Selective Staining of Fibrous Connective Tissue Capsules and Lymphatics

An Evaluation of “Interstitial” Fluids

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Summary

Ringer’s solution containing ferrocyanide ion was infused into the arterial system of limbs. Some of these ions filtered across the blood capillary wall into the adjacent extracapillary fluids, from which diffusion caused these ions to enter the wall of the enclosing epimysial capsule. Ringer’s solution containing ferric ion was then injected into the parenchyma (not intravascularly). In muscles, the intensely blue ferric ferrocyanide (Prussian blue) precipitate appeared on the walls of the fibrous connective tissue capsules that enclosed each small cluster of muscle cells, and in the lymphatics of the extracapsular clefts. No Prussian blue appeared inside the capsules. These results indicate that “interstitial” fluids are divided into two discrete pools: (a) an intracapsular pool of capillary ultrafiltrates, and (b) an extracapsular pool in the trabecular clefts. Certain implications concerning the mixing of the tissue fluids, the estimate of capillary filtration rates and some of the functional differences between intravascular and intramuscular injections are discussed.

Collagenous fibers and their associated ground substances are nearly ubiquitous in animal tissues. Much of the collagen is arranged in sheets that enclose specific structures. For example, each muscle fiber is enclosed in a delicate endomysial sleeve of collagen. A larger fibrous connective tissue capsule (epimysium) encloses each cluster of approximately 100 muscle fibers (1). Similar connective tissue encapsulations organize glands, bundles of nerve fibers (2) and other tissues into modules (3). Larger and stronger fascial enclosures hold clusters of capsules together to form tissue compartments (4).

Three-dimensional fibrous sheaths appear as lines in tissue sections and their roles as enclosures are seldom noted. Despite their omnipresence, attention is usually focused on the identification
of parenchymal cell types, especially in the search for inflammatory or malignant cells, and the fibrous sheaths are generally dismissed as "background connective tissue". The increasing power of microscopes has further limited the attention given to the encapsulating function of collagenous sheaths. The fibrous enclosures in the greatly magnified sections in electron microscopy are seen only as incidental accumulations of collagen fibers. In these views, the body appears as archipelagoes of epithelial, muscular and other parenchymal cells in an almost totally ignored sea of "loose" fibrous connective tissues. Biochemists have intensively examined the connective tissues, but usually only of minced tissues in which geometric and functional relationships are destroyed.

Our studies on the local regulation of blood flow and fluid exchange have focused attention on the potential regulatory properties of the geometric and territorial relationships of the fibrous connective tissue capsules (5). Thus, each tissue module, or capillaron, is considered to consist of a cluster of approximately 100 parenchymal cells, the numerous blood capillaries that supply these cells, and the extracapillary fluids in which they are bathed, all enclosed in a compliant fibrous connective tissue capsule of limited permeability. This organizational arrangement can account on purely mechanical bases for a large number of vascular phenomena (6). Thus, the purely mechanical properties of the capillaron can be shown to (7) readily reproduce the hemodynamic patterns of critical closing, critical opening, basal flow, the autoregulations (arterial, venous, compression, autoregulatory escape, etc.), the hyperemias (post-occlusion, post-compression, exercise, post-exercise, post-autoregulatory escape, post-vasoconstriction, etc.) and many other phenomena of blood distribution and fluid exchange. Our analyses of these mechanical relationships of peripheral vascular phenomena have received relatively little attention, in part because the existence or functional integrity of the tissue capsules has been questioned.

To examine the potential role of the tissue capsules and to provide data that such capsules may contribute to the local regulation of blood flow and fluid exchange, we have developed a technique that differentially "stains" the fibrous connective tissues of the epimysial capsules and of the lymphatic vessels, without marking adjacent histologically similar connective tissues. The effect is produced by a two-phase technique that precipitates ferric ferrocyanide on membranes that separate the two discrete pools into which the two reactants are introduced.

The results also indicate a need to re-examine certain questions related to the nature of the interstitial fluid pool of the body, and of two general types of clinically evident edemas.

Methods and Rationale

Several methods for the demonstration of barrier sheaths including the intraparenchymal injection of pigmented plastic have been developed and tested (1, 4). Two-injection techniques have utilized the separate introduction of starch and of iodine. The most definitive technique was found to be the separate injection of ions which precipitates a characteristic blue-black pigment, Prussian blue. This technique and some results are described in the present report.

Ferrocyanide ion. The hind limbs of 14 dogs were injected in a two-stage procedure. Ringer's solution containing 1% to 4% potassium ferrocyanide, K₄Fe(CN)₆, was infused into the blood vascular system of isolated dog limbs.

Rationale: Ferrocyanide ion filters relatively freely out of the blood capillaries (8) and diffuses in the extracapillary tissue fluids contained inside the enclosing fibrous connective tissue capsules. The relatively impermeable fibrous capsular membrane impedes diffusion. Ultimately, some of the ion filters into and diffuses across the capsular wall, to enter the fluids in the extracapsular clefts.
**Ferric ion.** Ringer's solution containing 1% to 4% ferric chloride (Fe Cl₃) was then injected intraparenchymally (not intravascularly). The injection pressure required was only a few mm Hg, as in a clinical intramuscular injection.

Rationale: Except for the relatively few capsules which are impaled on the needle, intraparenchymally injected fluids are prevented from entering inside the capsular modules that make up each tissue (3). The ferric ions are therefore introduced primarily into the extracapsular (trabecular) clefts and fluids. Some of the ferric ion diffuses into the capsular wall.

**Precipitate.** The combination of ferric and ferrocyanide ions precipitates ferric ferrocyanide, Fe₄(Fe (CN)₆)₃, thereby marking the site with Prussian blue crystals:

\[
4 \text{Fe}^{+++} + 3 \text{Fe(CN)}_6^{-4} \rightarrow \text{Fe}_4(\text{Fe(CN)}_6)_3
\]

(Prussian blue)

This intense pigment is usually visible even without benefit of a microscope.

After fixation in 10% formalin, tissue specimens were sectioned at 6 microns. Since nuclear-fast red was used as a counter stain, the ferric-ferrocyanide precipitate was the only blue color in the sections.

**Results**

Gross inspection revealed localized regions marked by Prussian blue. In skeletal muscle, microscopic examination showed that the blue precipitate was present primarily on the walls of the fibrous connective tissue capsules that enclosed each cluster of muscle fibers (Fig. 1). This pattern marked the borders of each trabeculum with a double line, one line on each of the adjacent capsular walls. In some specimens, Prussian blue precipitate was also present on an inner capsular membrane between the fibers of the outermost layer of the muscle fibers in each cluster, immediately adjacent to the capsule (Fig. 2).

Prussian blue precipitate did not appear inside the area enclosed by the inner layer of the capsule. This indicated that the capsule consisted of a double sheet of fibrous connective tissue, and that a separate tiny intermediate pool separated the inner and outer layers of the capsule (Fig. 3). Blue precipitate was also observed inside some capsules along lines that extended from the outer capsule of the midportion of the capsule; some of these resembled a capsular hilum.

In the extracapsular clefts, Prussian blue pigment was present along fine fibers and in valved endothelized vessels that resembled terminal lymphatic capillaries (rootlets).

Similar patterns of distribution of Prussian blue were seen in the capsular walls and in the extracapsular lymphatic vessels in heart muscle.

**Discussion**

The results show that the two-phase injection process produces precipitation at highly specific sites.

Intravascular perfusion filters ferrocyanide ion across blood capillary membranes into the intracapsular fluid pool. This intracapsular pool bathes the parenchymal cells enclosed inside each fibrous capsule. Some of the ferrocyanide ion filters through the layers of the capsular wall and diffuses toward the extracapsular clefts. Injection of ferric ion into the parenchyma introduces this ion directly into the extracapsular (trabecular) clefts of the tissue. Precipitation occurs primarily at the capsular boundaries between the intracapsular pool and the extracapsular pool. This boundary appears to be due to a double layer of fibrous connective tissues, with a small intermediate pool between the two layers. The absence of precipitation inside the capsules...
Fig. 1. Dog skeletal muscle with Prussian blue reaction (black lines). A typical capsule containing muscle fibers is in the center of the slide. Prussian blue is precipitated primarily on the walls of the capsules. In the major cleft at left below, a dark line is in the usual site of lymphatic vessels. Occasional black lines extend into the capsule (hilum?). X50. 80 B Filter, Panatomic X (FX 135-20).

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demonstrates that the capsular apparatus effectively separates the intracapsular fluid from the extracapsular fluid pool.

The demonstration of the fibrous capsule indicates that capsular compliance and permeability may affect intracapsular pressure, and through this, the transmural capillary pressure. This transmural pressure appears to be a factor that may determine the caliber and conductance of the flexible, permeable blood capillaries, and thereby account for some of the vascular phenomena noted above.
Sepa"ration of the "interstitial" fluids into two discrete pools by the capsular barrier indicates that analysis of blood capillary filtration can be achieved only by simultaneous measurements of intravascular and intracapsular fluids. No satisfactory means for direct sampling of the intra-
capsular fluids has yet been developed.

In related studies in which liquid plastic has been injected intraparenchymally, the hardened plastic was found to have entered only the extracapsular clefts and the excess drained out of the lymphatic vessels of the tissue (1).

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Workers who consider that the fluid in the lymphatic vessels is a sample of the transcapillary filtrate, use the lymph/plasma concentration ratio (9) as an index of the surface area and the permeability of the blood capillary. However, the demonstration of a capsular barrier between the intracapsular pool and the extracapsular pool indicates that the surface area of the fibrous capsule and its permeability must also be taken into account in such analyses. This is necessary because the lymph vessels, being excluded by the capsule from the intracapsular pools, appear to be the specific drainage system for the fluids of the extracapsular clefts (3). Estimates based on a comparison of the concentrations of markers in the blood vascular system and in the lymphatic vessels must therefore evaluate the effects of at least three sets of membranes — the wall of the blood capillary, the walls of the capsule, and the wall of the lymphatic capillary. The membrane with the least product of surface area, permeability, and transmural pressure, will be the prime determinant of the concentration of a marker in the lymph. The very large number of blood capillaries in each capsule offers an enormous capillary surface area, probably much greater than the surface area of the capsule. The blood capillaries are also known to be very permeable to small particles such as ferrocyanide (8). The lymphatic rootlet is high permeable since it accepts even particulates such as air bubbles, mercury, liquid plastic, etc. The permeability of the smaller surface are and possibly the lesser permeability of the capsule may therefore determine the nature of the materials that pass from blood to lymph. This capsular membrane separates the interstitial fluid into two discrete pools. Data which previously have been utilized as indices of capillary permeability may therefore require re-evaluation in terms of the permeability of the fibrous capsule.

The capsular separation of the interstitial fluids into an intracapsular pool and an extracapsular pool also suggests that local and general fluid accumulations and of the edemas may require re-evaluation. Studies of albumin metabolism are strongly suggestive of the presence of two extravascular pools — one which is rapidly and one which is slowly exchangeable with the intravascular space (10).

The present study demonstrates a method for the production of a precipitation reaction that demarcates epimysial connective tissue capsules from adjacent connective tissues. The results illustrate the functional integrity of the epimysial capsule which, in addition to other territorial functions, separates the interstitial fluids into discrete intracapsular and extracapsular pools. The results are consistent with the belief that specific fibrous connective tissues which have received little previous physiological attention may play an important role in fluid exchange and distribution. Through this means, it may affect local regulation of blood flow and fluid exchange and account for a variety of vascular phenomena.

The present results may also provide insights into the mode of uptake of drugs into the vascular system. Compounds given intravascularly are immediately distributed throughout the body. Compounds injected intramuscularly would appear on the basis of the present data to be deposited in the extracapsular trabeculae where they may remain until taken up into the lymphatic drainage system. Only after the injected material passes through the collecting trunks does it enter the blood stream and become available for general distribution. This suggests that the uptake of intramuscularly injected materials may be affected primarily by factors which control lymphatic flow.

References
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Impeded Interstitial Fluid Movement: A Factor in Pancreatic Oedema

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Summary

Pancreatic oedema was induced by physiological saline infused into the superior pancreatico-duodenal artery of 34, chloralose anaesthetized dogs. Both lymph flow from cannulated pancreatico-duodenal lymphatics and intralymphatic pressure in the non-transected ones increased significantly. The increase in pressure may be due to the regional lymph nodes obstructing increased lymph flow. The development of gross pancreatic oedema preceded the peak values of pancreatico-duodenal lymph flow and pressure. This suggested impeded fluid movement along tissue interstices and from tissue interstices into the pancreatic lymphatics. The progression of the oedema ran roughly parallel with the increase in fluid pressure measured by a perforated capsule implanted two weeks earlier into pancreatic tissues supplied by the artery.

The results suggest that both the rise in lymph flow and pressure during the development of oedema in lobular organs like the pancreas are rather the consequences and not the causes of oedema.

Introduction

As established by Rusznjak and his coworkers (1), oedema develops, i.e. fluid accumulates in the tissue interstices, when the lymphatic system is unable to transport from the interstitial space the fluid not reabsorbed by the blood capillaries. This concept failed to consider that in lobular organs like the pancreas, the fluid filtrated through capillary endothelium has first to pass fibrous connective tissue capsules, then the extracapsular interlobular connective tissue to reach the lymphatics (2, 3).

Theoretically, if the rate of capillary filtrate formation exceeds the migration rate of interstitial fluid, fluid will necessarily be retained in the interstitial space before entering the lymphatics. This may be called praelymphatic insufficiency of interstitial fluid movement. Oedema due to praelymphatic insufficiency of interstitial fluid movement appears without increase in lymph flow, as in bile-induced acute experimental pancreatitis (4). Another sign of impeded interstitial fluid movement is that oedema may develop earlier than lymph flow reaches its peak. Such impeded fluid movement was demonstrated after the release of experimental acute cardiac tamponade (5) and in pancreatic oedema induced by increasing capillary filtration (6).