The Apolipoprotein E Knockout Mouse: A Model Documenting Accelerated Atherogenesis In Uremia

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Abstract. Rodents do not develop spontaneous atherosclerosis. Currently, there is no good animal model to study the effect of uremia on atherosclerosis. This study evaluated whether apolipoprotein E knockout (ApoE−/−) mice are useful to study the effect of renal dysfunction on cardiovascular risk. ApoE−/− mice have decreased serum apolipoprotein E and exhibit lipid abnormalities and atherosclerosis even on a low-cholesterol diet. Ten-wk-old ApoE−/− mice were subtotally nephrectomised (SNX ApoE−/−; n = 8), uninephrectomised (UNX ApoE−/−; n = 5), or sham-operated (sham ApoE−/−; n = 5) and compared with their genetic controls (SNX C57/BL6; UNX C57/BL6; sham C57/BL6). After 12 wk, BP was measured intraarterially, blood samples were taken, and the experiment was terminated by perfusion fixation. The heart weight was determined, and quantitative morphologic analysis of intramyocardial arteries and aortic changes was performed. At the end of the experiment, heart weight and relative left ventricular weight were comparable in all groups. Intraarterial BP was somewhat higher in ApoE−/− mice compared with controls. Baseline serum cholesterol and triglyceride levels were higher in ApoE−/− mice than in C57/BL6. Atherosclerotic plaques were not present in sham or UNX C57/BL6, but minimal plaque formation was noted in some SNX control animals. In contrast, beginning plaques were seen even in untouched ApoE−/− mice, and strikingly increased plaque formation was noted in UNX and SNX ApoE−/− mice. Maximal plaque diameter (cross-section) was 37 ± 7 μm in SNX C57/BL6, 191 ± 90 μm in sham ApoE−/−, 323 ± 66 μm in UNX ApoE−/−, and 457 ± 17 μm in SNX ApoE−/−. The plaque morphology corresponded with that of early plaques characterized by foam cells and virtual absence of lymphocytes or smooth muscle cell infiltration. In conclusion, even mild renal dysfunction, i.e., after uninephrectomy, causes a dramatic increase in plaque size and aggressive morphology (foam cell rich soft plaques) in the animal model of the ApoE−/− mouse.

After the seminal communication of Lindner et al. (1), the high prevalence and possibly accelerated development of atherosclerosis have been well documented in patients with renal failure. Because of the multitude of classical and non-classical atherosclerosis risk factors, e.g., hypertension, dyslipidemia, hyperhomocysteinemia, increased oxidative stress, it has remained controversial whether the high burden of atherosclerosis in uremia can be explained by classical risk factors or whether uremia per se aggravates or accelerates atherosclerosis.

With the exception of diabetic patients with renal failure (2), there has been little documentation that atherosclerotic complications evolve more rapidly in patients with renal failure, and it has generally been difficult to reproduce atherosclerosis in animal models of renal failure.

The apolipoprotein E knockout mouse (ApoE−/−) is a well-established model to study atherogenesis (3,4). We reasoned that it might also constitute a suitable model to address the issue whether atherosclerosis evolves more rapidly or with a different morphology in renal failure.

Material and Methods

Animal Model

The ApoE−/− and their genetic background mice (C57BL/6) were obtained from the Bomholtgard Breeding and Research Center (Silkeborg, Denmark) at the age of 8 wk. Two weeks later, they were subjected to uninephrectomy (UNX) or subtotal nephrectomy (SNX) in a single session (resection of the right kidney, weight-controlled removal of 75% of the cortex of the left kidney) (5). Controls were subjected to sham operation (sham), i.e., decapsulation of the kidney. The animals received a low-cholesterol diet (Altromin 1324; Altromin Co., Lage/Lippe, Germany) containing 19% protein, 0.24% NaCl, and 5% fat (0.4% saturated, 1.29% unsaturated, 3.31% polyunsaturated fatty acids out of this). Food intake in the corresponding groups of ApoE−/− and C57BL6 mice was kept identical using a pairfeeding protocol. The experiment was terminated 12 wk after surgery under 0.2 ml of ketamine (Ketanest, 10%; WDT, Garbsen, Germany) and 0.02 ml of xylazine (Rompun, 2%; Bayer Co., Leverkusen) anesthesia. Blood samples were taken, and retrograde perfusion fixation via the abdominal aorta was performed using a 3% glutaraldehyde solu-
tion at body temperature or ice-cold NaCl for immunohistochemistry, respectively (6). After determination of heart weight, the orientator technique was applied and eight semithin sections (0.8 µm) per heart were cut and stained with methylenemnin and basic fuchsin (7). Approximately 3 mm distal of the aortic valve at the side of the aortic arch, a 2-mm-long segment of the aorta was prepared and carefully embedded in paraffin. Three-micrometer-thick sections were cut and stained with HE, PAS, Sirius red, Kossa, and Sudan. In addition, several sections were cut for immunohistochemical investigations (see below).

BP Measurement
Mean arterial BP and heart rate were measured in awake resting mice at the day of perfusion fixation as described previously (8). A polyethylene catheter (outer diameter, 400 µm) was inserted into the left carotid artery, and mice were placed in Plexiglas tubes. Catheters were connected to a pressure transducer (PRC-21K, amplifier MIO-0501, FMI) for continuous recording at 100 Hz (80586, DAS-0216, Keithly-Metrabyte; Lab Tech Notebook 10.2.1, Labtech) for 15 min.

Biochemistry
At the time of perfusion, fixation blood samples from the aorta were taken in EDTA tubes. Enzymatic investigations were performed using a Hitachi 917 747/400 analyser and Boehringer Mannheim reagents: serum creatinine and urea were measured. Serum cholesterol and triglycerides were measured by enzymatic methods using commercially available tests from Boehringer Co. (Mannheim, Germany).

Morphologic Evaluations
Heart. Wall thickness of small intramyocardial arteries was determined on eight isotropic uniform random (IUR) semithin sections per animal at a magnification of 400:1 by planimetry, as described in detail previously (5).

Aorta. Maximal plaque cross-section, plaque area per aortic circumference, and aortic wall thickness were determined by planimetry using an automatic image analyzing system (IBAS II; Kontron Co., Eching, Germany) combined with a counting grid eyepiece for defined movement over the section plane (Zeiss Co., Oberkochen; Germany) at a magnification of 400:1. Contours of the aortic plaques and profiles were marked manually with a cursor, and maximal diameter (cross-section) and plaque area per aortic circumference, as well as the thickness of aortic wall, were calculated.

Immunohistochemistry
Immunohistochemistry was performed on paraffin sections using antibodies against collagen IV (polyclonal rabbit, 1:100; Biotrend 2150–1470, Köln, Germany), fibronectin (polyclonal rabbit, 1:100; Sigma F3648, Deisenhofen, Germany), α-smooth muscle actin (polyclonal rabbit, 1:100; Sigma A5691, Deisenhofen, Germany), osteopontin (polyclonal rabbit, 1:100; Sigma A5691, Deisenhofen, Germany), osteonectin (polyclonal rabbit, 1:100; Immunodagnostik A4226.2, Benheim, Germany), nitrotyrosine as an index of oxidative stress (polyclonal rabbit, 1:400; Upstate Biotechnology 06 to 284, Lake Placid, NY), macropHages/moocytes (monoclonal rat, 1:5; Serotec MCA 519, Eching, Germany), proliferating cell nuclear antigen (PCNA, monoclonal mouse, 1:10; Biogenex, MU252UC, Hamburg, Germany), and the avidin-biotin technique (9). An antibody to localize RAGE-antigen (receptor for advanced glycation end products) was a kind donation of M. Shearman (Merck Sharpe and Dome, Essex, England); it was an established polyclonal goat anti-RAGE antiseraum, developed by immunizing goats with Escherichia coli–expressed recombinant human RAGE.

The sections were examined by light microscopy at a magnification of ×400 using a semiquantitative scoring system (scores 0 to ++ +). All antibodies had been tested for specificity in the mouse before. Negative controls were performed by omitting the primary antibody. To reduce run-to-run variations in the staining intensity, all stainings of the same antibody were performed in the same run for all Apoe−/− and C57/BL6 controls (6,9). All analyses were performed in a blinded manner, i.e., the observer was unaware of the experimental protocol.

Statistical Analyses
Data were normally distributed and are given as x ± SD. Differences between groups were analyzed using Kruskal-Wallis test or ANOVA followed by a post hoc Duncan multiple range analysis.

Results
Description of the Model
Model details are given in Table 1. The body weight was not significantly different in the C57/BL6 controls and Apoe−/− mice. Systolic BP was somewhat higher in sham Apoe−/− (136 ± 13 mmHg) compared with sham C57/BL6 controls (114 ± 3 mmHg). Compared with the untouched animals, BP was not altered after SNX in either C57/BL6 (112 ± 7 mmHg) or Apoe−/− (136 ± 13 mmHg). Serum creatinine concentrations increased marginally after SNX in C57/BL6 (0.45 ± 0.09) and Apoe−/− (0.53 ± 0.08) compared with the values in the respective baseline groups (0.23 ± 0.06 and 0.33 ± 0.04). The sham Apoe−/− mice showed comparable triglyceride values (43 ± 10 mg/dl) than the C57/BL6 (41 ± 17 mg/dl), whereas cholesterol values were approximately 10 times higher in Apoe−/− mice (647 ± 109 mg/dl) than in C57/BL6 (73 ± 13 mg/dl). Triglyceride and cholesterol concentrations were significantly higher after UNX and particularly after SNX in Apoe−/− mice than in C57/BL6. We have to acknowledge, however, that our animals were not strictly fastened before perfusion fixation (9:00 a.m.). Therefore, reliable determination of L-LDL and H-LDL was not possible. We would like to emphasize that the changes of the LDL fractions in Apoe−/− were characterized in detail in a previous study (10).

Findings in the Muscular and Central Elastic Arteries
Wall thickness of intramyocardial arteries (as a representative example of muscular arteries) was not significantly different in Apoe−/− compared with C57/BL6 control mice. In the aorta, no plaque formation was found in C57/BL6 controls with sham operation or UNX; in three of eight SNX C57/BL6 control animals small plaques were found. In contrast, in Apoe−/− mice, some plaques were seen even in sham animals. In sham Apoe−/−, the average number of plaques per aortic circumference was 4.25 ± 1.06 compared with 4.5 ± 1.09 in UNX Apoe−/− and 4.6 ± 0.55 in SNX Apoe−/−. Maximal plaque thickness (from cap to bottom; cross-section) was, however, significantly higher after UNX and SNX. The total aortic plaque area, summing up all individual plaques per aortic circumference, was progressively higher from sham to UNX to SNX Apoe−/− animals (Table 2).

Using light microscopy, the plaques in Apoe−/− mice were
characterized by numerous foam cells, absence of necrosis, minor lipid deposits (as documented by Sudan stain), scarce cholesterol clefts, absence of invasion of vascular smooth muscle cells (as documented by α-SMA staining), and virtually absent lymphocyte infiltration. Taken together, the morphology corresponded to that of an incipient plaque. By immunohistochemistry, there were no major differences in the aortic media between sham op and SNX Apoe/−/− mice, with the exception of somewhat more marked staining for anti-collagen IV and fibronectin. Striking changes, in particular a markedly higher expression of nitrotyrosine in SNX Apoe/−/− were noted in the non-plaque-bearing intima (Table 3). In plaques of SNX Apoe/−/− as compared with C57/BL6 SNX, striking but discontinuous staining for nitrotyrosine as an index of oxidative stress was also noted. Figure 1 provides a representative example of a plaque in a SNX Apoe/−/− mouse with strong staining for nitrotyrosine (A) also in the unaffected intima and strong staining for RAGE (B), osteopontin (D), and some staining of fibronectin (C). The Kossa stain consistently showed absence of calcified deposits.

The aortic wall thickness was similar in sham op C57/BL6 controls and in sham Apoe/−/−; it was significantly higher, however, in SNX animals, both C57/BL6-controls and in sham Apoe/−/− (Table 2). Thickening of the aortic wall was associated with hyperplasia and hypertrophy of media smooth muscle cells and with increased extracellular matrix deposition.

**Discussion**

To the best of our knowledge, the Apoe/−/− mouse is the first model without dietary intervention that shows more severe and more rapid development of atherosclerotic plaques in a
Table 3. Results of immunohistochemic investigations of the non-plaque–bearing aortic wall (by semiquantitative scoring)\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Nitrotyrosine</th>
<th>RAGE</th>
<th>PCNA</th>
<th>Macrophages</th>
<th>Osteopontin</th>
<th>Collagen IV</th>
<th>Fibronectin</th>
<th>(\alpha)-SMA</th>
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<td>Control (C57BL/6) SNX ((n = 5))</td>
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<td>media</td>
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<tr>
<td>Apoe(^{-/-}) SNX ((n = 5))</td>
<td>+++</td>
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*0, no expression; +, moderate expression; ++, strong expression; ++++, extremely strong expression. RAGE, receptor for advanced glycation end products; PCNA, proliferating cell nuclear antigen.*

Figure 1. Representative immunohistologic findings in aortic plaques and the aortic wall of SNX Apoe\(^{-/-}\) mice. Negative controls are given as inserts at lower left corner. m, aortic media; p, plaque; l, aortic lumen; o, outside. (A) Representative immunohistologic findings of nitrotyrosine protein expression. Please note strong staining for nitrotyrosine in the center of the plaque (arrow). (B) Representative immunohistologic findings of RAGE protein expression. Strong RAGE expression is also found in the center of the plaque as well as in the endothelium around the plaque. (C) Representative immunohistologic findings of osteopontin protein expression. Osteopontin is strongly expressed in the foamy plaque areas and in the aortic intima and parts of the underlying and surrounding media. (D) Representative immunohistologic findings of fibronectin protein expression. Fibronectin expression can be seen at the bottom of the plaque, in its outer surface and partly also in the aortic media.
atherogenesis is a high priority. We propose that the Apoe−/− genes on survival of patients with renal failure, elucidation of diabetic mice could be abrogated by scavenging of AGE by RAGE, the receptor for advanced glycation end products. Furthermore, the plaques showed massive expression of RAGE, the receptor for advanced glycation end products (AGE), but no evidence of cell proliferation (indicated by negative staining for PCNA) or infiltration by macrophages/smooth muscle actin. In view of the recent interest in accelerated calcification of plaques in uremia, we emphasize the consistent absence of staining for calcium in our mouse model, but we admit that the duration of the experiment was probably too short to give rise to plaque calcification. An overriding role of oxidative stress is suggested by the strongly positive staining for nitrotyrosine, which was not confined to the plaque per se, but was also found in the morphologically intact surrounding intima.

Because of the immense impact of atherosclerotic complications on survival of patients with renal failure, elucidation of the uremia-specific factors aggravating and accelerating atherogenesis is a high priority. We propose that the Apoe−/− mouse provides a suitable model to analyze the cellular and molecular mechanisms that are responsible for this complication of renal disease.

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