Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice

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Abstract Murine strains susceptible to atherosclerosis provide valuable models to study factors involved in atherogenesis. In some murine models, limited hypercholesterolemia can be achieved and lesions develop primarily in the aortic origin, in the vicinity of the aortic valve. In other models such as LDL receptor-deficient and apoE-deficient mice, diet-induced or spontaneous hypercholesterolemia and atherogenesis are much greater. To determine whether lesion formation in the aortic origin, where particular pathogenic conditions may exist, correlates with lesion formation throughout the entire aorta, we measured the extent of atherosclerosis in both areas in 8 apoE- and 11 LDL receptor-deficient mice fed cholesterol-rich diets for 3–6 months, as well as in 9 C57BL/6 mice fed an atherogenic diet for a year, using two different morphometric methods. Both apoE-deficient and LDL receptor-deficient mice developed extensive lesions throughout the aorta, and in these models a significant correlation was observed between the extent of lesions in the entire aorta (measured as percent of surface area) and that at the aortic origin (measured as averaged lesion area per cross-section) ($r = 0.77$, $P < 0.0001$). In contrast, the plasma cholesterol levels achieved in C57BL/6 mice were much lower, and atherosclerotic lesions were found almost exclusively in the aortic origin. These results demonstrate that in murine models developing extensive aortic lesions, both morphometric methods provide valid and complementary information on the degree and distribution of atherosclerosis, and suggest that under severe atherogenic conditions lesion formation throughout the aorta is determined by the same pathological factors, in each model. Comparison of the extent of atherosclerosis in the entire aorta between genders also showed that male LDL receptor-deficient mice had significantly more lesions than females (29.2 vs. 14.8%, $P < 0.005$, n = 16). A similar trend was also seen in apoE-deficient mice.–**Tangirala, R. K., E. M. Rubin, and W. Palinski.** Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. *J. Lipid Res.* 1995. **36:** 2320–2328.

Supplementary key words arteriosclerosis • morphometry

Murine models are increasingly used to study the pathogenic mechanisms of atherosclerosis (1–3). Several genes determining susceptibility to atherosclerosis have been identified in mice (4–6), and inbred mouse strains that develop lesions in response to high-fat diets containing cholic acid have been characterized (7). In addition, transgenic and gene-knockout techniques have provided a number of murine strains with altered lipoprotein metabolism and atherosclerosis (8–11). However, the observation that lesion formation in C57BL/6 mice and in many gene-targeted strains seems to occur first or predominantly in the aortic origin, where particular hemodynamic conditions may exist, raised the question whether these lesions represent a model of atherosclerotic lesions in other species. Recently, two murine models, apolipoprotein E (apoE)-deficient and low density lipoprotein (LDL) receptor-deficient mice, were generated that develop extensive atherosclerosis throughout the entire aorta (12–14). ApoE-deficient mice are characterized by spontaneous hyperlipidemia and atherogenesis. In LDL receptor-deficient mice, extensive hypercholesterolemia and lesion formation can be induced by high-fat diets. Lesion formation in these mice does not require the addition of cholic acid to the diet. The morphology, cellular compo-

Abbreviations: LDL, low density lipoprotein; apo, apolipoprotein; PBS, phosphate-buffered saline.

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sition, and predilection sites of atherosclerotic lesions in these mice have been well characterized and show many similarities to those found in other animal models of atherosclerosis (15–19). In view of these similarities and their extensive lesions, apoE- and LDL receptor-deficient mice may be particularly suitable models for the investigation of atherogenic mechanisms, as well as for anti-atherogenic intervention studies.

To date, the extent of atherosclerosis in murine models has usually been determined from cross-sections of the aortic origin, using modifications of a technique described by Paigen et al. (20). This method uses sequential sections through the heart and aortic origin, and determines the lesion area in a constant number of sections, beginning from an anatomical reference point such as the aortic valve leaflets. An improved variation of this method using computer-assisted evaluation of 60 progressive sections covering the initial 1.2 mm of the aortic origin has recently been reported (21). Even though this method is labor-intensive, it has proven to be very useful in evaluating differences in atherogenicity in those strains or under experimental conditions in which lesion formation is predominantly found in the aortic root. However, such a method would not be practical for the quantification of lesions in strains with extensive atherosclerosis involving major portions of the aorta.

Recently, we described a morphometric method that quantitates the extent of atherosclerosis in the entire murine aorta (17). This method utilizes computer-assisted image analysis of color images of Sudan-stained flat preparations of the aortic tree (from the heart to the iliac bifurcation) and determines the percentage of surface area affected by atherosclerosis.

To date, it is unknown whether the size of lesions in the aortic origin correlates with the extent of atherosclerosis in the entire aorta. To address this question, we quantified atherosclerosis in apoE-deficient mice, LDL receptor-deficient mice, and C57BL/6 mice fed a variety of atherogenic diets. The gender of the animals, the particular diet used for each strain, and the length of the intervention period were selected in such a way as to induce substantial degrees of atherosclerosis in the three models. Furthermore, gender-specific differences in atherosclerosis of the entire aorta were studied in apoE- and LDL receptor-deficient mice.

METHODS

Mice

The influence of gender on atherogenesis was determined using data on the extent of atherosclerosis in the entire aorta from 16 LDL receptor- and 15 apoE-deficient mice. These mice constituted the control groups of two anti-atherogenic intervention trials that will be published separately. The genetic background, the age of the animals at the start of the intervention period, the composition of the diets, and the length of the intervention were identical to those of the mice subsequently used for comparative morphometric analysis. Homozygous apoE-deficient mice (hybrids with a C57BL/6 × 129ola background) from a colony established from breeders provided by Dr. Jan Breslow (12) were fed a cholate-free rodent diet (Harlan Teklad, Madison, WI) containing 0.15% cholesterol for 6 months. Homozygous LDL receptor-deficient mice (with a C57BL/6J × 129Sv background) from a breeding colony established from animals provided by Jackson Laboratories (Bar Harbor, ME) were fed the same diet, supplemented with cholesterol to a final concentration of 1% for 6 months.

Comparative morphometry of atherosclerosis was carried out in 8 female apoE-deficient and in 11 male LDL receptor-deficient mice fed the diets described above for 3 months or 6 months. All apoE- and LDL receptor-deficient mice were 5–6 months old at the start of the dietary intervention. The average body weight of apoE-deficient mice at the time of killing was 37.1 ± 2.7 g. LDL receptor-deficient mice weighed 24.7 ± 0.9 g (n = 3) and 28.9 ± 1.7 g (n = 8), after 3 and 6 months on the diet, respectively. In addition, we also studied a group of 9 female C57BL/6 mice, age 14 months (Charles River, Wilmington, MA) that had been fed a high-fat diet containing 1.25% cholesterol, 15% fat, and 1% cholate (ICN, Costa Mesa, CA) for 12 months in the laboratory of Dr. Rubin. The average body weight of C57BL/6 mice at the time of killing was 29.0 ± 0.7 g.

The total plasma cholesterol and triglyceride levels were determined by an automated enzymatic procedure (Boehringer Mannheim Diagnostics), at 4–6 week intervals during the dietary intervention period, and immediately prior to killing the mice.

Preparative methods

The aortic tree of mice was prepared as previously described (17). After a 20-min perfusion with phosphate-buffered saline (PBS) containing 20 μmol/l BHT and 2 μmol/l EDTA, pH 7.4, via a canula inserted into the left ventricle, the aorta was fixed by perfusion with formal–sucrose (4% paraformaldehyde, 5% sucrose, 20 μmol/EDTA, pH 7.4). The aorta was exposed, minor branching arteries (e.g., the intercostal arteries) were cut off, and the adventitia was removed in situ as far as possible. The aorta was then opened longitudinally, from the heart to the iliac arteries, while still attached to the heart and major branching arteries. The primary incision followed the ventral side of the aorta and the inner curvature of the arch. To obtain a flat preparation

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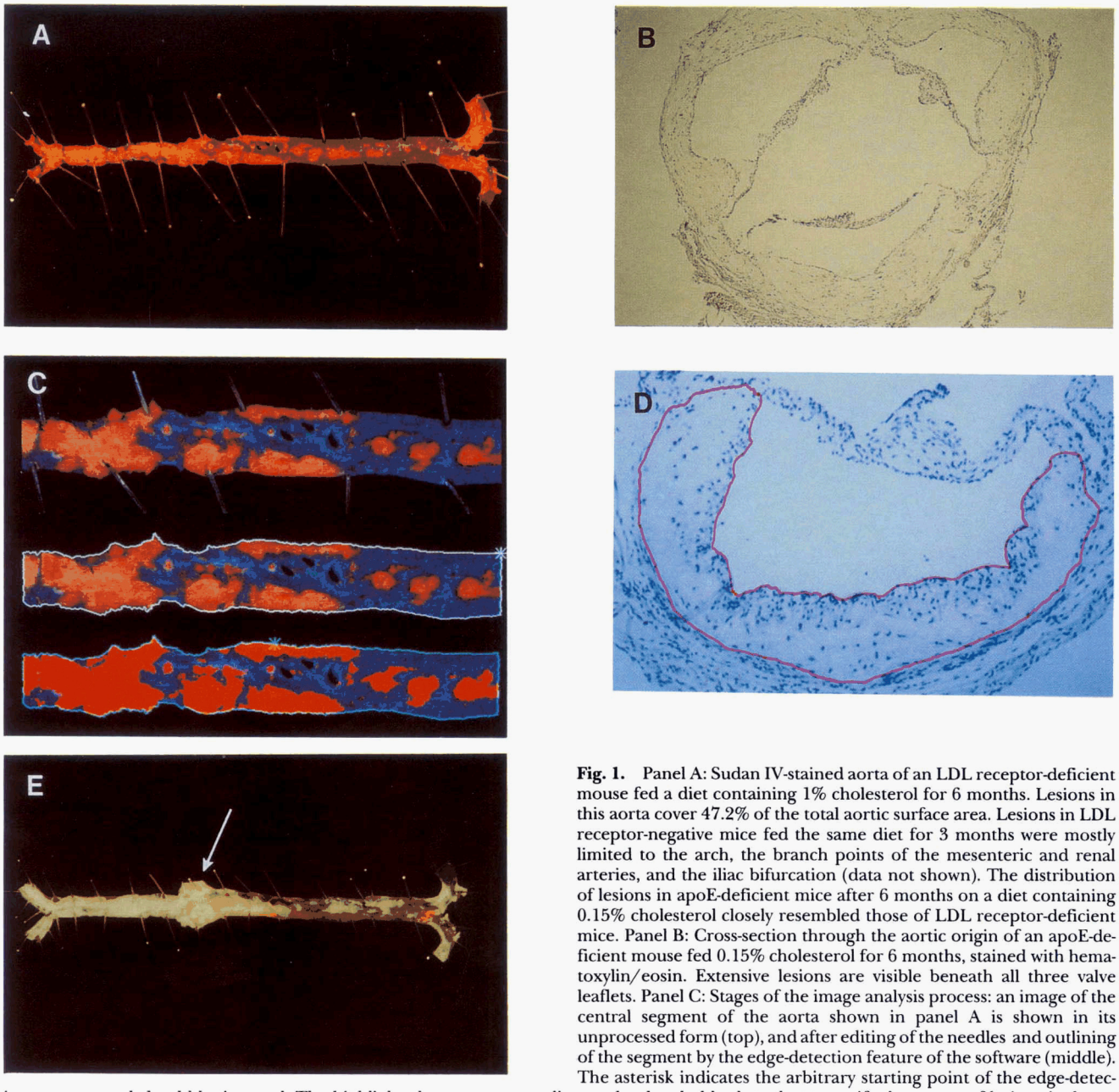

Fig. 1. Panel A: Sudan IV-stained aorta of an LDL receptor-deficient mouse fed a diet containing 1% cholesterol for 6 months. Lesions in this aorta cover 47.2% of the total aortic surface area. Lesions in LDL receptor-negative mice fed the same diet for 3 months were mostly limited to the arch, the branch points of the mesenteric and renal arteries, and the iliac bifurcation (data not shown). The distribution of lesions in apoE-deficient mice after 6 months on a diet containing 0.15% cholesterol closely resembled those of LDL receptor-deficient mice. Panel B: Cross-section through the aortic origin of an apoE-deficient mouse fed 0.15% cholesterol for 6 months, stained with hematoxylin/eosin. Extensive lesions are visible beneath all three valve leaflets. Panel C: Stages of the image analysis process: an image of the central segment of the aorta shown in panel A is shown in its unprocessed form (top), and after editing of the needles and outlining of the segment by the edge-detection feature of the software (middle). The asterisk indicates the arbitrary starting point of the edge-detection process, and should be ignored. The highlighted area corresponding to the threshold selected to quantify the extent of lesions is shown separately, at the bottom (rather than overlapping with the processed image, as during the imaging), to allow comparison with the lesions shown in the middle image. At this stage of the analysis the aorta (viewed through a stereo microscope) is used to verify that the highlighted threshold correctly reflects the actual lesions. Panel D: Image analysis of one segment of a cross-section of the aortic origin of an LDL receptor-deficient mouse fed 1% cholesterol for 6 months. The red line indicates the lesion area beneath one leaflet quantified by the software. Panel E: Unstained aortic tree from an apoE-deficient animal fed 0.15% cholesterol for 6 months showing a gross dilatation at the level of the renal arteries (arrow). Aneurysms were frequently observed in both apoE- and LDL receptor-deficient animals, and unusually extensive and advanced lesions distal to an apparent aneurysm were found in some cases in which the animal had survived the event.

for imaging, a second incision was made along the outer curvature of the arch. The remaining branches were then cut off, and the aorta (from the heart to approximately 3 mm distal to the iliac bifurcation) was removed and pinned out on a black wax surface in a dissecting pan, using 0.2-mm diameter stainless steel pins (Fine Science Tools, Foster City, CA). Each pin was placed either well within a lesion or well away from lesions, so that it could easily be edited out in the processed image. The aortas were then subjected to an additional fixation with formal–sucrose for 12 h, and stained with Sudan IV (17) (**Fig. 1A**). The above-described preparation of the aorta is a relatively simple procedure and can be completed in about an hour.

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The upper half of the heart was dissected, fixed overnight in formal–sucrose, and paraffin-embedded. Sequential 7-μm thick sections were cut from the apex towards the base of the heart until the aortic valve leaflets appeared. From this point, 17 sections representing every second serial section over a distance of 238 μm were collected and stained with hematoxylin and eosin (Fig. 1B).

Quantitation of atherosclerosis

The extent of atherosclerosis in the entire aorta was quantitated by an improved variation of the morphometric method previously described (17). To obtain better resolution, three images of segments of the Sudan IV-stained mouse aorta were captured with a Sony DXC-960MD three-chip color video camera. A Sudan-stained aorta and one such partial image are shown in Figs. 1A and 1C (top). Image analysis was performed on 24-bit color images, using Optimas 4.0 image analysis software (Bioscan, Seattle, WA), an Oculus TCX true color frame grabber with 4 megabytes of frame buffer memory (Coreco, St-Laurent, Quebec) and a separate VGA image monitor for the processed image. After retouching the needle holes in the captured image, the edge of the segment was traced using an automated feature of the software (Fig. 1C, middle). The extent of atherosclerosis was determined using a selection of threshold ranges in the three basic colors (Fig. 1C, bottom). The total aortic surface area (corrected by subtracting the area of the orifices of branching arteries) and the lesion area were then calculated by the program. The extent of atherosclerosis was expressed as the percent of surface area of the entire aorta covered by lesions.

The threshold ranges were initially determined on a few randomly selected arteries. Several atherosclerotic areas were defined in each of these arteries, and the threshold ranges of each area were established by the program. The lowest and highest delimiting values obtained from these determinations were then used as the "default" threshold. The accuracy of the default threshold was verified by comparing the shape and extent of the highlighted threshold area in the processed image (Fig. 1C) to that of the actual lesions in the arteries (viewed through a stereo microscope). The selection was also verified by a second investigator blinded for the identity of the image analyzed. The "default" threshold could be used for the subsequent analysis of most arteries, substantially reducing operator input. Nevertheless, during image analysis, the actual aortas were always used for reference, to ensure that the threshold truly reflected atherosclerotic lesions, and adjustments to the threshold ranges were made for arterial segments for which the default threshold could not be used, e.g., because of differences in staining intensity.

The extent of atherosclerosis in the cross-sections of the aortic origin was determined with the same image analysis system. Three images were captured of each hematoxylin/eosin-stained cross-section through the aortic origin, each roughly comprising the area under one valve leaflet, using the same video camera mounted on a Nikon Mikrophot FX microscope (Nikon, Garden City, NY). The luminal edge of lesions was traced with the help of an edge-detection feature of the program (Fig. 1D). The intima–media border was easily distinguishable in the hematoxilin/eosin-stained sections, and was traced manually, as the abluminal limit of the lesions does not lend itself to automated edge-detection (advanced murine lesions frequently disrupt the internal elastic lamina and involve the media). This approach was given preference over a threshold-based quantification of oil red O-stained lesions, as varying cell composition and lipid content of lesions does not guarantee staining of all lesion areas. The size of the lesions was then determined by the program. Lesion areas from the three images were added to obtain the lesion area for the entire cross-section, and results were reported as the average of all 17 aortic sections analyzed. As operator-input is more critical in the measurement of the cross-sections, all determinations were performed by the same operator.

Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA) and Student's unpaired *t*-test. Data shown are mean ± SEM. Correlations and confidence intervals for regression lines were determined by Plot-it software (Scientific Programming Enterprise, Haslett, MI).

RESULTS

Comparison of atherosclerosis in male and female apoE- and LDL receptor-deficient mice

Gender-specific differences in atherosclerosis have been described in several species, including mice. To determine whether this is also true for the extent of atherosclerosis in the entire aorta of apoE- and LDL receptor-deficient mice, we first analyzed data from 16 LDL receptor-deficient and 15 apoE-deficient mice that had been exposed to the same diets and experimental conditions as the apoE- and LDL receptor-deficient mice subsequently used for comparative morphometry. As shown in **Table 1,** male LDL receptor-deficient mice showed significantly more atherosclerosis than females (29.2 ± 3.1% of the aortic surface vs. 14.8 ± 3.2%; $P < 0.005$), whereas the average plasma cholesterol levels of males and females were similar. The difference in the extent of aortic atherosclerosis was found both in the

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TABLE 1. Comparison of the extent of atherosclerosis, the average plasma cholesterol levels, and body weights in apoE- and LDL-deficient mice

Variable	ApoE-Deficient		LDL Receptor-Deficient	
	Male (n = 7)	Female (n = 8)	Male (n = 8)	Female (n = 8)
Atherosclerosis (%)				
Entire aorta	27.4 ± 6.3	17.1 ± 1.6 NS	29.2 ± 3.1	14.8 ± 3.2[a]
Arch	46.9 ± 8.0	51.6 ± 2.0 NS	62.2 ± 3.3	47.7 ± 3.5[b]
Thoracic + abdominal	23.8 ± 7.4	9.2 ± 1.8[c]	22.5 ± 3.1	8.1 ± 3.9[b]
Average total plasma cholesterol (mg/dl)	1916 ± 187	2489 ± 275 NS	895 ± 116	992 ± 80 NS
Body weight (g)[d]	43.5 ± 4.8	39.2 ± 1.7 NS	28.9 ± 1.1	22.8 ± 0.9[b]

Data are given as mean ± SEM; NS, not significant.
[a] $P < 0.005$.
[b] $P < 0.01$.
[c] $P < 0.06$.
[d] At the end of 6 months dietary intervention.

arch and in the thoracic and abdominal aorta. ApoE-deficient mice also showed a trend towards more lesions in males than in females. However, the variability between males was much greater than that in females or in LDL receptor-deficient mice, and the differences did not reach statistical significance. It is also noteworthy that the extent of aortic atherosclerosis observed in males of both strains was similar, although the average plasma cholesterol levels were much higher in apoE- than in LDL receptor-deficient mice.

Based on these data, we decided to study the correlation between lesion formation in the aortic origin and in the aortic tree in mice of the same gender, in order to reduce the variability within each strain. However, the extent of atherosclerosis in the aortic tree could be expected to be very similar in male apoE- and male LDL receptor-deficient mice, as shown in Table 1. To avoid data clustering, which would have made it difficult to establish a possible correlation between the extent of lesions at both sites, we therefore opted to study male LDL receptor-deficient and female apoE-deficient mice.

Comparative morphometry of apoE-deficient, LDL receptor-deficient, and C57BL/6 mice

The average total plasma cholesterol values of eight LDL receptor-deficient and eight apoE-deficient mice after 6 months on high-fat diet (1937 ± 108 mg/dl and 1038 ± 84 mg/dl, respectively) and significantly exceeded those of C57BL/6 mice (194 ± 50 mg/dl). The average plasma cholesterol level of the three LDL receptor-deficient mice fed the same diet for 3 months was 1569 ± 348 mg/dl.

The extent of atherosclerosis in the aortic origin and in the entire aorta of the three murine models is shown in **Fig. 2.** All nine C57BL/6 mice maintained on a high-fat diet for 12 months developed sizable atherosclerotic lesions in the aortic origin. In all animals, lesions were found in every one of the 17 cross-sections of the sinus of Valsalva, and consisted mostly of fatty streaks and transitional lesions underneath one or several of the valve leaflets. The average lesion area per section was 121,091 ± 9,257 μm^2 (mean ± SEM). However, only two of the nine mice displayed atherosclerotic lesions in the aortic tree (less than 0.5% of the surface area, in each case), localized primarily in the aortic arch. In contrast, both LDL receptor-deficient and apoE-deficient mice showed extensive lesions throughout the entire aorta (Figs. 2 and 1A). Atherosclerotic lesions covered 18.9 ± 1.2% of the aortic surface of apoE-deficient mice fed 0.15% cholesterol (n = 8), and 29.2 ± 0.7% of the aortic surface of LDL receptor-deficient mice fed 1% cholesterol for 6 months (n = 8). The three LDL receptor-deficient mice killed after 3 months of dietary intervention showed 8.5 ± 0.2% of atherosclerosis. Although the number of animals in the 3-months group was too small for a meaningful statistical analysis, these results are consistent with our experience that in these mice atherosclerosis generally progresses with time.

LDL receptor-deficient and apoE-deficient mice also showed extensive atherosclerosis in the aortic origin (Figs. 2, 1B, and 1D). The size of these lesions was much greater than that observed in C57BL/6 mice (282,849 ± 10,506 μm^2, and 808,491 ± 66,333 μm^2, in LDL receptor-deficient mice after 3 and 6 months, respectively, and 640,530 ± 76,306 μm^2 in apoE-deficient mice after 6 months). Furthermore, their lesions were generally more advanced, frequently showed a necrotic core, and often extended to the valve leaflets.

To examine whether the extent of atherosclerosis in the entire aorta (expressed as percent of surface area) reflected that in the aortic origin (expressed as average lesion area per section), we analyzed the correlation between these two parameters. An analysis of the LDL receptor-deficient mice (n = 11) showed a very good correlation between the two parameters ($r = 0.83$, $P < 0.001$). Analysis of the apoE-deficient mice (n = 8) yielded a correlation line with similar slope, but did not quite reach statistical significance ($r = 0.64$, $P < 0.088$).

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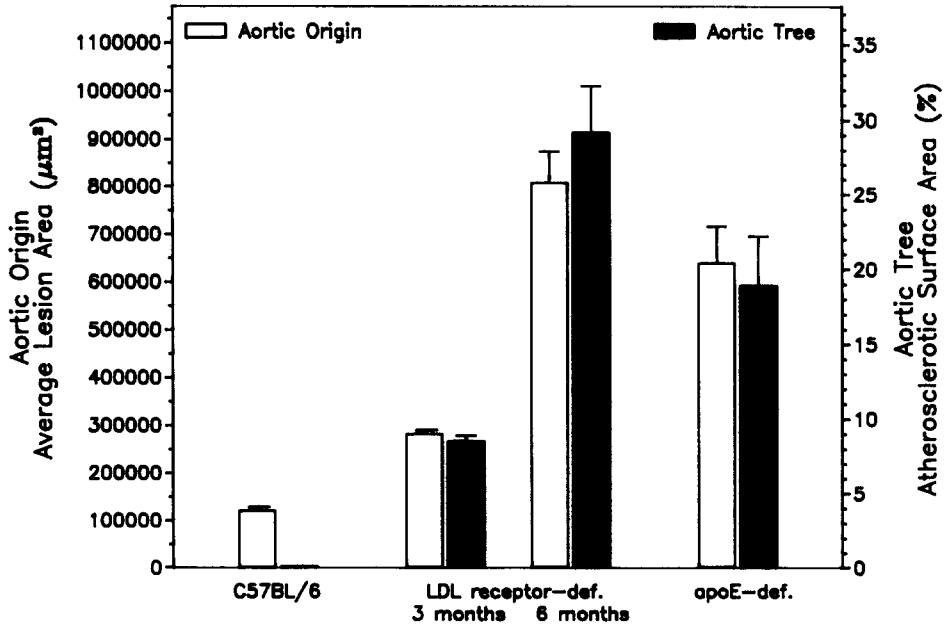

Fig. 2. Extent of atherosclerosis in C57BL/6 mice fed an atherogenic diet for 1 year (n = 9), LDL receptor-deficient mice fed 1% cholesterol for 3 or 6 months (n = 3 and n = 8, respectively) and apoE-deficient mice fed 0.15% cholesterol for 6 months (n = 8). The average lesion size in cross-sections through the aortic origin (open bars) is indicated in μm² (left scale), the extent of atherosclerosis in the entire aorta (closed bars) is shown as a percentage of the surface area (right scale). Values are mean ± SEM.

When data from all LDL receptor- and apoE-deficient animals were analyzed together, a significant correlation between the extent of lesions in the aortic origin and that in the entire aorta was observed ($r = 0.77$, $P < 0.0001$) (Fig. 3). Similar results were also obtained when the three LDL receptor-deficient mice examined after 3 months were excluded ($r = 0.65$, $P < 0.005$). In both LDL receptor- and apoE-deficient mice fed atherogenic diets for 6 months, neither the extent of lesions in the aortic origin nor that in the entire aorta correlated with the average plasma cholesterol levels.

DISCUSSION

Murine models of atherosclerosis have gained broader acceptance with the advent of gene-targeted mice that develop extensive lesions throughout the aorta and show many characteristic features of atherosclerosis found in other animal models and humans (12–19). In the present study, we demonstrated that in two murine models with extensive aortic atherosclerosis, i.e., apoE-deficient and LDL receptor-deficient mice, the extent of atherosclerosis in the entire aorta correlates with the size of lesions in the aortic origin ($P < 0.0001$) (Fig. 3). These results suggest that in each of these models the extent of atherosclerosis in the aortic origin and elsewhere in the aorta is determined by the same pathologic factors. The degree of correlation between the extent of lesion formation in the aortic root and that in the entire aorta observed in the pooled data from both strains is remarkable, considering that the genetic background and the lipoprotein composition of apoE- and LDL receptor-deficient mice are different.

The plasma cholesterol levels achieved in C57BL/6 mice were much lower than those achieved in the other two murine models. Despite the greater duration of the dietary intervention only two of the nine C57BL/6 mice developed minimal lesions in the aortic tree, whereas all of them showed consistent atherogenesis in the aortic origin. The almost complete absence of lesions in the aortic tree of C57BL/6 mice was somewhat unexpected, as such lesions have previously been reported (20). It is conceivable that a threshold level of hypercholesterolemia must be exceeded to induce the progression of lesions to advanced stages and the development of lesions in the thoracic and abdominal aorta. However, it cannot be ruled out that when this threshold is not reached, the formation of lesions in the vicinity of the aortic valve is determined, at least in part, by pathologic mechanisms particular to the aortic origin.

The data obtained in the present study provided an opportunity to compare the two methods of quantifying atherosclerosis in mice. The method commonly used consists of the quantification of lesion size at a single predilection site, the aortic origin (20). As lesions at this site seem to appear at an earlier time than lesions elsewhere in the aorta in many genetically susceptible and gene-targeted strains, this method makes it possible

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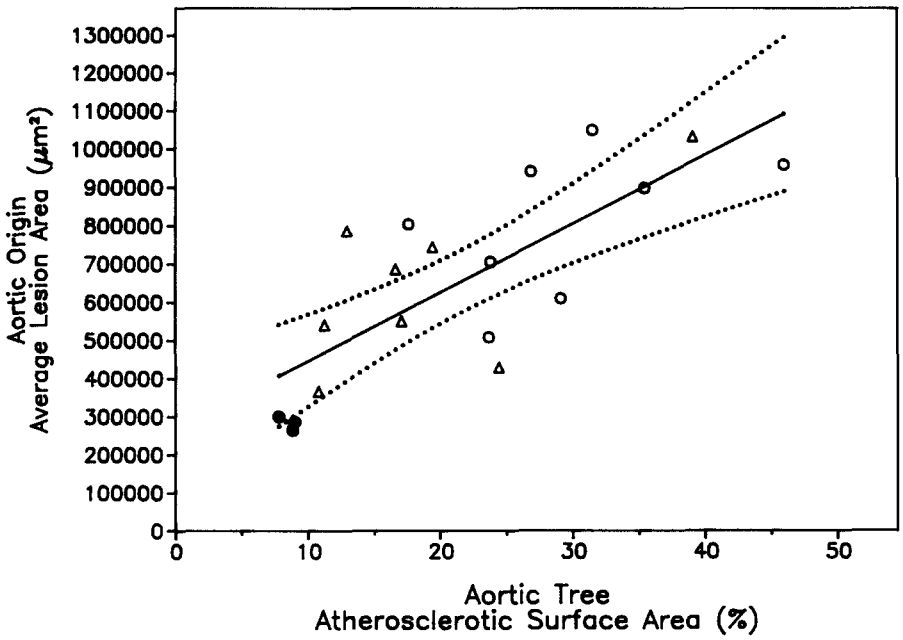

Fig. 3. Correlation between the extent of atherosclerosis in the aorta tree and in the aortic origin. Individual values shown are LDL receptor-deficient mice after 3 months (closed circles) or 6 months on atherogenic diet (open circles) and apoE-deficient mice after 6 months (open triangles). Dotted lines indicate the 95% confidence interval of the regression line. The coefficient of correlation was $r = 0.77$ ($P < 0.0001$).

to assess lesion formation after a relatively short dietary intervention or under experimental conditions that do not induce extensive atherogenesis. Furthermore, sectioning of the heart poses few problems, whereas preparation of the entire murine aorta was perceived as technically more challenging (20). The main advantage of this method is that the lesion volume can easily be calculated from the lesion areas. In addition, cross-sections may provide the basis for a three-dimensional reconstruction of lesions. A potential problem of this quantitation method lies in the possibility that anatomical differences of the sinus of Valsalva, turbulent flow patterns with associated areas of low shear stress in the vicinity of the aortic valve, and more pronounced pressure differences than in the distal aorta may enhance atherogenesis at this particular site. Furthermore, the extent of atherosclerosis in the aortic origin, or any other small segment of the aorta, may not reflect the extent of atherosclerosis in the entire aorta. Although specific predilection sites for lesions have been identified in mice, substantial variability has been observed at some of these sites, e.g., the orifices of intercostal arteries (17). Similar variability may occur in the aortic origin. To increase the accuracy of the measurement, some authors therefore extend the sectioned area to more than a millimeter (21), a valid but very labor-intensive approach.

Recently, a morphometric method was developed that makes it possible to determine the extent of atherosclerosis in the entire aorta (17). This method is an adaptation of that frequently used for intervention studies in other animal models (22–26) and for epidemiological studies in humans (27, 28). By utilizing Sudan-stained flat preparations of the aorta, this method provides valuable additional information on lesion distribution along the aorta. It allows one to determine the number and shape of lesions, to identify predilection sites, and to detect atypical lesions in individual animals. For example, it was established that predilection sites in both apoE- and LDL receptor-deficient mice consisted of the arch, the orifices of the brachiocephalic trunk, the common carotid artery, the subclavia, the branch points of the mesenteric and renal arteries, and the iliac bifurcation. Distribution and size of lesions at these sites were very similar in animals with the same overall extent of atherosclerosis. In animals with very extensive atherosclerosis, the additional atherosclerosis was found predominantly in the abdominal aorta (Fig. 1A). In contrast to other animal models such as the LDL receptor-deficient rabbit, lesions near the orifices of intercostal arteries were far less consistent in mice. These lesions often showed significant protrusion into the lumen, but extensive atherosclerosis in the thoracia aorta occurred relatively late. Furthermore, advanced lesions in mice frequently involve the media, and the occurrence of aortic aneurysms has been observed in both apoE- and LDL receptor-deficient mice. As shown in Fig. 1E, such aneurysms may have profound effects on lesion forma-

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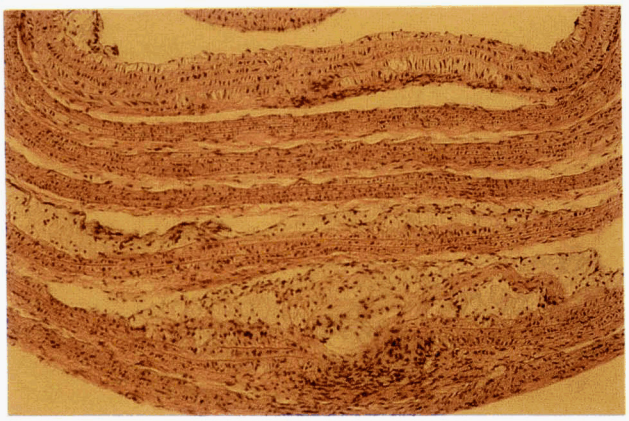

Fig. 4. Partial image of a section through an aorta from an apoE-deficient mouse prepared by a variation of the method described in reference 29. The thoracic and abdominal segments of this aorta were longitudinally rolled on themselves ("cake-rolled"), paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Thus, each "coil" represents a longitudinal section through the entire length of the aorta. Several lesions of different stages are visible in this section, including an advanced atheroma involving the media. In such preparations, lesion areas can be determined by the same procedure described for cross-sections through the aortic origin, and lesion volumes may then be calculated from results obtained in all serial sections. However, foam cell-rich murine lesions may be disrupted by this procedure.

tion distal to the rupture site, which may not be recognized as unusual by a method using cross-sections.

The computer-assisted evaluation of an entire aorta is a fast (approximately 1 hour per aorta) and, when performed with a color image analysis system, relatively accurate procedure with limited operator dependence. It may therefore be the method of choice for intervention studies on large numbers of animals, in murine models that develop significant aortic atherosclerosis. Furthermore, it allows comparison with the results obtained in other species. The main drawback of this method consists of the failure to assess the three-dimensional nature of lesions. As the height of lesions is not measured, no differentiation between early and advanced lesions of similar area is possible. However, the assessment of atherosclerosis in flat preparations of the entire aorta could be complemented by the determination of lesion areas and volumes in longitudinal sections through the thoracic and abdominal aorta by the method proposed by Daley and colleagues (29). This method uses cross-sections through aortas longitudinally rolled on themselves. As shown in Fig. 4, the approach originally described for rabbits can also be used to measure lesion areas in the thoracic and abdominal segments of murine aortas. However, is not suitable for the arch, an important predilection site of murine atherosclerosis.

While neither of the two methods by itself provides a complete quantitative assessment of atherosclerosis, a very thorough quantitative evaluation of murine atherosclerosis can be achieved by combining both techniques. The correlation between the lesion size in the aortic origin and that in the entire aorta also suggests that the selection of arbitrary end points for studies involving apoE- and LDL receptor-deficient mice is justified. For each set of experimental conditions (i.e., extent and duration of hypercholesterolemia), a site may be selected for the quantitation of atherosclerosis in which atherosclerosis has reached a stage that allows detection of either progression or regression. Predilection sites such as the arch and the branch points of the mesenteric and renal arteries, but most of all the aortic origin itself are clearly more suitable to measure the extent of lesions under conditions of less extreme hypercholesterolemia, whereas the aortic tree may be particularly suitable for regression studies requiring extensive atherosclerosis and more advanced lesions.

Finally, the present study showed that gender differences in the extent of atherosclerosis in the entire aorta are remarkably pronounced in LDL receptor-deficient mice (males: 29.2 ± 3.1%, females: 14.8 ± 3.2%). These mice may therefore be a suitable model in which to investigate the role of estrogens in atherogenesis.

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REFERENCES

1. Breslow, J. 1993. Transgenic mouse models of lipoprotein metabolism and atherosclerosis. *Proc. Natl. Acad. Sci. USA.* **90:** 8314–8318.
2. Witztum, J. L. 1993. Murine models for study of lipoprotein metabolism and atherosclerosis. *J. Clin. Invest.* **92:** 536–537.
3. Rubin, E. M., and D. J. Smith. 1994. Atherosclerosis in mice: getting to the heart of a polygenic disorder. *Trends Genet.* **10:** 199–203.
4. Paigen, B., D. Mitchell, K. Reue, A. Morrow, A. J. Lusis, and R. C. LeBoef. 1987. Ath-1, a gene determining atherosclerosis susceptibility and high density lipoprotein levels in mice. *Proc. Natl. Acad. Sci. USA.* **84:** 3763–3767.
5. Paigen, B., M. N. Nesbitt, D. Mitchell, D. Albee, and R. C. LeBoeuf. 1989. Ath-2, a second gene determining atherosclerosis susceptibility and high density lipoprotein levels in mice. *Genetics.* **122:** 163–168.
6. LeBoeuf, R. C., M. H. Doolittle, A. Montcalm, D. C. Martin, K. Reue, and A. J. Lusis. 1990. Phenotypic characterization of the Ath-1 gene controlling high density lipoprotein levels and susceptibility to atherosclerosis. *J. Lipid Res.* **31:** 91–101.

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7. Paigen, B., B. Y. Ishida, J. Verstuyft, R. B. Winters, and D. Albee. 1990. Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice. *Arteriosclerosis.* **10:** 316–323.
8. Warden, C. H., C. C. Hedrick, J. H. Qiao, L. W. Castellani, and A. J. Lusis. 1993. Atherosclerosis in transgenic mice overexpressing apolipoprotein A-II. *Science.* **261:** 469–72.
9. Marotti, K. R., C. K. Castle, T. P. Boyle, A. H. Lin, R. W. Murray, and G. W. Melchior. 1993. Severe atherosclerosis in transgenic mice expressing simian cholesteryl ester transfer protein. *Nature.* **364:** 73–75.
10. Linton, M. R. F., R. V. Farese, Jr., G. Chiesa, D. S. Grass, P. Chin, R. E. Hammer, H. H. Hobbs, and S. G. Young. 1993. Transgenic mice expressing high plasma concentrations of human apolipoprotein B-100 and lipoprotein[a]. *J. Clin. Invest.* **92:** 3029–3037.
11. Young, S. G., R. V. Farese, Jr., V. R. Pierotti, S. Taylor, D. S. Grass, and M. F. Linton. 1994. Transgenic mice expressing human apoB-100 and apoB-48. *Curr. Opin. Lipidol.* **5:** 94–101.
12. Plump, A. S., J. D. Smith, T. Hayek, K. Aalto-Setala, A. Walsh, J. G. Verstuyft, E. M. Rubin, and J. L. Breslow. 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell.* **71:** 343–353.
13. Zhang, S. H., R. L. Reddick, J. A. Piedrahita, and N. Maeda. 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science.* **258:** 468–471.
14. Ishibashi, S., M. S. Brown, J. L. Goldstein, R. D. Gerard, R. E. Hammer, and J. Herz. 1993. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest.* **92:** 883–893.
15. Nakashima, Y., A. S. Plump, E. W. Raines, J. L. Breslow, and R. Ross. 1994. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the aortic tree. *Arterioscler. Thromb.* **14:** 133–140.
16. Reddick, R. L., S. H. Zhang, and N. Maeda. 1994. Atherosclerosis in mice lacking apoE. Evaluation of lesional development and progression. *Arterioscler. Thromb.* **14:** 141–147.
17. Palinski, W., V. Ord, A. S. Plump, J. L. Breslow, D. Steinberg, and J. L. Witztum. 1994. ApoE-deficient mice are a model of lipoprotein oxidation in atherogenesis: demonstration of oxidation-specific epitopes in lesions and high titers of autoantibodies to malondialdehyde-lysine in serum. *Arterioscler. Thromb.* **14:** 605–616.
18. Ishibashi, S., J. L. Goldstein, M. S. Brown, J. Herz, and D. K. Burns. 1994. Massive xanthomatosis and atherosclerosis in cholesterol-fed LDL receptor-negative mice. *J. Clin. Invest.* **93:** 1885–1893.
19. Palinski, W., R. K. Tangirala, E. Miller, S. G. Young, and J. L. Witztum. 1995.Increased autoantibody titers against epitopes of oxidized low density lipoprotein in LDL receptor-deficient mice with increased atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1569-1576.
20. Paigen, B., P. A. Morrow, D. Holmes, D. Mitchell, and R. A. Williams. 1987. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis.* **68:** 231–240.
21. Purcell-Huynh, D. A., R. V. Farese, D. F. Johnson, L. M. Flynn, V. Pierotti, D. L. Newland, M. R. F. Linton, D. A. Sanan, and S. G. Young. 1995. Transgenic mice expressing high levels of human apolipoprotein B develop severe atherosclerotic lesions in response to a high-fat diet. *J. Clin. Invest.* **95:** 2246–2257.
22. Carew, T. E., D. C. Schwenke, and D. Steinberg. 1987. Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks slowing the progression of atherosclerosis in the WHHL rabbit. *Proc. Natl. Acad. Sci. USA.* **84:** 7725–7729.
23. Björkhem, I., A. Henriksson-Freyschuss, O. Breuer, U. Diczfalusy, L. Berglund, and P. Henriksson. 1991. The antioxidant butylated hydroxytoluene protects against atherosclerosis. *Arterioscler. Thromb.* **11:** 15–22.
24. Sparrow, C. P., T. W. Doebber, J. Olszewski, M. S. Wu, J. Ventre, K. A. Stevens, and Y. S. Chao. 1992. Low density lipoprotein is protected from oxidation and the progression of atherosclerosis is slowed in cholesterol-fed rabbits by the antioxidant N,N″-diphenylphenylenediamine. *J. Clin. Invest.* **89:** 1885–1891.
25. Sasahara, M., E. W. Raines, A. Chait, T. E. Carew, D. Steinberg, P. W. Wahl, and R. Ross. 1994. Inhibition of hypercholesterolemia-induced atherosclerosis in Macaca nemestrina by probucol. I. Intimal lesion area correlates inversely with resistance of lipoproteins to oxidation. *J. Clin. Invest.* **94:** 155–164.
26. Fruebis, J., T. E. Carew, and W. Palinski. 1995. Effect of vitamin E on atherogenesis in LDL receptor-deficient rabbits. *Atherosclerosis.* **117:** 217-224.
27. Cornhill, J. F., E. E. Herderick, and H. C. Stary. 1990. Topography of human aortic sudanophilic lesions. *Monogr. Atheroscler.* **15:** 13–19.
28. Wissler, R. W. 1991. USA multicenter study of the pathobiology of atherosclerosis in youth. *Ann. NY Acad. Sci.* **623:** 26–39.
29. Daley, S. J., E. E. Herderick, J. F. Cornhill, and K. A. Rogers. 1994. Cholesterol-fed and casein-fed rabbit models of atherosclerosis. Part 1. Differing lesion area and volume despite equal plasma cholesterol levels. *Arterioscler. Thromb.* **14:** 95–105.

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