Endothelial Luminal Membrane-Glycocalyx:
Functionalities in Health and Disease

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Endothelial Luminal Membrane-Glycocalyx:
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ABSTRACT

This book focuses on the structural, biochemical, and diverse functional properties of the endothelial luminal membrane glycocalyx (ELMG), an organelle which constitutes the endothelial cell "membrane." It is intended to provide the newcomer with a broad, basic, and brief perspective of the luminal endothelial vascular membrane, and for the more established investigator, a basic overview and integrated perspective of the "universe" we explore.

The endothelium is an assortment of heterogeneous regulatory cells whose cytoplasm and cell membranes are joined, forming functional units. There is a tremendous amount of literature on the endothelial cell, constituting seemingly isolated and distinct fields of encapsulated research. However, the multifunctional properties of some molecules give rise to an overlap of findings, frequently ignored between the different fields.

The book is divided into three parts. The first part concentrates on the structure of the ELMG, with emphasis on morphological and biochemical composition. The importance of the chemical composition to the physiological functions of the ELMG, such as sieving properties, pharmacology, and flow sensing, is the focus of the second part of the book. Finally, some of the pathologies associated with ELMG dysfunction are explored in the last section.

The aim is to provide basic and well-established knowledge in the various individual fields, identify the current concepts in each area, and discuss their respective strengths and weaknesses (including hidden problems). Finally, the overall goal is to integrate areas where overlap is clearly indicated, bringing them all together to provide the first ever basic, integrative, panoramic bird's-eye view of the field.

KEY WORDS

endothelial luminal membrane glycocalyx (ELMG), endothelium, endothelial cells, lectin–oligosaccharide interaction, flow sensing, sieving properties, endothelial pharmacology, glycocalyx pathology

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Preface

More than 35 years of intensive research has shown that the "endothelium" throughout the vascular tree is an assortment of heterogeneous regulatory cells whose cytoplasm and cell membranes are amalgamated forming functional units. These units are responsible for very diverse processes, all of which constitute deceptively independent fields of basic research and pathologies, such as selective solute permeability, blood coagulation, atherosclerosis, tissue inflammation, pus formation, metastasis, and regulation of hormone-dependent and/or flow-dependent diverse parenchymal functions and metabolism. In blood vessels, in some portions, the endothelial cell monolayer is extremely thin and is not an inert physical diffusional barrier, but a chemically complex and selective dynamic interface for the transfer of diverse physiological molecules and even cells across it.

It is increasingly clear that *in situ,* the abovementioned limited list of processes of the functional endothelial entity are initiated and may even be restricted to the complex endothelial luminal membrane-glycocalyx (ELMG), an organelle, which is dynamic and continuously varying. The luminal endothelial membrane, mistakenly is assumed to be "minimally thin," but, it is not a membrane bilayer, it is a membrane with attached structures with complex chemistry that stretches up to 1 μm toward the lumen and constitutes a multifunctional unit. The ELMG is either the site of reception of diverse stimuli and/or enzymatic molecular transformations and/or the site of initial signaling that regulates the mentioned processes. There is a tremendous amount of literature on the endothelial cell focused on each of the abovementioned processes constituting seemingly isolated and distinct fields of encapsulated research, with experts in apparently different disciplines. However, the multifunctional properties of some molecules give rise to overlaps of findings, frequently ignored between the different fields. This indicates that there are suspected and missed interrelationships between the fields, and their knowledge and awareness would enrich them. These interrelationships need to be defined as an attempt to integrate the basic knowledge of the ELMG.

Because research on the endothelial luminal membrane has evolved into apparently independent and complex research fields, it is time to attempt to select what are the basic findings of each field, the solid foundations for future work, and the time is ripe to attempt to define what they have in common, to bring them together and try to integrate them. This creates the

need to: (a) describe the basic and well-established knowledge which defines each individual field; (b) identify the current concepts, their strengths, weaknesses, and unresolved problems; (c) propose ways of revising prevalent concepts based on newer knowledge and methodologies; and d) integrate areas where overlap is clearly indicated. For this purpose, a panoramic perception of the whole field, a basic integrative view, is the purpose of this book.

Furthermore, regarding terminology: there are several variations in the terminology used by researchers to refer to the luminal structural layer of the endothelium, which is the focus of this book. It has been alternately referred to by authorities in the field as the "endothelial glycocalyx," the "endothelial luminal structural layer," or the "endothelial surface layer." We need to define and agree on a term for this organelle that best defines its structure and functions. First, the term "luminal" must be included because there is an abluminal and a luminal structure, each with distinct functions. Second, the space confinements of the "endothelial glycocalyx" have not been clearly defined, and we need to keep in mind that the glycocalyx fiber matrix and cell membrane are structurally and functionally part of one another; a concept that is excluded in the term "endothelial glycocalyx." Furthermore, most people usually think of the glycocalyx as being limited to only the mesh/brush above the membrane, excluding the cell membrane. For these reasons, we prefer the term ELMG because it specifies the luminal side of the endothelium and includes both the matrix and membrane as a structural and functional unit.

In this book, we attempt to keep the number of references to a minimum necessary to provide basic information and give credit to early foundations of current concepts. References, except for a few *in vitro* studies, are restricted to studies *in vivo* where ingenuity of the researchers allows deriving concepts at the cellular level. This philosophy is motivated by the fact that endothelial cell culture, besides studying the cell as an isolated homogeneous unit, causes numerous alterations of the cell phenotype. It is intended to provide the newcomer with a broad, basic, and brief perspective of the field on the luminal endothelial vascular membrane and, to the more established investigator, a basic overview and integrated perspective of the "universe" we explore.

Acknowledgments

With gratitude to the Consejo Nacional de Ciencia y Tecnología (CONACYT) de Mexico for their economic support that made it possible for R. Rubio to spend a year at Tulane University Medical School and use their excellent library facilities. Thanks to Tulane University and especially to Dr. Gabriel Navar, Chairman of the Department of Physiology for providing me office space, necessary facilities, and generous access to all activities of the Department plus a friendly and stimulating environment. We are also grateful to Joe Cho of Morgan & Claypool Life Sciences Publishers for his guidance, wise suggestions, and efficient production of the book.

PART I

THE DISTINCT MORPHOLOGICAL, PHYSICAL, BIOCHEMICAL, AND STRUCTURAL PROPERTIES OF THE ENDOTHELIAL LUMINAL MEMBRANE-GLYCOCALYX (ELMG)

CHAPTER 1

The Endothelial Cell *in situ* is not an Isolated Cell, but a Complex of Cells

The lining of the entire vasculature is an apparent cell monolayer termed the endothelium. As will be explained, it is more than one-cell layer thick. The early morphological studies show three distinct classic endothelial cell types: continuous, discontinuous, and fenestrated; they are not a single cell entity, but rather a heterogeneous one. Studies now stress that there is not "an endothelial cell" but multiple cell types, because frequently their structure, biochemistry, and physiology vary and adjust to the organ in which the blood vessel resides. Even within the vascular tree of a given organ endothelial cells are diverse, depending on their environment. Little is known on what are the local factors that program the phenotype of the endothelial cell. In some vascular regions, endothelial cells are small flat-shaped cells and are very thin (~ 0.2 μm), except in the nuclear area where they may be ~3μm thick.

The two opposing sides—luminal and abluminal—of the endothelial membrane differ considerably in chemical composition; at the level of the nucleus, there are mitochondria, myofilaments, and well-developed endoplasmic reticulum necessary for multiple functions and secretions. However, the overall mass of the endothelial cell is small, and one could wonder if such a small cell can accomodate the multiple elements of massive synthesis necessary for the controlled secretion of a large list of paracrine agents and numbered functions. Nevertheless, it is likely that the endothelial cell does not function as an isolated entity, but in cooperation with other cell elements. These cooperative elements are likely the pericytes, smooth muscle, and endothelial cells themselves.

1.1 ENDOTHELIAL-PERICYTE, ENDOTHELIAL-SMOOTH MUSCLE, AND ENDOTHELIAL-OTHER CELL TYPE COMPLEXINGS: A COOPERATIVE, INTEGRATIVE UNIT

Extensive *in situ* evidence supports the concept that the endothelial cell is not anatomically nor functionally an independent entity. It is electrically coupled to other adjacent endothelial

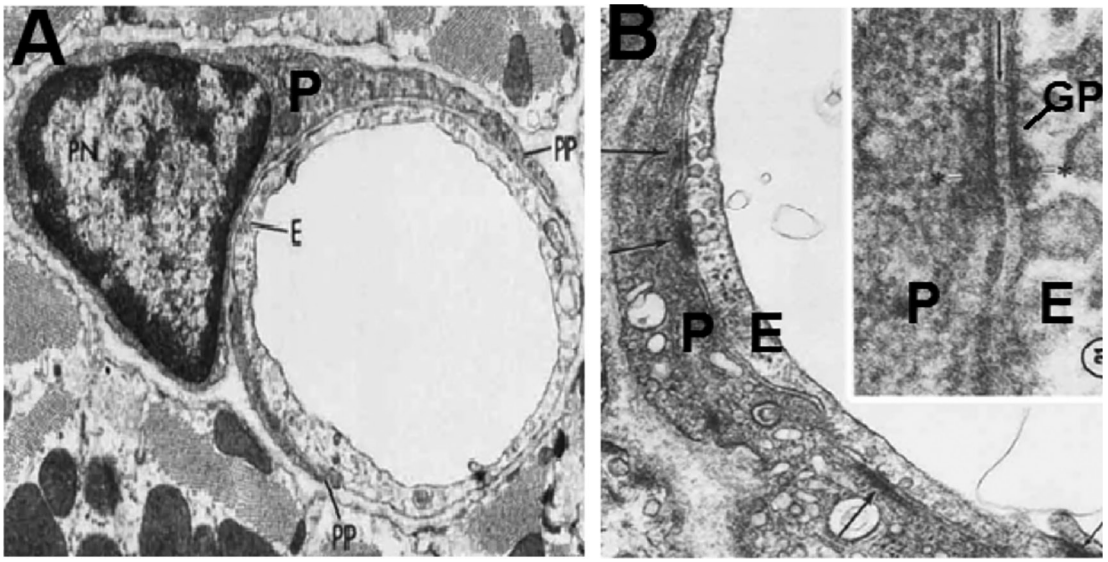

FIGURE 1.1: Relationship between pericytes (**P**) and a capillary endothelial cell (**E**). A) a pericyte clearly surrounds a capillary. B) A pericyte (**P**) is so closely adjacent to an endothelial cell (**E**) that both cells share the same basement membrane and, at some discrete points (arrows), their membranes fuse forming permeable channels, and gap junctions (**GP**). (From Forbes et al. With permission.)

cells and cell types like smooth muscle myocytes or pericytes, as well as to others like glial cells. Pericytes, in particular, are so close to the endothelial cell that there is no basement membrane between them. The pericyte, an octopus-like cell, surrounds the endothelial cell so that the cell membranes and cytoplasms of these two cells meet at discrete sites of contact, forming permeable channels called gap junctions (Figure 1.1A, B). Both cells share the same basement membrane.

Pericytes are polymorphic and their degree of envelopment of endothelial cells in the microvasculature is extensive and variable (Figure 1.1A, B). There are at least three types of pericytes: precapillary, capillary, and postcapillary venular. Pericytes are more massive than endothelial cells, are rich in mitochondria, endoplasmic reticulum, and myofilament networks, some of which are made of both smooth muscle and nonsmooth muscle isoforms of actin and myosin. At the arteriolar level, smooth muscle replaces pericytes. Locally and regionally, the three types of pericytes differ in their contractile and secretory capacities. Pericytes synthesize and secrete a wide variety of vasoactive autoregulating agonists such as TXA2, PGF2, Endothelin-1 plus many other paracrine messengers. Thus pericytes, like endothelial cells, are morphologically, biochemically, and physiologically heterogeneous.

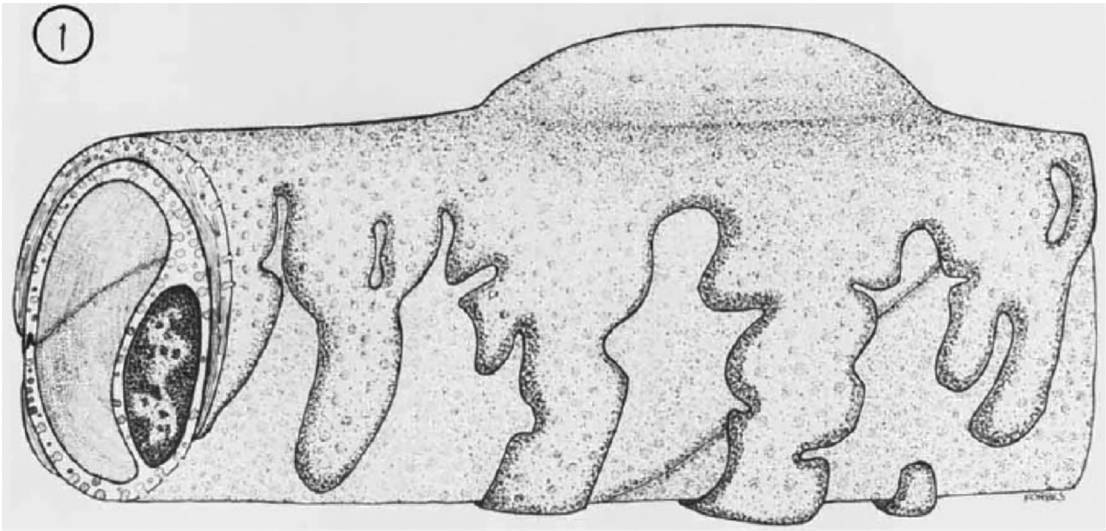

FIGURE 1.2: Drawing of a pericyte engulfing an endothelial cell through multiple branches. This drawing was constructed from transmission electron-microscope serial sections; Shepro later confirmed this image using scanning electron-microscopy of an arterial capillary. (From Forbes, et al. With permission.)

In situ, the ratio of pericyte to endothelial cells varies. For example, in retina it is 1:1, in lung 1:10, and for skeletal muscle 1:100. However, the area of overlap per capillary does not follow this ratio because in heart 80% of the capillary wall is covered by pericytes. This indicates that pericyte size and branching vary considerably (Figure 1.2).

In a very early ultrastructure study by Forbes et al. using serial electron microscopy sections, they generated a drawing as shown in Figure 1.2. These authors also stated:

> Myocardial pericytes occupy a stratum comparable to the smooth muscle-containing medial layer of larger vessels, and may perform a similar function; i.e., pericytes may modulate the caliber of the vessels with which they are associated. Performance of this role would require that these pericytes fulfill at least three morphological criteria: (1) the presence of suitably-arranged contractile cytoplasmic components, (2) the occurrence of attachments between pericytes and endothelial cells, providing a means by which pericyte contraction could be transmitted to the endothelial cylinder, and (3) the existence of some system controlling pericyte contraction or relaxation. The first morphological criterion appears to be met by the high degree of organization of the filaments of the myocardial pericyte; the second is supported by the presence of pericyte-endothelial junctions. The third criterion would be satisfied by the demonstration of autonomic innervations of myocardial pericytes.

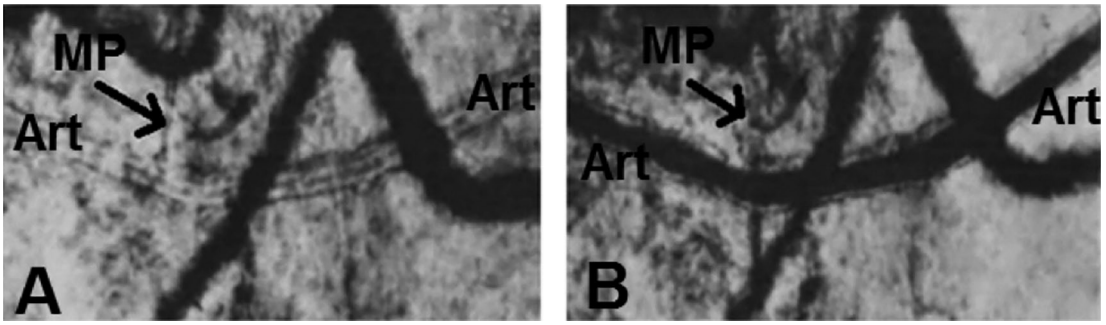

FIGURE 1.3: Very localized application of acetylcholine with a micropipette (arrow, **MP**) with a tip of a few microns just touching the outer surface of an arteriole (**Art**) causes a vasodilation that spreads much further beyond the site of application. **A**; Arteriole before acetylcholine brief application. **B**; Arteriole following the application of acetylcholine. From Duling and Berne (1970). Used with permission.

They demonstrated that pericytes are extensively innervated, as recently confirmed by modern studies.

Evidence of coupling between endothelial and immediately surrounding cells has been suspected since 1920. The demonstration that a highly localized and transient application of acetylcholine to arterioles caused propagated vasodilation that spread beyond the site of application (at 0.02 cm/sec) to a distance more than seventy endothelial cells in length (Figure 1.3) suggested that the arterial-wall cells must be coupled. These results opened the door for the ulterior demonstration of the role of gap junctions; intercellular pores, between endothelial cells, endothelial smooth muscle cells, and pericytes-endothelial cells.

Connexins are a large family of proteins and the predominant subtypes present in the vasculature are Cx37, Cx40, Cx43, and Cx45. Connexins are found in the membranes between adjacent cell types (Figure 1.4) and they allow ions and metabolite exchange between neighboring cells: an electrical and metabolic coupling—not a result of a passive syncytium but one that is dynamically modulated by extracellular signals such as angiotensin II.

Thus, studies in situ show that the "highly heterogeneous endothelium" is not made of an isolated small cell, but that anatomically, functionally, and metabolically it constitutes an operational multicellular unit whose function and metabolism depends on its coupling to pericytes and smooth muscle cells. This functional cluster of cells augments tremendously the synthesis and signaling capabilities of the unit: "the endothelial cell in situ." Furthermore, the endothelium is a dynamic and vigilant complex, constantly responding to microenvironmental signaling, altering its phenotype to serve the local demands of the tissue and consequently, it adapts to many different microenvironments.

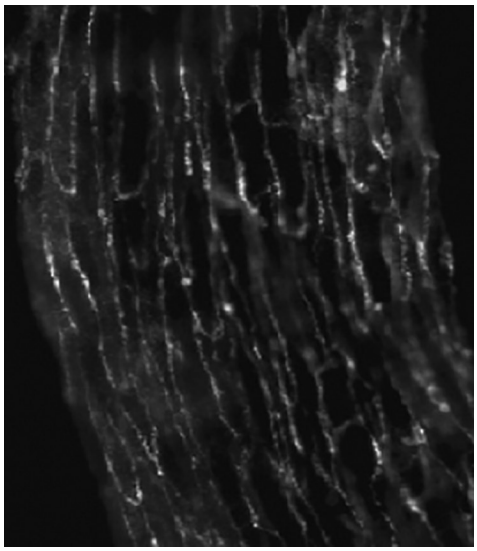

FIGURE 1.4: Immunofluorescent microscopy of connexin Cx40 in an arteriole. The distribution of Cx40 follows the contours of the endothelial cells. (From Looft-Wilson, et al. With permission.)

KEY REFERENCES

Majno G. Ultrastructure of vascular membrane. In: *Handbook of Physiology*. Section 2. Circulation. Vol III. American Physiological Society, Washington, DC, pp. 2293–2376, 1964.

This chapter provides an excellent overview of the diverse types of endothelium in different parts of the vasculature.

Simionescu M., Simionescu N. The ultrastructure of the microvascular wall. Functional correlations. In: *Handbook of Physiology*. Section 2. The Cardiovascular System. Vol. IV. Chapter 3. E. M. Renkin, C. C. Michel (eds.), American Physiological Society, Washington, DC, pp. 41–101, 1984.

This chapter provides a comprehensive ultrastructural and functional overview of the endothelial cell and its asymmetry, and presents the basis of many issues that are still current fields of study such as: heterogeneous luminal membrane electric charge distribution, molecular composition, and molecular movement across the endothelial wall.

Forbes M.S., Rennels M.L., and Nelson E. 1977. Ultrastructure of pericytes in mouse heart. *Am J Anat*. 149: 47–70. doi:10.1002/aja.1001490105

This paper provides one of the earliest and thorough electron microscopic descriptions of the spatial relationship between endothelial cell and its engulfing pericyte. It describes quantitative relationships and the presence of gap junctions between these two cell types. The high secretory complexity of the pericyte and the presence and orientation of contractile filaments in this cell are also discussed.

Shepro D., Morel N.M.L. 1993. Pericyte Physiology. *FASEB J*, 7:1031–1028. This review provides a quantitative description of the distribution of pericytes in different circulations.

A comprehensive description of functions, structures, and the highly secretory biochemistry of pericytes is given. The relationship and close association via gap junctions between pericytes and the endothelial cell is presented, identifying the proteins "connexins" that constitute the gap junctions.

Kroch A. 1920. Studies on the capillary-motor mechanism. I: The reaction to stimuli and the innervation of the blood vessels in the tongue of the frog. *J Physiol* (London), 53: 399–405.

This paper reported that local application of iodine to the web of the frog's foot resulted in a massive vasodilation which spread over a much larger area than could be accounted for by diffusion of the agent.

Duling B.R. and Berne R.M. 1970. Propagated vasodilation in the microcirculation of the hamster cheek pouch. *Circ Res*. 26:163–170. doi:10.1161/01.RES.26.2.163

This paper is the first demonstration that application of highly localized acetylcholine to an arteriole causes a vasodilation that spreads much further beyond the site of application and at a speed faster than that of Ach diffusion. Thus it is likely that vasodilation is conducted from cell to cell along the arteriolar wall through gap junction channels. This manuscript sets the basis for electrical and functional molecular coupling between endothelial-endothelial and endothelial-smooth muscle cells via channels later identified as connexins.

Little T.L., Beyer E.C., and Duling B.R. 1995. Connexin 43 and connexin 40 gap junctional proteins are present in arteriolar smooth muscle and endothelium in vivo. *Am. J. Physiol*, 268 (*Heart Circ. Physiol*, 37): H729–H739.

This paper established the distributions of connexin 43 (Cx43) and connexin 40 (Cx40) in smooth muscle and endothelium of resistance vessels, from three different vasculatures, and demonstrated the potential for cell-cell communication in both cell types. They indicated a greater level of coupling within the endothelium.

Looft-Wilson, Robin C., Geoffrey W. Payne, and Steven S. Segal. 2004. Connexin expression and conducted vasodilation along arteriolar endothelium in mouse skeletal muscle. *J Appl Physiol* 97:1152–1158. doi:10.1152/japplphysiol.00133.2004

This paper shows very clearly that, between neighboring endothelial-endothelial and endothelial-smooth muscle cells in skeletal muscle arterioles, there are the connexin protein subunits Cx40 and Cx37. These connexins may constitute the gap junction channels and provide the cellular pathway for conduction.

Puro D.G. 2012. Retinovascular physiology and pathophysiology: New experimental approach/new insights. *Progress in Retinal and Eye Research*, 31: 258–270. doi:10.1016/j.preteyeres.2012.01.001

This is a review on electrical coupling between endothelial-endothelial, endothelial-pericytes and endothelial-smooth muscle cells in retinal capillaries and arterioles. In isolated microvascular complexes from the rodent retina, using techniques such as perforated-patch-

clamping, fluorescent imaging and time-lapse photography, electrotonic and functional coupling between the three different cell pairs was demonstrated. Their analysis showed that this operational unit (capillaries and arterioles) is not simply a homogenous passive syncytium, but has a complex functional organization that is dynamically modulated by extracellular signals such as angiotensin II. i.e., locally generated voltage changes and connexin conductivity; the electrotonic architecture is dynamic, not static.

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CHAPTER 2

Morphological Structures: Diversity, Heterogeneity, and Appearance Depends on Staining Procedure

The luminal endothelial membrane is frequently mistakenly conceived to be "dimensionlessly thin," however, it is far from being a simple lipid bilayer with inserted insular proteins. It is a membrane with attached structures featuring complex chemistry and geometry that interact with one another. This layer stretches up to 1μm toward the lumen and constitutes a functional unit. Such a conceptual oversimplification frequently prevents correct interpretation of results.

The endothelial luminal membrane-glycocalyx (ELMG) is a physically, chemically, and geometrically complex structure constituted by a molecular layer about 0.3–1.0 μm thick (glycocalyx) that constitutes the cell "membrane." The ELMG participates in an increasing list of diverse endothelial functions. These functions are: selective transfer/diffusional mechanisms of molecules—even cells or parasites—from blood to interstitium; responses and transduction to circulating hormones; detection and transduction mechanisms of flow; mechanisms of ischemia-reperfusion; control of blood coagulation and thrombosis, and tissue inflammation and pus formation. Morphological studies frequently are oriented to provide structural evidence and quantitative structural data relevant to a possible function.

2.1 ELECTRON MICROSCOPY OF ELMG *WITHOUT* AN APPROPRIATE STAIN FOR THE GLYCOCALYX: NEGATIVE IMAGING OF THE ELMG

To visualize the ELMG structures with spatial precision requires electron microscopic imaging. The appearance of ELMG electron microscope images depends on the staining procedures

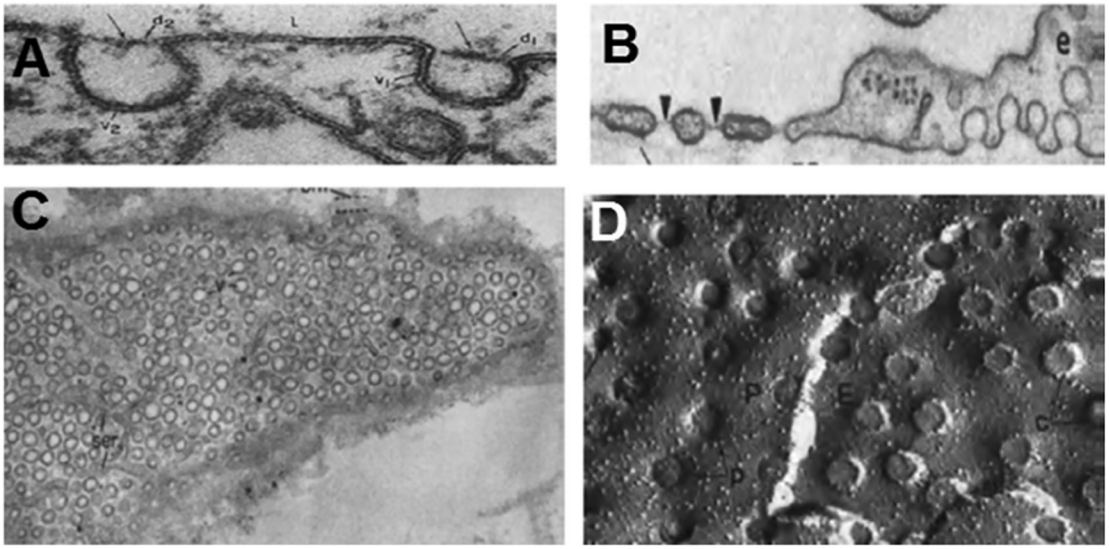

FIGURE 2.1: Luminal endothelial membrane without glycocalyx stained. **A**: Endothelium of a continuous capillary showing plasmalemmal vesicles (V1, V2) whose opening (stoma) is subtended by a diaphragm which consists of a single dense layer at D1. **B**: Fenestrated capillary. Endothelial cells (e) display numerous openings or fenestrae (arrowheads) closed by a thin diaphragm. **C**: Grazing section of a capillary endothelium showing the large density of plasmalemmal vesicles (v). **D**: Freeze-fracture preparation of fenestrated capillaries. Fenestrae appear as papillae (p) on the protoplasmic (P) face and as craters (c) on the external (E) face of the endothelial cell. (**A**, **B**, **C** from Bruns R.R., and Palade G.E., *Journal of Cell Biology*, 37, 244–276, 1968. **D** from Simionescu M., and Simionescu N., *Handbook of Physiology*, American Physiological Society, Washington, DC, 41–101, 1984.)

utilized. It is necessary to keep in mind that with the electron microscope one must use visible structures (whether naturally or artificially stained) that are electron opaque. The most common procedure is to stain thin tissue sections with salts of heavy metals, such as lead hydroxide/nitrate and uranyl acetate, before tissue fixation with OsO_4.

Such treated tissues that present a continuous endothelium show a smooth flat membrane lipid bi-layer with vesicles/coated pits (caveolae) interspaced by flat smooth membrane spaces of about 0.30 μm. The membrane vesicle is continuous with that of the plasma membrane via a neck stalk of about 0.02 μm long. Caveolae have an overall diameter of 0.07 μm, and frequently have a thin diaphragmatic membrane (~ 0.004 μm) covering its mouth (Figure 2.1A). This diaphragmatic membrane is not a continuity of lipid membrane and does not have the lipid bilayer appearance, indicating it has a different chemical composition. The portions of the luminal en-

dothelial membrane containing caveolae show a high density of these structures (Figure 2.1C), in the order of 50–100 caveolae/μm^2 with an estimated 5000–10,000 caveolae/cell.

The luminal endothelial membrane of fenestrated endothelium shows a scarcer presence of caveolae; instead, orifices (fenestrae) are found that appear closed by a thin ~ 0.004 μm thickness of nonlipid membrane, like that seen in the caveolae's mouth (Figure 2.1B). Fenestrae have a diameter of ~ 0.07 μm, their density varies from one vascular bed to another, they tend to appear in clusters, are more numerous at the venous side of capillaries, and their density can be as high as 32 fenestrae/μm^2. Thus, there are quantitative agreements between the caveolae of continuous endothelium and fenestrae of the fenestrated endothelium. Both have ~ 0.07 μm diameters, their mouths are sealed by ~ 0.004 μm thick nonlipid membranes, and their densities are of the same order of magnitude. This similarity becomes evident when one visually compares the images of Figure 2.1A and 2.1B. It is believed that the fenestrae diaphragms are vestigial structures of a collapsed trans-endothelial channel.

2.2 ELECTRON MICROSCOPY OF ELMG *WITH* AN APPROPRIATE STAIN FOR THE GLYCOCALYX: POSITIVE IMAGING OF THE ELMG

There is not a unique electron microscopic type of image for the ELMG. Its optical density (amount of probe bound) and thickness varies along the endothelial surface. This is because the obtained images of this fluffy layer depend on the vascular bed examined, the protocol followed, the stain utilized, and whether it is arterial, capillary, or venular endothelium. Thus, it is difficult to qualitatively and quantitatively analyze these images and to draw coherent functional inferences from them. Generating precise, repeatable, and reliable structural images of the ELMG has not been successful. Some of the diversity of images obtained, using different staining protocols, are shown in Figure 2.2.

Electron opaque cationic molecules are the most common stains used to make the glycocalyx visible. These probes, in interaction with other ions such as Mg^{++}, bind to anionic moieties that are most abundant in muco-polysaccharides, but also present in proteins. In addition, lectins—proteins with binding affinity to specific oligosaccharides—that are made electron-opaque, can be used to probe the ELMG. Furthermore, the states of dissociation of the binder cationic molecule and the binding anionic moiety are pH dependent. Thus, manipulation of pH and Mg^{++} concentration allows differential staining of the dissociable anionic moieties of mucopolysaccharides and proteins. This partially explains why glycocalyx images obtained with different probes vary.

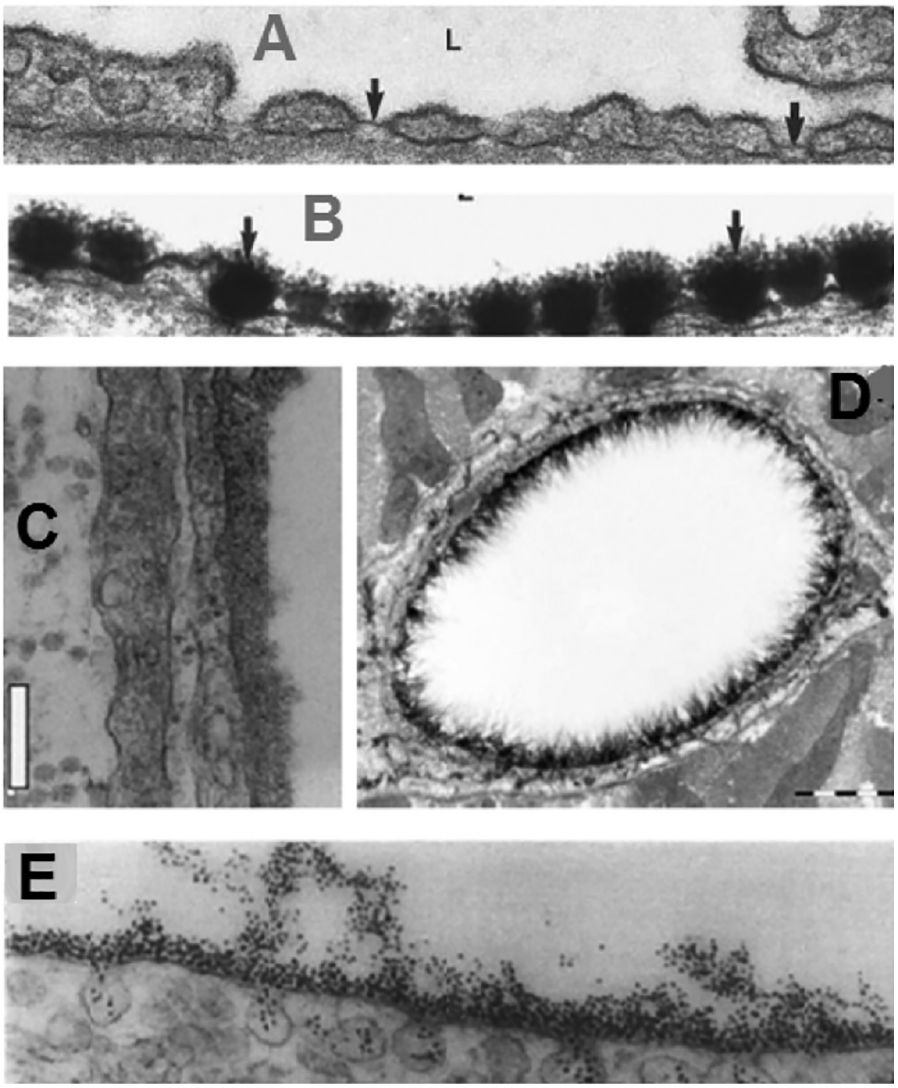

FIGURE 2.2: Diversity of electron micrograph images of the luminal endothelial membrane glycocalyx obtained when stained with different agents. **A**: Fenestrated capillary from the small intestine, post-fixed with OsO_4 and K_3Fe_2CN. The fenestrae are closed by a thin diaphragm (arrows), capillary lumen (L). (From Rostgaard J., and Qvortrup K., *Microvascular Research*, 53, 1–13, 1997. With permission.) **B**: Same preparation as in **A**, but tissue post-fixed with OsO_4, rinsed and immersed in tannic acid and stained with uranyl acetate. Fenestra now is a bush-like structure: filamentous plug, composed of 20–40 filamentous molecules. (From Rostgaard J., and Qvortrup K., *Microvascular Research*, 53, 1–13, 1997. With permission.) **C**: Capillary from the frog mesentery perfused with Ringers containing bovine serum albumin and ruthenium red. (From Squire

The cationic glycocalyx staining agents used are: tannic acid, ruthenium red, alcian blue, cationized ferritin, and lanthanum nitrate. Ruthenium red, an ammoniated ruthenium oxychloride, has been used by botanists for more than 100 years to stain pectin, a polylacturonic acid. Alcian blue is a copper phthalocyanine. Cationized ferritin is an iron containing protein to which multiple cationic groups have been added. Tannic acid, a mordant used in the dying process of cellulose fibers such as cotton, is often combined with iron or any other heavy metal. Lanthanum nitrate is known as a "super" calcium ion. These cationic stains have an electron opaque metallic core. The ELMG anionic binding sites correspond to many moieties in diverse polysaccharides: $-COO^-$ groups in sialic, glucuronic, and iduronic acids; $-SO3^-$ residues from glycosaminoglycans; and ionizable groups from protein amino acids, such as $-COO^-$ from aspartate and glutamate, $-S^-$ from cysteine, and $-NH_3^+$ from arginine and lysine.

This diversity of "stains" creates an equal or even larger diverse set of ELMG structural images that reflect the possible ELMG chemical compositions. However, artificial electron opaque images may even result from crystal formations of the "stain". The current interpretation of structural images and their quantitation assumes that the stain probe binds to a given molecular structure, and neither alters its geometric structure nor its dimension because the probe is assumed to be dimensionless.

2.3 FUNCTIONALLY DEDUCED IMAGING DIMENSIONS OF THE ELMG

In living blood vessels, two different experimental procedures are used to infer the dimensions, shapes, and arrangements of the presumed intertwining fibrous molecular structures and the dimensions of open spaces between structures in the ELMG. This subject will be presented in more detail in section II-1.

The first procedure limits its measurements to diffusion of fluorescent molecules within the ELMG (Figure 2.3). This procedure consists of rapidly injecting at zero time a fluorescent

J.M., et al., *J Struct Biol*, 136, 239–255, 2001. With permission.) **D**: Cardiac capillary. Tissue post-fixed with Alcian blue 8GX, followed by OsO_4 and lanthanum nitrate. (From van den Berg B.M., et al., *Circ Res*, 92, 592–594, 2003. With permission.) **E**: Frog mesenteric capillary perfused with 1 mg/ml polycationic ferritin solution. (From Turner M.R., et al., *Microvascular Research*, 25, 205–222, 1983.)

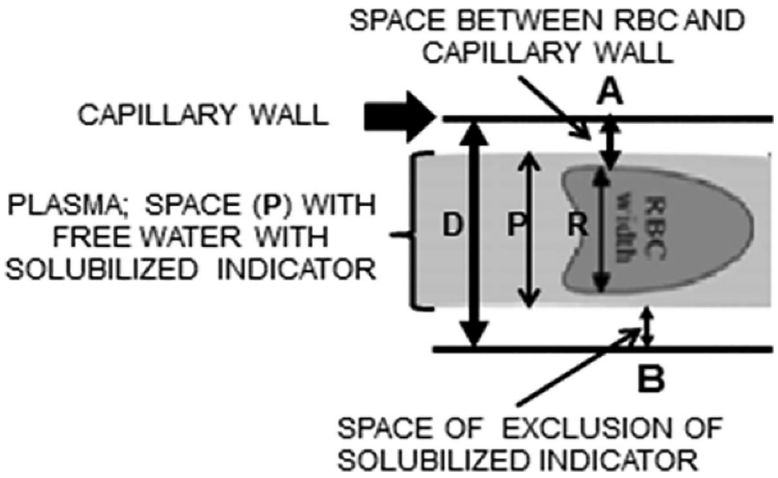

FIGURE 2.3: A. Schematic illustration of the method for measuring permeation of fluorescently labeled plasma solutes from the free plasma space (**P**) into the endothelial surface layer (**B**, glycocalyx). Depending on molecular size, charge, and structure, some fluorescent tracers can permeate into the endothelial surface layer (exclusion zone) over time. (Modified from Vink, H., and Duling, B.R., *Am J Physiol Heart Circ Physiol*, 278, H285–H289, 2000.)

probe that "instantly" uniformly fills up the core of plasma volume (**P**), but due to slow diffusion, it is transiently excluded from a small zone adjacent to the endothelial wall (see the exclusion zone **B** in Figure 2.3). Thereafter, the probe, depending on its size, electrical charge and chemistry, gradually diffuses circumferentially through the exclusion zone (glycocalyx thickness) toward the endothelial wall. The rate at which the probe moves through the ELM glycocalyx toward the endothelial wall is then determined.The probes used are molecules that are chemically different, or chemically similar, electrically charged or neutral, and all having the same or variable molecular size. The rates of diffusion of these probes, through the space of exclusion of the solubilized indicator, are determined and inferences are made concerning the physical properties of the ELMG matrix. Unfortunately, the complexity of the data obtained does not allow the generation of "a functional view of the matrix."

In the second procedure (Figure 2.4), a capillary is cannulated with a micropipette and perfused at a constant pressure with physiological saline containing a low concentration of red cells. Since red cells are very diluted, as saline flows through the vessel there are spaces full of fluid between red cells. At zero time, the capillary is rapidly and locally occluded far from one incoming red cell. The subsequent velocity of the incoming red cell (velocity = l/t) is a flow

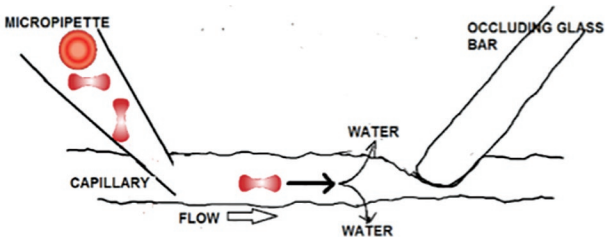

FIGURE 2.4: Diagram of a capillary showing the relations of the micropipette and occluding glass microbar. (Modified from Michell, C.C., et al., *Quarterly Journal of Experimental Physiology*, 59, 283–309, 1974.)

marker toward the point of occlusion and indicates the volume of water loss ($-\text{volume} = -\pi r^2 \cdot l/t$) because of filtering through the total surface area ($A = 2\pi r \cdot L$) and across the WHOLE capillary wall.

Thus, the rate of filtration of water across the whole complex wall of a single capillary can be measured at various capillary pressures. Rate of filtration is found to be a linear function of pressure, and the addition of a protein that binds to the lumen, such as cationic ferritin, reduces significantly the rate of filtration, presumably by "plugging the pores" of the ELMG, inferring that water permeability is largely determined by the ELMG. This procedure also measures passive permeation of different solutes (variable in molecular size, net electrical charge, chemical nature, and physiological significance) across the whole blood vessel wall and allows, from these measurements, to make inferences for blood vessel wall geometric structure—at least for the dimensions of open spaces.

Both procedures infer/ "see" that for ELMG, as a filter constituted of randomly arranged fibers with 0.6 nm radius, the fractional volume occupied by all the fibers in an ELMG unit volume is 0.1, and that such a matrix has the same hydraulic resistance to flow as that of parallel slits with a width of 15 nm. The problem is that the image analyzed depends on the nature of the probe used. (Figure 2.5)

Obviously, there are distinct operational and conceptual differences between these two functional procedures; the first circumscribes its measurements to only determine the rate of massive diffusion of a probe through the ELMG. The latter measures the rate of water loss from the capillary lumen into the interstitium. The second procedure has one explicit (I) and two implicit (II, III) assumptions:

I. The total path length for water movement between the lumen and free interstitium comprises at least three sections of diffusion in series:

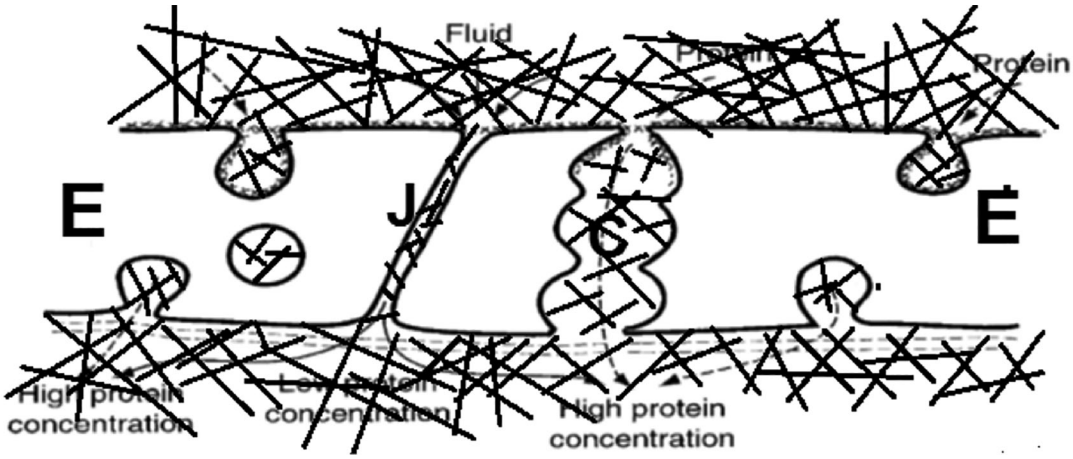

FIGURE 2.5: Fiber-matrix model of capillary permeability. Endothelium (**E**) is traversed by intercellular junctions (**J**) and trans-endothelial channels (**C**). Dimensions and numbers of these channels determine the area of permeable regions of the capillary wall, but molecular sieving properties of a capillary depend on the size of interstices of the fiber matrix enveloping the cell surface and the size of interstices of the fiber matrix filling channels that penetrate it. (Modified from Michel, C.C., and Curry, F.E., *Physiol Rev*, 79, 703–761, 1999.)

Section 1. ELMG.
Section 2. Parallel paths; intercellular endothelial gaps (J), trans-endothelial channels (C).
Section 3. Adventitial endothelial glycocalyx (basal lamina).

II. Along the diffusion path, the energetic cost to move water is constant and equal to that of water free in solution.

III. A given inert probe fully penetrates and equilibrates within the open spaces of the ELMG lattice, and reduces the lumen space for water passage. The probe, being inert, does not alter the chemical properties and structure of the matrix. Conversely, the matrix does not alter the physiochemical properties of the probe. Consequently, because of lumen reduction, the resistance to water flow increases.

KEY REFERENCES

Bruns R.R., Palade G.E. 1968. I. General organization of blood capillaries in muscle. *Journal of Cell Biology*, 37, 244–276.

This paper provides one of the earliest quantitative descriptions of caveolae. It describes their diameter, density, and presence of a membrane at their mouth opening. It shows the fusion between caveolae, leading to the formation of trans-endothelial channels. In fenestrated capillary endothelial cells, it demonstrates that numerous openings or fenestrae have a diameter similar to that of caveolae and that the fenestrae are closed by a thin diaphragm. It also shows that density of fenestrae is close to that of caveolae.

De Bruyn P.P.H., Michelson S. 1979. Changes in the random distribution of sialic acid at the surface of the myeloid sinusoidal endothelium resulting from the presence of diaphragmed fenestrae. *J Cell Biol*, 82, 708–714. doi:10.1083/jcb.82.3.708

This study demonstrates that the ELM, diaphragmed fenestrae (DF) and the rest of endothelial cell surface (EEC), have distinct physicochemical properties and that binding of ionic probes is pH dependent because both ELM structures and probes have different dissociation constants. Both EEC and DF sections bind the probes such as colloidal iron (CI), native ferritin (NF) and cationized ferritin (CF), and in these three cases the amounts bound are pH dependent. At low pH (1.8) these probes are cationic and binding is prominent in the EEC with none in the DF. At 3.5 pH probe binding increases in both EEC and DF. At pH > 5.0 binding of CF augments in both EEC and more prominently in the DF, but there is no binding for NF (pI = 4.5) because it is anionic at this pH. Removal of sialic acid groups reduced CF binding in the EEC only.

Rostgaard J., Qvortrup K., 1997. Electron microscopic demonstrations of filamentous molecular sieve plugs in capillary fenestrae. *Microvascular Research*, 53, 1–13, 1997. doi:10.1006/mvre.1996.1987

This paper is the first demonstration that filamentous structures constitute the glycocalyx. Fenestrated capillaries are far more permeable to water and small solutes than non-fenestrated capillaries; moreover, the permeability is highest in the capillaries with the most fenestrae. These investigators decided to use a method of fixation by perfusion which included the use of glutaraldehyde dissolved in an oxygen-carrying blood substitute (fluorocarbon), osmium tetroxide, and tannic acid. The investigation disclosed a prominent endothelial surface coat with bush-like filamentous sieve plugs in the fenestrae. Each

filamentous plug was composed of 20–40 filaments, each filament measuring 300–400 nm in length and 5–10 nm in thickness. A structure of this type was predicted by functional studies on water filtration by the capillary wall.

Simionescu M., Simionescu N. The ultrastructure of the microvascular wall. Functional correlations. In: *Handbook of Physiology*. Section 2. The Cardiovascular System, Vol. IV, Chapter 3. E. M. Renkin, C. C. Michel (eds.), American Physiological Society, Washington DC, 41–101, 1984.

This chapter provides a comprehensive ultrastructural and functional overview of the endothelial cell and its asymmetry; it presents the basis of many issues that are still current fields of study such as: heterogeneous luminal membrane electric charge distribution, molecular composition, and molecular movement across the endothelial wall.

Squire J.M., Chew M., Nneji G., Neal C., Barry J., and Michel C. 2001. Quasi-periodic substructure in the microvessel endothelial glycocalyx: A possible explanation for molecular filtering? *J Struct Biol*, 136, 239–255. doi:10.1006/jsbi.2002.4441

This paper provides electron microscopic data from diversely prepared and stained tissues. Based on sophisticated imaging analysis and quantitation, it indicates that filamentous structures may constitute the ELM glycocalyx. Their imaging analysis shows that there is an underlying three-dimensional meshwork of fibers, with diameters of about 12–20 nm with spacing between them of about 20 nm. The authors indicate that their measurements and observations are consistent with the fiber matrix model for filtration of water. This model suggests that a relatively regular fiber–matrix structure may be responsible for the ultrafiltration properties of the microvascular wall.

Turner M.R., Clough G., and Michel C.C. 1983. The effects of cationized ferritin and native ferritin upon the filtration coefficient of single frog capillaries. Evidence that proteins in the endothelial cell coat influence permeability. *Microvascular Research*, 25, 205–222.

This a functional and electron microscopy study that shows that water filtration across the isolated capillary is a linear function of intravascular pressure and that incorporation of protein (cationized ferritin) into the ELMG reduces the permeability of water. It is interpreted that the increased hydraulic resistance brought about by cationized ferritin appears to result from the addition of spherical obstacles (the ferritin molecules) to the endothelial cell surface coat or fiber matrix, reducing the surface of filtration. The authors assume that the ELMG is constituted by a fiber network with a fiber radius of $r_f = 0.6$ nm