Transport in rat vessel walls. II. Macromolecular leakage and focal spot size growth in rat arteries and veins

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Shou Y, Jan K-m, Rumschitzki DS. Transport in rat vessel walls. II. Macromolecular leakage and focal spot size growth in rat arteries and veins. Am J Physiol Heart Circ Physiol 292: H2881–H2890, 2007. First published January 5, 2007; doi:10.1152/ajpheart.00575.2006.—Transendothelial lipid transport into and spread in the subendothelial intima of large arteries, and subsequent lipid accumulation, appear to start plaque formation. We experimentally examine transendothelial horseradish peroxidase (HRP) transport in vessels that are usually, e.g., pulmonary artery (PA), or almost always, e.g., inferior vena cava (IVC), atherosclerosis resistant vs. disease prone, e.g., aorta, vessels. In these vessels, HRP traverses the endothelium at isolated, focal spots, rather than uniformly, for short circulation times. For femoral vein HRP introduction, PA spots have 30-s radii [~53.2 μm (SD10.4)]; compare aorta: 54.6 μm (SD8.75) and grow quickly from 30 s to 1 min (40%, P < 0.05) and more slowly afterward (P > 0.05). This trend resembles the aorta, suggesting the PA has a similarly sparse intima. With carotid artery (CA) HRP introduction, the 30-s spot (132.86 ± 37.32 μm) is far larger than the PAs, grows little (~28%, P < 0.05) from 30 to 60 s, and is much flatter than the artery curves. Transverse electron microscopy sections after ~10 min HRP circulation show thin, intense staining immediately beneath both vessels’ endothelia with an almost step change to diffuse staining beyond. This indicates the existence of a sparse, subendothelial intima, even when there is no internal elastic lamina (IVC). This motivates a simple model that translates growth rates into lower bounds for the flow through focal leaks. The model results and our earlier wall and medial hydraulic conductivity data explain these spot growth curves and point to differences in transport patterns that might be relevant in understanding the immunity of IVC to disease initiation.

focal horseradish peroxidase leaks; transport in vessel walls; theory for spot growth

atherosclerosis is a disease mainly of large arteries and the aortic valve (16, 18). It is the leading cause of death for individuals both above and below the age of 65 in the United States and in all Western countries. Atherosclerosis appears to begin with the delivery of low-density lipoprotein (LDL) cholesterol from the blood into the vessel wall, where it spreads and accumulates (24). Blood-borne monocytes enter the arterial intima in regions with high subendothelial lipid concentration, becoming macrophages that, along with media-derivered smooth muscle cells (SMC), attempt to scavenge the extracellular cholesterol. When overwhelmed, they progress to form foam cells, and this accumulation of lipid and necrotic cells appears to comprise the earliest lesions. Eventually, such lesions lead to stenoses that compromise the cross section for flow, causing the heart to be overworked, or they may rupture and release large particles in the blood that can become clots.

Large arteries, such as the aorta and the coronary arteries, are susceptible to atherosclerosis when exposed to prolonged high blood cholesterol. Smaller, lower-pressure arteries, such as the pulmonary artery (PA), and veins, such as the inferior vena cava (IVC), are generally spared. The human PA is only vulnerable under pulmonary hypertension. Interestingly, Schwenke (17) reported that, unlike in humans, the rabbit PA under normal pressures is as susceptible as rabbit aorta. Veins normally do not develop atherosclerosis. However, when veins are placed in arterial condition, i.e., high pressure, as in coronary bypass procedures, atherosclerosis often develops. The saphenous vein is frequently used in the bypass surgery. After 6–12 years, ~71% developed atherosclerosis with structures resembling arterial fatty streaks (13).

To understand the transport of large molecules into susceptible vessels, work has focused on the aorta. It has a continuous endothelial monolayer separating the lumen and the thin, sparse subendothelial intima. A complete internal elastic lamina (IEL) with fenestrae (spaced ~20–25 μm apart; see Ref. 10) separates the intima from the media, which consists mainly of continuous layers of elastin, extracellular matrix, and SMCs. Frank and Fogelman’s (6) ultrarapid freeze/rotary shadow etchings showed the detailed structure of the intima matrix. They displayed how LDL molecules bind to the intima matrix and accumulate.

Endothelial cells have junctions that are tight enough to severely restrict the transendothelial migration of molecules with the size of albumin (~5 nm diameter). How a molecule such as LDL cholesterol, with the size of 22 nm, can pass through these cellular junctions is an obvious question. Stemerman et al. (20) found that horseradish peroxidase (HRP) crossed the aortic endothelium in isolated spots, rather than uniformly. Lin and coworkers (14, 15) used Evan’s blue albumin conjugate (EBA) to demonstrate the association between macromolecular leakage and the junctions of dying or dividing endothelial cells. The result showed that 99% of the cells in mitotic (M) phase were associated with EBA leakage and that 80% of cells in M phase were permeable to Lucifer yellow-LDL. Conversely, using autoradiography of the 125I-labeled LDL, Truskey et al. (23) found that ~25% of the leakage sites found were associated with mitosis. Chuang et al. (4) injected HRP as a tracer in rats and measured HRP leakage spot growth in the subendothelial space of the aortas as a function of circulation time between injection and

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death. Their spots grew extremely fast, reaching ~200 μm diameter in ~2 min. Such rapid growth seemed incompatible with a diffusion-dominated process for any reasonable diffusivity.

These results motivated our group’s two-dimension, convection/diffusion model for tracer transport into the arterial wall (9). The key ingredients of this model were the use of Frank and Fogelman’s (6) ultrarapid freezing-rotary shadow etchings that revealed the extremely loose intimal matrix structure and Lark et al.’s (11) results showing that the intima had a radically different proteoglycan structure from the adjoining media. An ultrastructure-based, ab initio theory for intima transport parameters yielded values one to two orders of magnitude higher than the media values. With these parameters, the model yielded a convection-dominated rapid tracer spread in the intima that accounted for the large observed spots (9).

We now ask, what parts of this transport picture in the large arteries change when one shifts to less-susceptible or immune vessels? The typical pressure drop the aorta experiences far exceeds that of the PA, which again far exceeds that of the IVC (100:16:5 mmHg). As such, one might expect HRP tracer spots to grow much more slowly in the lower-pressure vessels. However, in part I of this series (henceforth, Part I; see Ref. 19), we found that the hydraulic conductivities \( (L_p) \) of these vessels follow a nearly inverse trend to the transmural pressure drops \( (Δp) \). The product \( L_pΔp \) leads to surprisingly similar overall water fluxes in all three vessels. This might suggest similar tracer growths, despite their very different \( L_p \) values. The endothelial layers of all three of these vessel types appear to have similar conductivities; thus, the difference in wall values derives from very different medial conductivities, likely a combination of their thicknesses and porosities. Thus easier drainage into the vessel media for the lower pressure vessels might again suggest slower spot growth than in the aorta.

Below (see Fig. 5) we carry out these measurements and experimentally resolve these issues. The specific questions that we ask are: Is macromolecular leakage into these vessels’ walls uniform or localized? If it is localized, how quickly do tracer leakage spots grow? Is such growth convection or diffusion dominated? The results will be the basis of a crude theory here and should serve to motivate and test more detailed theories for tracer transport into these vessel walls that will, hopefully, provide insight into their differing disease susceptibilities.

Before proceeding, we note that the basic structure of the PA is similar to that of the aorta, with an endothelium, continuous IEL, and a media comprised of continuous elastic sheets, extracellular matrix, and SMCs, but the PA has a thinner vessel wall (78.9 ± 3.3 μm) than the aorta (145 ± 9.7 μm). The vein has a different structure. Because it is usually exposed to low pressures (~5 mmHg), it is not as elastic as the other two vessels. Hence, the vein has a very incomplete IEL, only scattered discontinuous elastin, and less SMCs. Instead, the vein contains a large amount of collagen fibers (see RESULTS) (19).

## METHODS

To see if leakage is focal or uniform and, if the former holds, to measure the rate of leakage spot growth, we follow the protocols used by Steimerman et al. (20) and Chuang et al. (4), repeated in brief below, applied to these other vessels. All protocols below were Institutional Animal Care and Use Committee approved.

**HRP tracer spot experiment.** We anesthetized 16 healthy male Sprague-Dawley (SD) rats, weighing 250–300 g, with 1% (wt/vol) pentobarbital sodium (30 mg pentobarbital sodium/kg body wt of rat; Sigma) by intraperitoneal injection. After 0.5 ml of HRP (type II; 10 mg/100 g rat wt dissolved in 0.5 ml 0.15 M NaCl; Sigma) intravenous injection through a femoral vein (FV) cannulation, four rats were killed 0.5, 1, 2, or 4 min after each circulation time with overdoses of pentobarbital sodium. Before death, we injected 0.5 ml heparin (1,000 U/1 ml; Elkins-Sinn) to prevent blood clotting. We excised the aorta, PA, and IVC and fixed them in 1% and 2% (vol/vol) glutaraldehyde (Sigma) for 1 h each and incubated the tissues in 3,3’-diaminobenzidine (DAB; Sigma) and 0.02% \( H_2O_2 \) in Tris buffer \( (pH \ 7.4) \) at 37°C for 60 min. The time between rat death and vessel fixation was 10–20 min. After preparing the tissues for en face examination, we counted the brown DAB reaction product spots under the microscope (Olympus BX51) and measured the spot areas using Image-Pro Plus. We then calculated the radius \( r = \sqrt{A/π} \) of the circle with the same area \( (A) \). An additional 16 rats (4 for each circulation time) underwent the identical procedure, but with HRP injected through a carotid artery (CA), rather than a FV, cannulation, and only the IVC was excised.

**Transmission electron microscopy.** Eight SD rats weighing 250–350 g were FV cannulated as described above. Six rats received HRP as above, and two each were killed after 7, 10, and 13 min HRP circulation; two rats were killed without having received HRP. After the DAB reaction on vessels excised from the HRP-injected rats, the tissues were postfixed in 2% (vol/vol) osmium tetroxide (OsO4; Ted Pella) for 90 min. The specimens were en bloc stained with 2% (wt/vol) uranyl acetate (a nuclear stain, Electron Microscopy Sciences (EMS), PA) for 15 h at 60°C. The specimens were rinsed and dehydrated in a series of ethanol. The dehydrated specimens were embedded in Epon 12 (Ted Pella). Pale gold to silver sections were cut with a diamond knife on a LKB III ultramicrotome and stained with lead citrate (stains cell membrane) for examination under the electron microscope (model 902; Zeiss).

**Light microscopy with orcein stain.** An overdose of 1% pentobarbital sodium was injected to kill the rat. The blood vessels of interest, i.e., the PA, IVC, and aorta, were removed and fixed with 1% and 2% (vol/vol) glutaraldehyde solution for 1 h each, followed by PBS solution to wash out the fixative. The tissue was postfixed with 2% (vol/vol) osmium tetroxide (Ted Pella) for 90 min. After fixation, the tissue was rinsed with distilled water three times (~1 min each), dehydrated with ethanol in the series 30, 50, 75, 85, 95, and 100% (vol/vol) and propylene oxide (Ted Pella), and finally, after infiltration, embedded in Epon 12 (Ted Pella).

We sectioned the Epon blocks to a thickness of ~200 nm (purple reflection; see Refs. 2 and 10) using a glass knife with an MT-1 ultramicrotome (DuPont) and adhered the sections to a glass slide with 2% (wt/vol) gelatin (Ted Pella). We then applied orcein stain to specifically highlight the elastic fibers in the vessel walls (10). The slides were dried and observed under light microscopy (Olympus BX51). The vessel wall thicknesses were measured by using NIH ImageJ. For the aorta and the PA, which contain large amounts of elastin, we used a 0.4% (wt/vol) orcein solution (0.4 g orcein, dissolved in 100 ml 70% ethanol, with 0.6 ml of concentrated HCl then added), whereas, for the IVC, which contains much less elastin, we used a 1% orcein solution.

**Silver stain.** We anesthetized three healthy male SD rats, weighing 250–300 g, with 1% (wt/vol) pentobarbital sodium (30 mg pentobarbital sodium/kg rat body wt; Sigma) by intraperitoneal injection and cannulated their right CA and jugular vein. After a 0.5-ml heparin injection in the left FV, an overdose of pentobarbital sodium was used to kill the rats. The aorta was perfused through the cannulated CA with heparinized 1% (vol/vol) glutaraldehyde at 100 mmHg and then the perfusate was changed to 5% glucose for 2 min and a 1:1 mixture of 3% CoBr and 1% \( NH_4Br \) for 1 min. The aorta was removed, its adventitia was carefully peeled with a fine forceps, and its aorta was cut longitudinally and mounted on a glass slide for en face observation.
The IVC was perfused through the jugular vein with the same solutions at 10 mmHg, but with a perfusion time five times longer than the aorta. The PA was excised and fixed in heparinized 1% (vol/vol) glutaraldehyde for 2 h. We then incubated it with 5% glucose for 2 min and a 1:1 mixture of 3% CoBr and 1% NH4Br (GFS Chemical, Columbus, OH) for 1 min. We carefully removed the adventitia of the IVC and the PA and prepared the tissues as described above. All vessels were observed under the light microscope (Olympus BX51). The sizes of the endothelia were measured with NIH ImageJ.

**Crude model for spot growth.** To analyze the data, we propose a very crude model, pictured in Fig. 1. Suppose an idealized geometry of a flat endothelium that is parallel to a flat IEL, because the intima is far thinner than the vessel radius. The model makes two important assumptions. First, tracer enters the subendothelial intima via convection only through a localized leak, e.g., a leaky circular junction around an endothelial cell, centered at the origin, and not uniformly. Because the aortic intima is significantly more porous than the media, the model guesses that the other vessels are somewhat similar in this respect, even if the mismatch is not as extreme. Thus, second, the void fraction, and thus the hydraulic conductivity, of the subendothelial intima far exceed those of the adjacent media. The spot grows because there is far less (intimal) resistance to lateral flow parallel to the intima, away from the leak, than for normal flow in the denser media. This spread occurs purely passively by virtue of the advance of the HRP front by virtue of water flows in and out of the intima because the pressure drop across the local endothelial leak is lower than across nonleaky portions of the endothelium. This gradient drives this advection. As detailed below, the present moving-front model is not a force balance and therefore does not account for the pressure field; it is simply a balance of the resulting water flows.

Water enters the subendothelial intima through all of the cell-cell junctions and exits through (for the arteries, the IEL and) the media, both of which are characterized by a homogenized, area-averaged hydraulic conductivity detailed below. That is, we do not consider junctions other than the leaky junction explicitly. Below is a simple conservation argument for the incompressible water flow that expands the spot. It follows the growth of a spot containing HRP at sufficient concentration to be detectable. This value is not specified and may vary between batches of HRP. We simply follow the radial advancement of the HRP front by virtue of water flows in and out of the intima and ignore any dilution effects.

The volume of an assumed circular tracer spot in the intima layer is $V_{\text{spot}} = \pi R^2 l$, where $R$ is the radius of a spot and $l$ is the thickness of the intima. The rate $dV_{\text{spot}}/dt$ that the spot size changes arises from an imbalance between the inflow $Q_{\text{leak}}$ of water through the leak, the inflow across the endothelial surface $Q_{\text{E}}$, and the outflow $Q_{\text{M}}$ to the media below

$$2\pi R l \frac{dR}{dt} = Q_{\text{leak}} + Q_{\text{E}} - Q_{\text{M}},$$

with Starling’s law $Q_{\text{E}} = L_{\text{PE}} \Delta P_{\text{E}} (\pi R^2 - A_{\text{leak}})$ and $Q_{\text{M}} = L_{\text{PM}} \Delta P_{\text{M}} \pi R^2$ relating $Q_{\text{E}}$ and $Q_{\text{M}}$ to the product of their respective driving forces, $\Delta P_{\text{E}}$ and $\Delta P_{\text{M}}$, and hydraulic conductivities, $L_{\text{PE}}$ and $L_{\text{PM}}$, much of which is accessible to experimental measurements (see Part I). $L_{\text{PM}}$ is the conductivity of the media and, for the arteries, the IEL, lumped together. $A_{\text{leak}}$ is the area of a leak, which, if comprising only the junctional area, is quite small. We shall estimate $Q_{\text{leak}} = L_{\text{leak}} \times \Delta P_{\text{E,leak}} \times A_{\text{leak}}$ from fitting the experimental data below, since the intimal pressure (and thus $\Delta P_{\text{E,leak}}$) at the leak is, as yet, unknown. *Equation 1* makes the usual assumption that there are no osmotic differences across the endothelium or the IEL and assumes that the radial velocity in the intima is one dimensional, i.e., depends only on $R$. This latter assumption is reasonable for a porous media (Darcy) flow, which satisfies potential flow equations (*8*), provided that $l/R << 1$ and we are not too close to the leak. Far from the leak site, a steady-state balance implies that the filtration flux across each sublayer is equal to that across the entire wall, viz., $L_{\text{PE}} \Delta P_{\text{E}} = L_{\text{PM}} \Delta P_{\text{M}} = L_{\text{P}} \Delta P$, the latter term representing whole wall quantities. (Part I uses this to calculate the average intimal pressure.) If one (crudely) neglects the variation in $L_{\text{P}}$ with radial position (the present model is just a water balance, without a force balance, and is therefore too simple to include this variation) and applies this relation everywhere, one gets

$$\pi R^2 \frac{dR}{dt} = Q_{\text{leak}} - L_{\text{P}} \Delta P A_{\text{leak}} = K',$$

where $K'$ is a constant. In reality, since $L_{\text{P,leak}}$ is so much larger than $L_{\text{PE}}$ (typically by a factor of $\sim 10^3$; see Ref. *9*), $\Delta P_{\text{E}}$ is much smaller (and $\Delta P_{\text{M}}$ is larger) near the leak than far from it, and the resulting pressure variation is what drives the radial flow in the intima. Thus this model underestimates the outflow $Q_{\text{M}}$ across the IEL in the leak region and thereby leads to either an underestimated $Q_{\text{leak}}$ or an overestimated spot growth, with the former the case for a fitting of $R$ vs. $t$ (time) to data as below. Clearly the model will be best far from the leak. Nevertheless, $Q_{\text{M}}$ still slightly exceeds $Q_{\text{E}}$ because of the differences in area. *Equation 2* attributes tracer spot growth to the

![Fig. 1. Schematic of the crude model. Water enters the intima through both the leaky and normal endothelial cell (EC) junctions, but macromolecules enter only through the leaky junctions. The intima is far less dense than, and therefore presents far less flow resistance than, the media. Water can exit the intima through the internal elastic lamina (IEL) or, because of the intima’s low flow resistance, progress laterally in it, causing the advection front to grow. The crude model balances flows, follows the advection front, and does not follow the details of the forces or of the concentration profile.](http://ajpheart.physiology.org/10.1152/ajpheart.00654.2006)
excess fluid that enters through the leaky junction over what would have entered had the junction been normally permeable: the larger this mismatch, the faster the spot grows.

The solution to Eq. 2 is

\[ R = \sqrt{K(t - t_0)} + R^2(t = t_0), \]

where \( K = K' / \pi l \), and the initial spot size \( R(t = t_0) \) is the radius of an endothelial cell (slightly different for each vessel) when the spot results from the junctions around a single leaking cell. The circulation time \( t_0 \) then represents the time at which the tracer arrives from the point of injection to the vessel in question. By doing a least-squares fit of \( R^2(t) - R^2(t = t_0) \) vs. \( t \), we get values for \( t_0 \) and for \( K \) which, with values for \( L_p, \Delta P, \) and \( \lambda_{leak} \), yields \( Q_{leak} \). Because, as noted in the last paragraph, this fitting just gives a lower bound on \( Q_{leak} \), and because the spot radius is most sensitive to \( Q_{leak} \) initially, the fitting procedure also underestimates the initial growth. As a consequence, this crude model should therefore lead to spurious negative values of \( t_0 \). At later times, though, one expects the model to fit reasonably well.

**Statistics analysis.** Paired Students’ \( t \)-tests are used for comparison between tracer spot sizes for one vessel type at different times or between vessel types (\( P < 0.05 \), statistically significant).

**RESULTS**

**Ultrastructures of PA and IVC.** The ultrastructure of the PA is shown in Fig. 2A, an electron micrograph (EM) without stain.
(Fig. 2A, left) and a light micrograph (LM) with orcein stain, specific for elastic tissue (Fig. 2A, right). It consists of a continuous endothelium, intima, IEL, and media with elastic tissue and SMCs. The intima is confined to a thin space between the endothelial cell and IEL. It contains some collagen fibers (6). The orcein stain in Fig. 2B confirms the continuous elastic in the PA media. Figure 2C shows the ultrastructure of the IVC. It has an abundance of collagen fibers. Figure 2D shows it has almost no, i.e., a very discontinuous, IEL and only sparse elastin. Figure 2, E and F, presents the ultrastructure in EM and in LM of the aorta for later comparison.

**Electron microscopic cross sections of the PA and IVC with HRP.** With 7–13 min HRP circulation before death, Fig. 3A shows how HRP reaction product distributes in the extracellular space of the PA in transverse sections (compare Fig. 3C for the aorta). For such long circulation times, HRP also passes through the normal endothelial cell junctions, as seen in Fig. 3. There are a few indications of cell vesicles near the junction. HRP reaction product forms a thin, discrete, dark layer (~200 nm) between the endothelium and the IEL, concentrating in and outlining the existence of the intima layer. A noticeable amount of tracer leaks through IEL fenestrae and appears beneath the IEL as well. Figure 3B shows the corresponding tracer-product distribution in the IVC. In addition to appearing in the normal junction and cell vesicles, a thin, dark layer of the tracer product aligns beneath the endothelial cell despite the absence of a continuous IEL. The line has the thickness of ~135 nm.

**Endothelial cell radii measurement.** The endothelial cell area (A) was measured from silver nitrate staining by dividing the total area examined by the number of (assumed circular) cells of area $A = \pi R^2$, where $R$ is the effective average cell radius. PA endothelial cells have radii ~12 μm. The IVCs have radii ~11 μm.

**Discrete HRP spots and their growth with increasing circulation time.** Figure 4 presents en face views of the PA and IVC endothelia after 120 s HRP circulation. The isolated brown spots, surrounded by much lighter yellow areas, indicate focal leakage of HRP at these short circulation times. This indicates that molecules (e.g., LDL) larger than HRP likely

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**Fig. 3.** A: ultrastructure of the rat pulmonary artery with horseradish peroxidase (HRP) tracer (7 min). The arrow points the fenestra with HRP tracer. v, Vesicle. The bar represents 0.4 μm. B: transmission electron microscopy of rat inferior vena cava with HRP tracer (7 min). The arrow points to the outline of the HRP tracer in the subendothelial intima region (bar represents 0.4 μm). C: transverse section of aorta electron micrograph. The arrow points the normal cellular junction with HRP tracer (5 min). The tracer outlines the subendothelial intima. The bar represents 0.5 μm. Reprinted from *Atherosclerosis* 118, Chen YL, Jan KM, Lin HS, Chrens, “Ultrastructural studies on macromolecular permeability in relation to endothelial cell turnover.” 89–104, 1995, with permission from Elsevier Ireland Ltd. (3).
traverse the endothelium only through a subset of these rare leaky spots.

We now examine how far focal tracer spots have grown at 0.5, 1, 2, and 4 min circulation times for the PA and IVC. Each data point represents the average of approximately three to four spots per rat vessel times four rats. Figure 5A shows the result for the PA. At 30 s, the spot size is 53.2 ± 10.4 µm. It grows 40% (P < 0.05) to 74.7 ± 8.6 µm at 1 min and then slows down gradually. The spot sizes become 85.8 ± 11.0 and 109.5 ± 8.7 µm at 2 and 4 min, respectively; the increase in the spot size in this period is only 28% (P < 0.05). The PA exhibits spot size growth that is surprisingly similar to that of the aorta [see Fig. 5A for our PA and aorta results and Chuang et al.’s (4) aorta curve. Each set is within the error bars of the other]. Model results focuses on this result in more detail.

Figure 5C shows the growth of tracer spots in the IVC for both FV and CA cannulations. Both curves exhibit focal spots that are uniformly much larger than in the aorta. The FV cannulation, which was used for the aorta and PA spot size growth measurements, is far upstream from these arteries but adjacent to the IVC. As such, there is almost no time delay or tracer dilution resulting from circulation from the point of tracer introduction until its appearance in the IVC. The spot size is very large (192.56 ± 62.8 µm), even at 30 s, and increases only slightly at later times to 206.59 ± 80.03, 210.96 ± 10.45, and 214.32 ± 69.09 µm; spot growth with circulation time does not appear to be significant (P > 0.05).

With CA cannulation, the tracer is introduced upstream from the IVC, and there is a time delay between tracer introduction and its appearance in the IVC. At 30 s, the radius of the spot is ~132.86 ± 37.32 µm, although the SD is uniquely large at this point. The spot size increased 28% (P < 0.05) at 60 s to 170.09 ± 51.8 µm. At the later times, the spot grows more slowly to 158.23 ± 15.77 (P > 0.05) and 184.78 ± 30.1 (P > 0.05) µm.

Model results. We choose l = 100 nm (9). Our observation is that the endothelial cell radii are similar in all three vessels, and we therefore use $R_{\text{leak}} = 15$ µm throughout. $L_p$ and $\Delta P$ are available for each vessel from Part I. Despite the crudeness of the model, its root-$t$ dependence fits the data adequately for all three vessels for the data points measured [see the continuous curves in Fig. 5, B and C; r values are 0.898, 0.964, and 0.79 for the aorta (our data), PA, and IVC (CA cannulation) fits, respectively] although, as noted in METHODS, not at short times.

For both arteries, where we anticipate that the spots begin from the leaking junctions around a single cell, we fix $R(t_0) = 15$ µm. This underestimated short time growth is expected to lead to spurious negative values for the time $t_0$ at which the tracer arrives in the vessel. For our aorta data, it yields $t_0 = -55$ s compared with Huang et al.’s (9) much more thorough theory’s value of approximately +25 s. For the PA, the arrival time $t_0$ comes out to ~52 s, very slightly less than that of the aorta, as one would expect. For the IVC, however, if one assumes that the spot originated from the junctions around a single endothelial cell, one finds $t_0 = -321$ s. This suggests that, for the IVC, we should not fix $R(t_0)$ but rather extrapolate the IVC curve back to various potential $t_0$ values and examine the corresponding initial spot radii. For $t_0 = $ approximately +25 s, $R(t_0)$ is 9 cell radii; a similar underestimate $t_0 = $ approximately −56 s as in the arteries yields $R(t_0) \sim 8.5$ cell radii. Both values are clearly far too large but, nevertheless, suggest that the initial radius of the spot exceeds one cell.

From the model fits, the PA has $K = 43.8$ (aorta, our data), 40.8 (PA), and 60.5 (IVC) µm²/s. For the aorta and PA, $R(0)$ is likely one cell radius (taken as 15 µm). If the leak consists of a leaky junction around a single cell, whose thickness is ~20–30 (pick 25) nm (9), $A_{\text{leak}} = 2\pi R(0)\Delta R \approx 2.4$ µm². So, for the aorta (PA, IVC), where $L_p\Delta P = 31.6$ (27.1, 24.3) × 10⁻³ µm/s, $Q_{\text{leak}} = 14$ µm³/s (13, 21). In all of these cases, when the leak is just a junction of very small area (i.e., around one cell for the aorta and PA; the junctions of just a few cells for the IVC), the flow through the junction if it were not leaky, $L_pA_{\text{leak}} \sim 0.1$, is negligible with respect to $Q_{\text{leak}}$, the (lower bound for the) actual flow through the leaky junction, and with respect to $\pi K$. 

Fig. 4. A: en face view of the pulmonary artery endothelia with 120 s HRP circulation. The dark brown spot indicates the focal HRP leakage. Bar = 50 µm. B: en face view of the IVC endothelia with 120 s HRP circulation. The brown spot indicates the focal leakage. Bar = 50 µm.

Macromolecular Leakage Spot Growth in Rat Vessel Walls
DISCUSSION

The goal of this paper is to study the transport of macromolecules from the lumen into the walls of PA and the IVC and to see how it differs from that into large, atherosclerosis-susceptible vessels such as the aorta. Specifically, we investigate whether this transport is focal or uniform and, if focal, how quickly the localized spots spread. This may indicate whether or not such spread is convection dominated, as in the large arteries. It may also shed light on whether and how the critical transport processes in these vessels distinguish benign transport from those that may trigger atherosclerosis. We interpret vessel-vessel differences in terms of differences in vessel structures and filtration properties such as $L_p$, measured in Part I.

Ultrastructures. Large arteries such as the aorta typically suffer time-averaged transmural pressures of $\sim 100$ mmHg and have a low hydraulic conductivity, leading to a transmural water flow that is orders of magnitude slower than its downstream flow (see Part I, Ref. 19). As noted in the introduction, focal endothelial leakage allows macromolecules such as LDL or HRP to enter the vessel wall via a convection-dominated process. Huang et al.'s (9) structure-based (6) ab initio transport parameter theory found that the filtration resistance of the intima is one to two orders of magnitude smaller than that of the media. The vessel ultrastructure of a thin, ultraporous intima and a thick, dense media facilitates the rapid spread of these molecules in the intima (3, 4, 20) and allows them to remain there at high concentrations long enough to bind to intimal extracellular matrix. These are believed to be the earliest in a cascade of events leading to lesion formation. We begin by examining if any of these structural features carry over to less-susceptible or immune vessels.

The PA has a physiological pressure of $\sim 16$ mmHg, with a hydraulic conductivity $(1.69 \times 10^{-7} \text{cm}^2\text{s}^{-1}\text{mmHg}^{-1})$ that is roughly proportionally lower than that of the aorta $(3.16 \times 10^{-8} \text{cm}^2\text{s}^{-1}\text{mmHg}^{-1})$, thereby leading to a comparable transmural water flow. $L_p\Delta P$ for the PA is $\sim 2.7 \times 10^{-6} \text{cm}^2\text{s}^{-1}\text{mmHg}^{-1}$ vs. $2.9 \times 10^{-6} \text{cm}^2\text{s}^{-1}\text{mmHg}^{-1}$ for the aorta. [Note that Deng et al. (5), using canine common CA, found 3.22 cm/s (SD0.69) $\times 10^{-6}$.

Baldwin et al. (1) used two populations of rabbit aortas and found the values 3.5(SD1.3) and 3.8(SD1.1) $\times 10^{-6}$ cm/s at 100 mmHg. Tedgui and Lever (21) measured 1.71(SD5.6) cm/s at 70 and 7.2(SD2.4) $\times 10^{-6}$ cm/s at 180 mmHg in rabbit thoracic aorta. Our numbers are easily within the error bars of Deng et al. and Baldwin et al., despite the difference in species and, in Deng's case, in vessel choice. Tedgui and Lever's pressures were quite different from our "physiological pressure," but their $L_p$ values, both with intact and denuded endothelium, superimpose directly on our data for $L_p$ vs. transmural pressure in Part I; Ref. 19.]

The structure of the PA resembles that of the aorta. It consists of continuous endothelium, superimposed directly on our data for $L_p$ vs. transmural pressure in Part I; Ref. 19. The transmural water flow increases roughly proportionally to $L_p$ for the PA compared with the aorta. For the aorta, $K = 45.5$ $\mu$m $^3$/s, $Q_{\text{transinta}} = 34.1$ $\mu$m $^3$/s; for the PA, $K = 43.2$ $\mu$m $^3$/s, $Q_{\text{transinta}} = 38.07$ $\mu$m $^3$/s.

Fig. 5. A: spot size of the rat aorta and PA. The tracer spot grows $\sim 35\%$ from 30 to 60 s for the aorta ($P < 0.1$), and the growth slows down afterward. Compared with Chuang et al. (4), the results were in good agreement, with each set of measurements within the error bars of the other. The PA follows a trend remarkably similar to the aorta ($P > 0.1$) and increases 40% from 30 to 60 s ($P < 0.1$); $n = 4$ rats. B: fit of spot size data for the rat aorta and PA to the crude model. The model (dotted line; $r = 0.898$ for aorta, $r = 0.964$ for PA) underestimates the growth of spots in the aorta. For the aorta, $K = 45.5$ $\mu$m $^3$/s, $Q_{\text{transinta}} = 34.1$ $\mu$m $^3$/s; for the PA, $K = 43.2$ $\mu$m $^3$/s, $Q_{\text{transinta}} = 38.07$ $\mu$m $^3$/s. C: spot size comparison of the aorta and IVC. The IVC of both femoral vein (FV cannulation; $n = 4$) and carotid artery (CA cannulation; $n = 4$) cannulation shows a relatively flat trend ($P > 0.1$). The CA cannulation shows $\sim 28\%$ spot size growth from 30 to 60 s ($P > 0.1$). The 30-s IVC spot is $\sim 1.7$ times that of the aorta ($P < 0.1$). The model (dashed line; $r = 0.790$) predicts the spot growth of the IVC for $K = 78.53$ $\mu$m $^3$/s and $Q_{\text{transinta}} = 45.91$ $\mu$m $^3$/s.
lium, intima, and IEL (which serves as an additional filtration barrier; see Ref. 3), with a thick media consisting of continuous strips of elastin separated by SMCs and extracellular matrix [see Figs. 2, A and E (tracer free), and 3, A and B (with tracer)]. The intensity of the immediately subendothelial HRP accumulation, an \( \sim 200-\text{nm stripe} \), in Fig. 3A suggests that the PA intima is also much sparser than its media and of comparable size to that of the aorta. Tompkins et al. (22) measured the concentration distributions resolved in \( \sim 10 \) sections across the vessel wall for various vessels, including the PA, of squirrel monkey. He found a much higher tracer concentration in the first layer near the lumen that falls dramatically with the depth, which is consistent with the PA having a sparse intima. This will be important to the interpretation of the observed spot sizes below.

The IVC is the largest and most accessible vein in this small animal model. It has a low physiological pressure of 5 mmHg, but also the highest \( L_p \), thereby resulting in a similar transmural water flow to the other two vessels. Its structure, however, is much looser and less compact than the other vessels (Fig. 2), consistent with its much higher \( L_p \). Its media elastic tissues are very sparse (they can only be seen with a much higher concentration of the elastin-specific stain orcein than needed for the arteries) and discontinuous, and it shows only rare fragments of an IEL. Its media is the thinnest and has an abundance of collagen fibers (Fig. 2C), which accounts for its much lower distensibility (Fig. 2D). Consequently, the IVC lacks a definite boundary that highlights an intima layer (Fig. 3C). Our finding of intense HRP staining just beneath the endothelium with a sharp drop in intensity below argues that the IVC appears, despite the lack of an IEL, to have an intima layer that is similarly sparse and similar in thickness to those in the arteries. The key point is that, despite the fact that the arteries/media are thicker and denser than the IVCs, the IVC appears to have an intima, and this intima is far sparser than the IVC media. This would explain Tompkins et al.'s (22) high LDL concentration in the first decile of the IVC wall near the endothelium. This is consistent with the fact that endothelial cells synthesize an intimal extracellular matrix that is very different from that made by the SMCs in the media, and these syntheses do not appear dependent on an IEL (11).

**Tracer spots and their growth.** In both vessels, the tracer HRP (\( \sim 5 \) nm diameter; see Ref. 3) crosses the endothelium at rare, isolated leaks, rather than uniformly, at short circulation times of \( <5 \) min. It follows that the much larger LDL (\( \sim 22 \) nm diameter) should follow the same trend, and, because of the similarity of all three vessels' endothelia, it should do so even at longer times. Figure 3A shows that, despite the lower driving transmural pressure \( \Delta P \) in the PA, the spot growth with tracer circulation time in the PA and in the aorta (taken in our laboratory and that of Chuang et al.; Fig. 5A) is strikingly similar. This similarity concerns both the trend and the actual spot sizes at the same circulation times (\( P > 0.1 \)). [Note that Chuang et al.'s (4) rat aorta data appear very slightly higher than ours but remains within its error bars and retains its similar shape. There is likely some systematic investigator-dependent judgment in defining the spot edge.] Given the similarity in the ultrastructures and in the sizes of the transmural water flows in these two vessels, this suggests that tracer entry into the PA wall and its intimal spread are likely convection dominated, as in the large arteries. These similarities also render the result less baffling than one might have thought based solely on their transmural pressures.

The crude model proposed above allows for some quantitative comparisons. First, the adequate fit of the root-\( t \) model at longer times is consistent with the spot growth being convection dominated, as is its similarity to the growth in the aorta, which was shown to be too fast to be consistent with pure diffusion for any reasonable diffusivity (9). Because the root-\( t \) model underestimates \( Q_{\text{leak}} \), it gives a curve that is initially flatter than the data and, consequently, as already noted, a spuriously negative/meaningless time of tracer arrival in the vessel of \( \sim 52 \) s for the PA and \( \sim 55 \) s for the aorta. Finally, from the model's fit, the PA has \( K, Q_{\text{leak}}, \) and \( L_p \Delta P A_{\text{leak}} \) values very similar to the aorta, that is, comparable spot sizes correspond to comparable \( A_{\text{leak}} \) and comparable convective flows through isolated aortic and PA endothelial leaks.

We now turn our attention to the IVC. Figure 5C shows the experimental spot-size curves for both FV and CA cannulations. We measured the spot growth for the aorta, the PA, and initially for the IVC using FV cannulations. However, unlike for the arteries that were far downstream of the FV, the IVC proximity to the FV left essentially zero time delay between tracer injection, and its arrival in the target vessel and the HRP tracer was not yet diluted in the surrounding blood when it arrived there. As such, the time for the tracer to reach the IVC for the curve with FV cannulation was circa 25 s shorter than for the other vessels, and the spots appear larger because the local lumen concentration feeding the spot is higher in the FV cannulation. Since, even if convection is important, diffusion may not be negligible in IVC transport and since the measured size of the spot depends on the concentration level of detectability of the HRP reaction product, a higher local lumen HRP concentration makes the spots look larger (see Fig. 5). To correct this problem and to access earlier circulation times, we performed CA cannulation experiments.

The FV-cannulated IVC spot size curve in Fig. 5C is relatively flat, with only a slightly lower 30-s value, and it is uniformly much larger than the aortic and PA curves. The CA cannulation curve is, as expected, a bit lower, and its 30-s value differs from the subsequent values more than for the FV cannulation, consistent with the \( \sim 25 \) s longer it took for the tracer to reach the vessel than in the F-V cannulated curve. It is also essentially flat, except for this (significantly) lower value at 30 s (whose value has the largest SD) that differs from the 4-min value by a far smaller percentage than in the arteries. The much larger magnitude of the curve relative to the aortic curve is consistent with Chuang et al.'s (4) finding that veins tend to have clusters of dying cells, rather than single cells, that leak. Even when a single cell is dying, one can imagine how a cluster of neighboring cells might burrow underneath the dying cell to slough it off without leaving a large hole. This might stretch their junctions temporarily and lead to leakage of the cluster of cells, rather than just a single cell (4, 7, 25). In any case, although the root-\( t \) model gives spurious negative \( t_0 \) values, the large size of \( t_0 \) relative to the arteries suggests a much larger initial leak radius (the model suggests a clearly too large radius of \( \sim 127.5 \mu \text{m} \) \( \approx 8.5 \) cell radii) than one endothelial cell radius, which is consistent with this explanation.

What appears to be the asymptotic spot size for the IVC is 39% larger than its 30-s value, as opposed to 105 and 106% for...
the aorta and PA, respectively. The contrast is even larger when one compares the apparent asymptotic spot sizes with the estimated sizes of the leaks themselves in the normalized form \((|R(t) - R(0)|/R(0))\) gives 6.5 for the aorta, 6.3 for the PA, and 0.45 for the IVC, with \(R(0)\) coming from the crude model). Whereas these precise values are not significant because of the inaccuracy of the model at short times, they show that the IVC curve is nearly flat relative to the aorta and PA curves. That the IVC spots grow neither as fast nor as far as in the other vessels, despite the IVC’s apparent thin, very porous subendothelial intima and overall transmural water flow that appears comparable to those of the arteries, requires discussion. The first relevant fact is that, even though Tompkins found higher tracer concentrations in the decile of IVC wall nearest the lumen than in the subsequent deciles, the mismatch was not as stark as in the aorta; the IVC media concentration was an order of magnitude higher than that of the aorta. Consistent with this interpretation, the decile nearest the lumen, which contains the very thin intima but mostly media, for the IVC (and for the PA) has a tracer concentration two to three times that in the first decile of the aorta. This again indicates that the contribution of tracer in the media portion of this decile is far smaller in the aorta than in the other vessels. Moreover, Part I in Ref. 19 found that the \(L_p\) of the IVC media was substantially larger than in either of the arteries in question (45 times the aorta, 10 times the PA). This was a consequence of the thinner (only a factor of 2) and evidently much more porous IVC media, consistent with the much higher water content (81% of the IVC relative to the PA (76%) and the aorta (64–69%). As a result, it would seem likely then that, despite the large transmural water flow, the overriding water flow direction in the IVC intima is normal, rather than parallel, to the endothelium. This dictates more modest spot growth. Moreover, the IVC media has a much greater distribution volume (0.051 for albumin) than that of the aorta (0.009; see Ref. 12). Because the mismatch in intima/media void spaces is not as stark as in the arteries, it suggests that the tracer can more easily distribute between the two regions and even efflux from the tissue into the extravascular space. Therefore, it does not build up to as high concentrations in the IVC intima.

The kinetics of the binding of free lipid to extracellular matrix in the intima depends on the local intimal lipid concentration, and not on the total lipid in the wall. Thus a better distribution of lipid between intima and media may, indeed, be the reason why, despite the similarity in overall water flow between the three vessel types and despite the large spots in the PA and in the IVC, only the large, high-pressure arteries accumulate lipid in their intimas and are susceptible to lesions under normal conditions. This line of argument has motivated our current development of more detailed convection/diffusion theories for tracer transport into the PA and IVC walls and to confocal microscopy studies of tracer spots. These theoretical and experimental studies seek to determine the spatially distributed (as a function of radial distance from the leak and of depth into the wall) intima tracer concentrations in these vessels, as opposed to the spot size measurements that correspond to (projections) integrals of the tracer concentration over the direction normal to the endothelium.

Applying Eq. 3 to the CA cannulation data gave values for the lower bound, \(Q_{\text{leak}}\), on the flow through the IVC leaky site that is slightly less than double that of the other vessels, despite the far larger initial leak size. Because the IVC leak is likely the result of a cluster of (dying) leaking cells, we calculate the flow per leaking cell as \(q_{\text{leak}} = Q_{\text{leak}}/N_{\text{leak}}\) where \(N_{\text{leak}}\) is the number of leaking cells in the cluster. This value will clearly be much smaller for the IVC than for the arteries. These values for \(q_{\text{leak}}\) and \(Q_{\text{leak}}\) simply quantify the slower (fractional) growth rate of the leak, since the model assumes that the water and macromolecules entering the vessel intima through the leak travel mainly radially in the intima and are responsible for the spot’s growth. On the other hand, if the IVC indeed has clusters of cells that leak, then the area beneath this cluster where the actual \(\Delta P_m\) is larger (and \(\Delta P_E\) is smaller) than the model assumes is more significant. That would suggest that the model-estimated values for \(Q_M\), and thus \(Q_{\text{leak}}\), are quite conservative. A larger actual \(Q_M\) would suggest that a significant portion of \(Q_{\text{leak}}\) indeed flows normal, rather than parallel, to the endothelium in the IVC, contrary to the model’s assumption. This is consistent with the porosity mismatch between the intima and media being far less extreme in the IVC than in the arteries. We anticipate that it might also lead to a better distribution of macromolecules between the intima and media, thereby avoiding the high intimal lipid concentrations that lead to extracellular lipid liposome formation (6). A more detailed model that solves the filtration and convection diffusion problem will be needed to sort out the filtration flow and macromolecular advection in the IVC, and a model that solves the liposome formation kinetics will take this result and calculate lipid accumulation rates in the IVC and compare it with those in disease-prone vessels.

In conclusion, this paper has reported HRP tracer transport experiments to gain insight into the way in which macromolecules enter into the walls of blood vessel, as occurs in the early stages of LDL accumulation processes. We examine the growth of tracer spots with tracer circulation time in the rarely atherosclerotic PA and in the resistant IVC and compare with growth in the susceptible aorta. The goal is to see what differences in such processes tell us about vessel disease susceptibility. This paper discusses the results in conjunction with the differences in ultrastructures and hydraulic conductivities among these vessels.

Although the PA experiences a lower transmural pressure (16 mmHg) than the aorta (100 mmHg), the rapid convection-driven growth in the PA of a tracer spot from a radius corresponding to a single leaky endothelial cell to roughly nine cell radii in ~4 min is similar to that in the aorta. The similar ultrastructures of the two vessels suggest a similar line of reasoning. In the aorta, the tracer spreads in the subendothelial intima layer that is bordered by a continuous, presumably less permeable IEL. The similarity in spot growth at 1⁄6 the pressure driving force indicates that the PA must be more permeable than the aorta. This is consistent with measurements in Part I of Ref. 19 showing that the \(L_p\) of the PA is nearly an order of magnitude larger than that of the aorta. The combination of \(L_p\) and transmural pressure data led to similar overall transmural water flows and thus to the plausibility of similar rates of tracer spot growth. A crude root-\(t\) model infers from the similar spot growths that the flow through the leak into the intima is similar in both vessels.

The IVC experiences a very low transmural pressure of ~5 mmHg. Its spot growth is quite different from those in the arteries. The spots appear to originate from a cluster of leaking
cells, as Chuang et al. (4) noticed, and appear to grow only a small percentage in 4 min. Despite its absence of a continuous IEL or, in fact, much elastin, we have found evidence of a sparse, subendothelial intima in the IVC, as in the arteries. Part I (19) found that the balance between a much higher LP and a lower ΔP in the IVC nevertheless leads to an overall transmural flow that is comparable to those in the arteries. If the intimal water flow near an isolated leak is mainly parallel to the endothelium, as is the case in the arteries, the simple root-t model infers from the data that the total water flow through the leak into the intima in the vein is roughly double that into the arteries, but the flow per leaking cell is smaller. Alternatively, because the IVC media is more porous (and thus has a much higher LP; Part I of Ref. 19 and see Ref. 12) than the artery media, the water flow in the intima may not be predominantly in the direction normal to the endothelium. If that is indeed the case, then the root-t model has underestimated the flow and advection through the leak. These considerations and the larger media porosity of the IVC suggest an explanation for the immunity of IVC to disease initiation. Even though there seems to be more tracer swept into the IVC than into the artery walls in each leak, the macromolecules swept in would have a much easier time escaping/effluxing the intima and, ultimately, the wall before being detained. Resolution or confirmation of this insight awaits a more detailed modeling effort for the IVC.

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