Distribution of Lipid Deposits Around Aortic Branches of Mice Lacking LDL Receptors and Apolipoprotein E

Christopher J. McGillicuddy, Martin J. Carrier, Peter D. Weinberg

Abstract—Mice with inactivated genes are increasingly used as models of human atherosclerosis. The aim of the present study was to determine whether the characteristic age-related distributions of lipid deposition seen around human arterial branches are replicated in such mice. Lesions occur downstream of branch ostia in immature human aortas, but these regions are spared in adult vessels, with lesions occurring more frequently at the sides or upstream of the branches. We determined the pattern of lipid staining around 102 intercostal branch ostia from apolipoprotein E/low density lipoprotein receptor double-knockout mice aged 9 to 20 weeks by using en face microscopy and a frequency-mapping technique. Lesion prevalence was high in the ostium and the region immediately surrounding it. Frequencies were 2.12 ± 0.30 (mean ± SEM, n = 11) times higher upstream than downstream (P < 0.01), but the pattern did not resemble the adult human pattern: there were no peaks in frequency at the sides or upstream of the branch, and there was no sparing downstream. Furthermore, a patch of sparing upstream of the branch was seen, which has not been reported for human vessels, and there was no trend toward a more upstream pattern with age. We conclude that knockout mice may not be a suitable model in which to investigate localizing factors. (Arterioscler Thromb Vasc Biol. 2001;21:1220-1225.)

Key Words: apolipoprotein E LDL receptor knockout mice atherosclerosis distribution

Many recent studies have used mice with inactivated genes as models of human atherosclerosis. Inducing disease in normal mice, even of susceptible strains, requires prolonged administration of high-cholesterol diets with toxic additives, and the resulting lesions tend to be restricted to fatty streaks in the aortic root.1–4 The genetically modified mice, in contrast, develop more advanced, more widely distributed lesions on Western or normal mouse diets while retaining the advantages of small size and fast breeding. Furthermore, they allow investigation of the effects of single gene products on lesion development.

The true value of the new models, however, depends on the extent to which their disease resembles that occurring in human arteries. The present study addresses this similarity; more specifically, it is concerned with the nonuniform distribution of disease within the vasculature. The patchy occurrence of human atherosclerosis has attracted considerable attention because it demonstrates the existence of significant local risk factors. The determination of lesion distributions in models is an important test of the similarity to human disease and also indicates whether the models can be used to investigate localizing factors. The issue is particularly important for knockout mouse models because of their widespread and increasing use and because they could be used for investigating local risk factors at the single gene level.

The distribution of lesions has previously been studied in apoE knockout5–7 and LDL receptor (LDLR) knockout8 mice. In both models, disease preferentially affects the following: the aortic root, coronary ostia, and proximal coronary arteries; the lesser curvature of the arch and branches off it; the brachiocephalic trunk, carotids and subclavians; the branch ostia of the abdominal and thoracic aorta (the abdominal ostia being affected early and the thoracic ostia being affected much later); and the iliac bifurcation.4,8–13 However, the distribution of lesions around branches has not been systematically examined. This is a significant omission: it is not sufficient to demonstrate that disease occurs near branch points because it can have diametrically opposed distributions within such regions under different circumstances, probably reflecting important differences in underlying mechanisms. Thus, lesions occur downstream of branch ostia in immature human aortas14 but develop in a more lateral or upstream distribution at later ages, with the downstream region becoming spared.15–17 The downstream distribution has been observed in some animal models,18,19 and the more upstream distribution has been observed in others.20–22

In the present study, we examined whether the downstream distribution or a more upstream one, or a switch with age from the former to the latter, occurs in mice. We studied the origins of the intercostal arteries because these sites have been extensively studied a range of other species, because they are the only locations where age-related distributions paralleling those at human branches have been demonstrated in an animal model,23,24 and because there are large numbers...
in each aorta. We used apoE/LDLR double-knockout mice because their disease develops sufficiently quickly that it can be mapped in young animals. Furthermore, they do not need to be fed a cholesterol-enhanced diet (which can increase variability), and they have a lipoprotein profile more human than that of the apoE single-knockout mouse.

Methods

Animals

All animal procedures complied with Home Office and local regulations. Homozygous apoE/LDLR double-knockout mice were obtained from a closed colony at the William Harvey Research Institute, London, UK. The colony originated with animals (B6,129 background, white-bellied agouti type, No. 002245) from Jackson Labs (Bar Harbor, Maine). They were fed a normal mouse diet, RM39(e) (Special Diet Services), and tap water ad libitum and were housed at 21°C on a 12-hour light cycle.

Eleven males aged 9 to 20 weeks were used in the present study. To confirm their broad similarity to the animals used in previous studies, total plasma cholesterol levels were determined in 2 mice by using a commercial enzymatic kit (Boehringer-Mannheim) and averaged 534 mg/dL. This value lies in the middle of the range (430 to 620 mg/dL) obtained in previous studies of the double-knockout mouse or of the apoE single-knockout mouse (from which there is no significant difference). Plasma cholesterol concentrations show no obvious changes with age.

Surgery

Mice were immobilized with ether and then given 120 μg/g IP of pentobarbital (Sagatal, Rhone Merieux). The abdomen and thorax were opened by 1 observer (C.J.M.); intersession variability is not recorded in every square. All mapping was conducted by 1 observer (C.J.M.); intersession variability is not significant. A hole made in the right atrium allowed drainage of the fluid. The descending thoracic segment of the aorta was processed by a modification of our previous techniques. Briefly, it was immersed for 1 hour in 4% (vol/vol) glutaraldehyde and 5% (vol/vol) formalin to form highly autofluorescent structures and then for 2 hours in a 1% (wt/vol) solution of oil red O in 60% (vol/vol) triethyl phosphate to stain lipid. It was subsequently destained for 15 minutes to remove the 1% solution and then transferred to PBS (0.15 mol/L, pH 7.4).

Histology

The descending thoracic segment of the aorta was processed by a modification of our previous techniques. Briefly, it was immersed for 1 hour in 4% (vol/vol) glutaraldehyde and 5% (vol/vol) formalin to form highly autofluorescent structures and then for 2 hours in a 1% (wt/vol) solution of oil red O in 60% (vol/vol) triethyl phosphate to stain lipid. It was subsequently destained for 15 minutes and then transferred to PBS (0.15 mol/L, pH 7.4).

After the stubs of the intercostal arteries had been trimmed to within 0.5 to 1.0 mm of their origins, the segment was opened along its ventral surface and flattened between a microscope slide and a coverslip, with the luminal surface uppermost. It was then examined by epifluorescence microscopy (×4 objective and standard filters for fluorescein isothiocyanate, Zeiss). Emission from the lipid stain coverslip, with the luminal surface uppermost. It was then examined by epifluorescence microscopy (×4 objective and standard filters for fluorescein isothiocyanate, Zeiss). Emission from the lipid stain was measured across the map for each mouse; these means were then combined to provide mean frequencies for the various groups of mice described below. Such frequency-mapping techniques, unlike the polar coordinate method, can include lesions within the ostium, those not directly in contact with the ostial lip, and those extending beyond the mapped region.

Results

Some branches could not be mapped because disease obscured the ostial perimeter. This finding is consistent with previous observations that raised lesions develop in knockout mice and cause severe stenoses in smaller arteries. There was a nonsignificant tendency (P = 0.107 by ANOVA) for the frequency of lesions to increase with age. In total, 102 branches could be mapped, with a mean of 9.3 (range 6 to 15) in each mouse.

Disease Severity and Distribution in All Mice

Disease was detected in all aortas but not at every branch. To calculate a representative frequency, values were averaged across the map for each mouse; these means were then themselves averaged for all mice, giving a value of 15±4% (n = 11). The mean frequency for the 100 grid squares (10×10 array) centered on the branch was also averaged for all mice. A 3-fold higher value (46±7%) was obtained, indicating that disease was associated closely with the branch.

The map for all mice is shown in Figure 1A. The disease is clearly centered on the branch, with high frequencies in the ostial itself. Frequencies were also high upstream, at the sides, and downstream of the ostium. Images of branches with typical patterns of staining are shown in Figure 1A through 1D. Some of the disease appearing within the ostia may in fact have developed on the walls of the intercostal arteries (which could have been pushed up into the ostium when the aorta was flattened on the microscope stage), and its density may have been exaggerated because the vessels were not fixed at pressure; however, it is clear from published photomicrographs of sections through pressure-fixed branches that intercostal ostia in knockout mice do become almost completely blocked by disease originating from the ostial margins.

To determine whether there was a higher frequency upstream or downstream of the branch, the mean frequency in the 100 grid squares (10×10 array) upstream of the branch center and the mean for the equivalent 100 squares downstream of the center were calculated for each mouse. The ratio of the upstream to downstream values, averaged for all mice, was 2.12±0.30 (n = 11). This ratio was significantly different from unity (t = 3.77, P = 0.01), indicating that there was consistently more disease upstream of the branch. However, unlike the upstream pattern in human vessels, there was no sparing downstream of the branch. The frequency in the downstream region was at least as high as the mean for the whole map compared to 15±4%.
Effect of Age on Disease Severity and Distribution

To determine whether the pattern and severity of disease changed with age, the analysis described above was repeated for the 55 branches from the 5 mice aged 11.5 weeks (mean age 12.0 weeks) and for the 47 branches from the 6 mice aged >16 weeks (mean age 18.0 weeks). The resulting maps are shown in Figures 3A and 3B, respectively.

An increase in disease frequency with age is visible in the maps, as expected. This impression was confirmed quantitatively by the mean frequencies of $8 \pm 2\%$ ($n=5$) for the young group and $21 \pm 5\%$ ($n=6$) for the older group ($t=2.12$, $P=0.031$), by the means for the central 100 grid squares of $30 \pm 7\%$ for the young group and $57 \pm 10\%$ for the older group ($t=2.08$, $P=0.033$), and by the peak frequencies of $60\%$ (young group) and $95\%$ (older group).

Furthermore, when the mice were considered individually rather than in 2 age groups, there was a trend for the mean disease frequency to increase with age (Figure 4A), which approached statistical significance ($P=0.062$ by ANOVA after logarithmic transformation), and there was a stronger trend for the frequency in the central 100 squares to increase with age (Figure 4B); the variation in this region was lower, and the trend reached significance ($P=0.038$ by ANOVA after logarithmic transformation).

Inspection of the maps for the young and older groups did not reveal any obvious differences in distribution. To examine the distribution quantitatively, the ratio of the frequency for the 100 grid squares upstream of the branch to the equivalent downstream frequency was calculated. The values obtained were $2.30 \pm 0.39$ for the young group and $1.97 \pm 0.46$ for the older group; the difference was not significant ($t=0.54$, $P=0.61$).

The ratio was also examined in individual mice rather than in the 2 groups, and this also failed to show an effect of age ($P=0.439$ by ANOVA). Not only was there no evidence for a switch from a downstream to an upstream distribution with age, but if anything, there was a trend in the opposite direction (Figure 4C). Only 1 outlier (an older mouse with an overall), even though it was less than half the frequency upstream.

Many branches showed a characteristic detail in the pattern of staining in the upstream region: a crescentic or triangular region of apparent sparing occurred immediately upstream from the ostium and was surrounded by a line or larger patch of stain. An example is shown in Figure 2A. This detail in the pattern of staining was not visible on the map of average frequencies for all mice (Figure 1A) because it did not occur at every branch and because its location was somewhat variable. However, it was clearly visible in the maps for some individual mice (eg, Figure 2B).
anomalously low frequency of disease as well as an atypical distribution) was markedly at variance with this trend; the tendency for a more downstream pattern in older mice became significant (P<0.008 by ANOVA) if the data from this mouse were removed. However, the ratio remained >1 (ie, there was more disease upstream) in all mice except for one 17-week-old mouse.

The characteristic detail in the pattern of staining upstream of the ostium, described above, was seen in branches from the youngest and the oldest aortas and at intermediate ages.

Discussion
Three patterns of lipid staining around branches of human arteries have been reported. In fetuses, neonates, and infants, lipid deposition occurs preferentially downstream of branch points, with less being observed upstream.1 This distribution resembles that conventionally associated with the cholesterol-fed rabbit.18,19 Adult human arteries, in contrast, are characterized by sparing downstream of branches.15–17 Recently, Sloop et al29 described 2 distinct variants of the adult pattern at intercostal branches. In the first, lipid deposition occurs lateral to the ostia, with sparing upstream and downstream along the center line. In the second, which occurs at later ages, a streak of lipid extends upstream from the inflow tract, with sparing at the sides and downstream of the branch. The latter pattern is a mirror image of the conventional rabbit one, in which lesions occur downstream and at the sides of the branch but in which the inflow tract and regions upstream of it are spared.26 (However, patterns comparable to adult human patterns have now been seen in older rabbits.23,24)

In the present study, we found no definite downstream, lateral, or upstream distribution around intercostal branch ostia of apoE/LDLR double-knockout mice; all 3 regions were affected. The frequency of lipid deposition was 2-fold higher in the upstream region than the downstream region, but it is our interpretation that this result alone does not indicate a pattern resembling those occurring in adult human arteries. There was no peak in frequencies lateral to the ostia, nor was there a streak emerging from the upstream lip of the branch. Most importantly, there was no consistent sparing downstream of the branch; although such sparing was seen at some branches, the mean frequency in the downstream region was higher than that for the map as a whole. The overall impression was of disease centered on and completely surrounding the branch, albeit with a bias toward the upstream location.

Only a relatively limited range of ages was studied, but this was as much as the model would permit. In the younger mice, there was little disease, whereas in the older mice, there was so much that a substantial percentage of the ostia could not be mapped. Within this range, we found no evidence of the switch with age from a downstream to a more upstream distribution seen in rabbit and human aortas. If anything, the reverse trend was apparent, although this effect became significant only with post hoc selection of data.
We are not aware of any previous studies to determine systematically the distribution of lipid deposits near arterial branches in mice. However, our observation of a lack of a clear downstream, lateral, or upstream pattern is consistent with impressions gained from illustrations in earlier reports. Thus, a sketch of the location of disease in apoE knockout mice shows lesions upstream of branches in the aortic arch but downstream of the origins of the celiac and superior mesenteric arteries; a photograph of disease in an apoE knockout mouse shows lesions completely surrounding 2 of the 3 affected intercostal ostia. One photograph of the aorta from an LDLR knockout mouse shows lipid deposition upstream and at the sides of branches, whereas another shows it upstream and downstream of the large abdominal branches. These observations in single-knockout mice seem to rule out the possibility that the lack of a distinctively upstream or downstream distribution in the present study arose because of the use of the double-knockout.

The conclusion that the distribution of lipid deposition around branches does not resemble the adult human pattern is supported by the previous observations that disease is prevalent in the aortic root and proximal coronary arteries of knockout mice. These are not common sites of disease in most mature human subjects, although, as noted by Ishibashi et al, they are affected in familial hypercholesterolemic patients. Further evidence for the disparity is the characteristic detail seen in the pattern of staining upstream of branches in the present study; this detail has not been reported for human vessels. In summary, although we expected to find a downstream or an upstream distribution or a switch from a downstream to an upstream distribution with age, none of these was found. The distribution that was mapped did not resemble any previously described pattern of lipid staining. Although the results were obtained in the apoE/LDLR double-knockout mouse over a limited range of ages, they may be relevant to mouse models in general. Anecdotal evidence suggests that the same pattern occurs in apoE or LDLR single-knockout mice, and there are no obvious differences between studies that have used widely different ages. Thus, knockout mice seem inferior to some other species for investigating the pattern of human lipid deposition. In particular, they seem less useful for this purpose than rabbits, which show age-related changes in lesion distribution and type that parallel those seen in human aortas, and in which age-related alterations of NO-mediated permeability properties of the wall provide a plausible explanation. It is possible that the distribution of disease in mice may be different because the hemodynamic stresses are different. For example, the lower Reynolds numbers and higher Womersley numbers may lead to distinct inflow patterns into branches. Alternatively, the underlying properties of the wall or the disease process itself may differ. Until these issues are resolved, the present results suggest that inferences concerning human disease should be drawn with caution. Unless the difference in mechanical stresses can alone account for the discrepancy in distributions, the mouse would not be a useful species in which to investigate the roles of genes putatively involved in disease localization.

Two aspects of the distribution of disease in knockout mice merit further investigation. The first aspect is the possibility that different patterns occur around branches at different points along the aorta. Nakashima et al have indicated that disease occurs upstream of branches in the aortic arch but downstream of those in the abdominal aorta. The present finding of upstream and downstream disease at branches between the arch and abdomen fits this trend. Also consistent with this, we found a greater tendency for lesions to develop upstream of branches in the thoracic than in the abdominal aorta of mature rabbits. Hence, there could be a gradient of some critical localizing factor down the aorta.

The second aspect is that the distribution of lipid staining in the mouse resembles descriptions of the pattern of raised lesions around intercostal branches in aged human aortas. In such vessels, disease completely surrounds the ostium, giving the appearance of a volcano. It may be significant that the mouse lesions are fibroproliferative and lead rapidly to stenoses, unlike those of other models. The mouse lesions stain for lipid when observed en face, whereas advanced human disease does not, but this could reflect the much higher cholesterol levels in the mouse. There is long-standing uncertainty concerning whether fibroproliferative lesions have a different distribution and etiology from fatty streaks. If they do, knockout mice may provide a better model for the former.

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References


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