Endothelial Cell Perturbation and Low-Density Lipoprotein Quantitative Autoradiography

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INTRODUCTION

The mechanism of the initiation and formation of the atherosclerotic plaque remains unclear. The response to injury hypothesis suggests that endothelial cell loss induces intimal thickening and lipid accumulation, which are early steps in plaque formation. Many studies have confirmed that experimental de-endothelialization can induce an atherogenic response. A number of recent studies with experimental hypercholesterolemic animals, however, have observed more subtle morphological changes in the early stages of atherogenesis without the overt loss of endothelial cells. Apparent evidence of endothelial loss was not seen until plaque development was notable. Moreover, only a limited number of studies have demonstrated nonmechanically induced endothelial cell desquamation with exposure of the subendothelium. Furthermore, dead endothelial cells may remain a part of the intact monolayer until they are sloughed without exposing the underlying subendothelium. The infrequency of naturally occurring de-endothelialization suggests that endothelial cell loss may not be a common cause for initiation of the atherogenic process. Instead of cell denudation, endothelial dysfunction without cell loss could be the first event in atherogenesis.

The endothelium is a barrier to macromolecular transport into the arterial wall. Loss of this barrier function represents one form of endothelial dysfunction. Regions of enhanced permeability to the protein binding azo-dye Evans blue are seen in normal pigs and rabbits. Areas of increased Evans blue permeability are correlated with elevated uptake of labeled albumin, labeled fibrinogen, and labeled cholesterol. Endothelial cell morphology is slightly altered in these regions, but there is no evidence of de-endothelialization.

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FOCI OF ENHANCED ENDOTHELIAL PERMEABILITY

Utilizing horseradish peroxidase (HRP) as a macroscopic marker of enhanced permeability, Stemerman noted numerous spots of intense brown color scattered over the surface of the aorta of normocholesterolemic rabbits. These regions are found to be focal sites of enhanced permeability to HRP. Feeding rabbits a lipid-enriched diet increased the area covered by HRP foci by fivefold while raising the total cholesterol from 50 to 400 mg/dl. Others have also observed variability in vessel wall permeability to HRP at the electron microscope level. The regions studied by Stemerman and coworkers are scattered over the rabbit aortic luminal surface, appearing as foci (on average 200 μm in diameter, range 150–650 μm) of brown HRP reaction product. HRP-stained regions are most prevalent in the arch and upper abdominal aorta. The number of spots per square centimeter range from about 150 in the arch to 50 in the upper abdominal aorta and 15 HRP sites per square centimeter in the descending thoracic aorta. The corresponding fractional area coverage is roughly 3.0% in the arch, 1.5% in the thoracic aorta, and 0.5% in the descending thoracic aorta. Staining is more common proximal and distal rather than lateral to aortic ostia.

Stemerman et al. measured the uptake of ¹²⁵I-labeled LDL within sites of low and high HRP permeability. Following 10 min of ¹²⁵I-labeled LDL circulation and 1 min of HRP circulation, animals were sacrificed, perfusion fixed with glutaraldehyde, and sites of low and high HRP permeability were excised from the descending thoracic aorta. Samples of the high permeability foci included both the intima and the underlying media from each site and were roughly 0.1 mm² in luminal surface area. Intimal-medial samples from low permeability regions were typically 15 mm² in luminal surface area. ¹²⁵I-labeled LDL concentrations were up to 50 times greater in foci of enhanced HRP permeability when compared with low permeability zones.

QUANTITATIVE AUTORADIOGRAPHY

A method of absolute quantitative autoradiography, which permits the measurement of ¹²⁵I-labeled LDL concentrations within 1 μm cross sections of tissue, has now been incorporated into the study of foci of enhanced HRP/LDL permeability. The use of 1-μm cross sections as samples permits high-resolution studies of radiolabeled tracer uptake into small areas. Additionally, with autoradiography, the distribution of a radiolabeled tracer within a sample can be observed directly. Arterial wall tissue samples are excised from rabbits in which ¹²⁵I-labeled LDL is injected 30 min and HRP 1 min prior to sacrifice. Samples of HRP high and low permeability sites are embedded in epoxy resin for ultramicrotomy. One micron sections are cut and autoradiographed along with gelatin standards of known ¹²⁵I-labeled LDL radioactivity concentration. The autoradiographic grain densities measured over the tissue samples are translated into ¹²⁵I-labeled LDL concentrations by comparison with a calibration curve calculated from the gelatin standards. In this way, the concentration of ¹²⁵I-labeled LDL across the arterial wall within a one micron section is measured. Autoradiography reveals ¹²⁵I-labeled LDL concentrations one to two orders of magnitude higher in HRP foci than in low permeability regions. The highest concentrations are localized directly beneath the endothelium and are an order of magnitude greater than measured previously with whole sample counting. The region of elevated ¹²⁵I-labeled LDL concentration, after 30 min tracer circulation, measured about 100 μm in diameter and penetrated about 30 μm into the media. The endothelium in these unstained autoradiograms appeared unaltered.
In two animals, in addition to an injection of HRP and \(^{125}\text{I}-\)labeled LDL as described above, the lower third of the descending thoracic aorta was de-endothelialized and Evans blue dye was injected 35 min prior to sacrifice. The grain density pattern in de-endothelialized regions is strikingly different from either the white (low permeability) or brown HRP (enhanced permeability) sites. There is a high grain density over nearly half the media with a slow decrease toward the luminal adventitial border. Grain density measurements translated into \(^{125}\text{I}-\)labeled LDL concentration profiles reveal markedly increased concentrations in brown HRP regions and blue de-endothelialized regions when compared to white areas. The magnitude and shapes of the brown and blue profiles, however, are different, suggesting that different uptake mechanisms are operative. With the endothelium removed, the concentration near the lumen averages about 7% of the plasma value, and decreases continuously moving away from the lumen into the underlying media. By contrast, the concentration near the lumen in the brown regions approaches 25% of the plasma value and drops rapidly in the inner 20% of the media. The shape of the profile in the HRP foci is consistent with predominantly diffusive transport in the media, whereas the profile shape suggests that convection may dominate when the endothelium is removed. The high concentration near the lumen in the brown foci suggests that (1) the rate of transendothelial transport is extremely high compared to normal, and (2) LDL is sequestered in the subjacent media to a greater extent than usual, perhaps by binding to tissue components or smooth muscle cell receptors with subsequent internalization.

**ENDOTHELIAL CELL TURNOVER**

The increased presence of IgG permeable cells\(^{23,24}\) and elevated uptake of Evans blue in vivo\(^{46}\) have been correlated with increased cell proliferation. Evidence for foci of endothelial growth activity has been described.\(^{41-43}\) Any of these findings contribute in part to understanding the observation of foci of enhanced HRP-LDL permeability. Preliminary tritiated thymidine uptake studies were performed in HRP injected animals to determine if HRP brown staining correlated with endothelial growth activity.

Twelve animals were injected with \(^{3}\text{H}-\)labeled thymidine 17, 9, and 1 hour prior to sacrifice. Samples for en face autoradiography were dissected from the aorta and stripped of adventitial and medial tissue without damaging the intima. The autoradiograms from these studies revealed no consistent association between HRP uptake and tritiated thymidine uptake. These results indicate that only a subset of all replicating endothelial cells permit subsequently enhanced transport of macromolecules the size of HRP. A second possible interpretation is that the period within the cell cycle during which the region around a replicating cell is permeable occurs long before or after DNA synthesis.

**ENDOTHELIAL CELL PERTURBATION IN CULTURE**

Study of the mechanism(s) of focal endothelial cell dysfunction is difficult in vivo. We have recently investigated the feasibility of studying the response of the endothelium to protracted exposure of high low-density lipoprotein concentrations.\(^{44}\) We have used an all-human system to culture endothelium in the presence of atherogenic levels of LDL for up to one month. These experiments, thus far, show no direct overt cytotoxicity associated with such treatment. There was a notable steady change in
eicosanoid generation as measured by release of increased prostacycline, determined by measures of 6-keto PGF$_1\alpha$ from these cells as compared with controls. Thus LDL imparts a disturbance to normal EC function in vitro without induction of overt cytotoxicity.

**SUMMARY**

The focal entry and accumulation of LDL within the arterial wall of the normal animal may represent an early stage in the development of the atherosclerotic plaque. Concentrations of LDL 10 to 100 times normal medial concentrations might be difficult to clear from the arterial wall, permitting accumulation of lipid. Elevated LDL concentrations, in proximity to smooth muscle cells, appear to stimulate SMC proliferation. High LDL concentrations might also enhance mononuclear cell adhesion to endothelium. Since LDL has a high affinity for heparin and heparin for growth factors, LDL accumulation may be a mechanism for the concentration of such materials in the intima. The observation of markedly enhanced macromolecular permeability foci could be related to several potential mechanisms of initiation of atherosclerosis. This observation is of particular note when the focal occurrence of atherosclerosis is considered. Although atherosclerosis is seen as a generalized thickening of the intima, it is the focal narrowing of the lumen that is often responsible for the stenosis which produces symptoms such as angina or myocardial infarction.

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