early lesional stages from vulnerable patients were analyzed after cardiovascular events occurred. Prospective data that would support the concept that incipient apolipoprotein deposits in the arterial wall identify presymptomatic patients who will develop symptomatic atherosclerosis later in their life are difficult to obtain. Despite this limitation, our findings encourage the use of lipoproteins containing apoE or apoB as targeting devices to deposit radioisotides or drugs to the diseased arterial intima, precisely to the site where atherosclerosis starts. In the non diabetic subset of patients, this may offer a promising diagnostic or therapeutic approach to reach not only advanced lesions but also healthy appearing arteries or, in the context of secondary prevention, less affected vascular beds.

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