Perspective

Endothelial Glycocalyx and the Revised Starling Principle

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Introduction

It is not often a textbook paradigm that has withstood time for 100 years is put to the test. In 1894, E. H. Starling found discrepancies in data presented by Heidenhain that suggested "lymph was to be looked upon as a secretion rather than a transudation."[1] Starling was unable to reproduce many of Heidenhain's experimental data and realized that lymph filtration was not an active process of secretion. Instead, as we learn from any physiology textbook, it is the equilibrium between hydrostatic and oncotic pressures.[2] However, though this is not completely wrong, it is incomplete. These pressure gradients were applied to the overall difference between the lumen of the microvasculature relative to the underlying interstitial space.[3] Eventually it became clear the endothelial glycocalyx (eGCX) has matrix properties restricting larger macromolecules to the vessel lumen. As such, new theories developed challenging the idea that simple filtration was regulated through variable gaps between the cells.[4] A revised Starling Principle, proposed by Michel and Weinbaum (independent research), suggested the Starling forces only be applied across the eGCX since it is now considered the molecular sieve for plasma proteins.[5,6] When the eGCX is experimentally removed, the hydraulic permeability would rise dramatically. This, they claim, is due to the eGCX which streamlines the flow of plasma away from the paracellular clefts, thereby reducing hydrostatic pressure.[7] Additionally, the eGCX contains a steep solute concentration gradient due to its thickness and diffusion resistance.[7] To summarize, hydrostatic and oncotic pressure gradients between the microvessel lumen and the interstitium are dependent on the eGCX.

The importance for us research scientists is not the revision itself, but the idea that the eGCX holds the power to change our understanding of a fundamental principle for which much of our current knowledge on edema and vascular health is based. Since the presence of the eGCX has not been considered in other physiological studies, unexplained phenomena could be attributed to eGCX function.

Brief History of eGCX discovery

On the path to discovering the eGCX, two developments occurred that eventually merged and led to the revised Starling Principle: (1) The invention of the electron microscope; and (2) Continued research on fluid exchange as well as mathematical models developed to predict or compare experimental data on the Starling forces.

The electron microscope was co-invented in 1931 by Max Knoll and Ernst Ruska. After refinements and advancements, the first transmission electron microscope (TEM) was made commercially available in 1939. With this, the fine structure of cells could be visualized; the eGCX could be seen and its function speculated upon. The mid 1950's saw the first mentionings of a homogenous fuzzy coating on the surface of endothelial cells, George Palade being one of them.[8] In 1966, Luft was the first to use ruthenium red to specifically mark the glycocalyx for electron microscopy.[9] Before naming the glycocalyx, there were various descriptions including the cell wall, cell surface layer, mucous coating, cuticle or red cell antigen, as well as others.[8,10] In 1963, Bennett suggested a unifying term, 'glycocalyx,' as the general name

for this "extracellular, sugary coating, wherever it may be found." Its Greek translation is "sweet husk."[10] As indicated in the introduction, the endothelial glycocalyx (Figure 1) is a meshwork of long glycosaminoglycans (GAGs) linked to membrane bound proteins (proteoglycans) as well as glycosylated proteins (glycoproteins). Glycoproteins are usually what we envision as cell surface receptors, selectins, integrins, and other functionally dynamic proteins at the cell surface.[11] Proteoglycans play more of structural role to the glycocalyx and are made of a core protein anchored to the cell membrane with long GAGs attached to them.[12] Glycoproteins and proteoglycans are synthesized and assembled in a series of steps as they are vesicularly shuttled from the endoplasmic reticulum to the golgi apparatus and finally to the cell membrane.[13] Other GAGs could be considered soluble since they are not assembled as part of protein synthesis but rather assembled extracellularly and later bound to surface proteins or receptors. One example is hyaluronic acid (HA), also called hyaluronan, linked to the endothelial surface receptor, CD44.[14,15] This HA/CD44 interaction is now known to be a contributor responsible for what is termed the molecular sieve characteristic of the glycocalyx.[14] This means long HA GAGs weave through the eGCX just above the cell surface and create a fence-like meshwork which contribute to size exclusion of plasma molecules.[16] These soluble GAGs are also responsible for the seamless meshwork that bridge the luminal surfaces of one endothelium to the next, thereby creating a semi permeable filter to large solutes.[6]

Starling Forces

As you can see, the endothelial glycocalyx (as we know it) has a very short, half-century history. Interestingly, we can look at historical biological findings and see where the glycocalyx had influence. The rest of this paper will take a look what led to the revision of the Starling

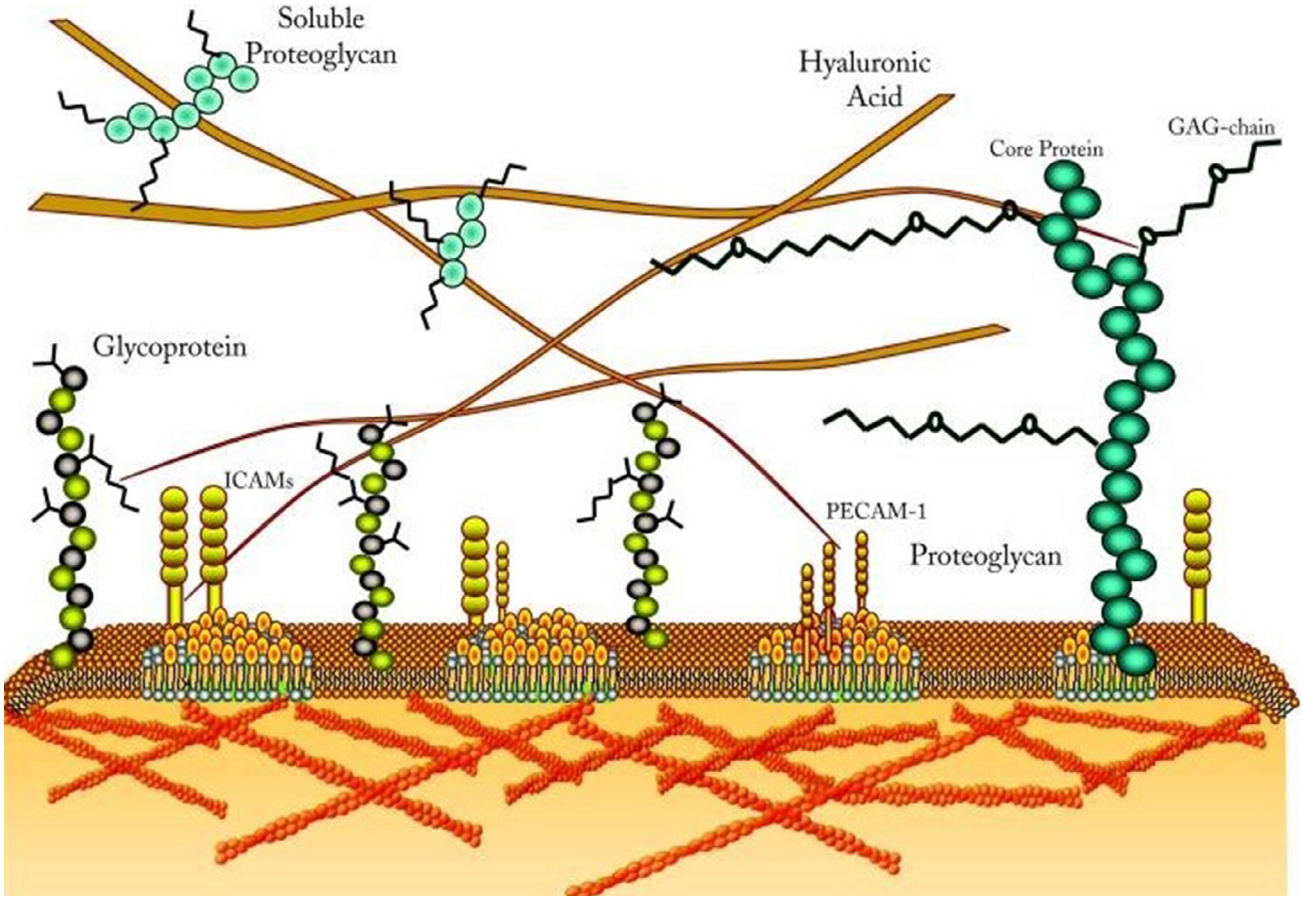

Figure 1: Glycocalyx structural components.
Figure reference: Yuan SY, Rigor RR. Regulation of Endothelial Barrier Function. San Rafael (CA): Morgan & Claypool Life Sciences; 2010. Chapter 2, Structure and Function of Exchange Microvessels. Available from: http://www.ncbi.nlm.nih.gov/books/NBK54123/

Principle influenced by the eGCX. This aspect is interesting because the research involving fluid exchange was in progress before the eGCX was appreciated, while it was this work on fluid exchange that led to understanding the enormous role of the eGCX.

The original Starling Principle refers to the balance between hydrostatic and oncotic pressures relative to the microvascular wall. Hydrostatic pressure is the fluid pressure exerted on the vessel wall, a force generated as a function of the contracting ventricles of the beating heart. Since the vasculature leaks between endothelial cells, the hydraulic pressure forces water out into the surrounding tissue space until the pressure meets the resistance of the interstitium and lymphatics. The oncotic pressure is created by the imbalance of protein concentration on either side of a semi permeable membrane. A membrane permeable to water, but not large proteins, will cause a pressure increase where the proteins are more concentrated as the water attempts to equalize the concentration. The oncotic pressure favors movement of water into the vasculature where the protein concentration is higher. According to Starling, when these forces are combined, the net force will cause water to filter out of the higher pressure capillaries while causing absorption back into the vasculature at lower capillary pressures.[17] The latter has been shown not to be the case (discussed below).[18]

Starling's research on fluid exchange began in 1892 and his conclusions were published in 1896. Using the isolated canine hind limb he demonstrated the forces governing the movement of plasma fluid in and out of the blood stream. Amazingly, he hypothesized that this movement was regulated by gaps in the intercellular space but in the same sentence admitted that there was no basis for this conclusion since at the time there was no evidence in support of this theory.[17] Even so, this was the prevailing dogma for about 100 years.

The familiar equation that exemplifies Starling's findings is as follows:

$$J_v/A = L_p (\Delta P - \Delta \pi)$$

J_v/A is the net filtration volume per area, L_p is the permeability coefficient for plasma fluid, ΔP is the difference in hydrostatic pressure and $\Delta \pi$ is the difference in oncotic pressures. The actual equation was not created by Starling himself. It was an evolutionary process that follows the history of understanding the nature of these forces.

Revised Starling Principle

I. Confirming Starling's findings

We know the variables in the Starling equation to be derived from the principles that Starling set forth, but the definitive measurements were not made possible until Landis, in 1927, confirmed Starling's findings.[3] Landis began his investigations on capillary permeability in 1925 by modifying a micro-injection apparatus involving a micro pipette of 4 to 8 micrometers in diameter, which allowed the direct measurement capillary pressures relative to lymph pressure.[19] These methods are still used in modern experiments for precise measurements of intracapillary pressures.[20] In 1948, Pappenhiemer showed the relationship between hydrostatic pressure and oncotic pressure of the microvessels using isogravimetric studies further bolstering Starling's hypothesis.[21]

II. Reflection coefficient- determining how, what and why solutes traverse the barrier

The simple Starling Equation mentioned above does not take into consideration the restrictive properties of the barrier to solutes. Meaning, the barrier will allow the passage of water at a certain rate and the same can be said about the passage of solutes, especially solutes determined to be of similar size to the inclusion area of the barrier. Pappenheimer's 1948 study considered the direct measurements of Landis' experiments and the idea that the barrier filtered solutes, thought at the time to be the space between the cells where tight junctions are found. From this, he discovered there were discrepancies in calculations of permeability and the actual measured values. This led Pappenheimer to develop both the Pore Theory, and, from Staverman's osmotic reflection coefficient, a mathematical model that described the permeability of solutes.[22,23] A solute with complete restriction would have a value of 1 (100% reflection) while a solute with no restriction would have a value of 0. A solute with 100% reflection at the barrier would exert its maximal oncotic pressure on that barrier.

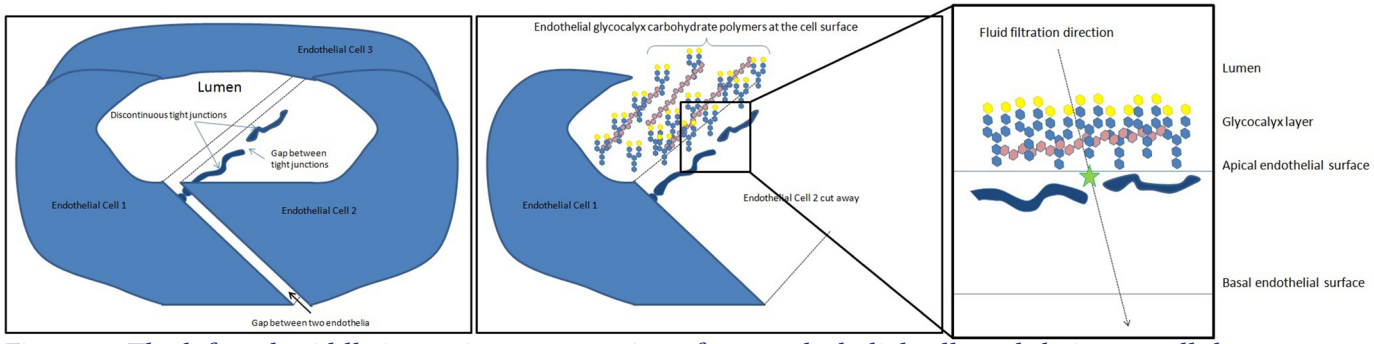

Figure 2: The left and middle image is a cross section of two endothelial cells and their paracellular space. The right image is a depiction of the protected space (green star) between the endothelial glycocalyx and the endothelial tight junctions. Figure reference: Adamson, R.H., *et al.*, Oncotic pressures opposing filtration across non-fenestrated rat microvessels. J Physiol, 2004. 557(Pt 3): p. 889-907.

Pappenheimer's calculations were based on a cylindrical pore of a certain length and radius. We now think of these pores (or simply the restrictive space through which solutes pass) as a two dimensional fence-like meshwork of the eGCX rather than a series of parallel channels so the natural behavior was in disagreement with mathematical predictions. Over the next decade, Curry, Michel and others called into question the model for the reflection coefficient based on pore theory. [24] This prefaced the realization that the relatively newly identified glycocalyx could have relevance to this puzzle.

III. Closer to implicating the eGCX

Up to this point, the glycocalyx had not been considered to be a regulator in the filtration of plasma fluids, despite speculation of this function in the late 1950s and early 1960s. Studying electron micrographs, Palade made his prediction based on the basal lamina and noticed a homogenate surface coating much like the basement membrane.[8,10] Most suspicion had been directed to the intercellular clefts between the cells because researchers recognized discontinuous tight junctions could have restrictive properties and thus considered it as the filtering barrier for fluid and solutes (discussed later).[24] In 1980, Michel published "A Fiber Matrix Model of Capillary Permeability." This shed the pore theory and suggested "the endocapillary layer is a three-dimensional network formed by the fibrous chains of the membrane glycoproteins of the endothelial cell coat reinforced by the absorption of plasma proteins."[4,25] This may be the hypothesis that changed the historical importance of the eGCX. This model takes into consideration a random array of fibers of specific diameter, length and density for a given area. These fibers are later shown to be hyaluronan as described in the introduction.[26]

Challenging the Starling Principle

I. Steady state filtration- 1st challenge to Starling Principle

From the fiber-matrix model, Michel and Phillips were able to develop the idea of steady-state fluid filtration in 1987. This theory suggests that when the Starling forces have balanced, there is always filtration. The only exceptions are in organs where reabsorption is a function of that organ- such as the kidney and gut.[5,18,27] This challenges Starling's finding that reabsorption occurs on the venous side of capillary beds due to the drop in hydrostatic pressure while the oncotic pressure remains the same. This situation results in a net force that favors absorption of fluid back into the vasculature.[3,17,21] The problem is that Starling, and others since, were measuring transient reabsorption as a result of changing experimental conditions. Michel and Phillips showed that when they dropped the arterial pressure, there was a temporary adjustment period during which they found absorption. But within a few minutes, the flow stopped and finally filtration resumed, even at the "new" lower capillary pressure while no change to plasma proteins had been made.[18]

II. Not the cleft – It's the eGCX

From Starling's writings it was assumed that the major factors regulating filtration were tight junctions in the gaps between endothelial cells.

Starling himself clearly stated this was an assumption.[17] Using Weinbaum's Junction-matrix model[28], Adamson and Michel[29] concluded experimentally that tight junctions account for 90% restriction in the continuous frog mesentery capillaries. This left 10% of the space between the cells unrestricted to larger proteins and water. Additionally, these junctions were determined to be 150 nm by 20 nm, much too large to restrict albumin of 7 nm in size (the major plasma protein that contributes to oncotic pressures). They found the restrictive properties of this 10% to contribute only slightly to rates of filtration and solute movement.[5,29] This means Starling's century old assumption has been found to have little influence on regulating filtration.

From this finding and Michel's fiber-matrix theory, Weinbaum proposed a 2-dimensional model in which there are distinct zones about the eGCX that vary in concentration gradients.[6] He suggested the steady-state filtration is due to washout of protein between the eGCX and tight junctions(Figure 2). Once on the tissue side, the back-diffusion of proteins is prevented by the funneled flow of fluid through breaks in the tight junctions. At steady hydrostatic pressures, a small amount of proteins filter through the semi permeable glycocalyx, but then are immediately washed out of this sub-glycocalyx space due to the flow of plasma funneled through the gaps between tight junctions. This sets up a relationship between fluid flux and protein flux. At any capillary hydrostatic pressure greater than interstitial pressure, washout will occur because protein permeation is slower than the flow of fluid in a steady state situation. An abrupt change in pressure or permeability could affect the flux of protein, but only temporarily until equilibrium between solute flux and washout is re-established.[6] Considering this, there might be some question as to the influence of hydrostatic pressures on fluid flux. In 1963, Guyton showed that interstitial pressures were largely negative averaging -2 cm H_2O.[30] Therefore, in the absence of pathology, the interstitium would always accept new fluid driven by capillary hydrostatic pressure down a pressure gradient.[30]

III. Tissue protein concentration not a factor-2nd Starling Principle challenge

Regarding the movement of macromolecules, based on Weinbaum's 1998 model and experimentally demonstrated by Adamson in 2004, protein concentration in the tissue, at physiological levels, doesn't affect the rate of filtration.[6,20] This is thought to be due to the washout of the protected space (Figure 2) beneath the eGCX described earlier. This space, where the concentration of protein is a function of hydrostatic flow, is said to uncouple the oncotic effect of interstitial concentration from the vessel lumen.[31] Therefore, the protein concentration in the interstitium has little influence on fluid flux.

Summary

In conclusion, the eGCX holds the key to understanding the forces that regulate plasma filtration and the fundamental principles of edema. It may be that studies on vascular permeability would be reconsidered if the GCX was neglected as an experimental condition. Further evidence suggests that the health of the glycocalyx could play a mechanical or signaling role in vascular permeability.[32] One hypothesis states that the breakdown of the glycocalyx is a first step in barrier disruption to miss-regulated fluid flux. Studies involved in leukocyte rolling and adhesion explain that the breakdown of the eGCX is important in the process of leukocyte migration through the vascular wall.[33] This shows that the eGCX has a role in this complicated process during a normal immune response. Annecke *et al.* have demonstrated that ischemia can degrade the eGCX in guinea pig coronary arteries.[34]

In terms of lung biology, perturbation of the eGCX, for example in high altitude pulmonary hypoxia, could lead to pathological pulmonary edema, hypertension and ultimately right heart failure.

Most textbooks dedicate a single paragraph to a description of the GCX as the carbohydrate rich coating on the cell surface. Fewer go on to describe the vesicular shuttling and synthesis of some GCX components, but never suggest this structure is physically and biochemically relevant to vascular function, essentially leaving the reader with its function unknown. In the same textbook, you would find a chapter on the Starling forces which explains the four factors affecting fluid filtration. What is lacking in

this description is that fluid filtration is always maintained in a steady state condition rather than reabsorbed at lower pressures, nor would you find that the interstitial protein concentration does not affect the filtration rate. These two major diversions from the original Starling Principle make enormous contributions in our understanding of edema, but as of yet they are not a part of mainstream education.

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