Local Variation in Arterial Wall Permeability to Low Density Lipoprotein in Normal Rabbit Aorta

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Normal rabbits were injected intravenously with horseradish peroxidase (HRP) and 125I-labeled human low density lipoprotein (LDL), and the aortas were perfusion-fixed. Subsequent visualization of HRP in the aortas was produced by reaction of the tissue with diaminobenzidine and hydrogen peroxide. The luminal surface of the aortas showed many small punctate foci of brown reaction product to the HRP, which represented penetration of the HRP into the vessel wall. The foci were scattered over the luminal surface, and most of the focal areas were less than 1 mm in diameter. The concentration of LDL was up to 47 times greater in these focal areas than in surrounding noncolored regions not showing increased permeability to HRP. Small circumscribed foci of heightened permeability to LDL may predispose to the local accumulation of lipids and ultimately to the formation of atherosclerotic plaques.

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Two salient features of atherosclerotic lesions are the focal nature of plaque formation and the accumulation of cholesterol, cholesterol esters, triglycerides, and other lipids. The process by which atherosclerotic lesions originate is poorly understood. It is possible, however, that endothelial injury leading to increased arterial wall permeability could be a first stage in atherogenesis. In vivo studies with the protein-binding dye Evans blue have shown that the dye is taken up preferentially by certain regions of the aortas of rabbits, dogs, and pigs. In pigs these relatively broad regions of enhanced permeability have also been shown to incorporate 3H-cholesterol and 131I-fibrinogen at increased rates. In addition, these regions have been shown to be rich in apoprotein B.

Our report extends previous studies by identifying focal regions of increased permeability to horseradish peroxidase (HRP) in the normal rabbit aorta. These regions, which are less than 850 mm in diameter, are significantly smaller than those previously described for Evans blue (EB). Other studies have observed variations in HRP permeability within the rat aorta and among different arteries of the rat. The en face preparation used in this report and in our previous work adds to those electron microscope studies by demonstrating, on a macroscopic scale, the heterogeneity of the arterial wall.

Immunohistochemical studies of atherosclerotic plaques have demonstrated the presence of low density lipoprotein (LDL) apoprotein. Therefore, we examined the grossly normal arterial wall to determine if regions of enhanced HRP uptake correlate with sites of increased LDL permeability.

Methods

Human LDL was isolated from plasma according to the density gradient method of Hatch and Lees. LDL was iodinated with Na125I using a modification of the iodine monochloride method of McFarlane. The iodinated LDL was exhaustively dialyzed against a solution containing 10.99 g/liter NaCl and 1 mM ethylene diaminetetraacetate (pH 8.6). The injected radioactivity was at least 97% precipitable by 10% trichloroacetic acid in each experiment. Approximately 3% to 4% of the 125I in the injectate was lipid bound as determined by Folch extraction. All plasmas were obtained from normocholesterolemic human donors. The isolated LDL was

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characterized both by gel filtration and by immuno-electrophoresis.\textsuperscript{13} Radioactivity in the blood was TCA-precipitated and was similar to the results previously reported.\textsuperscript{13}

Five male New Zealand White rabbits were used in this work. Our animal care facility is accredited by the American Association for Accreditation of Animal Laboratory Care, and all procedures were reviewed and approved by the veterinary staff of the Division of Comparative Medicine. The animals in the experimental group (3 to 3.5 kg) were anesthetized with 50 mg/kg ketamine given intramuscularly as well as ether administered by mask. The left carotid artery and both femoral arteries were cannulated, and a catheter was inserted into the right marginal ear vein. The carotid cannula was connected to a bottle of fixative (25 g/liter glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4) positioned 180 cm above the table; the femoral cannulas were connected to a waste bottle. These lines were kept clear with normal saline.

At 0 time, 2.5 to 5 mCi of \textsuperscript{125}I-LDL was injected through a nitrocellulose filter (pore size, 0.22 \mu m, Millipore) into the ear catheter. At 5 minutes, a sample of blood was drawn through the right femoral cannula. At 9 minutes, 80 mg/kg of HRP (Type II, Sigma) in 5 ml of saline was injected through the filter into the ear vein.\textsuperscript{7} At 10 minutes, the rabbit was killed by injection of 1 ml of pentobarbital. The aorta was then immediately perfusion-fixed in situ at 100 mm Hg via the carotid cannula, and the femoral cannula was used to collect the effluent. After 10 minutes of perfusion under pressure, the flow rate was cut back. At 40 minutes, the thoracic aorta was removed and placed in fixative. After 30 minutes the aorta was transferred to a solution of 70 g/liters sucrose in 0.1 M sodium cacodylate (pH 7.4) and stored at 4\degree C. Blood samples were centrifuged at 2400 rpm at 4\degree C for 20 minutes, and plasma supernatants were pipetted off and in stoppered tubes at 4\degree C. Later, the plasma samples were assayed for \textsuperscript{125}I-LDL concentrations and TCA precipitability. A separate control group of three rabbits was treated identically except for the omission of the LDL injection. An additional group of rabbits was treated identically, but without prior infusion of either LDL or HRP.

Each aorta was trimmed, pinned out, and reacted with diaminobenzidine (DAB) as previously described\textsuperscript{2} to form a brown reaction product at the site of the HRP. The foci of brown staining were as large as 850 \mu m in diameter in the arch and averaged about 200 \mu m (range, 150 to 300 \mu m) in diameter in the descending thoracic aorta. The adventitia was peeled away from the stained aorta, leaving the intima and the entire media for tissue sampling. Tissue was taken from the descending thoracic aorta, except for the regions between the paired intercostal arteries. Each piece of tissue, having typically about 0.3 cm\textsuperscript{2} luminal surface area, was inspected microscopically for red blood cells and was discarded if there was observable blood contamination. The brown (HRP-stained) foci and white (unstained) regions (Figure 1) from the thoracic aortas of rabbits in the experimental group were carefully separated. The brown foci were carefully sliced out of the tissue by sharp dissection using an 11 Bard-Parker scalpel blade. All white regions were pooled, except those that were trimmed away from the brown foci. Only a thin border of white tissue outlined the brown foci as seen through a dissecting scope at \times 30 magnification.

The pooled brown focal areas and the pooled white regions from each animal were counted in a gamma counter for at least 500 minutes to assure accurate measurements. The tissue samples were then dried and weighed. Some tissue samples were examined to determine the amount of TCA nonprecipitable radioactivity in those samples. TCA nonprecipitable counts were not found in the glutaraldehyde fixed tissue. Therefore, all the measured radioactivity in the glutaraldehyde-fixed tissue was protein-bound. Other work in our laboratory has also indicated that relatively few counts are lost with our perfusion fixation technique or in any succeeding processing steps.\textsuperscript{16}

Results

Morphologic results from the current study were similar to those previously reported. Foci of increased permeability showed only minor topographic changes as compared with white areas.\textsuperscript{7} There was at no time any loss of endothelium, and no separation between endothelial cells was seen by transmission electron microscopy. No difference in the numbers of HRP-positive areas of their morphology was found in control animals. Aortas not exposed to HRP showed no brown regions.

Table 1 presents the tissue sample dry weights and the corresponding \textsuperscript{125}I-LDL radioactivity concentrations. The measured concentrations were corrected for decay to yield the \textsuperscript{125}I-LDL concentration on the date of the experiment. The total weight of tissue measured for each animal is low since much tissue had to be discarded to assure separating HRP-stained tissue from unstained tissue. Plasma concentrations, C_{po}, represent the TCA-precipitable counts, which were never less than 97\% of the total counts measured in the plasma. A dimensionless tissue concentration, C_{T}/C_{po}, based on the dry tissue concentration, C_{T}, was then calculated from these data. This dimensionless concentration is also presented based on wet weight (calculated by assuming a wet weight/dry weight ratio of 3.74:1) to permit comparison to our group’s previous data.\textsuperscript{13} Results from the pooled focal areas indicate that the spontaneously arising focal areas that are delineated as highly permeable to HRP are also permeable to LDL. The ratio of glutaraldehyde-fixed radioactivity in brown regions to that in white regions averaged 35:1 with a range of 10:1 to 43:1.
Figure 1. En face photomicrograph of sites of enhanced horseradish peroxidase (HRP) uptake in three regions of the rabbit thoracic aorta. Flow is from top to bottom in all photographs. A. Region of aorta from which photographs were taken. B. Region around ostia in the aortic arch. C. Region around the second and third pairs of intercostal ostia. D. Region around the fifth and sixth pairs of intercostal ostia. The presence of HRP within the tissue is revealed by incubating the excised aorta in a solution of diaminobenzidine and hydrogen peroxide in sodium cacodylate buffer. Arrows in C and D point to typical brown spots of HRP-reaction product. These punctate sites have diameters ranging from about 100 to 200 μm in the descending thoracic aorta, and up to about 850 μm in the arch. They contain much greater concentrations of ^{125}\text{I}-\text{LDL} (up to 47-fold) than surrounding nonspotted regions when the radiolabeled LDL has been allowed to circulate 10 minutes before sacrifice.
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Table 1.  I-LDL Permeation: White vs Brown Regions

<table>
<thead>
<tr>
<th>Exper no</th>
<th>Tissue type (brown = HRP perm.) (white = low perm.)</th>
<th>Dry wt (mg)</th>
<th>cpm (on day of exper)</th>
<th>Cpo × 10^-7 (on day of exper)</th>
<th>Ct/Cpo × 10^3</th>
<th>Ct/Cpo × 10^3</th>
<th>Ratio Ct/Cpo' s</th>
<th>Brown/White</th>
</tr>
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<tr>
<td>1</td>
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<td>0.49</td>
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<td>23.0</td>
<td>0.84</td>
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<td>160</td>
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<td>1.1</td>
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<td>1.8</td>
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</tr>
<tr>
<td></td>
<td>Average</td>
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<td>4.4</td>
<td>35:1</td>
<td></td>
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<td></td>
<td></td>
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</table>

Uptake of radioactive label by the aortic wall. Cpo = glutaraldehyde-fixed tissue radioactivity concentration in cpm/mg dry weight or in cpm/μl wet tissue (calculated by assuming media water content is 75% by volume and density is 1.07 g/ml). Cpo = TCA precipitable plasma radioactivity concentration in cpm/μl. Each brown and white pair is the pooled data from one rabbit.

Discussion

Other studies9, 9 have observed general regional variations in HRP permeability at the electron microscopic level; however, the en face preparation used in this study demonstrates that there is a pattern to these variations in HRP permeability. These foci of enhanced permeability were scattered over the entire endothelial surface of the descending thoracic aorta, which appeared unstained except for these foci. There was a tendency for foci to be concentrated around the ostia of aortic branches.

The brown foci described here differ from regions of increased aortic permeability demonstrated in other studies in which EB dye was injected intravenously into normal animals whose endothelia were undisturbed.1-3 The uptake of EB identified broad regions of moderately increased permeability to macromolecules, since EB is an azo dye that circulates mainly bound to albumin. These EB-stained regions were mainly found in the aortic arch and in the thoracic aorta; smaller areas of increased uptake were found around the ostia of aortic branches.

In contrast, our current experiments with HRP demonstrate much smaller and more sharply defined foci of greatly increased permeability. The differences in the staining patterns obtained in these two different systems may reflect interspecies variations in macromolecular transport in arterial wall, differences in transport properties between HRP and serum albumin, or differences in affinity for binding to arterial wall tissue components. Another important difference between our work and previous work that used EB as a marker is that EB was allowed to circulate for 3 hours, while HRP is allowed to circulate for only 1 minute before animal sacrifice. This short circulation time also does not allow for the development of any HRP-related hypotension. Presumably, the short circulation time of HRP and the immediate, rapid fixation of aortic tissue reduces lateral transport of HRP within the aortic wall, giving a sharply defined pattern of uptake.

The pattern of enhanced HRP uptake is reminiscent of regions of trypan blue staining associated by Bjorkerud and Bondjers17 with cell injury or death. Similarly, Gerrity and co-workers18 described a subset of Ruthenium red-permeable cells within EB-stained regions which they postulate to be injured or dead cells. They suggested that these injured and dead cells could function as "ultralarge pores." If HRP-permeable foci are areas of cell injury or death, then the presently observed HRP/LDL enhanced permeability could be a result of these ultralarge pores.

The ratio of LDL concentration in HRP-permeable foci to that in low permeability areas reaches almost 50:1. This concentration is more than an order of magnitude greater than the ratio of cholesterol content in the Evans blue-stained regions versus non-stained areas.7, 9 The difference may represent dilution of the cholesterol in the more diffuse EB-stained regions, although comparison may be inexact because of the vast differences in molecular sizes.18

The LDL concentrations reported here are also much higher than those predicted by Pfeffer et al.20 This may be due to the accumulation of LDL beneath dysfunctional endothelium. Thus, accumulation of LDL could be a result of increased binding of LDL within HRP-permeable regions. Alternatively, there may be more extracellular space available to LDL beneath dysfunctional endothelium. Additional studies are currently being undertaken to examine these possibilities.
We do not know the mechanism of these permeability foci. Several studies\textsuperscript{10, 15, 18, 21, 22} have noted generally altered cellular morphology in regions of increased EB and HRP uptake. Similar irregularities were reported in regions of hemodynamic stress about flow dividers in cholesterol-fed rabbits\textsuperscript{23, 24} and pigeons.\textsuperscript{25} More specifically, alterations in endothelial intercellular junction morphology were associated with increased EB permeability in pigs.\textsuperscript{26} Increased HRP uptake in rats,\textsuperscript{27} increased permeability to colloidal carbon in experimental renal hypertensive rats,\textsuperscript{28} and hemodynamically stressed regions of the aorta in hypercholesterolemic rabbits.\textsuperscript{24} Other studies, however, showed no difference in intercellular junctions when areas of low permeability to HRP\textsuperscript{7, 26} or EB\textsuperscript{26} were compared with high permeability regions. Widened endothelial junctions, therefore, cannot explain all incidents of increased permeability.

Altered pinocytotic transport could also cause increased permeability in endothelial dysfunctional areas. Schwartz and co-workers\textsuperscript{29} found greater transendothelial flux of ferritin in EB-stained regions when compared to unstained areas. Since ferritin has been observed to cross endothelium by only vesicular transport, an increase in transendothelial ferritin transport implies alteration in pinocytosis. Endothelial injury, widening of intercellular junctions, and increased pinocytotic transport may all be related to endothelial turnover or proliferation. Focal endothelial changes in cell replication and turnover have been noted.\textsuperscript{30-32} Davies and co-workers\textsuperscript{33, 34} have demonstrated higher rates of pinocytosis in growing endothelium. Other investigations have noted altered intercellular junctions in regions of endothelial regrowth.\textsuperscript{35-38} Lastly, regions about flow dividers and other hemodynamically stressed regions, exhibited high rates of \(^{3}H\)-thymidine uptake when compared to adjacent, nonstressed regions.\textsuperscript{39-43} Areas subjected to hemodynamic stress often exhibit cellular morphology changes and elevated permeability.

Atherosclerosis is a disease characterized by focal areas of lipid accumulation and intimal smooth muscle cell proliferation. Although the mechanisms of these processes are not understood, evidence suggests that LDL may be an important mediator of lipid deposition in plaques.\textsuperscript{10} LDL may also contribute to atherogenesis by acting as a mitogen for arterial smooth muscle cells.\textsuperscript{44} We have described here a focal increase in permeability to both HRP and LDL in intact endothelium of normal rabbits. If the increase in permeability reflects a pathologic process, then the dysfunction must be fairly subtle; gross endothelial damage or desquamation is not required to mediate large local increases in uptake of LDL. The highly focal pattern of increased LDL uptake observed in this study, occurring spontaneously in normal rabbits, is consistent with the putative role of LDL in atherogenesis and with the focal pattern of early plaque formation.

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