Macromolecular Transport across Arterial and Venous Endothelium in Rats
Studies with Evans Blue-Albumin and Horseradish Peroxidase

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Atherosclerotic lesions are characterized by lipid infiltration in regions with high rates of endothelial cell turnover. The present investigation was designed to elucidate the route of macromolecular transport across vascular endothelium. The aorta and vena cava of male Sprague-Dawley rats were perfusion-fixed after the intravenous injection of Evans-blue albumin (EBA) or horseradish peroxidase (HRP). Fluorescence microscopic examination of en face preparation of the aorta stained with hematoxylin allowed the identification of endothelial cells that underwent mitosis, together with the localization and quantification of fluorescent spots for EBA leakage. The HRP specimens were subjected to histochimical treatment, and HRP leakage was seen as brown spots under the light microscope. Silver nitrate stain was added in both EBA and HRP studies to outline cell boundaries and to visualize stigmata, stomata, and dead cells. In the aorta, almost every dividing cell showed junctional leakage to albumin and HRP, with clustering of leaky spots around the branch orifices. Time-dependent studies showed gradual increases in the diameter and number of these heterogeneously sized leaky spots, which finally fused to sizes corresponding to the "blue areas" for EBA or "brown areas" for HRP. Compared with arteries, veins had fewer mitotic cells, but more dead cells and diffuse dye-staining areas, indicating a more rapid transport of macromolecules. The leaky spots in the artery were associated mainly with mitotic cells, dead cells, and stigmata, whereas those in the vein occurred primarily at regions with dead cells. These results suggest that the preferential association of the enhanced transport of macromolecules with mitosis in the arterial as compared to venous endothelium and the differential behavior in transmural transport between arteries and veins may form the basis for the predilection of atherosclerosis in arteries.

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dependent changes, 2) comparison between branching and nonbranching areas, and 3) comparison between arteries and veins.

Methods

Evans Blue-Albumin Study In Arteries

EBA conjugate was prepared by mixing 0.5 g of bovine serum albumin (obtained from Sigma Chemical, St. Louis, MO) and 0.1 g of Evans blue (Sigma) in 50 ml of normal saline. The EBA study was performed in arteries of 10 young, healthy male Sprague-Dawley (SD) rats on a normal diet and weighing 250 to 350 g. After pentobarbital anesthesia (Abbott Laboratories, North Chicago, IL; 3 mg/100 g, i.p.), the femoral arteries of both sides, the right femoral vein, and the right common carotid artery were cannulated by using PE 50 or PE 90 polyethylene tubings. EBA conjugate (5 ml) was injected through the femoral vein over a 3-minute period and was allowed to circulate for a total period of 10 minutes in five rats, 45 minutes in four rats, and 2 hours in one rat. Ten seconds before the termination of each circulation period, 0.3 ml of heparin (5000 USP units/ml; China Chemical & Pharmaceutical, Taipei, R.O.C.) was injected through the femoral vein to prevent coagulation, and this was followed by 0.5 ml of an overdose of pentobarbital to stop the heart. The arterial system was perfused through the cannulated common carotid artery with 60 ml of heparinized saline from a reservoir of the perfusion apparatus set at a pressure of 110 mm Hg, with the femoral arteries of both sides serving as exits for the perfusate. Then the perfusate was changed to a 1:1 mixture of 20% formaldehyde/4% glutaraldehyde (60 ml). Thereafter, the reagents for silver nitrate staining were perfused in the following order: 5% glucose for 2 minutes, 0.05% silver nitrate (Sigma) for 1 minute, and a 1:1 mixture of 3% CoBr (Strem Chemicals) and 1% NH₄Br (Merck) for 1 minute. The thoracic aorta was then removed, and the connective tissues, including the adventitia, were carefully peeled away with a fine forceps under a dissecting microscope. The aorta was cut open longitudinally along the ventral curvature and was sectioned into several segments. Each segment was stained with Hank’s hematoxylin (Merck) for 45 seconds, was mounted onto a glass slide with glycerol gelatin (Merck), was covered with a coverslip, and was examined en face with a fluorescence microscope (see below). Another coverslip was carved with a diamond knife under the dissecting microscope into a grid composed of squares of 1 mm². The grid was then placed on top of the coverslip, and a square was positioned in the center of the field at 100 x magnification under the fluorescence microscope. A micrometer was added in the eyepiece to subdivide this square into 400 (20 x 20) smaller squares (Figure 1). In this way we were able to record the coordinates, size, and endothelial morphology of every spot. The distribution of the spots in relation to the branch orifices of the intercostal arteries was plotted. Five times the diameter of the intercostal regions, and the remaining luminal surface area of the aorta outside the circles was referred to as the nonbranching region. In each of these two regions, the number of spots and the surface areas examined were determined for the calculation of the number density of spots per unit area as well as for the ratio of the number densities in these two regions.

Horseradish Peroxidase Study In Arteries

Ten SD rats weighing 250 to 350 g were cannulated as described above. HRP (type II, Sigma; 8 mg HRP in 1 ml of normal saline per 100 g of body weight) was injected through the femoral vein and allowed to circulate for 30 seconds in four rats, 1 minute in one rat, 2 minutes in four rats, and 4 minutes in one rat. As in the EBA study, the aorta underwent perfusion fixation and silver nitrate staining. After the adventitia had been peeled off, the aorta was incubated in a mixture of 0.15% 3,3'-diaminobenzidine (DAB, Sigma) and 0.02% H₂O₂ in Tris buffer (pH 7.0) at 37° C for 60 minutes. Then the aorta was cut open longitudinally and was stained with hematoxylin; the specimen was used for en face observation under the light microscope. In some of the HRP experiments, the aortic specimens were processed for electron microscopy. During perfusion fixation of the aorta, glutaraldehyde was also added to the outer surface of the vessel. One hour later, the excised aortic specimen was postfixed for 1 hour in 1% osmium tetroxide, was stained with 2% uranyl acetate, was dehydrated in ascending ethanol series, and was embedded in Epon. Thin sections were viewed in a Jeolco C100 electron microscope.

Horseradish Peroxidase Study In Veins

Ten other SD rats weighing 350 g were anesthetized with pentobarbital, and the right femoral vein was cannulated.
HRP was injected into the femoral vein and was allowed to circulate for 1 minute. Perfusion fixation and silver nitrate staining were performed as in the artery study described above, except for the following aspects. First, the perfusion pressure was maintained at 20 mm Hg instead of 110 mm Hg, and the perfusate entered the femoral vein and exited from the cut open jugular vein. Second, 0.05% silver nitrate was perfused for 5 minutes instead of 1 minute. The infradiaphragmatic segment of the inferior vena cava was excised and immersed in 2% glutaraldehyde for another 30 minutes. The remaining procedures were the same as in the artery study.

**Fluorescence Microscopy**

A Nikon epifluorescence microscope (MICROPHOT-FX) equipped with a diascopic phase-contrast attachment was used for identifying and mapping the distribution of EBA leaky spots in en face preparations of the thoracic aorta. The EBA fluorescence was studied with a combination of an excitation filter at 450 to 490 nm, a dichroic mirror at 510 nm, and a barrier filter at 520 nm. Color slides were made with Kodak Ektachrome P800/1600 professional color-reversal film 5020 for photomicrographs.

**Criteria for Identifying Mitotic Figures**

With hematoxylin staining, different stages of cell division in the mitotic (M) phase of the cell cycle were identified according to the following morphological criteria. Cells with visible, condensed chromosomes and an intact nuclear envelope were defined as being in the prophase. Cells with chromosomes aligned at a metaphase plate halfway between the poles and without a nuclear envelope were defined as being in the metaphase. Cells with separated chromatids pulled toward the poles were defined as being in the anaphase. Cells with chromatids clustered at each pole, a contractile ring creating a cleavage furrow, and a reformed nuclear envelope were defined as being in the telophase.

**Criteria for Identifying Dead Endothelial Cells, Stomata, and Stigmata**

In en face preparations of both aorta and vena cava, silver nitrate staining produced a network of dark brown lines corresponding to endothelial cell borders. Cells showing intense surface silver staining were defined as the dead cells. Focal black deposits on or between the cells seen by light microscopy, either as ring-shaped structures with a clear center or as solid dots with or without a halo of finer granules, were identified as stomata and stigmata, respectively.

**Results**

**Evans Blue-Albumin and Horseradish Peroxidase Studies in Arteries**

As found in our previous study, nearly all mitotic cells found on en face preparations were associated with the presence of EBA leaky spots. The present study shows that the same is true for HRP. Of all the EBA leaky spots, approximately one-third were associated with dividing cells (Figures 2 and 3A) and 5% with dead cells (Figure 3B). The remainder (more than one-half) of the spots were associated with ordinary, nondividing cells (Figure 3C); in about half of them a dark silver nitrate staining band exhibiting the morphology of stigmata could be seen either along the periphery or in the center of the cell (Figures 4A and 4B). Sometimes combinations of more than one morphological characteristic, e.g., dividing cell and stigmata (Figures 4C and 4D), and dividing cell and dead cell (Figure 4E), could be found.

Image analysis of the EBA fluorescent spots with an image processor system (Model IP 512, Image Technology, Woburn, MA) revealed a graded concentration distribution profile, with the peak located at the center of the spot (Figure 5). The average diameter of the EBA fluorescent spots increased as the EBA circulation time was prolonged (Figure 6A). The average number of EBA spots per unit area also increased with time (Figure 6B). For HRP, the rate of increase in spot size was much faster (Figure 7). There was a considerable degree of heterogeneity for spot size with each dye circulation time (Figure 8). The heterogeneity was much greater in the HRP study than with EBA. Small spots and diffuse HRP staining regions coexisted in the same sample of en face observations after an HRP circulation time as short as 30 seconds. As time increased, the spot size increased rapidly, and finally the spots fused together, forming diffuse HRP staining of wide areas of the aorta at about 4 minutes after its intravenous injection.

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Distribution profile of the association of leaky spots with nondividing cells, dividing cells, and dead cells. About one-third of the leaky spots examined on en face preparations under the microscope were associated with dividing (mitotic) cells, and 5%, with dead cells. More than one-half of the spots were associated with nondividing cells. EBA=Evan's blue-albumin.
Figure 3. En face preparation of rat thoracic aorta. Silver nitrate and hematoxylin staining. Bars=20 µm. A. A dividing cell in the anaphase with condensed chromosomes (arrows) in the center of a fluorescent Evans blue-albumin (EBA) spot (bright area). ×550 B. A dead cell with intense surface silver staining (arrow) associated with a horseradish peroxidase brown spot. ×360 C. Ordinary, nondividing cells associated with a fluorescent EBA spot (bright area). ×550

When the spatial distribution of EBA fluorescent spots was determined in relation to intercostal branch orifices, the spots were found to exist in higher density around branch orifices. Quantitative calculation showed an average of two times more spots per unit area in the branching than in the non-branching area, but no preference of spot distribution was found with respect to the upstream or downstream side of branch orifices (Table 1). There was a tendency for the spots to distribute spirally along the longitudinal direction of the aorta, curving around the branch orifices (Figure 9).

Extensive electron microscopic examinations of thin sections of aortic specimens in HRP experiments yielded the finding of one endothelial cell in mitosis (Figure 10), as revealed by the nuclear pattern of this cell. The junction to the right of this dividing cell shows abnormal widening and is filled with the electron-dense HRP reaction product (Figures 10B and 10C), thus providing direct experimental support of the cell turnover–leaky junction hypothesis.15 (This picture was reproduced with permission from a Proceedings paper which we reported at a meeting.14) Due to the rare occurrence of mitotic cells (1/104) compounded by the difficulty of obtaining the mitotic nucleus and leaky junction on the same thin section, it is estimated that more than one thousand grids need be examined to find one such picture.

Horseradish Peroxidase Study in Veins

HRP staining in veins was rather diffuse in comparison with that in arteries. After a 1-minute dye circulation time, the HRP staining area in veins was similar to that in arteries with a dye circulation time of 4 minutes (Figure 7). Dead cells (Figure 11) constantly appeared in the diffuse tracer staining regions. Mitotic cells were occasionally seen, but no association of HRP staining with these cells could be identified in the vein.

Discussion

An analysis of the various pathways for transendothelial transport of macromolecules has led to the hypothesis that the endothelial cells that are involved in cell turnover have poorly formed or leaky junctions.13 As described in our previous studies, 99% of mitotic cells are associated with leaky spots for EBA,8 and 80% of mitotic cells are associated with leaky spots for Lucifer yellow-labeled LDL.18 However, mitotic cells account for only 23% of the total EBA leaky spots8 and 42% of the LDL leaky spots15; thus, macromolecular leaky spots are also found around nonmitotic cells. According to the cell turnover–leaky junction hypothesis, one possible explanation for the finding of spots not associated with mitotic cells is that the intercellular junctions become disrupted before the meta-
Figure 4. En face preparation of rat thoracic aorta. Silver nitrate and hematoxylin staining. Bars = 20 μm. A. Dark silver nitrate staining band (arrow) in the center of the cell in a fluorescent Evans blue-albumin (EBA) spot (bright area). ×550 B. Dark silver nitrate staining spots (arrows) in the periphery of the cells in a horseradish peroxidase (HRP) brown spot. ×360 C. Appearance of a dividing endothelial cell in the metaphase with condensed chromosomes aligned at a metaphase plate (arrow), and a stigmata (black dot, short arrow) within the same fluorescent EBA spot (bright area). ×550 D. Appearance of a dividing endothelial cell in the metaphase (arrow), and stigmata (black dots, short arrows) within the same HRP brown spot. ×360 E. Coexistence of a dividing cell in the metaphase (arrow) and a dead cell with intense silver staining (short arrow) in the same fluorescent EBA spot (bright area). ×550

phase and need time to reform completely after the late telophase.14 Another possibility is that EBA leakage also occurs in association with endothelial cell death and during denuding or nondenuding desquamation.

The increase in the number of EBA leaky spots as dye circulation time is prolonged (Figure 6B) indicates that the junctions of different cells become leaky to macromolecules at various times. That is, because endothelial cells are in different stages of the cell cycle, there would be a progressive increase in the number of mitotic cells whose junctions become leaky to EBA. The increase in average spot size as dye circulation time increases (Figure 6A) reflects the continuous influx of EBA and its lateral diffusion process in the media. At 2-hour dye circulation time, diffuse dye-staining regions are seen compatible to the so-called blue area.3,18 The heterogeneously sized spots at each dye circulation time can also be explained by the asynchrony of cell mitosis with attended junction leakiness and the continuous influx and
Figure 5. Image analysis of an Evans blue-albumin fluorescent spot (micrograph of Figure 3A) with the IRIS system. Note the graded concentration distribution profile with a peak located at the center of the spot.

Lateral diffusion of the macromolecules. The HRP study demonstrated the same trend as that observed in the EBA study, but the lateral diffusion in the subendothelial space was more rapid. The spreading velocity of HRP was approximately 50 μm/min in the first minute and 10 μm/min between 2 and 4 minutes; in contrast, the spreading velocity of EBA was approximately 0.2 μm/min.

Certain sites in the circulation are more prone to the development of atherosclerosis than others. In humans, these sites include the inner curvature of a curved flow, the upstream side of the side branch, the downstream side of the main branch, and the inner sides of the bifurcation. It has been suggested that this focal nature of atherogenesis is determined by hemodynamic factors, e.g., shear stress, and disturbed flow at the vessel wall. The number and density of EBA spots was found in the present study to be twice as high in a branching region as compared to a nonbranching region, suggesting that the concentration of macromolecules transported into the subendothelial space would be correspondingly higher in the branching region than the nonbranching region. If the local concentration of atherogenic molecules is an important factor that initiates local atherosclerotic changes, this regional variation in macromolecular transport in relation to local hemodynamic pattern may provide a physical basis for the focal pattern of atherogenesis. While the mitotic cells were nearly all leaky to HRP and EBA, a lower percentage (80%) of these cells were leaky to LDL. These results suggest that...
there is some degree of size restriction of macromolecular passage through the leaky junction and that there are quantitative differences in the behavior of LDL from the smaller macromolecules. Further investigations on hemodynamic influences and the interactions among endothelium, macrophages, and platelets in these foci.

Table 1. Evans Blue-Albumin Spot Distribution in Branching and Nonbranching Regions

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<th>4</th>
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<td>45</td>
<td>45</td>
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*There were 111 spots on the upstream side and 100 spots on the downstream side.
†The means±SEM for A/B was 1.9±0.2 at 10 minutes and 2.2±0.3 at 45 minutes.
Figure 10. Electron micrographs of rat aortic endothelium. A. An endothelial cell undergoing cell division, × 5000 B. was taken from another thin section of the same cell and shows a junction widened to a bizarre shape (arrow) on the right side of the dividing cell (× 4700). C. An enlargement of the same junction, which is filled with horseradish peroxidase (× 15 500). Reproduced from reference 14, with the permission of Plenum Press.
may help to elucidate the mechanism leading to the final occurrence of atheromata plaques in loci of macromolecular leakiness.24,25

One of the important features of atherosclerosis is that it occurs in arteries but not in veins. Both types of vessels are exposed to blood with a comparable lipid composition and most other chemical constituents, but the hemodynamic factors (e.g., pressure, flow pattern, etc.) and the wall properties (e.g., wall thickness, wall composition, etc.) are markedly different. Fluid mechanical considerations suggest that the preferential occurrence of atherosclerosis in the thick-walled arterial vessels may be explained by the transport phenomena within the wall.26 Theoretical modeling shows that the thickness of a vessel is an important determinant of the hydraulic resistance, which in turn would influence the macromolecular transport across the vessel wall.25,26 The finding of a faster transendothelial transport through the thin-walled veins in comparison with the thick-walled arteries is in agreement with the theoretical prediction of a lower resistance to the macromolecular transport.25,26

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References


Figure 11. Diffuse horseradish peroxidase staining region in the inferior vena cava. Several dead cells (arrows) are seen in this region. En face preparation with silver nitrate and hematoxylin staining. × 550


Index Terms: albumin • atherosclerosis • endothelium • horseradish peroxidase • leaky junctions • macromolecular transport • mitosis • permeability
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