Two Patterns of Lipid Deposition in the Cholesterol-Fed Rabbit

Sally E. Barnes, Peter D. Weinberg

Abstract—A central feature of arterial lipid deposition is its nonuniform and variable distribution. In immature human and rabbit aortas, spontaneous lesions occur most frequently downstream of branch points, but they tend to occur upstream of the same branches at later ages. In cholesterol-fed rabbits, the juvenile pattern has been seen regardless of age. These distributions may be determined by transport properties of the arterial wall, because uptake of plasma macromolecules is elevated downstream of aortic branches in immature rabbits and upstream in mature ones, except during cholesterol feeding, when the juvenile pattern is seen in adult vessels. The effect of cholesterol could reflect its inhibitory influence on the nitric oxide (NO) pathway because the adult transport pattern is NO dependent. Using protocols expected to preserve NO function and the mature pattern of transport during hypercholesterolemia, we made 2 attempts to induce upstream disease in rabbits. In trial I, plasma concentrations of cholesterol were kept within the normal human range for 15 weeks by using dietary levels of 0.05% to 0.2%. Although disease patterns reverse with age in human vessels exposed to these concentrations, lesions in both immature and mature rabbits occurred downstream of intercostal branch ostia. Trial II used older rabbits, a different base diet containing more vitamin E (96 mg/kg rather than 57 mg/kg), and higher levels of cholesterol (1%, administered for 8 weeks). For some animals, extra vitamin E (2000 mg/kg) was added to the diet. The mature pattern of lipid deposition was apparent around intercostal branches in the first group and was accentuated by the additional vitamin E, a change that was associated with a significant increase in the plasma concentration of NO metabolites. Spontaneous lesions, assessed on the base diet, were too rare to have influenced these distributions. This is the first report of upstream disease in the cholesterol-fed rabbit. The results support but do not prove the view that NO and transport are important in atherogenesis. (Arterioscler Thromb Vasc Biol. 1999;19:2376-2386.)

Key Words: rabbits ■ atherosclerosis ■ distribution ■ vitamin E ■ nitric oxide

The existence of disease-prone and disease-resistant sites near arterial branch points can be used to identify factors of potential importance in atherogenesis. A significant constraint is the need to account for not one but two distributions of disease: lipid accumulates primarily downstream of branches in immature human aortas,5 but in adult vessels these regions are spared, and sites upstream of branches are affected more frequently.2–4 Similar changes in distribution with age are discernible in the rare, spontaneous disease affecting rabbits.5

Of the many mechanical and biological attributes that vary around branches, corresponding age-related patterns have been demonstrated only for transport properties of the arterial wall. In juvenile rabbits, quasi-steady uptake of circulating macromolecules by the aortic wall is greater downstream of branches than upstream,6 while the reverse pattern is observed in mature rabbits.7 The spatial correlation with spontaneous lesions in rabbit and human arteries is consistent with such transport being a limiting step in the disease process. Both patterns of quasi-steady uptake appear to be determined by variations in the rate of macromolecule influx into the wall,8 and the mature pattern but not the immature one is dependent on the endogenous synthesis of nitric oxide (NO).9

These phenomena may be modified in the cholesterol-fed rabbit. Only the juvenile pattern of lipid accumulation has been described in this model.10–16 Adult rabbits have not been intentionally studied, but several trials lasted sufficiently long for maturity to have been reached by the end of the dietary intervention. If the distribution in cholesterol-fed rabbits is independent of age, as such results suggest, this would not necessarily contradict the proposed importance of transport because quasi-steady uptake in adult rabbits reverts to its immature pattern shortly after hypercholesterolemia is induced.17 The reversal probably reflects the dependence of the mature transport pattern on NO and the impairment of NO release and activity by hyperlipidemia.18

Although these effects of cholesterol on wall properties can account for existing data concerning the distribution of experimental atherosclerosis, they are reversible. Quasi-steady uptake in adult rabbits returns to its normal pattern...
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Protocols Used for the Different Groups of Rabbits in Trials I and II

<table>
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<tr>
<th>Protocol</th>
<th>Trial I Young</th>
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During a sustained period of mild hypercholesterolemia, and endothelium-dependent relaxation can be preserved, even in severely hyperlipidemic rabbits, by the administration of vitamin E or L-arginine. Consequently, if the postulated links between NO activity, transport processes, and disease localization are correct, it should be possible to obtain the upstream distribution of lesions in mature, cholesterol-fed rabbits.

Here we describe 2 attempts to induce such disease by using protocols based on these observations. In the first trial, plasma cholesterol concentrations of rabbits were kept within the normal human range for 15 weeks. Although these concentrations are consistent with an age-related switch in the distribution of human disease, immature and mature rabbits both showed the downstream pattern. In the second trial, older rabbits, 1% cholesterol, and a different base diet containing higher levels of vitamin E were used. The diet of some of the animals was supplemented with additional vitamin E. The adult pattern of disease was apparent on the first diet and was accentuated on the second.

Methods

Animals

All procedures complied with the Animals (Scientific Procedures) Act of 1986. Twenty-nine male New Zealand White rabbits (Harlan, Huntington, UK) were individually housed at 18±2°C on a 12-hour light cycle. They were fed experimental diets that were based on standard diets for long-term maintenance from 2 different manufacturers (see the Table for a summary of the protocols). Therefore, for at least 20 weeks before the start of each trial, animals were fed the diet from the appropriate manufacturer in its unsupplemented form to allow adaptation to any differences in basic constituents. Diets and water were administered ad libitum; food consumption and body weight were regularly monitored.

Protocol Used in Trial I

Six rabbits aged 52 to 62 weeks were used to determine whether humanlike concentrations of plasma cholesterol can give rise to the adult distribution of disease in rabbits that are mature from the start of dietary intervention. Additionally, 6 weanlings, 6 weeks old, were used to determine whether the juvenile pattern is induced by these concentrations in rabbits that remain immature throughout the trial. This control was considered necessary, even though there is no theoretical justification for expecting a pattern other than the juvenile one, because all previous low-cholesterol trials have mapped disease in animals that were mature by the end of the study.

The animals were fed a diet (RABMA, Special Diet Services), described in detail elsewhere, to which the manufacturer had added cholesterol (derived from sheep wool, Pecob) in soya oil. Spontaneous disease in young and old rabbits fed the unsupplemented base diet from this supplier has been described in a previous report and was not reassessed. The diet was administered over a period of 15 weeks, except that 1 rabbit from each group was killed after 13 weeks to monitor the progression of disease; these 2 animals did not differ in any significant way from the remainder of their respective groups, and data obtained from them are included in the analyses described below unless otherwise stated.

A dietary cholesterol concentration of 0.2% wt/wt was used for all animals during an initial period of 6 weeks, while intake stabilized and trends in plasma lipid levels became apparent. By mixing supplemented and normal diets in varying ratios, the dietary level was subsequently adjusted within the range 0.05% to 0.2% cholesterol on a weekly basis for each animal, so that concentrations of total cholesterol in plasma, averaged over the trial, were (1) matched for the immature and mature groups, (2) kept within the normal human range, and (3) maintained, as far as practicable, at similar levels in all animals.

Protocol Used in Trial II

In the second trial, effects of raised plasma concentrations of vitamin E on lesion distribution were investigated. A more severe hyperlipidemia was induced than in trial I, so that levels of vitamin E, which is transported within plasma lipoproteins, could be elevated by a substantial amount. The concentrations of cholesterol and vitamin E and the method for adding them to the diet were based on those used in previous studies demonstrating protection of the NO pathway during hyperlipidemia. Plasma lipid levels rose in an identical way in the 2 cholesterol-fed groups (see below), and consequently, there was no requirement for the cholesterol content of the diet to be adjusted during the trial. To obtain disease frequencies similar to those in trial I despite the higher lipid levels, the period of intervention was reduced from 15 to 8 weeks.

The base diet (9603 TRB, Harlan Teklad) came from a different manufacturer than the one used in trial I, and according to the manufacturer, it contained 96 mg/kg vitamin E, of which 91 mg/kg was added in the oxidation-resistant acetate form. Corresponding values for the diet used in trial I, again determined by the manufacturer, were 57 and 30 mg/kg. These values suggest that the switch in base diet alone should raise plasma concentrations of vitamin E compared with those in trial I.

Three groups of rabbits were fed different variants of this diet. Group 1 was fed the base diet alone, because the level and distribution of spontaneous disease on it had not previously been assessed. The diet was treated with the vehicle used in the experimental groups. For group 2, the diet was supplemented with 1% wt/wt cholesterol (derived from sheep wool, Sigma Chemical Co) and for group 3, it was supplemented with 1% cholesterol and 0.2% wt/wt vitamin E (±-tocopherol acetate, Sigma). These additions were made in ether (inhibitor-free spectrophotometer grade, Sigma; ~3 volumes ether per 1 volume cholesterol), which was then evaporated from the diet in a stream of air overnight.

The distribution of disease in immature rabbits fed comparable levels of cholesterol has previously been investigated and was not reassessed; only mature animals were used. Groups 1 and 2 consisted of 5 rabbits each. Group 3 initially comprised 7 animals, but only 5 were considered in the main analysis, since 1 was killed three quarters of the way through the trial to assess the progression of
disease and a second showed anomalous concentrations of cholesterol and vitamin E in plasma; the latter animal is discussed separately. The ages in groups 1, 2, and 3 were not significantly different, averaging 28±4.9, 27±6.5, and 24±6.1 months, respectively (mean±SD, P>0.05 by ANOVA).

Analysis of Plasma Samples
Plasma was prepared from blood samples that had been collected into EDTA. Blood was taken at weekly (trial I) or fortnightly (trial II) intervals. Samples were taken at a higher frequency in trial I to assess the level of dietary cholesterol required. Additionally, terminal samples were taken from the heart just before aortic cannulation. The 10 animals fed for 15 rather than 13 weeks in trial I and all of the animals in trial II had been fasted for 16 hours before terminal blood collection.

Lipids
All samples were analyzed for total cholesterol by using a commercial kit (CHOD-PAP, Boehringer Mannheim). Assays were performed in triplicate. In trial I, because of the possibility that lipid profiles might differ substantially between young and old animals, the terminal samples were also analyzed for free cholesterol and triglycerides, in duplicate or triplicate, again using commercial kits (MPR1 and GPO-PAP, Boehringer Mannheim).

α-Tocopherol
Terminal samples in trial I and pretrial, mid-trial, and terminal samples in trial II were assayed for α-tocopherol by high-performance liquid chromatography and the protocol of Stewart-Lee et al with minor modifications. In brief, ethanol, iso-octane, and water were sequentially added to plasma. After centrifugation, the organic phase was collected, dried in N2, and redissolved in acetonitrile. Samples were applied to a Hypersil ODS column (3×100 mm, 5-μm particle size, HiChrom) by using a mobile phase of acetonitrile/water/tetrahydrofuran. Concentrations were determined from the absorbance at 280 nm by using an internal standard of δ-tocopherol (Sigma) and a calibration curve obtained for high-purity ±α-tocopherol (ICN).

Nitrite/Nitrate
Terminal plasma samples from trial II were analyzed for combined nitrite and nitrate concentrations, indicative of NO production and subsequent oxidation, by using the Griess reaction. The animals were given glass-distilled water rather than tap water for 3 days and were then fasted for ≥16 hours before blood collection for this analysis to avoid interference from dietary nitrates. Other samples from trials I and II, briefly described in the Discussion, were obtained without taking these precautions. In all cases, 50 μL of plasma was incubated for 15 minutes at 37°C with 10 μL each of 0.5 mmol/L FAD, 5 mmol/L NADPH, and 10 U/L nitrate reductase and for a further 5 minutes after the addition of 10 μL of 1000 U/mL lactic dehydrogenase and 1 mol/L sodium pyruvate. Fifty microliters of 5% (vol/vol) H3 PO4 and 1% (wt/vol) sulfanilamide and 50 μL of 0.1% (wt/vol) N-(1-napthyl)ethylenediamine were then added (all reagents were from Sigma). Absorbance at 550 nm was converted to the concentration of nitrite plus nitrate by using a linear regression coefficient obtained for standard solutions. All assays were conducted in duplicate.

Surgical Procedures
Animals were administered heparin (2000 USP units IV, Sigma) and then an overdose of pentobarbital (600 mg Sagatal IV, Rhone Merieux). A ventral midline incision was made, the abdominal organs were deflected, and the diaphragm was removed. The aorta was cannulated caudal to the origin of the inferior mesenteric artery, flushed by retrograde perfusion with 50 mL of Ringer’s solution (9.0 g/L NaCl, 0.2 g/L CaCl2, 0.2 g/L KCl, and 0.01 g/L NaH2PO4) from a reservoir 90 to 100 cm above the animal, and fixed in the same way for 24 hours.

Detection of Lipid Deposition
The methods used for detecting and mapping lesions were similar to those employed previously and are only briefly described. After removal of adventitial fat, aortas were stained overnight at 4°C in a

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**Figure 1.** Concentrations of total cholesterol in plasma during feeding trials. Mean±SEM of values occurring in individual animals (n=5 or 6) is shown for each group. Analyses were conducted in triplicate.

**Figure 2.** Concentrations of α-tocopherol in plasma during trial II. Mean±SEM of values occurring in individual animals (n=5) is shown for each of the 3 groups.
1% (wt/vol) solution of oil red O in 60% (vol/vol) triethyl phosphate and then destained in 60% triethyl phosphate for 30 minutes.

The thoracic aorta was opened along its ventral wall, and the luminal surface was examined using an epifluorescence microscope, a ×4 objective, and standard filters for fluorescein (Zeiss). The glutaraldehyde-stimulated autofluorescence of the normal wall appeared yellow or green and contrasted with fluorescence from the overlying lipid stain, which was red. Photomicrographs of the aortic wall surrounding intercostal branch ostia were taken on color print film. The ostia of upper subcostal arteries, here considered equivalent to intercostals, were photographed in the same way. Between 12 and 18 branches from each animal were examined, depending on the length of aorta recovered.

The abdominal aorta was opened along its dorsal wall and pinned onto a custom-made mount that was curved to avoid the distortion that can be caused by flattening pressure-fixed vessels.12 The luminal surface around the celiac ostium was examined as described above. Other branches were not studied, because spontaneous lesions, which can be frequent enough in the abdominal aorta to bias the distributions seen in cholesterol-fed animals, were mapped only at the celiac ostium in our previous study.5 Montages were constructed from photomicrographs taken at different locations and foci around the branch.

Lesion Mapping

Staining was mapped by placing grids over the photomicrographs or montages. The grid consisted of a rectangular array of squares. For the area around each intercostal ostium, 18×28 squares were used, each having a length equivalent to 0.12 mm before magnification. For the celiac branch, the grid had 20×30 squares and the line spacing was increased 2.75 times to scale up for the larger ostial diameter. At the end of trial I, the 2 age groups had celiac ostia of similar size.

Lipid staining was recorded when it occupied >50% of the area of a grid element. The small spots of stain that constituted a significant proportion of the lesions in our study of spontaneous disease4 and that were mapped separately therein were seldom seen. They were therefore included only when they met the criterion of occupying >50% of a grid element.

All maps were constructed by 1 person (S.E.B.) to eliminate interoperator variation; variability between mapping sessions is not significant.1 Grids for intercostal branches were combined using the ostial center as a datum. For each animal, the percentage of branches having disease was calculated for every grid element The resulting maps from all animals in each group were then averaged. For the single celiac ostia, a simple group average could be calculated for each grid element, and the center of the flow divider, not the center of the ostium, was used as a datum.

Statistics

Data are presented as mean±SD unless otherwise stated. The sample size was usually 6 rabbits per group in trial I, except for analyses of lipids in terminal plasma samples, for which only the 5 rabbits fasted before blood collection were included; it was also 5 rabbits per group in trial II. Comparisons between pairs of means were made by Student’s unpaired t test; the equivalence of >2 groups was tested by ANOVA. The least-significant-difference method was used for multiple comparisons between means, and the significance and linearity of trends were assessed by ANOVA applied to regression.23

Results

Cholesterol Consumption and Plasma Lipid Concentrations

The immature and mature groups of trial I entered the study with significantly different concentrations of total cholesterol in plasma, the former averaging 56±9 mg/dL and the latter 24±6 mg/dL (P<0.001; data not obtained for 1 young animal), consistent with previous observations that concentrations drop from weaning to maturity.5 Subsequent changes are shown in Figure 1. The initial discrepancy was exacer-

bated during the early part of the trial because consumption of the cholesterol-enriched diet at a sustainable rate started within a day for all of the young animals but took an average of 1 week for the adults. Levels of dietary cholesterol were modified after 6 weeks to compensate for this discrepancy. After another 6 weeks, further adjustments were made to equalize the 2 groups.

This strategy was successful in making the average and terminal plasma lipid levels comparable for the 2 groups: there were no significant differences in any of the parameters assessed. Total cholesterol concentrations, averaged over the trial period, were 143±18 and 152±64 mg/dL for the young and old groups, respectively (P<0.7). Corresponding concentrations in the terminal plasma samples were 161±34 and 245±202 mg/dL (P<0.3). The nonsignificant trend toward a higher level in the old group almost entirely reflected the influence of 1 rabbit, which became a hyperresponder in the final 4 weeks. Terminal triglyceride concentrations (young, 69±12 mg/dL; old, 56±28 mg/dL) and free cholesterol concentrations (young, 32±10 mg/dL; old, 43±37 mg/dL) were also not significantly different between the 2 groups (P>0.3 and P>0.5, respectively). The total cholesterol consumption required to achieve these similar profiles differed by a small but significant amount, averaging 20±2 g for the young animals and 17±2 g for the old animals (P<0.05).

Plasma cholesterol concentrations for trial II are also shown in Figure 1. There were no significant differences among the 3 groups on entry into the trial (P>0.05), the average concentration for all 15 rabbits being 21±7 mg/dL, a similar value to that obtained for the mature animals in trial I. In group 1, which was fed the maintenance diet treated with vehicle alone, cholesterol concentrations did not show any significant trends during the course of the trial (P>0.05). In groups 2 and 3, which were fed the maintenance diet supplemented with cholesterol and the maintenance diet supplemented with cholesterol plus vitamin E, respectively, plasma concentrations rose throughout the trial (P<0.05 for both) without significant deviation from linearity (P>0.05 for both). There were no significant differences between the 2 groups in any of the cholesterol consumption or uptake parameters assessed. The cholesterol intakes averaged 48±12 g (group 2) and 48±10 g (group 3) (P>0.9). Corresponding concentrations in plasma, averaged over the trial, were 560±225 and 417±90 mg/dL (P>0.2), and terminal levels were 1065±259 and 990±298 mg/dL (P>0.6).

Plasma Concentrations of α-Tocopherol

Concentrations of α-tocopherol in the plasma of rabbits from trial I were below the threshold of detection, conservatively estimated at 0.7 µmol/L, in all of the young rabbits and in 4 of the 6 mature rabbits. In the remaining mature rabbits, values of 2.9 and 4.6 µmol/L were obtained.

Concentrations in trial II were substantially higher and always detectable (Figure 2). There were no significant differences between the groups on entry into the trial (P>0.05), the average value for all 15 animals being 8.5±4.6 µmol/L. In group 1, which was fed the diet treated with vehicle alone, there was no significant trend in this concentration during the course of the trial (P>0.05). In group 3, which was fed the diet supplemented with cholesterol plus vitamin E, plasma concentrations rose throughout.
Figure 3. Prevalence of disease around intercostal ostia in A, Trial I young rabbits; B, trial I adult rabbits; C, trial II controls; D, trial II rabbits fed cholesterol; and E, trial II rabbits fed cholesterol and supplementary vitamin E. In F, disease prevalence in trial II rabbits fed cholesterol and supplementary vitamin E has been divided by that in trial II rabbits fed cholesterol. G, Disease prevalence in 1 anomalous rabbit from the trial II group fed cholesterol and supplementary vitamin E, which had low plasma concentrations of cholesterol and α-tocopherol. H, Disease prevalence in 1 rabbit from the trial II group fed cholesterol, clearly demonstrating lesion sparing in a
the trial ($P<0.01$) without significant deviation from linearity ($P>0.05$). Intermediate plasma concentrations were seen in group 2, which was fed the maintenance diet supplemented with cholesterol alone. Both the average and the terminal concentrations of $\alpha$-tocopherol (18.4±7.8 and 20.1±10.6 $\mu$mol/L, respectively) were significantly higher than those in group 1 (5.4±1.4 and 2.9±1.1 $\mu$mol/L, respectively, $P<0.01$ for both) and significantly lower than those in group 3 (56.8±25.4 and 88.0±52.4 $\mu$mol/L, respectively, $P<0.02$ and $P<0.05$). However, $\alpha$-tocopherol levels reached a plateau during the first 4 weeks, and consequently there was no significant tendency for concentrations to rise throughout the trial as a whole ($P>0.05$).

**Plasma Concentrations of Nitrite and Nitrate**

In trial II, terminal plasma samples from groups 1, 2, and 3, which were fed the control diet, the cholesterol-only diet, and the cholesterol plus vitamin E diet, respectively, had concentrations of 31±9, 39±8, and 51±10 $\mu$mol/L. The values for groups 1 and 2 were not significantly different from each other ($P>0.05$), but the value for group 3 was higher than the group 2 value at $P=0.05$ and higher than the group 1 value at $P<0.01$.

**Lesion Distribution Around Intercostal Ostia in Trial I**

The map obtained for the juvenile intercostal ostia is shown in Figure 3A. Lesions were seen at all 96 branches examined. For the most affected site, lesions occurred in 77% of these branches. The average for the whole map was 16%. The staining was mainly distributed within an arrowhead shape surrounding the distal half of the ostium, with the tip pointing downstream. The same regions were the most frequently affected in old rabbits from our previous study of spontaneous lesions; the frequency of staining at the most lesion-prone site was 3%, and the average for the whole map was 0.2%. Although lesions were too rare to draw firm conclusions about their distribution, they appeared to lie along longitudinal axes displaced laterally from the ostium. The same regions were the most frequently affected sites in old rabbits from our previous study of spontaneous disease, in which considerably more disease was detected. Conversely, all of the fatty streaks in the weanlings from that study occurred within a small triangular area downstream of the branch. It therefore seems likely that the distribution shown in Figure 3C is fundamentally the same as that which we previously reported for old animals.

The equivalent map for adult animals is shown in Figure 3B. Lesions were observed at 81 of the 90 branches mapped (90%), and the peak and mean frequencies were, respectively, 29% and 8%. The mean is substantially below the juvenile value, but this difference was not significant ($P>0.05$) because more than half of the adult lesions were attributable to the hyperresponder, and the variance was therefore high. The average distribution was almost identical to that at the juvenile branches. The exact shape of the arrowhead differed only slightly, and the fundamental features were clearly the same. Illustrative photomicrographs of juvenile and adult branches are shown in Figures 4A and 4B.

At a few adult branches, lesions were observed with a pattern resembling that seen for spontaneous lipid deposition in old rabbits; staining occurred upstream but not downstream of the branch. Such branches, because of their rarity, had a negligible influence on the overall distribution. They did not seem to have a particularly high occurrence in any single animal, all rabbits on average showing the juvenile pattern. This anomalous distribution may reflect the occasional presence of spontaneous lesions, the frequency of which is substantially lower than the mean frequency observed in this trial. Alternatively, it may indicate a small tendency for the induced disease to occur in the adult rather than the juvenile pattern. In either case, the overwhelming trend was for lesions to occur in the locations normally affected in cholesterol-fed rabbits.

**Lesion Distribution Around Intercostal Ostia in Trial II**

The map obtained for group 1 animals, which were fed the base diet treated with vehicle alone, is shown in Figure 3C, and a photomicrograph of the most affected branch is shown in Figure 4C. Lipid deposition was rare, being seen at only 5 of the 72 branches examined (7%); in fact, all of the staining was observed in a single animal. Terminal plasma concentrations of cholesterol, vitamin E, and nitrite plus nitrate in this rabbit were typical of the group as a whole. The prevalence of staining at the most lesion-prone site was 3%, and the average for the whole map was 0.2%. Although lesions were too rare to draw firm conclusions about their distribution, they appeared to lie along longitudinal axes displaced laterally from the ostium. The same regions were the most frequently affected sites in old rabbits from our previous study of spontaneous disease, in which considerably more disease was detected. Conversely, all of the fatty streaks in the weanlings from that study occurred within a small triangular area downstream of the branch. It therefore seems likely that the distribution shown in Figure 3C is fundamentally the same as that which we previously reported for old animals.

The equivalent map for group 2 animals, which were fed 1% cholesterol in their diet, is shown in Figure 3D, and an example is shown in Figure 4D. Lesions were seen at 61 of the 74 branches examined (82%). The peak and mean frequencies were, respectively, 37% and 15%. The mean was 84 times the control value, ruling out a significant influence of spontaneous lipid deposition. Lesions again occurred most frequently along longitudinal axes displaced laterally from the branch, and the incidence was particularly high at the sides of the ostium itself. The overall pattern was clearly more similar to that reported for spontaneous adult disease than to the downstream pattern induced in trial I.

The map for the animals of group 3, which were fed 1% cholesterol and 0.2% added vitamin E, is shown in Figure 3E. An example is shown in Figure 4D. Staining was seen at 63 of the 78 branches examined (81%). The peak and mean frequencies for the map were, respectively, 32% and 9%, again ruling out a measurable influence of spontaneous lesions. The mean was 60% of the value for group 2; this difference is not significant ($P>0.3$). The basic pattern was similar to that seen in group 2 and not to that seen in trial I. There was, however, an additional tendency for regions upstream of the branch to develop lesions more frequently than those downstream.
To examine this discrepancy further, the group 3 map was divided by the group 2 map. An identical pattern in the 2 groups would have given a value of 0.60, the ratio of the mean prevalences, in every square of the resulting grid. In fact, the values were neither uniform nor randomly distributed (Figure 3F). Instead, the sites that were relatively more affected in group 3 than in group 2 occurred in clusters upstream and at the sides of the ostium, where ratios of more than triple the average were not uncommon, and tended not to occur downstream of it, ratios less than a third of the average being frequent in this region. Because this is also true for spontaneous adult disease compared with the equivalent juvenile disease, we conclude, despite the existence of exceptions, that the adult pattern was more accentuated in animals fed the vitamin E supplements. An apparent anomaly, discussed below, is that the group 3 animals also showed more frequent staining near the lip of the flow divider itself, particularly at its center.

One of the rabbits fed cholesterol and vitamin E was not included in the analysis presented above because it responded to the diet in a different way from the remaining animals during the second half of the trial. Despite a food intake that was 16% above the group mean and that was maintained at a high level for the entire 8 weeks, its plasma cholesterol concentration dropped during the last 4 weeks; all other animals showed substantial rises. The terminal concentration was only 281 mg/dL, a value that was 28% of the mean for the rest of the group and >2 SDs below it. The terminal α-tocopherol concentration was 41.6 μmol/L, or 47% of the group mean. Two weeks before the end of the trial, both the cholesterol and α-tocopherol concentrations had been even lower. The rabbit showed no obvious signs of ill health, and it lost only 6.6% of its body weight during the trial, comparable to the 8.2% mean loss for the remaining rabbits. The distribution of staining in this animal (Figure 3G) was strikingly similar to that seen in mature animals from trial I (Figure 3B) and markedly different from that observed in all of the remaining rabbits in this group.

Lesion Distribution Around Celiac Ostia in Trial I

Staining was detected at all celiac branches examined. There was no difference in prevalence between the 2 age groups. The frequency averaged over the entire grid was 27% for juveniles and 30% for adults, and the level at the most affected site was 100% in both cases. These values are not much greater than those observed in rabbits fed the un-supplemented base diet. In adults, the mean frequency of spontaneous lesions was 10% and the maximum, 60%. In juveniles, the mean frequency was ~10-fold lower but rose to 67% around the distal margin of the ostium. Hence, a significant contribution from spontaneous lesions is likely.

The distribution near the celiac branch differed from that at intercostal ostia. In both juvenile and adult aortas, lesions were observed downstream, at the sides, and upstream of the branch. Maps are shown in Figures 5A and 5B and montages in Figures 6A and 6B. The distribution in young animals resembled a combination of the arrowhead pattern seen at intercostal ostia and a streak approaching the branch from the upstream direction, along the ventral wall; nonlesion areas were seen between these 2 regions. Parallel features were discernible in the pattern of spontaneous juvenile lesions at this branch. Only minor changes were apparent in adult animals. Lesions were somewhat less frequent at the sides of the branch, and the highest prevalences were equally distributed between upstream and downstream regions rather than only in the latter.
Lesion Distribution Around Celiac Ostia in Trial II

Staining was detected at 3 of 5 celiac branches (60%) from control rabbits that were fed the base diet treated with vehicle alone. The peak frequency observed at any site was 60% and the mean was 13%. Lesions again occurred downstream, at the sides, and upstream of the ostium, but the highest prevalences were almost exclusively located in the upstream region (Figure 5C). These values and distribution are essentially identical to those previously reported for adults fed the trial I base diet.5

In the groups fed the diets enhanced with cholesterol or cholesterol plus additional vitamin E, lesions were detected at all branches examined, and the peak frequency in each case was 100%. Mean frequencies were 47% and 34%, respectively, the ratio between them being similar to that obtained at intercostal branches. In both cases, as in trial I, a significant contribution from spontaneous lesions is likely. The distributions (Figures 5D and 5E) were indistinguishable from each other and from the pattern of adult spontaneous disease. Montages from the 3 groups are shown in Figures 6C through 6E.

Discussion

Anitschkow26 and colleagues demonstrated early in this century that areas of aortic wall downstream of the origins of intercostal arteries are especially prone to lipid deposition in rabbits fed a cholesterol-enhanced diet. The nonuniform distribution of lesions was thought to be a useful way of establishing mechanisms and a test of the relevance of the model to human disease; attention was drawn to the similar pattern occurring in young children. Parallels between the distributions in hyperlipidemic rabbits10–16 and children1 were later confirmed by quantitative mapping techniques. However, it also emerged that disease has a different distribution in adult human arteries. In these vessels, lesions occur more frequently upstream of branch points than downstream.3–4

In the present study, attempts were made to induce the upstream distribution of lesions in cholesterol-fed rabbits. These attempts were based on the concept that age-related variations in arterial transport properties determine the two patterns of disease and that the adult pattern requires an intact NO pathway. In the first trial, dietary cholesterol levels of...
young and mature rabbits were manipulated to keep average plasma concentrations of total cholesterol within the normal human range. The second trial used older rabbits, higher cholesterol concentrations, a base diet containing more vitamin E and, for some animals, additional vitamin E supplements.

The main finding was that the upstream distribution of disease can be induced at intercostal branch ostia of adult rabbits. In trial I, the classic Anitschkow pattern was seen in both young and old rabbits, as in previous studies, but this pattern was abolished in trial II. Those rabbits fed the diet supplemented with cholesterol alone developed a lesion distribution with several features characteristic of spontaneous disease in mature human and rabbit vessels. When vitamin E consumption was further enhanced, all such features were observed; the maps for these rabbits closely resembled the pattern of sudanophilia in published photographs of adult human aortas.27

The patterns found at the celiac branch are more difficult to interpret. Our previous study showed that the difference between juvenile and adult patterns of spontaneous disease is less clear-cut at this ostium than at intercostal branches.5 Possible reasons for this discrepancy include the influence of large neighboring branches, relatively small variations in shear stress28 and transport properties29,30 around the ostium, and the different architecture of the abdominal aortic wall. In the present study of induced disease, the changes in distribution were again more subtle than at intercostal ostia. This may have arisen not only for the above reasons but also because the frequency of spontaneous lesions at the celiac branch is high enough to affect the patterns seen in hypercholesterolemic animals. The pattern in the cholesterol-fed rabbits of trial II was indistinguishable from the spontaneous adult one, although frequencies were higher; it may therefore reflect a combination of spontaneous and induced lesions, both having the mature distribution. Similarly, the juvenile rabbits of trial I had a pattern that resembled the spontaneous juvenile one, which could therefore reflect a combination of spontaneous and induced lesions both having the immature distribution. The adult rabbits of trial I had an intermediate pattern, possibly because preexisting spontaneous disease of the adult pattern combined with induced lesions of the juvenile type. This interpretation, only one of several possibilities, would imply parallel switches in underlying mechanisms, but different resulting lesion patterns, at celiac and intercostal branches.

The highest lesion frequencies near intercostal ostia in trial I occurred at sites with the lowest frequencies in trial II. Thus, the two patterns appear to be not just different but opposite in some sense. This view is supported by several maps for individual rabbits from trial II, such as shown in Figure 3I. Although this rabbit had a mean lesion prevalence of 16%, one of the highest for a mature animal, there was a marked absence of staining in the triangular region downstream of the branch corresponding to the most affected area in trial I. Some images of individual branches from trial II, particularly the one shown in Figure 4F, display a pattern that seems the inverse of that conventionally associated with cholesterol-fed rabbits. The absence of staining downstream of ostia in the second trial cannot plausibly be attributed to the development of more advanced disease with lipid-free caps in this region, because all experimental groups, despite their dissimilar staining patterns, had mean prevalences within the narrow range of 7% to 16%. Values for adult animals in trial I and for rabbits fed vitamin E supplements in trial II, the groups for which the difference in distribution was clearest, were 8% and 9%, respectively. In fact, lesion-free areas occurred downstream of ostia in animals that had little lipid deposition. The concept of 2 mirror-image patterns is further supported by the greater prevalence of lesions on the flow-divider lip in

![Figure 6. Montages of photomicrographs showing disease around typical celiac ostia from A, trial I young rabbits; B, trial I adult rabbits; C, trial II controls; D, trial II rabbits fed cholesterol; and E, trial II rabbits fed cholesterol and supplementary vitamin E. In all cases, aortic wall area is shown en face, with mean flow from top to bottom. Stained areas appear dark (see legend to Figure 4); contrast for control rabbits is higher in the color print, and disease is apparent above and below ostium. Bar=5 mm.](http://atvb.ahajournals.org/DownloadedFrom.png)
rabbis given additional vitamin E (Figure 3F), because this site normally has a paradoxically low lesion frequency in hypercholesterolemic animals.16,31,32

Although both upstream and downstream lesion distributions were clearly induced around intercostal ostia, the causes of the switch between them were less well established. Within trial II, those rabbits receiving cholesterol and vitamin E supplements tended to develop disease with a more upstream pattern than those receiving cholesterol alone. We attribute this difference in distribution to vitamin E, because the groups were otherwise well matched, and only their plasma concentrations of α-tocopherol differed. Surprisingly, however, the largest shift toward the mature pattern occurred between mature rabbits in trial I and those in trial II that were fed the diet supplemented with cholesterol alone. It is plausible that this shift was also caused by vitamin E, which was present at higher concentrations in the base diet of trial II than in that of trial I. Nevertheless, several alternative explanations are possible because many other factors differed between the 2 trials. For example, the mature rabbits in trial II were older than those in trial I, they came from a different colony, and they were administered higher levels of cholesterol in a different vehicle (the Table). Furthermore, many nutrient components had different concentrations in the 2 base diets. One rabbit fed vitamin E supplements developed lesions with an anomalous downstream pattern, yet it differed from the remainder of its group only in having lower plasma concentrations of cholesterol and α-tocopherol, suggesting that these factors played a critical role. Clearly, systematic investigation of this issue is required.

An upstream distribution of lesions had been expected in trial I because quasi-steady transport properties of mature animals return to their normal adult pattern within 11 days of such mild hypercholesterolemia.17 Since this occurred on the diet also used in trial I, the results do at first sight appear to contradict the proposed importance of transport in disease. However, the pattern of uptake occurring beyond 11 days of hypercholesterolemia is unknown. Thus, the majority of lesions detected in trial I will have developed after the duration of dietary intervention for which transport data are available. Furthermore, the spatial correlation between variations in macromolecule influx and in quasi-steady uptake breaks down during hypercholesterolemia (A. Sekhki and P.D. Weinberg, unpublished observations, 1994), introducing the possibility that the intimal accumulation of lipids may also not be correlated with quasi-steady uptake. More detailed studies of transport during more prolonged hypercholesterolemia are required, as are investigations of the influence of vitamin E supplements on uptake, an area in which there are currently no data at all.

Finally, we consider whether the presence or absence of an upstream pattern was related to the maintenance of an intact NO pathway. Vitamin E, if it did cause some or all of the switch in distribution, need not have done so by affecting NO; it has many actions. Its ability to inhibit the activation of protein kinase C (PKC) caused by lipoproteins33 and other factors,34–36 an ability apparently unrelated to its antioxidant properties,33,35,36 is of particular interest, since PKC is elevated in endothelial cells by shear stress37,38 and has numerous effects in addition to its inhibitory influence on NO production.39 The most convincing evidence for the proposed mechanism was that terminal plasma concentrations of nitrite and nitrate were higher in the trial II animals given vitamin E and cholesterol supplements than in the otherwise well-matched group given cholesterol alone, correlating with the tendency to develop lesions with a more pronounced upstream distribution. However, the anomalous rabbit that took up only small amounts of vitamin E and developed the downstream pattern did not conform to this trend, since it had a plasma concentration of nitrite and nitrate that was almost identical to the mean for the rest of the group. Some additional support derives from samples collected at the end of trial I and midway through trial II. Nitrite and nitrate levels were 30% lower in the former, again consistent with the view that NO production determines the distribution of lesions. Unfortunately, the animals had not been fasted and had been drinking tap water when these samples were collected, and it was subsequently found that this can elevate plasma concentrations by 100 μmol/L. The result is therefore weakened by the need to assume equal contributions from dietary sources.

Plasma concentrations of nitrite and nitrate can at best indicate the production of NO; they cannot account for changes in its rate of degradation. Evidence obtained with other techniques supports the view that vitamin E protects NO activity in hypercholesterolemia,19–20,40 but these techniques also have limitations. The measurement of endothelial-dependent relaxation of arteries ex vivo removes the tissue from the chemical, physical, and systemic environment that causes impairment or protection. Furthermore, the measurement of agonist-induced relaxation is unsatisfactory, since it is the flow-dependent release of NO that has been implicated in determining transport properties of the arterial wall,9 and this is mediated by a different signaling pathway41 and is inhibited to a different extent by hypercholesterolemia.42 Even if a suitable indicator of the flow-dependent, NO-mediated relaxation of large arteries were to be developed for use in rabbits and it did confirm an influence of vitamin E, correlations with the distribution of lesions could simply reflect coincidental effects of the change in dietary protocol. However, now that an upstream lesion distribution has been produced, the issue of its NO dependence can be addressed more rigorously by blocking NO synthesis as the lesions develop. Until results from such experiments are obtained, the involvement of NO must be regarded as plausible but unproven.

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References

Two Patterns of Lipid Deposition in the Cholesterol-Fed Rabbit
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