Contrasting Patterns of Spontaneous Aortic Disease in Young and Old Rabbits

Sally E. Barnes, Peter D. Weinberg

Abstract—The pattern of spontaneous lipid deposition around aortic branch ostia was mapped in New Zealand White rabbits aged 1 month or 2 to 6 years. The young rabbits were studied within 1 day of weaning, and the older rabbits had been maintained on a low-protein, low-fat diet. Plasma concentrations of total cholesterol for the two groups averaged 75 and 18 mg/dL, respectively. Aortas were fixed in situ at a pressure of 90 to 100 cm H2O, stained with oil red O, and photographed en face under an epifluorescence microscope. Areas of staining contrasted in color with the fixative-stimulated autofluorescence of nondiseased tissue and were mapped by placing grids over the photomicrographs. Disease around intercostal ostia was rare, but two distributions were established by combining data from many branches. In weanlings, staining was seen within a triangular area downstream of the branch. In old animals, this area had the lowest frequency of disease; lesions tended to occur downstream of the spared region, along axes lying to either side of it, and at the lateral and upstream margins of the ostium. Disease was less rare at celiac branches. It occurred mainly downstream of the ostium in weanlings, whereas upstream sites were most affected in old animals, although significant disease remained at the juvenile locations. Earlier reports have described similar age-related distributions of disease in human aortas, consistent with a common underlying mechanism. The distributions also correlate with the spatial variations in arterial transport properties established in previous studies, and may be determined by them. (Arterioscler Thromb Vasc Biol. 1998;18:300–308.)

Key Words: spontaneous disease ▪ rabbits ▪ arterial branches ▪ age ▪ atherosclerosis

Lipid-rich lesions have a characteristic, age-related distribution in human aortas. Sudanophilia initially develops downstream of branch ostia in fetuses, neonates, and infants, but these sites are spared of disease in adult vessels, with regions upstream of branches being affected most frequently. In rabbits fed a cholesterol-enhanced diet, however, there is no evidence for a reversal with age: the juvenile pattern of sudanophilia is observed even in animals maintained on the diet well into maturity. These distributions may be related to variations in the net uptake of plasma macromolecules by the arterial wall. In immature rabbits, uptake of circulating albumin is greater downstream of aortic branches than upstream, but the opposite pattern is seen after the age of sexual maturity. It is plausible that similar age-dependent variations in transport properties occur in human arteries and determine the juvenile and adult patterns of disease. The apparent discrepancy with the consistently juvenile pattern of diet-induced lesions in rabbits could reflect modification of these transport properties by hypercholesterolemia, rather than an inherent difference between the species. Consistent with this view, the immature pattern of albumin uptake is seen in older animals fed a cholesterol-enhanced diet for 1 week.

It is a corollary of this hypothesis that if spontaneous lesions occur in rabbits fed a normal diet, then the lesions should be distributed in the same age-related patterns observed in human vessels. This possibility has been investigated in the present study. Aortas of weanling and old rabbits were examined for the presence of spontaneous lipid deposits by using sensitive detection techniques based on fluorescence microscopy in conjunction with a probability mapping method similar to that of Kjaernes et al. Lesions were detected in both age groups, and their distributions resembled those seen in human arteries of the same developmental stage. A preliminary report of this work has been published.

Methods

Animals and Diet

All animal procedures complied with Home Office and local regulations. Disease was studied in 16 male New Zealand White rabbits obtained from a variety of sources (Interfauna, Huntington, UK; Froxfield, Hants, UK; Porcellus, Sussex, UK). Six were supplied on the day of weaning and were used the next day, when their ages were 31 to 33 days. The remaining 10 were aged 2 to 6 years (7 were ≥5 years) and were acquired 1 to 3 years prior to use. Further details are given in the Table.

While in our facility, all the rabbits were individually housed at 18±2°C on a 12-hour light cycle. They were given tap water ad libitum and 200 g/d of a standard laboratory diet for long-term studies of rabbits (RABMA, Special Diet Services). This diet was high in fiber and low in protein and other nutrients, and, to prevent weight gain,
had only 7.8 MJ/kg of digestible energy. The manufacturer’s analysis, which assumed a nominal 10% water content, gave values of 2.2% crude oil (of purely vegetable origin), 13.1% crude protein, 19.8% crude fiber, 7.3% ash, 47.6% nitrogen-free extract (mainly carbohydrate), 38.5% dietary fiber, 21.5% starches, and 7.4% sugars. Minerals and fat- and water-soluble vitamins were included.

Vegetarian diets with low protein contents are deficient in several essential amino acids unless supplemented in some way. Most of the protein in the present diet was derived from soy, but supplementation with small quantities of animal protein and methionine altered the amino acid composition to the balance required for long-term maintenance. These levels do not resemble the concentrations used to induce hypercholesterolemia.

Surgical Procedures

Heparin (1000 USP units, Sigma Chemical Co) was introduced via the marginal ear vein and allowed to circulate for 1 minute. An overdose of pentobarbital (600 mg, Sagatal, Rhone Merieux) was then given by the same route. The thorax and abdomen were opened along the midline, the diaphragm removed, and the viscera displaced. Blood from the heart was collected into EDTA. Terminal concentrations of total cholesterol in plasma were subsequently measured with a commercial kit (CHOD-PAP, Boehringer Mannheim).

The aorta was cannulated caudal to the origin of the inferior mesenteric or renal arteries and flushed in situ 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 Na₂HPO₄) from a reservoir 90 to 100 cm above the vessel. The aorta was then fixed in the same way with 600 mL of Karnovsky’s fixative (4% glutaraldehyde and 5% formaldehyde). In the weanlings, a drain was placed in the right ventricle of the heart, since the volume of fluid used was much larger than the blood volume and the fixative otherwise failed to enter the aorta at a sufficient rate. This procedure was not required in old animals. Pressure was maintained for ~10 minutes. The aorta from the point of cannulation to the descending arch was excised and placed for at least another 30 minutes in the same fixative.

Staining and Microscopy

Each aorta was equilibrated with PBS (pH 7.4, 0.15 mol/L), freed of fat from its adventitial surface, and stained overnight at 4°C with a 1% (wt/vol) solution of oil red O in 60% (vol/vol) triethyl phosphate. The aorta was then destained for 30 minutes with 60% (vol/vol) triethyl phosphate and reequilibrated with PBS. The thoracic aorta was opened along its ventral wall, placed luminal surface up in a Petri dish containing PBS, and lightly covered with a microscope slide. Fluorescence from tissue was stimulated using an epifluorescence microscope, a 4× objective, and standard filters for fluorescein (Zeiss). These staining techniques and filters were found to give better color contrast than those previously employed to examine diet-induced disease. The glutaraldehyde-stimulated autofluorescence of the normal wall appeared yellow-green, whereas the overlying lipid stain appeared red. Regions 2.4 × 3.6 mm² in area and centered on intercostal branch ostia (or phrenic ostia, here considered as intercostals) were photographed with color print film.

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The abdominal aorta was opened along its dorsal wall. The larger branches of this region could not be examined with the technique used for intercostal ostia because flattening of the pressure-fixed vessel caused significant distortion across the field of interest. Instead, segments were pinned onto a custom-made mount that was curved to maintain approximately their in vivo geometry. PBS was placed on

### Characteristics of Weanling and Old Rabbits

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<th>Weight, kg</th>
<th>Plasma Cholesterol, mg/dL</th>
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IC indicates intercostal.
the tissue to prevent drying. To obtain an adequate field of view and allow for nonplanar geometry, montages were constructed from many photographs obtained by the techniques described above but taken at different locations and foci.

**Lesion Mapping**
The frequency-mapping techniques have been presented in detail elsewhere and are only briefly described. For intercostal ostia, a grid of 17×28 squares was placed over each photomicrograph, and the presence or absence of lesions was recorded for all squares other than those overlying the ostium itself. The line spacing of the grid was equivalent to 0.12 mm before magnification. For ostia of the abdominal aorta, a grid of 20×30 squares was used, montages rather than single photomicrographs were examined, and the line spacing was approximately scaled to account for their greater diameters, being 2.75 times larger for the celiac branch. Age was not taken into account when deriving these approximate scale factors. The results from all intercostal branches in an age group were combined by using the center of the ostium as a datum. For celiac branches, the center of the flow divider was used. A cumulative sum of lesion occurrence was calculated for every square by using commercially available spreadsheet software.

A second method was used to make more detailed maps of areas close to the ostial lip. Variations in the size or geometry of individual branches can lead to “blurring” of features in these regions when the first method is used, and small-scale but consistent trends in lesion frequency can consequently be overlooked. To avoid this problem, grids with an adaptive geometry were used to map lesions at each branch and were then distorted to a standard form so that data from all branches could be combined.

This technique is similar to the warping method of Ivey et al. Lines were drawn radiating at 20° intervals from the center of the branch. Distances equivalent to 0.12 mm before magnification for intercostal ostia and appropriately scaled distances for other branches were marked along these lines, starting at the edge of the ostium and moving outwards. Equivalent marks on adjoining radials were joined by straight lines, and the presence or absence of staining was recorded for each element of the grid formed in this way. Cumulative frequencies, obtained by combining the data from individual branches, are presented on a grid in which the branch ostium is idealized as a circle.

**Statistics**
Variation in the extent of disease recorded for the same branch at two different mapping sessions was assessed by a paired *t* test. Potential correlations of parameters (such as plasma cholesterol concentration) with the number of diseased branches in each animal were examined by applying an ANOVA to multiple linear regression. Spatial correlations of parameters (such as plasma cholesterol concentration) were remarkably stable. Terminal values were 100 ± 0.19 (mean ± SD; range, 94% to 104%) of those recorded 1 to 3 months before death and 101 ± 8% (range, 88% to 119%) of those recorded 5 to 7 months before death. This stability is evidence that a suitable diet was employed and that there was no serious illness.

The difference in plasma cholesterol concentrations between the groups is consistent with an earlier observation of a smooth decrease from 59 to 17 mg/dL as age increased from 6 to 24 weeks (A. Sebkhi and P.D. Weinberg, unpublished data, 1993) and may in part reflect the switch from a milk to a solid-food diet. Concentrations in the group of old rabbits were lower than those reported by others for similarly aged rabbits, presumably because of the meager diet.

**Mapping Criteria and Variability**
Two distinct types of staining were observed. In the first type, lipid was distributed in patches or streaks, as previously reported for diet-induced disease. These lesions were mapped if they occupied >50% of a grid square. In the second type, spots with a typical size of ~20 μm were seen. This disease was recorded if at least one such spot occurred within a square. Because the relation between the two types is unclear, they were mapped separately. Approximately equal numbers of intercostal branches were examined in each animal (Table). Consequently, within both age groups the maps from all such branches were combined without regard to the rabbit from which they were derived.

All maps were constructed by one person (S.E.B.) to eliminate interoperator variability. To assess variability between mapping sessions, streaks at one intercostal branch from each rabbit were remapped at a second session by using a blind protocol. Variability between the two sessions was generally low. For all 6 weanlings and 7 of the 10 old rabbits, the discrepancy in the number of squares considered to be diseased averaged only ±1.5 squares per grid (range, −7 to +9; *t* = 1.54, *P* = .1), and the maps were essentially superimposable. In the other 3 adult rabbits there was more disagreement, but the overall discrepancy was still not significant (*t* = 1.87, *P* = .1). In this group, the uncertainty always concerned the delimitation of diffuse staining, which appeared at the edges of the maps (see below). The more discrete patches of disease occurring near the ostia in both age groups were reliably identified. For the main study, adult disease has been mapped in a deliberately conservative manner, and the frequencies shown for these peripheral regions should therefore be regarded as minimum estimates.

**Disease Near Intercostal Ostia in Weanlings**
Disease was detected in the aortic wall near 23 of the 85 intercostal ostia examined in weanlings. It was not evenly distributed between animals. The fraction of branches affected in each animal was used as a rough index of disease severity, and it varied from 0% (2 rabbits) to 86% (Table). There was no statistically significant correlation between this fraction and either plasma cholesterol levels or body weight (*F* = 1.9, *P* = .3), but 95% of the intercostal branches with lesions came from the heaviest 50% of the group, a nonsignificant trend that is consistent with the dependence on weight found in an earlier study. Lesions affected branches in the proximal, middle, and distal segments of the descending thoracic aorta with approximately equal frequency; there was no evidence to suggest that some orifices were particularly likely to be involved.

Fig 1a and 1b, respectively, show the distribution of streaks and spots near the intercostal ostia of weanlings. Streaks occurred in a small, approximately triangular area downstream of the ostium and occasionally at its lateral margins as well. The fraction of branches affected at any grid square averaged 0.19 ± 1.13% (mean ± SD), and the fraction for the square with the highest frequency was 14.1%. Spots had a similar distribution, although they were more scattered. Squares correspond-
ing to those affected by streaks in Fig 1a had >10 times the density of spots than did the remaining area of the grid (P<0.001). This ratio underestimates the spatial correlation between the two types of disease by a few percent since, for a given branch, the presence of one type of lesion precludes the presence of the other at the same location.

A second plot of the streaks, in which variations of intercostal branch geometry have been taken into account, gives a more accurate picture of their distribution close to the ostial lip (Fig 2). It reveals the presence of a region of lower disease incidence between the downstream lip of the ostium and the upstream edge of the triangular area of heavier disease, a feature also seen in the distribution of spots (data not shown) and in the early stages of diet-induced disease. Small discrepancies between this plot and Fig 1a are caused by the nonuniform size of the grid elements and their displacement relative to those in the rectangular map. Photographs illustrating the patterns of streaks and spots are shown in Fig 3a and 3b, respectively.

**Disease Near Intercostal Ostia in Old Rabbits**

For the group of old rabbits, the fraction of intercostal branches affected by lesions in each animal was again used as an index of disease severity. This index did not correlate significantly with age, plasma cholesterol concentration, weight, or length of stay in our facility (F=3.9, 0.1>P>0.05), although the marginal probability suggests that a pattern would emerge in a larger sample. As with the weanlings, there was no evidence for a preferential involvement of specific ostia.
Within the group of old rabbits, cumulative lesion frequencies were mapped for the 7 rabbits showing milder disease, as defined by their having less than one third of their intercostal branches affected, as well as for all of the rabbits combined. The fraction of branches affected at any grid square averaged $0.44 \pm 0.06\%$ (mean $\pm$ SD; maximum value, 4.8%) in the less-diseased group and $1.73 \pm 0.15\%$ (maximum, 6.9%) for all of the old rabbits combined. These mean values are more than twofold and ninefold greater, respectively, than the mean for weanlings, but the maxima are lower, indicating a wider, more-uniform spatial distribution of disease in the adult animals.

Streaks in the less-affected adults (Fig 4a) were mainly clustered around the lateral and upstream margins of the ostium, the former having the highest frequencies. Occasional patches were also seen that did not appear to be related to the branch. When more severely affected animals were included (Fig 4b), the most conspicuous feature was the complete sparing of a triangular region downstream of the ostium, essentially the area affected in young animals. Frequencies were relatively high downstream of this area, around the lateral and upstream branch margins, and along the sides of the grid, the latter location corresponding to disease that occurred toward the lateral walls of the aorta and toward its dorsal midline.

Figure 3. Representative photomicrographs of the fluorescence from branch regions of aortic wall that were viewed en face and excited with blue light. Glutaraldehyde-induced autofluorescence of normal tissue appears green or yellow and contrasts with the red lipid stain. For intercostals (A–D), mean aortic flow is from left to right (bar=0.5 mm). For celias (E, F), flow is from top to bottom. A, Fatty streaks downstream of an intercostal ostium from a weanling; B, lipid-rich spots in the vicinity of a similar branch; C, fainter, more diffuse staining upstream of an intercostal ostium from a mildly diseased old rabbit; D, streaks upstream and at the side of an intercostal branch from a more severely diseased old rabbit; E, disease predominantly downstream of a juvenile celiac ostium (bar=0.5 mm); and F, disease surrounding an adult celiac ostium (bar=2 mm); shadowing and unusual texture are visible in the upper left quadrant of the image at the site of a raised lesion.
There was sporadic sparing of the wall in the upper third of the grid.

Corresponding maps of the patterns of spots (Figs 5a and 5b) showed the same general trends. Although the higher variability makes this similarity hard to discern by eye, spots were significantly more likely to occur in squares corresponding to those affected by streaks in Fig 4b than at other sites, the ratio being 1.8:1 (P=0.02) for the less-diseased adults and 2.6:1 (P<0.01) for the group as a whole. (These ratios underestimate the spatial correlation by a few percent, as explained above.)

The map of streaks in regions close to the branch, corrected for variations in ostial size and shape (Fig 6), did not reveal any sparing close to the lip in affected regions. Photographs of streaks in less- and more-diseased vessels are shown in Fig 3c and 3d, respectively.
The patterns of streaks seen at the celiac ostia of weanling and old rabbits are shown in Figs 7a and 7b, respectively. The average frequency of disease at any site was 1.39 ± 0.66.53% (mean ± SD) for juveniles. This was much lower than the maximum value (67%), indicating that staining was restricted to a small fraction of the total area. The maximum value for old animals was approximately the same (60%), but the average was 10.12 ± 13.79%, the highest value obtained in this study.

In the younger animals, disease occurred in an arrowhead pattern surrounding the distal half of the ostium, the tip being oriented downstream. Disease was also occasionally seen on the upstream lip. This juvenile pattern was accentuated in the old animals, but there was in addition an even higher level of disease upstream of the branch. It seems unlikely that the disease in the upstream location was an extension of that occurring downstream, since the two sites were separated by regions having a low prevalence. The frequencies shown in the upstream region may underestimate the severity of disease, since gross examination showed that raised lesions were present at this site in a number of the mature vessels and these lesions, probably because of their fibrous cap, do not stain well.13

The maps corrected for changes in branch dimensions showed no evidence of sparing of the ostial lip, either upstream or downstream, in the two age groups, and spots were rarely seen (data not shown). Photographs illustrating the patterns of streaks are shown in Fig 3e and 3f.

Data are not presented for other abdominal branches. The location of the inferior mesenteric and renal ostia varied significantly between animals. The branches were close together in some vessels while in others they were separated by many ostial diameters. Furthermore, on two occasions disease was present in a continuous band between the origins of the superior mesenteric artery and a renal artery and hence could not be ascribed to the downstream area of the first branch or the upstream area of the second. Inferior mesenteric ostia were well separated from other branches, but they were not collected from all animals, and in those that were obtained, there was a substantially lower frequency of staining than at the celiac branch, especially in the old animals. It was consequently not possible to draw firm conclusions about the pattern of disease, but there did appear to be less development upstream than at the celiac branch in adult vessels (data not shown).

**Discussion**

Spontaneous disease of rabbit arteries has been investigated less frequently than the disease induced by a cholesterol-enhanced diet, and considerable confusion therefore exists concerning its prevalence and character. Influential early reviews25,26 of studies involving several thousand rabbits concluded that spontaneous disease does occur, but that it consists almost exclusively of localized areas of medial thinning and calcification. These lesions bear some resemblance to Monckeberg’s sclerosis of human arteries but not to the changes seen in atherosclerosis. Recent work23,27 has appeared to confirm that there is no lipid deposition in the arteries of normocholesterolemic rabbits. This view is so widely held that controls are often omitted in investigations of diet-induced lesions.

A few studies, however, have claimed to demonstrate spontaneous disease of an atherosclerotic nature. Ophuls28 found lesions in a large female rabbit that were histologically similar to human disease, and Nazum et al29 found raised, lipid-rich plaques in the aortic intima, particularly near branch ostia, in rabbits aged 2 to 3 years. The value of these studies has been questioned26 because the history of the rabbits is unclear, the former having been “raised in the country”28 and the latter being used in the standardization of insulin and as control animals in various experiments.29 Less controversially, Soolojew30 found that lipid-rich lesions were prevalent in suckling rabbits aged 35 to 48 days, although none were found 1.5 to 2 months after weaning. Extending this work, Bragdon21 demonstrated the occurrence of lipid deposits throughout the
suckling period. These deposits regressed after weaning and seldom recurred before maturity, but lesions were present in the majority of rabbits older than 2 years.

At least some of the uncertainty concerning the occurrence of spontaneous lipid deposition can be explained by its apparent dependence on age, particularly since many studies have used rabbits between weaning and maturity, a period that seems to have the lowest prevalence of lesions. For this reason, weanling and old rabbits were examined in the present investigation. A second cause of the uncertainty may be that different techniques have been used to detect lesions, ranging from macroscopic examination of unstained tissue to microscopy of stained sections. In the present study, lesions were identified by their reaction with oil red O to gain sensitivity and avoid confusion with the lipid-free lesions resembling Monckeberg’s sclerosis. To obtain the highest probability of detection, this staining was examined by fluorescence microscopy and lesions were mapped near branch ostia, the sites most commonly affected by spontaneous disease. Frequency-mapping techniques were used to avoid the omission of some types of lesion that can occur with the polar coordinate method. This combination of procedures did result in the detection of disease.

The spontaneous disease was generally rare. In 1 old and 2 weanling animals, there was no detectable disease at all. Of the 229 intercostal branches examined, 26% were affected, but in the majority of these only a few grid squares were involved. The spatially averaged frequency of disease was 0.19% for young rabbits and 1.73% for old rabbits. Even when disease was present, staining was almost always considerably fainter than in rabbits fed a cholesterol-enhanced diet and would have been difficult to detect with the unaided eye. Disease was less rare near the celiac ostium. In both age groups, the frequency of lesions reached 60% at some locations, and the average value was 10% in old rabbits.

Despite the rarity of disease, it was detected often enough to reach firm conclusions concerning its distribution. At both ages, spots and streaks showed similar patterns, consistent with the former being a precursor of the latter, and they are considered herein to be aspects of the same disease. Lesions occurred downstream of intercostal branches in weanlings with a pattern that was indistinguishable from the lipid deposition seen in hypercholesterolemic rabbits and immature human vessels. These downstream sites, however, were the least affected regions in old rabbits. Branch–related disease in this group appeared to occur near the upstream and lateral margins of the ostium and downstream of the spared region. Similarly, in adult human aortas, there is a sparing of disease downstream of intercostal ostia, with a high prevalence on the upstream lip and midway between ostia. Whether the apparent disappearance of staining from downstream regions represents regression of disease or the development of structures that overlie it remains to be determined.

Disease was observed at the downstream and lateral margins of celiac ostia in weanling rabbits, with occasional lesions on the upstream lip. This pattern is identical to the immature human pattern, but the distribution in hypercholesterolemic rabbits is less well established; maximal disease has variously been reported to occur in the downstream, lateral, or

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**Figure 7.** Maps of the frequency of fatty streaks near A, 6 celiac ostia from weanling rabbits, and B, 10 celiac ostia from old rabbits. Representation is the same as that described in the legend to Fig 1, except that there are 20×30 squares covering a total area of 6.6×9.9 mm² and that the more elongated ostia occupy a region with a radius approximately 1.5–2.5 squares from the central “x” in weanlings and up to 4.5 squares in old rabbits.
upstream quadrants, possibly reflecting differences in feeding protocols. In old rabbits, the highest frequency of spontaneous lipid deposition occurred upstream of the ostium, as at celiac branches in adult human aortas. In contrast to the intercostal ostia, however, considerable disease occurred around the downstream half of the branch, a trend toward the juvenile pattern that seemed to be accentuated at the inferior mesenteric ostium. The reason for this discrepancy is unclear.

The spontaneous disease of old rabbits might be a useful model of atherosclerosis. Not only does its location coincide with that seen in adult human aortas but also raised lesions with minimal lipid staining were apparent at some celiac ostia. Although rabbits can live for >10 years in captivity, animals of the age used in the present study are difficult to obtain. It was for this reason that the adult group was more diverse than the weanling group. Elderly, exbreeder female rabbits are more readily available, and we have observed a similar pattern of spontaneous celiac disease in one such animal (data not shown).

The same strain of rabbit, similar age groups, and an identical diet were used in previous studies of macromolecule transport into the arterial wall. Uptake of circulating albumin was found to be substantially greater downstream of intercostal ostia than upstream in young animals, but a smaller difference in the opposite direction was seen at later ages. These patterns coincide with the distribution of spontaneous disease, and theoretically, the spatial correlation could reflect causation in either direction. However, the pattern of transport in weanling animals is too consistent to be explained by such rare lipid deposits, and the same pattern occurs, albeit with a decreased mean uptake, at later ages when spontaneous disease is thought to be nonexistent. The pattern of transport in older animals is reversed by inhibiting NO synthesis or abolishing blood pressure and flow on a time scale over which disease cannot possibly be influenced. Thus, it seems more likely that variations in transport lead to variations in lesion prevalence, and a similar mechanism may apply in human vessels, since these show the same patterns of disease.

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References

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