Wall permeability of isolated small arteries. Role of the endothelial surface layer
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chapter 7:

GENERAL DISCUSSION
7.1 MAJOR CONCLUSIONS OF THIS THESIS

We developed a new model to study vascular wall permeability in isolated arteries using confocal microscopy. By direct observation of fluorescent tracers near the arterial wall we were able to determine dimension and location of the permeability barrier to transport of these tracers. Thus we demonstrated the presence of a relatively thick (~2-3 μm) endothelial surface layer (ESL, also known as the glyocalyx) in arteries of 105-255 μm in diameter. Covering the luminal endothelial surface, the ESL forms the main permeability barrier for transport of anionic macromolecules. Dimension and barrier properties of the ESL were found to be dependent on molecular size, solvent ionic composition and the presence of albumin in the perfusate.

Very large anionic macromolecules (~150 kD) remained excluded from the ESL for at least 30 minutes, whereas smaller macromolecules (50 kD, 4kD) were able to penetrate the ESL in a size-dependent manner. Thickness of the ESL was found to be dependent on solvent ionic composition, ranging from ~0-1 μm at high solvent ionic strength, and ~2-3 μm at normal ionic strength, to ~6 μm at low ionic strength. Moreover, the barrier properties of the ESL were also dependent on solvent ionic composition, resulting in larger ESL permeation times (i.e. lower ESL permeation rates) at low ionic strength. Furthermore, the barrier properties of the ESL were also dependent on the presence of albumin in the perfusate. Interaction of albumin with the ESL attenuates solute movement into the ESL, without affecting its thickness. Finally, the ESL was found to be sensitive to phototoxic stress as induced by light-dye-treatment.

7.2 EVALUATION OF THE CURRENT APPROACH

Confocal laser scanning microscopy (CLSM) on isolated, cannulated small arteries enabled direct observation of various fluorescent molecules and their transport behavior inside these arteries. As fluorescent tracers we used fluorescein-isothiocyanate (FITC)-labeled dextrans (FITC-Δs) of different sizes and the endothelial membrane dye Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate). Rapid inflow of fluorescent tracers into the arteries was accomplished by application of double-barreled θ-pipets. Although θ-pipets (29-31;33) as well as confocal microscopy (3) have been applied in permeability studies before, the method we developed for the first time quantified fluorescence intensity profiles at the arterial wall, allowing for a direct measurement of the localization of permeability barriers for transport of the fluorescent tracers.
The localization of fluorescent tracers with respect to the position of the arterial wall is complicated by the optical properties of the confocal microscope system. We developed and verified correction procedures to circumvent this complication. The first method made use of fluorescence recordings of a small glass tube (167 μm inner diameter) filled with FITC-Δ that served as a model for an artery with uniform luminal filling and no wall fluorescence.

The second method employed the Dil labeling of the endothelial cells in the arterial wall. The Dil fluorescence profile at the wall served as an optical 'line-spread function' that characterizes the blurring in the confocal recorded images in the plane of focus perpendicular to the arterial wall. These procedures have been described and verified in chapter 2 and further applied in chapter 4 and 5.

7.3 THE ENDOTHELIAL SURFACE LAYER (ESL)

Using the approach described above, we found that a 2-3 μm thick ESL confines large anionic molecules (FITC-Δ148; 148 kD) to a core volume inside the isolated small arteries, while FITC-Δ50 (50 kD) was able to slowly penetrate this layer, and FITC-Δ4 (4 kD) was able to pass the ESL and accumulate in the arterial wall within 30 minutes (chapter 2).

These findings fit well within the evolving insights that vascular endothelium possesses a glycocalyx (ESL) forming an exclusion zone in which axial convective plasma transport is greatly impaired and that forms a barrier for radial transport of especially larger molecules through the vessel wall. Presence of the ESL was originally postulated to explain low levels of capillary hematocrit (16). Direct evidence for an effect of the ESL on capillary rheology was obtained from intravital microscopy demonstrating a gap between red blood cells (RBCs) and the endothelium in the order of 0.5 μm (9;55-57).

The ESL thickness found in the present studies is much larger than measured in capillaries. In capillaries RBCs are partly squeezing the ESL, because of their size in relation to the capillary diameter. The large white blood cells were found to completely squeeze the ESL (56). These findings suggest that the ESL is a highly flexible and dynamic layer. Squeezing obviously will occur to a lesser extend in larger vessels. The existence of an ESL that might be as thick as 7 μm in larger arterioles (13-101 μm) was postulated in a permeability study on arterioles treated with pronase and heparinase (30), enzymes known to degrade the ESL (16).

Direct evidence for the predominant barrier properties of the endothelial surface layer (ESL) in capillaries was provided by intravital microscopic recordings from Vink and Duling (56;57).
These authors demonstrated that molecular penetration rates in the capillary ESL depend on size and charge of the molecules. Molecules larger than 70 kD are excluded from the ESL regardless of their charge, while anionic macromolecules smaller than 70 kD slowly permeate into the ESL in a size- and charge-dependent fashion, and neutral or cationic tracers rapidly pass the ESL (57). The existence of an ESL in larger arterioles with barrier properties similar to those in capillaries, was postulated from a 2.3-fold increase in solute permeability after treatment of the arterioles with pronase and heparinase, indicating that the ESL may account for up to 60% of the total vessel wall permeability barrier to macromolecular solute exchange (30). Furthermore, it was demonstrated by enzymatic treatment that the ESL contributes as much as 60% of the hydraulic resistance of the capillary wall (1).

We found that large anionic FITC-A148 remained excluded from the ESL and is confined to a core volume inside isolated small arteries, while FITC-A50 was able to slowly penetrate this layer, and FITC-A4 passed the ESL and accumulated in the arterial wall within 30 minutes. Moreover, we found that the small cationic lipophilic tracer Dil rapidly passed the ESL before it was incorporated in the membranes of the endothelial cells of the arterial wall. Comparison of the width of the fluorescence column of the small cationic Dil and the large anionic FITC-A8, 1.5 min after simultaneous infusion of these tracers into the cannulated arteries, resulted in a difference that reflects the dimension of the ESL and therefore provided a second method to estimate the ESL thickness.

Transvascular transport of solute molecules can be described by diffusive and convective solute transport, and possibly a small contribution by active vesicular transport. In order to interpret the experimental FITC-Δ transport results in terms of effective diffusion coefficients, effective solute velocities for convective transport and the dimension of the ESL, we developed a model of the arterial wall in which solute transport was described by diffusive and convective transport equations. This model predicted that the ESL forms the main barrier to transport of FITC-Δs in which diffusive as well as convective transport are severely hindered. This was reflected in very low effective diffusion coefficients in the ESL and high reflection coefficients of the ESL to convective solute transport. The rest of the arterial wall (endothelial cells, internal elastic lamina, smooth muscle cells, adventitia and other adjacent tissue, lumped in one arterial wall model layer) had negligible influence on FITC-Δ transport characteristics (chapter 3).

Many models to predict transvascular fluid and solute transport have been described in literature. Only recent models appreciate the influence of the ESL on transvascular transport (11;13;24;25;30;34;51). Whereas most of the described models observe solute distributions and
transvascular solute transport on a sub-microscopic scale in the order of Ångströms or nanometers, we described solute distributions on a more macroscopic scale in the order of micrometers. Nevertheless these other models also predict the predominant role the ESL plays in vascular wall permeability and transvascular solute transport.

7.4 MODIFICATION OF ESL THICKNESS AND BARRIER PROPERTIES

A method to influence the barrier properties of the ESL is modulation of the effective charge density within the ESL. Literature on transvascular transport of charged molecules generally shows diminished transport of anionic molecules in comparison to neutral molecules (6;7;14;15;18;40;42;48;49), which supports the presence of fixed negative charges on the luminal endothelial surface. Sörensson and coworkers (48;49) have studied the influence of perfusate ionic strength on the charge selectivity of the glomerular capillary wall. These authors demonstrated that lowering ionic strength by 4.5-fold reduced the fractional clearance of several anionic tracers by ~1.5-fold, which was attributed to a reduction in radius of the small pores responsible for the exchange and to a reduction in the charge density of the glomerular barrier. Furthermore, Granger and coworkers (18) demonstrated that neutralization of the negative fixed charges on the intestinal capillary wall by infusion of polycations induces a ~6-fold increase in permeability to fluid and a ~4-fold increase in protein clearance. Nevertheless, the exact dimensions of the endothelial structures carrying these negative charges had not yet been determined satisfactorily (4;39;46;47;53).

We hypothesized that modulation of solvent ionic strength influences the effective charge density of the ESL and can thus result in altered barrier properties of the ESL to transport of anionic molecules. ESL volume is determined by a dynamic equilibrium of water movement into the ESL due to interactions between the charge carrying structures of the glycocalyx, free plasma ions and other charged molecules present in the perfusate and the hydrostatic pressure of the perfusate (13). Upon modification of one of these factors, the ESL will adapt its structural organization to a new dynamic equilibrium. Therefore, modulation of perfusate and superfusate ionic strength is likely to result in dimensional changes of the ESL as well as changes in ESL charge distribution, both affecting ESL permeability properties.

We found that ESL thickness was dependent on solvent ionic composition, being smaller at high ionic strength and larger at low ionic strength. These effects may be similar to the conformational changes in red blood cell (RBC) glycocalyx due to variations in ionic strength, as reported by
Wolf and Gingell (58). These authors demonstrated that the RBC glycocalyx is swelling roughly by a factor ~2.2 as ionic strength falls by a factor 4. The swelling effects at low ionic strength might explain the large exclusion zone to FITC-Δ50 we observed at low solvent ionic strength. As described in chapter 4, the dimension of the exclusion zone increased by a factor ~2.3 as ionic strength was decreased 2-fold, while ESL thickness decreased by a factor ~2.5 as ionic strength was doubled. Assuming that the total amount of charge carrying proteoglycan fiber-structures in the ESL is constant, this implies condensation of these fibers inside the ESL at high ionic strength and extension of the fibers at low ionic strength. Furthermore, we demonstrated an ionic-strength-dependent ESL permeation of FITC-Δ50. We found a longer ESL permeation time at low ionic strength in comparison to normal ionic strength, indicating that a reduction in ionic strength resulted in a greater impairment of FITC-Δ50 transport across the ESL (chapter 4).

The barrier properties of the ESL can also be influenced by modulating the protein composition of the perfusate. Many studies on the effects of proteins, such as albumin and orosomucoid, on vascular wall permeability to water and solutes have been reported, indicating that proteins lower vascular wall permeability (26-28;32;36;37;52). The exact dimension and location of the structures interacting with proteins had not yet been determined satisfactorily. In chapter 5 we tested the hypothesis that the effects of proteins on vascular permeability are the result of interactions of these proteins with the endothelial glycocalyx (11;19-21;32;35-37;41). Conformational changes in the endothelial cell glycocalyx due to the presence of albumin or plasma have been reported by Adamson and Clough (2), using cationized ferritin (CF) as a marker of the glycocalyx. These authors demonstrated that the presence of albumin impaired the access of cationized ferritin to the endothelial cell surface as compared to vessels perfused with protein-free Ringer, but albumin did not - in contrast to full plasma - affect total glycocalyx thickness as compared to vessels perfused with protein-free Ringer.

We found that the ESL thickness was not diminished upon removal of albumin from the perfusate. In contrast, the permeation rate of FITC-Δ50 into the ESL increased by a factor 1.5 in the absence of albumin. Thus albumin preserves the barrier properties of the endothelial surface layer (chapter 5).
7.5 THE ESL IS SENSITIVE TO OXIDATIVE STRESS

We applied light-dye-treatment for disruption of the ESL (chapter 2). Prolonged luminal fluorochrome illumination with a bright mercury lamp destroyed the 2-3 μm exclusion zone for FITC-Δ148 within a few minutes. We chose for LDT above a more physiological oxidative stress such as the application of oxidized low-density lipoproteins (ox-LDL) (9;10;55). The latter would require switching to yet an additional perfusion solution and a longer period for the oxidative stress to develop: 15-30 min for ox-LDL (9;10;55) versus a few minutes for LDT (56). The mechanisms for ESL disruption are comparable, in the sense that in both cases the disruption can be prevented by the radical scavengers superoxide dismutase and catalase (9;10;55;56). This indicates that the damage to the ESL is exerted by free radicals that are generated during the ‘bleaching’ of the fluorescent tracers (FITC-Δs).

Under normal physiological circumstances there is always a small amount of free radicals present. Under some conditions, an enhanced generation of free radicals can occur in healthy vessels. For example, pulsatile shear stress induces formation of free radicals (50). These small amounts of free radicals are under normal circumstances counterbalanced by constitutive radical scavengers present, such as superoxide dismutase and catalase or the endothelium derived nitric-oxide (NO). Therefore these levels of free radicals are unlikely to have damaging effects on the endothelium or the endothelial surface layer (ESL) per se. Rather, free radicals should be seen as part of normal cell signaling. However a disturbance in the balance between free radicals and radical scavengers may lead to deleterious effects and may therefore form an initial step in the occurrence of vascular pathologies, such as atherosclerosis, but also a disease such as preeclampsia (54).

7.6 ROLE OF THE ESL IN ATHEROGENESIS

It is obvious that an ESL of several micrometers in thickness has an effect on the barrier function of the endothelium as well as on the interaction of blood cells with the vascular wall. The ESL might play a predominant role in the regulation of physiological processes such as ligand-receptor-interactions, transvascular exchange of oxygen, nutrients or proteins, and protection of the endothelium against atherogenic stimuli.

As described above, we applied light-dye-treatment (LDT) to disrupt the endothelial surface layer (ESL). This resulted in a 2-3 μm shift of the fluorescence profile of large FITC-Δ148 towards the
endothelium, thus reflecting disappearance of the exclusion zone for FITC-A148.

The damaging effect of LDT on the ESL in chapter 2 is consistent with observations in capillaries where the ESL could also be damaged by oxidative stress induced by light-dye-treatment (56) or by oxidized LDL-cholesterol (9;10;55), by enzymes, such as heparinase, pronase, hyaluronidase or neuraminidase (1;16;17;22;30), by ischemia-reperfusion (5;12), or by inflammatory agents, such as tumor necrosis factor-α (23). From permeability studies it is known that damage to or removal of the ESL results in a marked increase in vessel wall permeability (1;30). Furthermore, it has been demonstrated that oxidative stress-induced ESL damage results in an increased adhesiveness of the endothelium to platelets and leukocytes (10;55).

Hence, the ESL can be seen as a line of first defense of the vascular wall and damage to the ESL might be the first onset to the occurrence of vascular pathologies. This view is supported by the notion that an increased vascular permeability is an early hallmark in many vascular pathologies, including atherosclerosis (38;43;44).

7.7 DIRECTIONS FOR FUTURE RESEARCH

7.7.1 Role of the ESL charge

To further investigate the role of the charge of the endothelial surface layer (ESL) on the distribution of charged tracers inside the arteries or the transport properties of these tracers over the arterial wall, one could apply differently charged fluorescent tracers, such as neutral or positively charged ones, next to negatively charged ones as has been done in the present studies. We have just seen that very small, positively charged, hydrophobic molecules such as DiI migrate very fast through the ESL. Presently we have no information available about the transport behaviour of large, neutral or positively charged molecules in arteries as we have been using. From capillary studies we know that molecular transport through the ESL is dependent on size and charge of the molecules (56).

Another interesting possibility would be to study transvascular transport of different physiologically relevant (macro)molecules, such as plasma proteins, hormones, nutrients or vasoactive agents, whether or not fluorescently labeled. Some of these molecules are known to carry a certain charge themselves, others could be manipulated by using differently charged fluorescent labels.

It has been demonstrated that permeability characteristics of venules towards albumin depend on the fluorescent dye used to label albumin (45), which is likely to be due to the charge of these fluorescent labels.
Furthermore, better understanding of the importance of ESL charge in the regulation of vascular wall permeability requires *in vivo* ESL charge modulation, for example by means of varying the ionic composition of the perfusate of a certain vessel under study. Obviously, it will be difficult to manipulate the perfusate ionic composition *in vivo*. One would have to manipulate the ionic composition of the blood or replace the blood by a physiological saline solution with a certain ionic composition. It might be expected that these kind of procedures would affect the functionality or normal physiological behaviour of many more structures, cells and organs than solely the ESL. Instead of systemic perfusion with such artificial solutions, we recommend application of saline solutions with various ionic compositions locally to individual vessels under study, for example by means of single vessel cannulation techniques or by micro-injection procedures.

### 7.7.2 Influence of plasma proteins on ESL barrier properties

The results of our albumin study (chapter 5) leave the question how different plasma proteins or serum would influence the ESL barrier properties. One possible approach to tackle this problem is the application of more plasma proteins or full serum to the perfusate in comparison to albumin alone. From many studies on the effects of plasma proteins, such as albumin and orosomucoid, on vascular wall permeability, we know that proteins lower permeability (26-28;32;36;37;52), but the direct effect of these proteins on the ESL have not yet been studied in a setting as presently described.

Furthermore, better understanding of the importance of the presence of plasma proteins on ESL integrity requires *in vivo* protein composition modulations as well. Again one would have to apply an artificial perfusate solution to modulate the protein composition, and again we recommend individual vessel perfusion or micro-injection techniques to establish these procedures.

### 7.7.3 Influence of atherogenic stimuli

Our model could serve very well to investigate the effects of various atherogenic stimuli on the vascular wall in general and more specifically on the ESL. Besides the damaging effect of free radical production by light-dye-treatment on the ESL, it would be interesting to test more physiological atherogenic stimuli such as the application of oxidized low-density lipoproteins (ox-LDL) or hyperlipidemic conditions (8), and to quantify their effects on ESL dimension and barrier properties. It has been shown that ox-LDL (transiently) degrades the ESL in capillaries, which leads to an increased adhesiveness of the endothelium to platelets and leukocytes.
Furthermore high-fat diet induced hyperlipidemia in ApoE3-Leiden mice also causes degradation of the endothelial glyocalyx, which is associated with an increased endothelial permeability to large chylomicrons (8).

Since atherosclerosis is mainly a disease of larger blood vessels, it is indispensable to study the effects of such atherogenic stimuli in larger vessels.

We feel that the study protocols on arteries of ~150-200 μm, presently descried could serve as a adequate model to further elucidate the process of atherogenesis.

7.8 REFERENCES


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7 General discussion