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PREPARATION OF ISOLATED BLOOD CAPILLARIES

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Abstract

Blood capillaries have been isolated from various tissue sources yielding suspensions of capillary segments. These have provided opportunities to study the cellular properties of capillary endothelium under conditions uncomplicated by the presence of stromal tissues and in which measured parameters can be attributed to endothelial cells. Fresh capillary isolates have been used directly as experimental systems but the yield of endothelium is quite low. Amplification of endothelial biomass has been accomplished by using freshly isolated capillaries as explants for primary tissue culture. It has not been previously possible, however, to obtain large amounts of capillary endothelium from a single preparation nor have different capillary types been isolated from the same tissue. The rete mirabile of the eel swim bladder is a copious source of capillaries of two types: thick-walled, continuous capillaries heavily invested with pericytes and thin-walled, fenestrated capillaries. These can be isolated in large numbers free of large blood vessels and contaminating stromal tissue. The two types of capillaries can be isolated from each other by perfusing magnetic beads into one type prior to isolation and separating them from the other type in a magnetic field. This provides a system in which the cellular properties of the two types of endothelium can be studied in vitro and, due to a common isolation procedure, direct comparisons can be made.

KEY WORDS Capillary, Endothelium, Isolation, Pericytes, Fenestrae, Junction, Venous, Arterial, Magnetic Beads, Rete Mirabile, Eel.

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Introduction

Capillary endothelium has come to be appreciated not only as the site of regulation of blood-tissue exchanges but also as performing many vital metabolic and regulatory functions. It has been difficult to study experimentally these properties in capillary networks in situ since tissue cells and other vessel types intrude upon endothelial-specific data. In order to obtain more precise information on endothelial function, capillaries have been isolated from several tissue sources of many animal species. The capillary isolates have been used as experimental systems in vitro either in the freshly-isolated condition or as sources of primary culture of capillary endothelial cells.

The usual protocol for isolating capillaries begins with excision of microvascularized tissue in such a manner that excludes the majority of larger vessel types present. The tissue is then subjected to slicing into smaller fragments, enzymic digestion with collagenase and/or mincing to dissociate tissue cells and connective tissue. This results in microvessel fragments of various lengths in suspension with free tissue cells and blood cells. Separation of capillaries from other cell types is accomplished by filtering or density gradient centrifugation.

The biomass yield of capillary endothelium is generally quite low due to the diffuse nature of most capillary systems and pooling of several isolates is usually necessary in order to obtain a sufficient number of cells for metabolic and biochemical studies. Using fresh capillary explants for primary tissue culture of capillary endothelial cells is yet another way of obtaining large quantities of cells. In most cases, however, fibroblasts, vascular smooth muscle from larger vessels, pericytes and blood elements constitute contaminating cell types in fresh isolates. Specific endothelial growth factors, inhibitors for growth of contaminating cell types or actual cloning is necessary to assure a pure population of capillary endothelial cells.

Isolation of Capillaries from Adipose Tissue

Adipose tissue is irrigated by a profuse system of continuous capillaries and has certain

inherent advantages as a source for capillary isolation. The rat epididymal fat pad provides a copious source of microvascularized fat and has been used to obtain capillary isolates (Wagner et al., 1972; Wagner and Matthews, 1975). Fat pads are excised from male rats, pieces of exclusively microvascularized fat are dissected from the pads and subjected to dissociation by collagenase. A vascular-stromal pellet is obtained by low speed centrifugation and the buoyant adipocytes float to the top and are discarded. A pure preparation of capillaries can be obtained by centrifugation of a Percol gradient and erythrocytes, stromal cells and larger vessels are pelleted (Williams et al., 1981). Isolated adipose capillaries have been used in the fresh condition to study endothelial transport of various tracers (Robinson and Wagner, 1984; Wagner et al., 1977, 1983; Williams et al., 1981, 1984). Capillaries can also be isolated from a variety of other fat tissues and have been utilized extensively as a source for cultures of capillary endothelium (Williams, 1987, a review).

Isolation of Heart Muscle Microvessels

Viable endothelial cells of microvascular origin have been obtained from heart muscle (Simionescu and Simionescu, 1978). This procedure involves removal of large superficial vessels of the heart ventricles, mincing the cardiac muscle to disrupt muscle cells, collagenization and homogenization of muscle fragments and separation of capillaries by velocity sedimentation through a serum albumin cushion. The final preparation contains 85-90% endothelial cells in small capillary segments consisting of 2-3 endothelial cells. The total yield of endothelial cells from one preparation is $1.0\text{-}1.5 \times 10^7$ as determined by DNA content.

Isolation of Capillaries from the Central Nervous System

Several laboratories have developed methods for the isolation of capillaries from brain (Joo and Karnushina, 1973; Orlowska et al., 1974; Brendel et al., 1974; Goldstein et al., 1975; Lai et al., 1975; Williams et al., 1980). Capillaries have also been isolated from the retina (Meezan et al., 1974) and spinal cord (Joo et al., 1982). These procedures generally involve excision and mechanical disruption of nervous tissue, separation of microvessels on a fine mesh of nylon and capturing of capillaries on glass beads which removes cellular debris. The preparations contain enzymic markers for central nervous system endothelial cells, are metabolically active and exhibit characteristic transport properties. They have thus provided useful systems for studying the transport and metabolic functions of the blood-brain barrier. Cultures of endothelial cells from the retina (Betz et al., 1983) and brain (Bowman et al., 1981; Robinson et al., 1986) have provided enhanced biomass which is more convenient for the study of their basic endothelial properties. Algers et al. (1986) have shown, however, that brain microvessels isolated by several current techniques contain pericytes, basal membrane, astrocytic end feet, neuronal nuclei and blood cell elements.

Capillary Isolation from Other Tissues

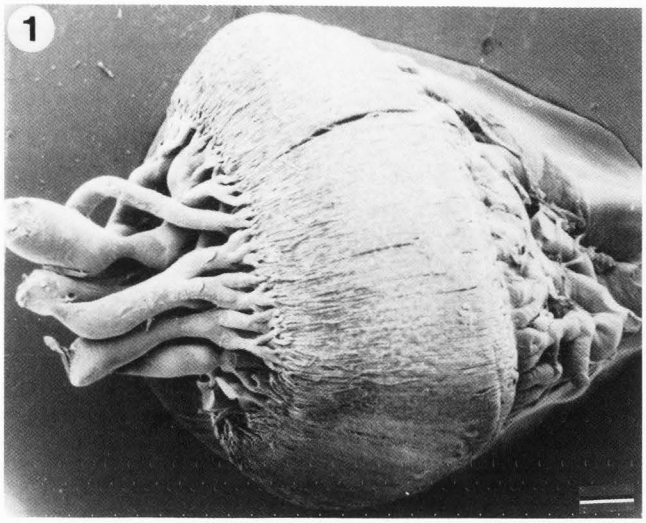
Capillaries have also been isolated from the dermis (Davison et al., 1983), foreskin (Sherer et al., 1980; Davison et al., 1980), adrenal medulla (Banerjee et al., 1985), decidual tissue (Johannison and Redar, 1984) and thyroid gland (Johannison and Bjorkman, 1983). A rather unique approach has been used to isolate microvascular endothelial cells from the lung (Ryan et al., 1982). The lung is perfused via the pulmonary arterial system with microcarrier beads. These lodge in the capillaries and in the presence of EDTA, the endothelial cells will detach from the capillary walls and attach to the surface of the beads. Beads laden with endothelial cells are then recovered retrogradely from the pulmonary arterial circulation. These can be used to initiate a primary culture of pulmonary capillary endothelium since the cells will migrate off the beads onto the surface of a culture dish.

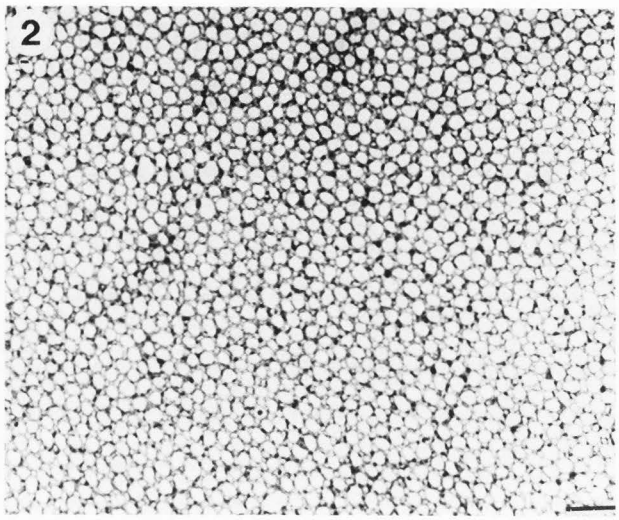
Limitations of Capillary Preparations

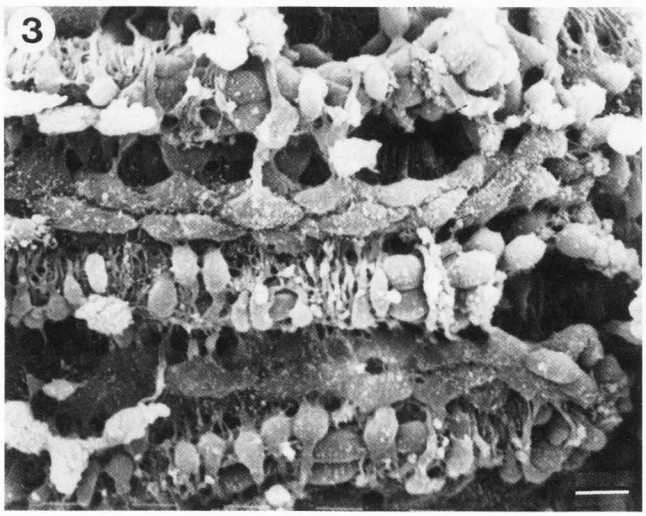
Capillary isolates have provided opportunities to study the cellular properties under conditions uncomplicated by stromal tissues. Many of these preparations, however, may be less than ideal since they may contain various cellular and noncellular contaminants and even other vessel types (arterioles and venules). Preparations containing a variety of vessel types might better be termed "microvessel isolates." It has also not been possible to isolate different morphological types of capillaries from the same tissue. The yield of capillary endothelium in many cases is quite low. Amplification of biomass can be accomplished by the growth of fresh capillary explants in culture. However, cultured endothelial cells are the progeny of those present in tissues, have a very uncharacteristic high mitotic index and may exhibit phenotypic drift. They man not, therefore, accurately reflect capillary endothelial function *in vivo*.

Capillaries of the Rete Mirabile

The retia mirabilia (red bodies) of the eel swimbladder are capillary portal systems arranged in discrete organs which function in countercurrent exchange of solutes and gases (Fig. 1). They contain close-packed arrays (Fig. 2) of straight, unbranched capillary segments which can be up to 0.5 cm long in larger eels. The capillaries are of two different morphological and functional types (Bendayan et al., 1974): Thick walled continuous "arterial capillaries" arise from the prerete artery and converge again to form the postrete artery. These are heavily invested with pericytes (Figs. 3 and 4). Arterial capillaries course parallel to thin-walled "venous capillaries" which arise from the prerete vein and converge again upon the postrete vein. They are devoid of pericytes (Figs. 3 and 4).







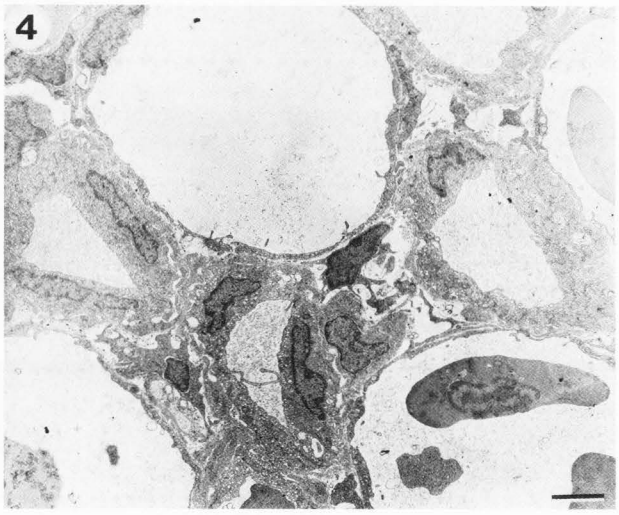


Figure 1. Scanning electron micrograph of the entire vasculature of the rete mirabile. The capillary bed is sharply demarcated from the larger vessels forming the poles of the organ. Bar = 500 μm

Figure 2. Light micrograph of a stained thick plastic section taken transversely through the capillary bed of the rete. The capillaries are closely packed with minimum interstitium and no stromal tissue. Bar = 100 μm

Figure 3. Scanning electron micrograph of capillaries in a rete which had been partially digested with collagenase, fixed, cleaned with 60°C, 8 N HCl and critical point dried. Arterial capillary segments are heavily invested with pericytes while venous capillary segments are devoid of pericytes and exhibit naked endothelial cells. Bar = 28 μm

Figure 4. Transmission electron micrograph of a thin section taken transversely through the capillary bed. Venous capillary segments are thin walled and fenestrated with large lumenae. Arterial capillary segments are thick walled, filled with vesicular profiles and have smaller lumenae. Bar = 2 μm

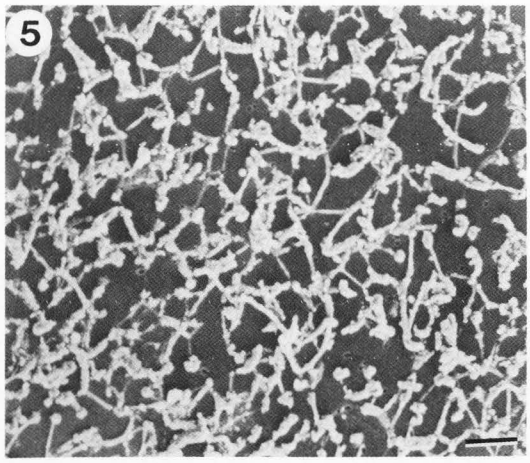


Figure 5. Scanning electron micrograph of a combined capillary isolate which had been captured on a nucleopore filter and critical point dried. Bar = 100 μm

The rete mirabile thus consists of two separate circulations in which blood flows in counterparallel directions in two distinct capillary types. Each rete (two per eel) contains about 10^5 capillaries of each type and is thus a copious source of capillary endothelium. It is difficult to imagine a higher concentration of capillary endothelium per unit volume (Fig. 2) of any other tissue. If the organ is exsanguinated by perfusion and the poles of the rete containing larger blood vessels are sliced off (Fig. 1), the remainder consists exclusively of capillary endothelial cells and pericytes.

Isolation of Capillaries from the Rete Mirabile

The high concentration of capillaries present in the rete mirabile and the nearly total absence of stromal tissue make it an excellent source for isolation of capillaries (Rasio, 1975). An efficient method for isolation has been devised (Froehlich et al., 1988). American eels (Anguilla rostrata) were anesthetized with MS-222, cannulated at the prerete artery and perfused with Kreb's ringer buffer (pH 7.4) containing 4% BSA and 0.9% glucose with outflow from the cannulated postrete vein. This exsanguinates the organ through a unilateral circuit coursing through both capillary types plus the swimbladder circulation. A solution of collagenase (type CLS, 6.0 mg/ml) and BSA (1.0 mg/ml) is then injected with a 30 gauge needle directly into the center of the rete and parallel to the capillary bed. This expands the interstitial spaces hydrostatically and administers collagenase directly into areas needed to dissociate the capillaries. The rete is then excised from the eel, connective tissue is trimmed from the surface of the organ and the poles are sliced off removing larger vessels from the preparation. The remaining capillary bed is then sliced into quadrants parallel to the long axis of the capillaries and incubated for 30-40 min. in collagenase at 37°C with gentle agitation. Gentle aspiration with a wide bore pipette (4 mm dia.) results in a homogeneous suspension of capillaries (Figs. 5 and 6). The isolate is then pelleted (100 x G, 3 min.) and washed once with Kreb's buffer resulting in a combined isolate of capillary segments of both types (Fig. 6). A combined isolate contains 110 μg DNA (assay of Labarca and Paigen, 1980) which is equivalent to 4×10^7 cells (Hindgardner, 1968).

The venous segments are devoid of pericytes and the walls are composed of long slender endothelial cells (Figs. 6 and 7). The arterial segments have thick, short endothelial cells and are invested with pericytes (Figs. 6 and 8). The isolates appear remarkably similar to their condition in the intact organ. The endothelial cell membranes are intact as evidenced by the exclusion of tannic acid and all but the broken ends of the capillaries exclude trypan blue attesting to their viability. They remain osmotically active since they swell in hypotonic media and shrink in hypertonic media. Initial attempts at culturing the endothelial cells from capillary explants have also proved successful.

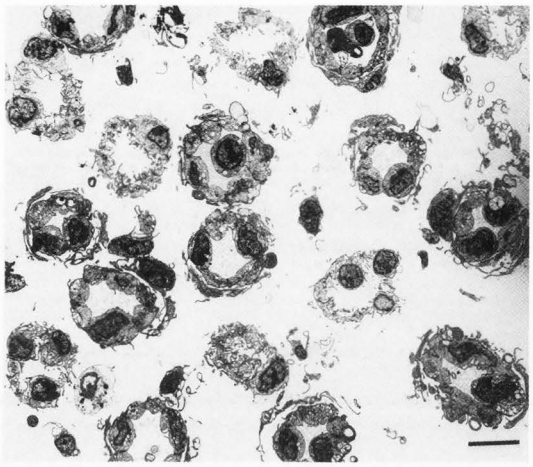


Figure 6. Transmission electron micrograph of a section through a combined capillary isolate. Arterial capillaries have thick endothelial cells and pericytes adhering to their abluminal surfaces. Venous segments are thin walled and are devoid of pericytes. Bar = 4.4 μm

Separation of Arterial and Venous Capillary Segments

The two separate circulations in the rete, each flowing through different capillary types, means that each circulation and associated capillaries can be perfused independently. This situation is advantageous for the separation of one capillary type from the other. Upon clearance of blood from the organ by perfusion through a unilateral circuit, an additional circuit can be established through the postrete vein with outflow at both caudal vessels. This isolates the venous from the arterial circuit (Fig. 9). Paramagnetic beads (Duke Sci. Avg. Dia. 1.0 μm) are then introduced into either the arterial or venous circulations depending upon which type of capillary is to be isolated. The beads fill one capillary type, turning the rete a light brown color, and ligatures are applied to both poles of the rete to prevent efflux of the beads. The rete is then excised from the surface of the swimbladder and the poles sliced off (Fig. 9).

Isolation of the entire capillary bed is accomplished as before and the combined isolate is washed, pelleted (100 x G, 3 min.) and resuspended in Kreb's ringer. The suspension is then placed in a short segment of glass tubing (5 mm I.D.) surrounded by circular permanent magnets (Edmund Scientific). The capillaries containing the magnetic beads are drawn to the sides of the glass tubing and capillaries without beads are collected in the effluent (Fig. 10). Additional refluxes through the tube concentrate a particular capillary type. These can be recovered by removing the magnets and aspirating the sides of the tube (Fig. 10).

The capillaries containing the magnetic beads are highly pure with regard to type since they are the only ones containing the beads (Figs. 11 and 12). Capillaries in the effluent exhibit some cross contamination due to extravasation of beads from some of the capillaries.

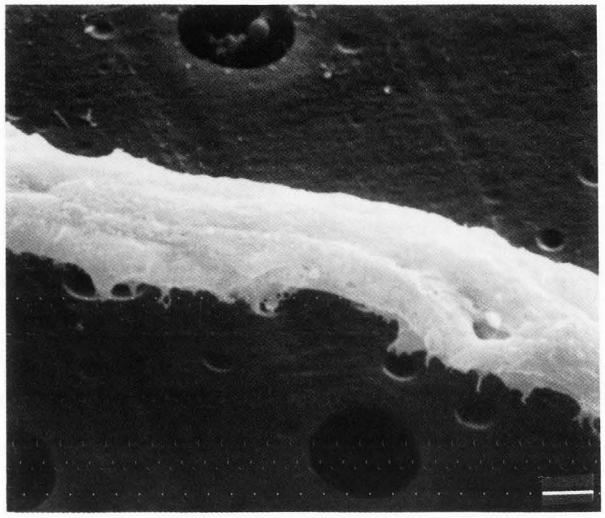


Figure 7. Scanning electron micrograph of an isolated venous capillary. The vessel walls are comprised of slender endothelial cells. Bar = 5.1 μm

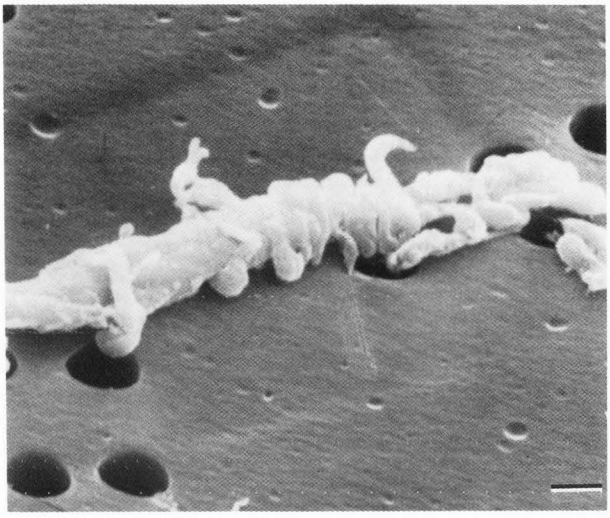
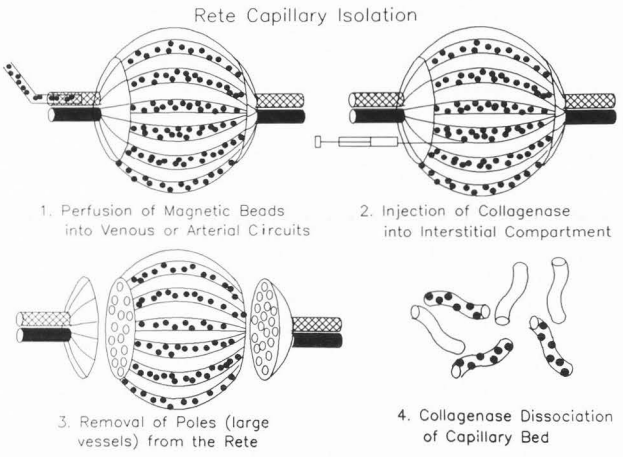


Figure 8. Scanning electron micrograph of an isolated arterial capillary. Pericytes remain adhered to the outer endothelial surface. Bar = 7.5 μm



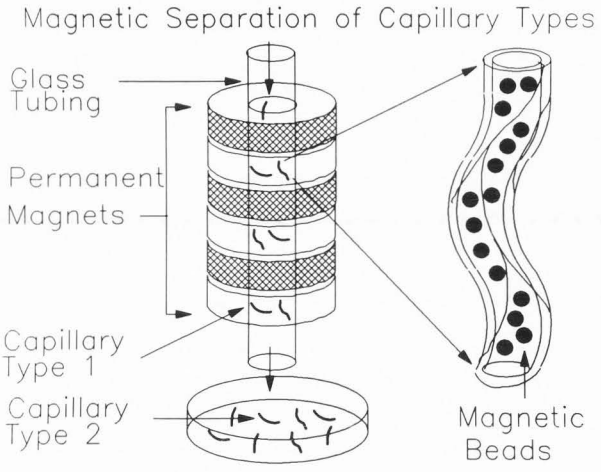


Figure 9. Schematic representation of the isolation procedure for rete capillaries. 1. Magnetic beads are perfused into either arterial or venous circulation. 2. Collagenase is injected into the interstitial compartment which expands it hydrostatically and separates individual capillaries from each other. This also administers collagenase to areas necessary for digestion of connective tissue. 3. The poles of the rete are sliced off removing large blood vessels and leaving a drum-shaped piece of tissue consisting exclusively of capillaries. 4. Incubation in collagenase results in dissociation of the capillary bed and results in a suspension of both capillary types.

Figure 10. Magnetic separator for purification of capillary types. A combined capillary isolate with one type containing magnetic beads is passed through glass tubing (10 cm long, I.D. 0.5 cm) surrounded by circular permanent magnets. Capillaries containing the beads adhere to the sides of the tubing and beadless capillaries are collected in the effluent. Bead-containing capillaries are recovered by removing the magnets and rinsing the sides of the tubing into a separate container.

This is especially true when the beads are infused into the venous segments since they are thin-walled and appear more fragile probably due to the absence of pericytes.

Counts of bead-containing and bead-free capillaries reveal the degree of enrichment of both types by magnetic separation. When beads are perfused into the arterial segments, the capillaries separated magnetically are 91% pure arterial segments (Fig. 12). However, when beads are placed into venous segments, the isolate contains only 60% pure venous segments.

The rete mirabile thus provides an ideal source for the isolation of capillaries since it is comprised almost entirely of capillaries with minimal contaminating tissue cells. This circumvents the problems of vessel and cell purity in other preparations and also provides for an initial high cellular biomass in the fresh isolate. Separate circulations through two different capillary types also make it possible to obtain highly enriched preparations of continuous or fenestrated capillaries. Such separations will make it possible to investigate the cellular and biochemical properties of these different capillary types.

Acknowledgement

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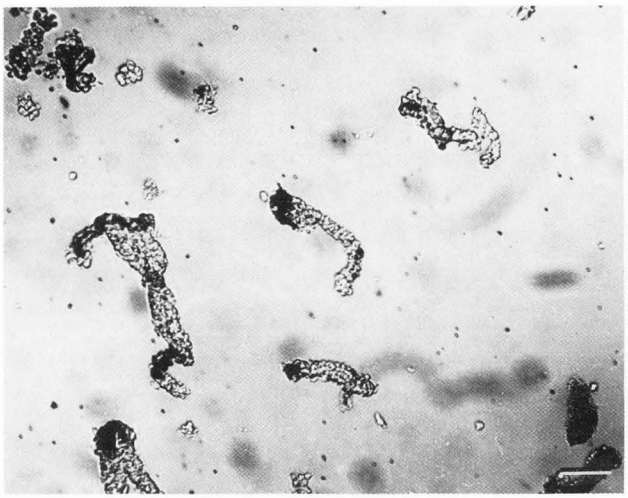

Figure 11. Arterial capillary segments which were separated from venous segments due to the presence of magnetic beads. Bar = 50.1 μm

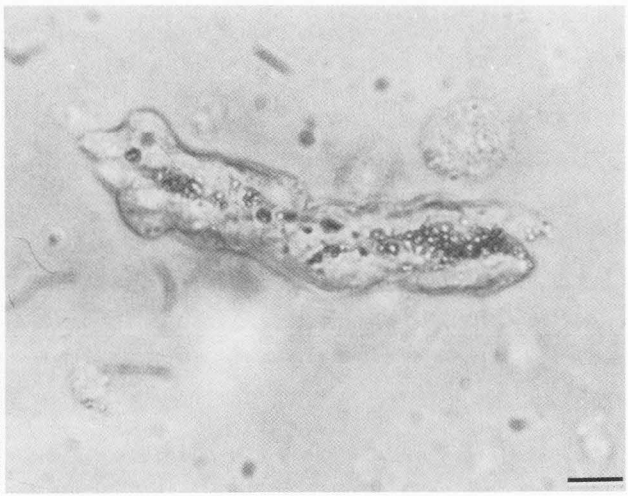

Figure 12. High magnification brightfield micrograph of an arterial capillary segment containing 1 μm magnetic beads in its lumen. Bar = 8.3 μm

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Discussion with Reviewers

Reviewer I: Figure 6 clearly shows that there are pericytes and blood cells associated with the isolated capillary fragments. Can single cell suspensions be prepared to determine the percent of cell mass which are endothelial cells as well as pericytes, blood cells or other cells?

Authors: There are no blood cells associated with this capillary preparation since the organ was completely exsanguinated prior to the isolation procedure. The single profiles seen are probably detached pericytes. The capillary segments are very resistant to dissociation since they are bound together with tight junctions. It would be very difficult to determine the proportions of fenestrated and continuous endothelial cells and pericytes based on random sections. We estimate, however, that pericytes may constitute up to 30-40% of the total cell population.

S. K. Williams: Is there any significance to the large number of pericytes associated with the arterial capillaries in the rete mirabile isolates?

Authors: Pericyte function in general has been a matter of several conjectures including regulation of capillary tone and/or pressure, regulation of permeability by opening and closing junctions and inhibition of endothelial mitosis and angiogenesis. Rete pericytes may serve one, all or none of these functions. We do not yet know their functions but their sheer number suggests an important role in the physiology of the rete.

Reviewer II: In the portion describing the vascular architecture of the rete, why not always talk about continuous and fenestrated capillary beds (as in the final paragraph) rather than arterial and venous?

Authors: Arterial and venous are used to designated the two types of capillaries primarily because reference must be made periodically to the specific circulations into which the beads were perfused.

Reviewer I: Is cell viability compromised by isolation of the capillary fragments and fractionation into two populations using paramagnetic beads?

Authors: The endothelial cell plasma membranes are intact since they exclude tannic acid. All but the broken ends of the capillaries exclude trypan blue which attests to their viability. They remain osmotically active since they swell in hypotonic media and shrink in hypertonic media. Initial attempts at culturing the endothelial cells from capillary explants have also proved successful.

Reviewer I: Do the paramagnetic beads remain in the capillary segments or do they come out spontaneously? If they remain in the fragment, may they not affect the physiological activity of the cells? If they do come out, what is the time required for them to do so and do all the beads come out?

Authors: As stated in the text, after isolation some of the beads definitely extravasate from the capillary segments which is the primary reason why the capillaries without beads in the first effluent are not pure with regard to vessel type. However, a good portion remain in the capillary lumenae and this results in a very pure magnetic isolate. Unless the beads are treated prior to use (for instance made anionic or cationic) they remain chemically inert and are unlikely to have adverse affects on the behavior of the endothelial cells.

Reviewer I: In Figures 11 and 12, how can one be certain that these are arterial capillary segments? A transmission EM should be included to confirm this statement.

Authors: A section for transmission EM such as Figure 6 in which all vessels are seen in cross section must be appreciated as a very fortuitous circumstance in which all the isolated vessels came to lie roughly parallel to each other. A similar preparation for the purified types could not be obtained. Oblique or grazing sections through the vessel types would in most instances not allow positive identification of them as one type of vessel or another. The primary reason for asserting that the vessels in Figures 11 and 12 are arterial segments is that they contain beads and those beads were originally only perfused into the arterial circulation. It would seem somewhat absurd to imagine that they had extravasated from one capillary type and migrated to the other type. The vessels in Figures 11 and 12 also have very thick walls characteristic of the arterial type.

S. K. Williams: What is the physiologic importance of capillaries in the eel rete mirabile? Do they exhibit functions similar to endothelial cells from other species such as prostaglandin production, angiotensin converting enzyme and factor VIII related antigen?

Authors: The primary function of the rete capillaries is in countercurrent exchange of gases, primarily oxygen. However, they have been shown to be able to transport solutes of various molecule sizes (Rasio, 1975) and transport lactic acid in the eel in order to sustain a partial pressure of oxygen across the capillary walls. We do not yet know whether they share the other biochemical functions exhibited by mammalian capillaries.

Reviewer II: Do you have any high magnification TEM micrographs of the pure isolate that is shown in Figures 7 and 8? This would allow one to unequivocally identify the vascular segments since the SEM micrographs shown do not allow identification of characteristic mural structures (i.e., diaphragmed fenestrae in the venous capillaries).

Authors: The isolated venous segments do retain their fenestrae, however, in a somewhat collapsed condition, they are not easy to find. The arterial segments are easily identified by their associated pericytes which can be seen with SEM. The reviewer is referred to Wagner et al., Microvascular Res. 34, 349-362 (1987) for a complete anatomical description of the ultrastructure of the rete mirabile.

S. K. Williams: The authors state the yield of capillary endothelial cells or "biomass" has been quite low in the past. However, numerous authors have reported the isolation of microvessels in large quantities from numerous microvascularized tissues. The yield of endothelial cells from a gm of tissue is at least an order of magnitude greater than the number of endothelial cells available from a cm length of a large blood vessel. What is known about the true yield (i.e., cells/gm of tissue) for the different tissues described?

Authors: Yield was used only to compare the rete with more diffuse capillary beds in that sense would refer only to endothelium per volume of tissue. In previous papers which have reported yield, this has been done on the bases of endothelial cell number derived from an analysis of DNA and the amount of DNA per endothelial cell. Protein content would seem to be a rather poor index of yield since it would also reflect extracellular material. Yield can also be improved by using more tissue which accomplishes the same thing as pooling of isolates.