Macromolecular transport in heart valves. I. Studies of rat valves with horseradish peroxidase

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1Department of Chemical Engineering and 2Department of Mechanical Engineering, City College of the City University of New York, New York, New York; 3Institute of Biomedical Science of the Academia Sinica, Taipei, Taiwan, Republic of China; and 4Department of Medicine, College of Physicians and Surgeons of Columbia University, New York, New York

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Zeng Z, Yin Y, Huang A-L, Jan K-M, Rumschitzki DS. Macromolecular transport in heart valves. I. Studies of rat valves with horseradish peroxidase. Am J Physiol Heart Circ Physiol 292: H2664–H2670, 2007. First published February 2, 2007; doi:10.1152/ajpheart.01419.2006.—The present study aims to experimentally elucidate subtle structural features of the rat valve leaflet and the related nature of macromolecular transport across its endothelium and in its subendothelial space, information necessary to construct a rational theoretical model that can explain observation. After intravenous injection of horseradish peroxidase (HRP), we perfusion-fixed the aortic valve of normal Sprague-Dawley rats and found under light microscopy that HRP leaked through the leaflet’s endothelium at very few localized brown spots, rather than uniformly. These spots grew nearly as rapidly with HRP circulation time before euthanasia as aortic spots, particularly when the time axis only included the time the valve was closed. These results suggest that macromolecular transport in heart valves depends not only on the direction normal to, but also parallel to, the endothelial surface and that convection, as well as molecular diffusion, plays an important role in macromolecular transport in heart valves. Transmission electron microscopy of traverse leaflet sections after 4-min HRP circulation showed a very thin (~150 nm), sparse layer immediately beneath the endothelium where the HRP concentration was much higher than that in the matrix below it. Nievellstein-Post et al.’s (Nievellstein-Post P, Mottino G, Fogelman A, Frank J. Arterioscler Thromb 14: 1151–1161, 1994) ultrarapid freezing/rotary shadow etching of the normal rabbit valve’s subendothelial space supports the existence of this very thin, very sparse “valvular subendothelial intima,” in analogy to the vascular subendothelial intima; focal horseradish peroxidase spots; convective transport

THE UPTAKE, ACCUMULATION, and transformation of lipoproteins, such as low-density lipoprotein (LDL), have been thought to be among the earliest steps of valvular lesion formation that lead to tissue calcification, sclerosis, and stenosis in heart valves (1, 4, 5, 8, 13, 16–22, 24, 28, 29, 32). The study of Tompkins et al. (26) appears to be the first, and possibly only, study that quantitatively examined the transport and distribution of macromolecules as a function of anatomic location within heart valves. After 10–30 min of in vivo circulation of 125I-labeled LDL or 125I-labeled rabbit serum albumin, Tompkins et al. (26) excised aortic valves of squirrel monkeys and rabbits. They used absolute quantitative autoradiography to measure transvalvular LDL and albumin concentrations as functions of the fractional distance into the valve from the arterialis to the ventricularis aspects, for various sets of serial sections taken perpendicular to the plane of the valve endothelium (see Fig. 2 in Ref. 26). Overall, the LDL concentrations near both the aortic and the ventricular aspects are higher than those in the middle region of the interposed matrix, indicating that tracer transport has likely not yet reached steady state and depends on the direction normal to the endothelia. Unfortunately, these profiles showed a very large variation in both shape and magnitude for sections taken from different monkeys, from different leaflets, and even from different spots on the same leaflet.

Tompkins et al. then set up a mathematical model to describe this transport and used it to analyze each of their transvalvular macromolecular concentration profiles. Three important assumptions underlie this model: 1) The only independent spatial variable is the depth into the valve from the aortic aspect normal to the endothelium, i.e., macromolecular transport is independent of the direction parallel to the endothelium. 2) Molecular diffusion, without convection, is the only important transport mechanism for macromolecules in heart valves. 3) One can treat the matrix interposed between the endothelial monolayers of the valve leaflet’s aortic and ventricular aspects as a single, homogeneous layer with uniform transport properties. This one-dimensional (1D) model contains three transport parameters, an effective diffusion coefficient for the interposed layer and an endothelial mass transfer coefficient at each of the valve’s aspects. They found best-fit values for these three parameters independently for each experimental concentration profile.

Because of the above-mentioned variability in the magnitudes and shapes of the measured transvalvular concentration profiles, the parameters fitted for the different profiles reflected this by exhibiting very large variations. Such variation seemed to signify large mass transport variations both between different leaflets of the same aortic valve and even between different regions of the same valvular leaflet. However, the scale of this variability is troubling. For example, the mass transfer coefficients varied for the monkey’s aortic endothelium from 5.1 × 10^-9 to 73 × 10^-9 cm/s and for its ventricular aspect from 4 × 10^-13 to 3 × 10^-3 cm/s, ten orders of magnitude. If we exclude the largest and smallest values, this parameter still varied by a factor of 20. It is therefore likely that this 1D model is too simple and is meant only to quantify, rather than to account for, these large variations. This motivates us to question the three assumptions that underlie this 1D model. Since
such assumptions are commonly made for transport in vessels and valve leaflets of any species, it is valid to question these assumptions and to experimentally investigate their validity, in particular, in the rat valve model, which presents fewer ethical issues than work on monkeys.

Let us review the transport in the more well-studied artery wall. The endothelium, a monolayer of endothelial cells (ECs), is a barrier that blocks macromolecules in the blood from penetrating into the tissue. Weinbaum et al.’s (31) mathematical model proposed that macromolecules cross the arterial endothelium through the intercellular junctions around rare ECs that temporarily leak while in turnover, rather than being ferried through ECs in vesicles. Theoretically, these wide-junction leaks could transport enough macromolecules across the endothelium that even a few leaky junctions could dramatically increase the total endothelial permeability to macromolecules (23), even though the en face area of these leaky sites could be as little as 10^-6 of the entire endothelial surface (31). After short-time LDL circulation, Stemeran et al. (23) found isolated local sites of elevated permeability, i.e., leaks, rather than uniform leakage in rabbit aorta, but they did not elucidate the nature of those spots. Lin et al. found, by scanning the entire rat aorta, that nearly 99% of all mitotic ECs identified by hematoxylin staining leaked Evans blue-albumin (EBA) (14) and 80% of all mitotic ECs leaked the much larger Lucifer yellow-LDL (LY-LDL) (15). However, mitotic ECs accounted for only ~23% (14) of the total number of leakage sites for EBA and 45% (15) for LY-LDL. Stigmatic, dying ECs and cells in the process of sloughing off dying ECs accounted for more leakage sites (3). Truskey et al. (27) used I25I-labeled LDL autoradiography to show that only 25% of leaks were associated with mitosis. Although all of this work focused on the arterial endothelium, it is tantalizing to suspect that focal leakage may be responsible for Tompkins et al.’s (26) observed variability, since, even though the valve has a very different structure and function from the artery wall, the valvular endothelium is contiguous with the aortic endothelium, the endothelium being one of the largest “organs” in the body (12). Chuang et al. (3) studied the growth of horseradish peroxidase (HRP) leaks in the rat aortic endothelium as a function of HRP circulation time before death. They found rapid spot growth that, in hindsight, was not consistent with a diffusion-only model for any reasonable diffusivity and hinted at convection’s role. Frank and Fogelman’s (6) ultrarapid freezing/rotary shadow etchings revealed that the aortic intima was far sparser than the adjacent media, and Huang et al. (10) showed that this meant that it presented far less resistance to flow and tracer advection than the media. Their convection-diffusion theory needed to utilize this structural feature to explain Chuang et al.’s (3) data and showed that, indeed, convection dominated the macromolecular transport in the aortic wall.

In the present study, we focused exclusively on the rat model and asked which of these features carries over to the valve leaflet. We asked whether HRP can penetrate the rat’s valvular endothelium uniformly, or just focally. In the latter case, larger molecules such as LDL, would, a fortiori, also only cross focally. We used HRP as tracer to interrogate details of the rat valve leaflet’s structure and the related nature and magnitude of the quasi-steady (that is, averaged over the cardiac cycle) water convection patterns into and within the leaflet’s matrix. It should be noted that, as in any tracer diagnostic test of structure or of steady flows (e.g., tracer studies to detect perturbed blood flow around faulty heart valves), such conclusions are not limited by the length of the tracer experiment, or the specific (passive) tracer. The experiments reported below directly address the three assumptions of Tompkins et al.’s model in the rat leaflet only. In the second paper in this series (Ref. 34; Part II, this issue)—not here—we shall guess that the monkey’s valve leaflets have similarities to the rat’s and attempt to justify this extrapolation by constructing a theoretical model that attempts to rationally explain, among others, Tompkins et al.’s studies (26), and their great variability, quantitatively with a single, unique set of parameters.

**METHODS**

All protocols were approved by the Institutional Animal Care and Use Committee.

We anesthetized healthy male Sprague-Dawley rats, weighing ~300–400 g on a normal diet, intraperitoneally with 1% pentobarbital sodium solution (Sigma; 7 mg/100 g body wt) and cannulated both of the rat’s femoral arteries and its left femoral vein with PE-50 polyethylene tubing. HRP (type II, Sigma; dissolved in 0.5 ml of normal saline and at a dosage level of 2 mg/100 g body wt) was injected through the femoral vein and allowed to circulate for 30 s in seven rats, 60 s in four rats, 120 s in four rats, and 240 s in five rats. One rat (control) received no HRP. Ten seconds before the termination of each circulation period, we injected 0.5 ml of heparin (Elkins-Sinn; 5,000 USP units/ml) through the femoral vein to prevent blood coagulation, followed by 2 ml of an overdose of pentobarbital to stop the heart. The chest was opened immediately. The heart was perfused through the left ventricle, punctured by a needle catheter with heparinized saline from a pressure reservoir set at the physiological pressure of 110 mmHg until clear fluid emerged from both femoral arteries, which served as egress sites for the perfusate. We then switched the perfusate to 30 ml of 2% glutaraldehyde. We harvested the heart and carefully dissected out the aortic valve’s leaflets under a dissection microscope. The valvular leaflets were then processed either for en face study under light microscope (LM) or for an ultrastructural study of the subendothelial matrix under the transmission electron microscope (TEM) (3, 9).

A further control followed the above procedure through the 30-s HRP circulation. We perfused this rat with PBS pressurized to 100 mmHg in the reservoir through the right femoral vein to wash out the blood. When clear fluid replaced blood exiting through the femoral arteries (~10 min), we cannulated the descending aorta, tied off the aortic bifurcations between the cannulation and the heart, and used PBS, pressurized at 100 mmHg, to close the valve and create a pressure gradient (HRP washout conditions) across it, for 1 h. We harvested the valve, fixed it, carried out the 3,3’-diaminobenzidine (DAB) reaction, and prepared slides as above.

*En face study of valve leaflets under LM.* We incubated the valve leaflets that had been subjected to HRP circulation at 37°C for 1 h in a mixture of 0.05 M Tris-HCl buffer (pH 7.0) containing 45 mg of DAB tetrahydrochloride (Sigma) and 20 µl of 30% H2O2. In some experiments the valve leaflets were stained with Harris’ hematoxylin (Sigma) for 45 s to mark the EC nuclei. We then mounted the valve leaflets onto a glass slide, covered them with coverslips, and examined them en face under LM (Olympus BX51). We calculated the effective radius $R$ of an HRP spot by outlining the brown spot with the cursor, calculating the area $A$ outlined, and setting $R = (A/π)^{1/2}$.

*Ultrastructural study of subendothelial matrix under TEM.* After 30 s (1 rat) or 4 min (1 rat) of HRP circulation, we processed the valve leaflets for TEM examination. They were further fixed in 2% glutaraldehyde for 30 min and then in a mixture of 1% tannic acid and 1.5% potassium ferrocyanate for 1 h to obtain enhanced contrast with a modified Karnovsky method (11). After rinsing was completed, we
postfixed the leaflets in 1% osmium tetroxide for 90 min. Subsequently, the leaflets were washed with distilled water, stained with 2% aqueous solution of uranyl acetate at 60°C for 15 h, dehydrated in a graded series of ethanol, infiltrated with propylene oxide, and embedded in Epon 812. The leaflets were properly oriented during embedding so as to be cut perpendicularly to the valvular endothelial surface. A diamond knife on an ultramicrotome (Reichert-Jung, Vienna, Austria) cut ribbons of ultrathin sections with a silver or pale gold reflection color. We collected them on Formvar-coated single-slot grids without poststaining and examined them in a JEM 1200EX electron microscope (JEOL, Tokyo, Japan).

RESULTS

Previous LM studies (2, 3, 14, 15, 23) of en face arterial endothelium preparations after short-time HRP circulation in normal rats found isolated brown spots resulting from HRP leakage. The present en face LM examination of the valve leaflet similarly found HRP brown spots in very few, localized parts of the whole leaflet and not uniformly. Figure 1 shows leaflet similarly found HRP brown spots in very few, localized leakage. The present en face LM examination of the valve normal rats found isolated brown spots resulting from HRP endothelium preparations after short-time HRP circulation in

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\[ \text{such HRP leakage spots in the valve leaflet after 30 (Fig. 1\text{A}) and 120 (Fig. 1\text{B})-s HRP circulation in a normal rat at two magnifications. Figure 1\text{B} shows an entire leaflet with spots. The overall color of Fig. 1\text{B} is darker than Fig. 1\text{A} because of the much longer HRP circulation time and thus larger amount of HRP that has penetrated into the tissue, some beginning to penetrate through the normal junctions. To show that the leaflet does not trap the reporter molecule HRP, we carried out a control experiment in which, after 30-s HRP circulation, we flushed HRP-containing blood from the heart and, using a PBS solution, applied a pressure gradient from the aortic side across the valve. The results (not shown), when viewed en face, showed a nearly white leaflet, far whiter than Fig. 1\text{A}, indicating that essentially all of the HRP was washed out. Since the EC monolayer tiles the valve leaflet surface, one can estimate a rough lower bound for the average area per EC by dividing the total area by half the total number of hematoxylin-stained nuclei, half because one sees the ECs from both aspects and a lower bound because a fraction of the nuclei might be from rare interstitial cells. If each EC is assumed to be roughly circular (even though circles do not tile a plane), we calculate average effective EC radii from patches from different valve leaflets of 11–13 \( \mu \)m, which is comparable to 15 \( \mu \)m for arterial ECs (33). We use a representative value of 11.7 \( \mu \)m below.

Figure 2\text{A} shows how the radius of HRP leakage spots in the valve leaflet increases with HRP circulation time. Two separate experimenters independently did these experiments, and the results are quite consistent. The spots grow rapidly in the first minute and gradually in the following 3 min. Finally, after 4 min of HRP circulation, the spots fuse together, forming wide areas of diffuse HRP staining region. The striking feature of this HRP leakage spot growth curve for the aortic valve is how similar it is in shape and magnitude to (only \( \approx -25 \)% slower) that in the thoracic aorta (3). Since the valve leaflet experiences a pressure gradient that can potentially drive a convective flow across it only when the valve is closed, and the valve is closed for roughly 70% of a cardiac cycle, Fig. 2\text{B} also replots the leaflet spot growth curve as a function of only the HRP circulation time during which the valve is closed. It should be noted that in the first minute the valve and aorta curves are extremely close, and then the former grows slightly slower. Since the tracer is injected into the femoral vein, there is a time lag before it reaches the blood surrounding the valve. Huang et al. (10) estimated this time as \( \approx 25 \) s for the aorta by extending the theoretical spot size curves that agree with the experimental points back to a nondimensional radius of 1. Clearly, the delay for the valve should be almost identical, and one would expect a valve theory to similarly extend backward to radius 1 for a similar time.

The number of rats, about 20, in the present investigation was not large enough for detailed quantitative and statistical assessment of the spatial distribution and frequency of HRP spots. In general, we found no preference for HRP spots among the three leaflets of the same aortic valve in a normal rat. However, in a single leaflet, most of the spots tended to localize in the pressure-bearing part of the leaflet and near the line of coaptation where the ECs are more easily damaged or killed because of the recurring coaptation at the cardiac frequency. Figure 2\text{B} shows a typical distribution of spots in a leaflet. In addition, one could easily find incomplete spots along the sample’s cutting edge, i.e., the line of attachment between the leaflet and the sinus of Valsalva. Of 47 leaflets examined, we found 81 HRP leaky spots and there were 0–3 HRP leaky spots per leaflet, including both aspects; since our observation was through a leaflet under LM, we did not distinguish on which aspect a leak appeared. Since human valve lesions occur almost exclusively on the pressure-bearing aortic face (29), Nievelstein-Post et al. (18) looked for lipid deposits mainly there. If convection, as we shall see, is responsible for the large spots, then one expects all of the larger spots to emanate from the aortic aspect, since convection drives tracer into aortic aspect leaks and out of the leaflet for ventricularis leaks. Any (rare) ventricularis leaks would tend to lower the average spot size at each circulation time relative to only aortic face leaks.

Figure 3\text{A} shows the TEM examination of the traverse sections of the valve leaflet after 4 min of HRP circulation,
serially sliced until we encountered regions associated with ECs whose junctions were leaky to HRP. The lumen is on the upper left, and an EC is aligned diagonally. The intense black color, representing HRP, appears particularly strong in a very thin region (roughly 150 nm thick) directly under ECs in Fig. 3A (but not in the control, Fig. 3B), followed by an abrupt change to a much lighter color, indicating that HRP concentration is much higher in this subendothelial thin layer. We have many similar figures from this rat and others from the rat exposed to only 30-s HRP circulation. The sections from the HRP-exposed rats that were not near localized leaks resemble those of the control.

**DISCUSSION**

*Is macromolecular transport in rat heart valve leaflets one-dimensional?* En face LM examination of normal rat aortic valve endothelium after short HRP circulation times showed only isolated brown spots (Fig. 1), indicating that HRP leaked across the endothelium focally and not uniformly. If these leaks are similar to those in the aorta, they are due to the enlarged junctions of leaky ECs relative to normal tight junctions (31). We chose short circulation times because at circulation times much larger than 4 min, the small HRP molecule, unlike the larger LDL molecule, is known to easily penetrate normal EC junctions in the aorta as well as isolated leaks (3), resulting in difficulty in distinguishing localized HRP spots from background. These spots tended to appear mostly in the leaflet regions noted above. If HRP, a much smaller molecule than LDL (3–5 vs. 22–23 nm in diameter), can only traverse the endothelium through isolated leaks at such short times, then the larger LDL molecule also likely can only pass through some of these isolated leaks. In the aorta, the larger LDL can only pass through localized leaks even at longer times. Once the tracer has crossed a localized leak, it has the potential to transport both normal to and parallel to the endothelium. Thus macromolecular transport in heart valves depends on both of these directions, i.e., it is at least two-dimensional (2D). As such, sectioning near a leak will give a very different LDL profile than sectioning far from a leak. Part II (Ref. 34, this issue) considers the possibility that the squirrel monkey’s leaflets are similar to the rat’s, and that such focal leakage may account for the variation observed by Tompkins et al. (26).

*Is molecular diffusion the only important mass transport mechanism in the rat valve leaflet?* Because Chuang et al.’s observations (3) of the rate of HRP spot growth in the rat aorta led to important insights into the mechanism of mass transport in the rat’s artery wall, we performed a similar study, with the same HRP circulation times of 30, 60, 120, and 240 s, on the rat’s aortic valve leaflet. En face LM examination of HRP spots in the aortic valve (Fig. 2) showed growth reflecting a continuous influx of HRP across leaky EC junctions and subsequent transport, strikingly similar in rate and extent to that in rat aorta (3), which had a large component parallel to the endothelium in the subendothelial matrix. The valve’s spots reached a radius of ~75 μm in 4 min, but the valve was closed, experiencing a pressure difference across it of ~90 mmHg for only ~70% of those 4 min and nearly 0 mmHg for the balance. In contrast, the transmural aortic pressure cycles from ~80 to 120 mmHg over the cardiac cycle. (In both the leaflet and the aorta, the transendothelial pressure drop is a fixed fraction of the total drop across the entire tissue. In the aorta, this fraction is ~1/2 (25) for these pressures; a value for this fraction in the valve is determined in Part II.) Figure 2B also plots a curve that rescales the time axis for the valve’s spot growth to take into account only the time the valve was closed. Its similarity to the aortic spot curve is even more striking, strongly suggesting that HRP transport through the leaky junction and in the subendothelial space in heart valves and in arteries may be similar. Huang et al. (10) showed that only convection in the subendothelial space with a strong component parallel to the endothelium in a subendothelial intima that was much sparser and therefore presented far less flow (and advection) resistance than the media could account for the observed rapid HRP spot growth.
that were far smaller than observation (10, 33). Huang et al. showed an extremely sparse intimal structure in an ab initio theory for the intimal transport parameters. They found that the intimal resistance is far lower than the media’s and that these nonuniformities lead to rapid intimal convection parallel to the endothelium, i.e., to tracer spot sizes in agreement with observation. Since the aortic valve leaflet apparently has no IEL, it is natural to ask whether the valve has a layer analogous to the artery’s subendothelial intima and, if so, if one needs to account for it. Or can a model that assumes a uniform, isotropic interposed matrix without any internal barriers such as an IEL, as in Tompkins et al.’s 1D model (26), but made 2D and including convection, account for the observed spot growth? One can reason that, in such a model, HRP traversing an interposed matrix without any internal barriers such as an IEL, account for it. Or can a model that assumes a uniform, isotropic layer? In a first attempt to explain HRP spot growth in the artery, Yuan et al. (33) proposed that the internal elastic layer (IEL) functions as the transport barrier that forces fluid crossing the endothelium to flow parallel to the endothelium in the arterial intima, thereby creating large tracer spots, rather than proceeding directly into the media. This assumption in water and permeability between the leaky and normal junctions then likely causes a subendothelial flow near the leak that is mainly parallel to the endothelium and pointing away from the leak. This parallel flow becomes negligible further from the leak and, augmented by the slower water flow through normal junctions, becomes normal to the endothelium. If a passive tracer is present, this flow advects it through releak and in a way to produce spots that initially grow quickly and then level off, as observed. A rational model for water and macromolecular transport in the rat leaflet must allow for this possibility.

Can the interposed matrix be treated as a single, homogeneous, isotropic layer? In a first attempt to explain HRP spot growth in the artery, Yuan et al. (33) proposed that the internal elastic layer (IEL) functions as the transport barrier that forces fluid crossing the endothelium to flow parallel to the endothelium in the arterial intima, thereby creating large tracer spots, rather than proceeding directly into the media. This assumption alone, under the presumption that the intima and media structures have similar intrinsic flow resistances, led to tracer spots that were far smaller than observation (10, 33). Huang et al. (10) used ultrarapid freezing/rotary shadow etchings (6) that showed an extremely sparse intimal structure in an ab initio theory for the intimal transport parameters. They found that the intimal resistance is far lower than the media’s and that these nonuniformities lead to rapid intimal convection parallel to the endothelium, i.e., to tracer spot sizes in agreement with observation. Since the aortic valve leaflet apparently has no IEL, it is natural to ask whether the valve has a layer analogous to the artery’s subendothelial intima and, if so, if one needs to account for it. Or can a model that assumes a uniform, isotropic interposed matrix without any internal barriers such as an IEL, as in Tompkins et al.’s 1D model (26), but made 2D and including convection, account for the observed spot growth? One can reason that, in such a model, HRP traversing an endothelial leak would spread at least as fast in the direction normal (the direction of the overall transleaflet pressure drop) to the endothelial surface as parallel to it, as Yuan et al.’s arterial study (33) found. One would thus expect it to yield significantly slower HRP spot growth for the valve than for the artery, in contrast to our observation (Ref. 3 and Fig. 2).

Is it possible that the valve leaflet, like the arterial wall, contains a very thin and sparse intima-like layer directly underneath the endothelium, whose flow resistance is much lower than the balance of the interposed region? If so, could such a region, even in the absence of an IEL, induce a convective flow to proceed preferentially in the direction parallel to the endothelium, and thus explain the observed HRP spot growth in the valve?

Figure 3 provides evidence to support the first of these hypotheses. It shows a TEM of a transverse section of a leaflet through a localized HRP leak after 4-min circulation. There is a very thin, ~150-nm, intensely black HRP layer, indicating a very high HRP concentration, immediately beneath the ECs, followed by an abrupt change to a much lighter color. Because a certain minimum level of peroxidase is needed to be detectable, the lighter areas need not be free of HRP or other, naturally occurring peroxidases, but their concentrations probably fall below this threshold. If the valve’s subendothelial matrix’s HRP-void space were uniform, one would have expected to observe a gradual decrease of HRP reaction product intensity with increasing depth into the valve’s matrix. Instead, this abrupt change immediately beneath the endothelium and the dark intensity in this thin region appear to indicate the presence of a thin, sparse arterial subendothelial intima-like layer that, in analogy to the aorta, likely has a much higher void space than the matrix below it. Consequently its permeability and effective macromolecular diffusion coefficient are likely much larger than those of the deeper layers. These conditions would favor lateral convective transport in this region as in the aortic subendothelial intima. Finally, the thickness of this very thin layer, roughly 150 nm, is comparable to that of the arterial subendothelial intima in the normal rat aorta, ~100–500 nm (10). Again, this is a structural conclusion that is independent of the HRP circulation time used as the diagnostic.

Further evidence for this proposition comes from Nievelstein-Post et al.’s (18) clear electron micrograph of an ultrarapid freezing/rotary shadow etching of a normal rabbit’s cardiac valve’s subendothelial space. Figure 4 shows that the subendothelial extracellular matrix immediately adjacent to the EC membrane, the diagonal middle layer in the figure, is much sparser than the matrix toward the upper right area of the figure that is further into the valve. This micrograph of the atrioventricular valve’s immediate subendothelial layer is surprisingly
The thickness of this layer is unknown, it is only an upper bound). In summary, we have used short-time tracer diagnostics and TEM to draw conclusions about the detailed structure of the rat aortic subendothelial intima-like layer in Ref. 6 (see, in particular, Fig. 5 of Ref. 6), indicating the likelihood that these similar structures have similar properties. In particular, the typical spacing between the dominant fibers and collagen is ~30–40 nm in Fig. 4, which is consistent with that in the aortic intima (6, 10, 18). In Ref. 10 we argue that the freeze etchings in Refs. 6 and 18 do not show the glycosaminoglycans (GAGs), but our calculations indicate that the GAGs do not act to hinder transport in the aortic intima. The same is likely true for Fig. 4 as well.] The apparent (because the crack plane’s angle is unknown, it is only an upper bound) thickness of this layer is ~200 nm, remarkably consistent with that estimated from our TEM examinations of the rat valve (Fig. 3).

It should be noted that the intense HRP staining of this region in Fig. 3 is not due to HRP trapping. This region is geometrically far less likely to be a large-molecule trap than deeper tissue’s far tighter matrix (Fig. 4). If it trapped HRP by rapidly binding HRP, then entering HRP would immediately bind to matrix adjacent to the leak, contradicting the observation (Fig. 3) of the ultrarapid growth of focal HRP spots to form a very large spot, unhindered by binding to matrix. As a final demonstration that there is no HRP trapping in the leaflet, we pressurized the leaflet with PBS at 100 mmHg for 1 h after 30-s HRP exposure and found the HRP successfully washed out. We chose this long washout time because for HRP to escape through the leaflet interior it must traverse the far thicker, far denser tissue between the endothelia and then escape through the very tight ventricular endothelium. Thus the kinetics of washout from the leaflet are likely far slower than those of its spread in this region, and we chose a washout time long enough for the likely appearance of a ventricular aspect endothelial leak to allow HRP escape [0–3 leaks per leaflet at any given time and mean aortic (the only region for which there are data) leak lifetime of ~1 h (3, 30)].

The above evidence strongly supports the hypothesis of a sparse, 150- to 200-nm-thick subendothelial intima-like layer in the valve, with much higher void space, Darcy permeability, and effective tracer diffusion coefficient than the balance of the matrix in the interposed region. In analogy to the artery, we call this layer, composed of a basement membrane plus loose connective tissue, the “valvular subendothelial (to exclude the endothelium) intima” despite the fact that, unlike the arterial intima, this region is not bounded by a continuous IEL. Although the valvular subendothelial intima comprises a tiny fraction of the ~15-μm-thick valve leaflet, it may, in analogy to the arterial subendothelial intima, play a critical role in macromolecular transport into and in the concentration distributions within the valve. Our new theoretical models in Part II investigate how its inclusion affects the prediction of short-term tracer spot growth in heart valves as observed, e.g., in the present paper. Figure 5, a diagram of the cross section of the valve leaflet, indicates the structural features that we have determined.

To date, the existence of a valvular intima has been noted by Nievelein-Post et al. (18), Haberland et al. (7), and Simionescu et al. (21), but no one seems to have yet addressed its structure, and therefore its importance in macromolecular transport in the cardiac valves. The likely reasons why it has escaped study are that 1) unlike large arteries, the heart valve has no IEL to highlight the existence of its intima; 2) there has been little quantitative study of short-time macromolecular transport in heart valves, where the importance of the valvular intima is most obvious; and 3) it is natural to suppose that such a thin layer cannot strongly influence the overall transport processes, especially for biologically interesting long times. As we shall see in Part II, this latter line of reasoning may be too facile.

In summary, we have used short-time tracer diagnostics and TEM to draw conclusions about the detailed structure of the rat aortic subendothelial intima and that the change in matrix sparseness is rather abrupt. Note that the apparent layer thickness of ~200 nm is consistent with the estimate from Fig. 3. [From Nievelein-Post P, Mottino G, Fogelman A, Frank J. An ultrastructural study of lipoprotein accumulation in cardiac valves of the rabbit. Arterioscler Membr 14: 1151–1161, 1994. (18).]

Fig. 4. Electron micrograph of an ultrarapid freezing/rotary shadow etching of the subendothelial space of the atrioventricular valve from a normal rabbit. Arrows indicate low-density lipoprotein (LDL) particles, also shown in inset. MEMB and CYTO, cell membrane and cytoplasm of endothelial cells, respectively. Image clearly shows that the matrix immediately adjacent to the EC membrane is much sparser than the denser matrix further into the valve interior and that the change in matrix sparseness is rather abrupt. Note that the apparent (because the crack plane’s angle is unknown, it is only an upper bound) layer thickness of ~200 nm is consistent with the estimate from Fig. 3. [From Nievelein-Post P, Mottino G, Fogelman A, Frank J. An ultrastructural study of lipoprotein accumulation in cardiac valves of the rabbit. Arterioscler Membr 14: 1151–1161, 1994. (18).]

Fig. 5. A schematic cross section of the valve leaflet showing the aortic and ventricular endothelia, with a focal leak deriving from the junctions around an individual EC on the aortic aspect. Directly adjacent to each endothelium is a thin (~150–200 nm thick) subendothelial intima (SI). At present, we simplify the balance of the matrix between the endothelia (the lamina fibrosa and lamina spongiosa) as a uniform, homogeneous medium.
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