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Laboratory Investigation

Isolation and characterization of microvessels from normal brain and brain tumors

Daniel L. Silbergeld[1] and Francis Ali-Osman[1,2]
[1] *Department of Neurological Surgery, Northwest Neuro-Oncology Research Laboratory, University of Washington Medical Center, RI-20, 1959 N.E. Pacific Street, Seattle, Washington 98195, USA;*
[2] *Department of Experimental Pediatrics, Section of Experimental Therapeutics, University of Texas, MD Anderson Cancer Center, 1515 Holcombe Avenue, Houston, Texas 77030, USA*

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Abstract

We describe a new technique for isolating microvessels from both brain and brain tumors. This method is relatively quick and provides a microvessel preparation free of contamination by other brain tissue. Using this method, structurally intact microvessels from normal rat brain and from a malignant rat astrocytoma were isolated and characterized with light microscopy, scanning electron microrscopy and transmission electron microscopy. In contrast to microvessels derived from normal rat brain, rat astrocytoma microvessels had endothelial cells with multilayered basement membranes, extensive microvilli on the cell surfaces, and a significant increase in the number of pinocytes in the cytoplasm. Furthermore, astrocytoma microvessel endothelial cells had pleomorphic electron dense nuclei with pale perichromatin, whereas the nuclei of endothelial cells of microvessels derived from normal brain tissue were finely granular and homogeneous with characteristically electron dense perichromatin. The morphologic characteristics of the astrocytoma microvessels are similar to the histologic changes seen in astrocytoma tissue in situ, and correlate well with the known altered functions of brain tumor neovasculature.

Introduction

Highly purified preparations of brain microvessels (MV) provide an important *in vitro* tool for the study of the blood-brain-barrier (BBB), for investigating cerebral endothelial cell biology [1–3], and for studying microvascular changes associated with malignant astrocytomas. Consequently, much effort has been directed at developing efficient methods for preparing MV from normal brain tissue [2–10]. While some of these methods yield MV preparations of good quality, others are less efficient and require relatively long periods of time. Furthermore, though suitable for isolating MV from normal brain, these techniques are generally inefficient for preparing MV from brain tumors, and thus are not useful for studies such as those related to the capillary permeability in brain tumors, tumor endothelial cell biology, and tumor angiogenesis.

The main objective of this study, therefore, was to develop and optimize a method for preparing highly purified MV that is applicable to both normal brain tissue and brain tumor tissue, and to utilize this technique to isolate and characterize MV from both normal brain and brain tumors. Using both whole rat brain and a transplanted rat anaplastic astrocytoma, we developed a MV preparation method that is relatively quick, and yields structurally intact MV with very little contamination by normal brain or brain tumor tissue components. The technique presented is unique not only

because of its applicability to both normal brain and brain tumor, but also, because compared to previously described methods, it is less labor-intensive, and does not require highly specialized instrumentation or skills. Using this method, we compared structural and ultrastructural characteristics of MV derived from normal brain and brain tumors. The differences observed correlate well with histologic alterations seen in the neovasculature of malignant astrocytomas in situ.

Materials and methods

Animals

Twelve-week old female Fisher 344 rats were sacrificed with ether and the whole brains, without meningens, were removed aseptically. A second set of rats was implanted intracranially with an N-ethyl-N-nitrosourea-induced rat glioblastoma cell line, R175A [11]. Briefly, the animals were anesthetized with ether, and using sterile techniques, a small burr hole was drilled in the right frontal bone. A stereotactic frame was used to guide a Hamilton needle into the deep right frontal lobe of the skull, where 2 μl of minimal essential medium (MEM, Gibco Laboratories, Grand Island, NY), containing 5×10^4 tumor cells, was implanted. The tumors were allowed to grow for four weeks after which the animals were sacrificed with an overdose of ether anesthetic. There were no deaths from tumor implantation or during the ensuing four week period of tumor growth. The resulting encapsulated tumors, which ranged in size from 6 mm – 10 mm in diameter, were easily dissected free from the normal brain parenchyma.

Preparation of MV

The tissue (brain or brain tumor) was grossly minced with crossed scalpels and then suspended in 15 ml MEM per gram of tissue. The resultant slurry was homogenized for 40 seconds using a Brinkmann Polytron (Model 10/35; Brinkmann Instruments Co., Switzerland) at a low speed setting (# 4), and then centrifuged at $250 \times$ g for 10 minutes at 4° C. The pellet was resuspended in 25% dextran (molecular weight 150,000 daltons, Sigma Chemical Company, St. Louis, MO) in MEM, and then centrifuged at $2000 \times$ g for 20 minutes at 4° C. The supernatant, containing cellular debris and myelinated material, was discarded. The pellet was washed and then resuspended in MEM. The suspension was passed through a 60 μm nylon mesh sieve which allows passage of most red blood cells and fine cellular debris while trapping the intact MV. The sieve was rinsed with fresh medium, placed in MEM, and vortexed to free the trapped MV. The remaining contaminating red blood cells were lysed with a lysis buffer [12]. The entire procedure is completed in two to three hours. A schematic outline of the procedure is shown in Fig. 1.

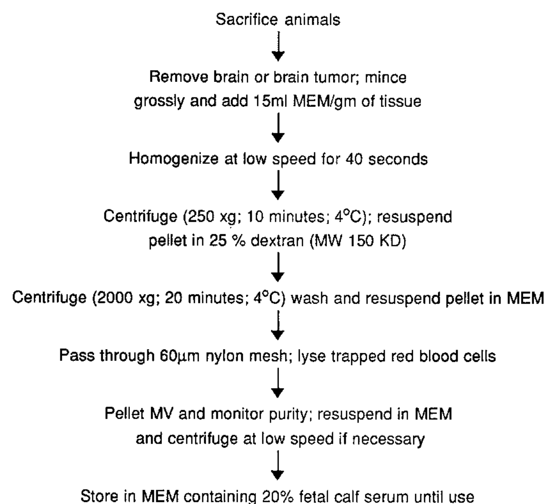

Fig. 1. Outline of MV preparation from normal brain and brain tumors.

Light microscopy

Aliquots of the MV suspension were mounted directly on glass slides and evaluated by inverted phase contrast microscopy. Other samples were fixed with 4% paraformaldehyde, embedded in paraffin, and sections were stained with hematoxylin/eoson (H/E) or reticulin, using standard procedures [13], and evaluated by light microscopy.

Scanning electron microscopy

Samples of freshly isolated MV were prepared for scanning electron microscopy (SEM) by fixing them in 2% paraformaldehyde/0.5% glutaraldehyde (in 0.1 molar sodium phosphate buffer) for 5 minutes and then in 3% glutaraldehyde for 24 hours. The MV were then pelleted by centrifugation (300 ×g for 5 minutes) and further fixed for 1 hour with 1% osmium tetroxide. After progressive dehydration with increasing concentrations of ethanol, followed by immersion in Freon TF, the MV were critical point dried using Freon 13. The MV were then mounted in colloidal silver, coated with gold/palladium (60%/40%), and examined using a JEOL JSM3 electron microscope (Japanese Electron Optical Laboratory, Japan) at 15 KV.

Transmission electron microscopy

For transmission electron microscopy (TEM), MV preparations were initially processed in the same manner as the SEM samples. After dehydration, the MV were infiltrated and embedded with epoxy resin (Mecast; Ted Pella Inc., Tustin, California), and stained with uranyl acetate and lead citrate. Thin sections were cut (silver to light yellow interference color) and then examined using a Phillips 410 transmission electron microscope (Phillips, Netherlands) set at 60 KV.

Results

MV preparation method

The series of steps in the procedure for MV isolation as outlined in Fig. 1 result in increasingly pure MV preparations. The use of a dextran gradient allows myelinated material and large tissue fragments to be removed. However, even after this stage of MV preparation, contaminating small tissue fragments and single cells are still present (Fig. 2a). After passage of the crude preparation through a 60 μm mesh, followed by washing and recovery, inverted phase contrast microscopy reveals intact microvessel with the only contaminants being single cells and fine debris (Fig. 2b). These are subsequently removed by red blood cell lysis and low speed centrifugation. H/E staining of paraffin-embedded MV sections after this stage of preparation is shown in Fig. 1c. SEM of normal cerebral microvessel preparations (Fig. 3a–c) demonstrates the high degree of purity obtained when utilizing this method, and shows arborization of intact MV. The MV walls are free of adherent debris and structural damage. The average yield of

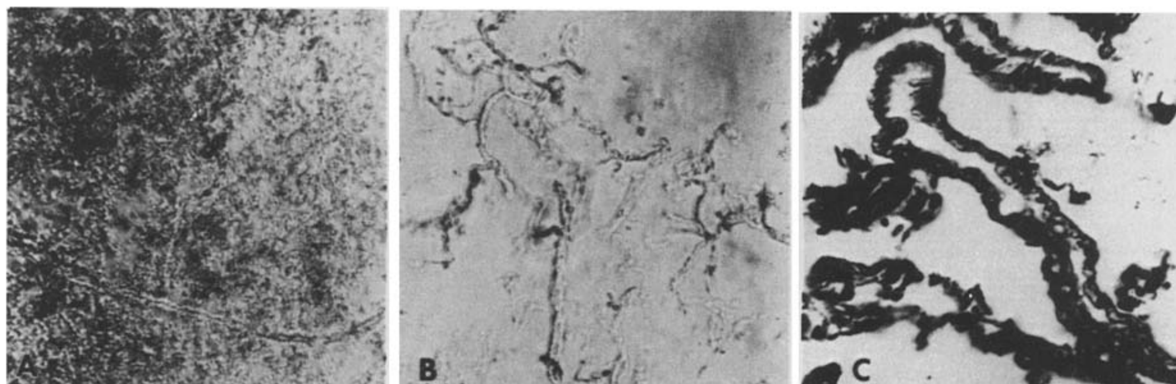

Fig. 2. A. Inverted phase contrast microscopy of MV preparation prior to dextran gradient centrifugation. Few vessels are recognizable. Much debris is seen. B. Inverted phase contrast microscopy of MV prior to sieving, with some debris still remaining. C. H/E staining of final MV preparation showing very little contaminating debris.

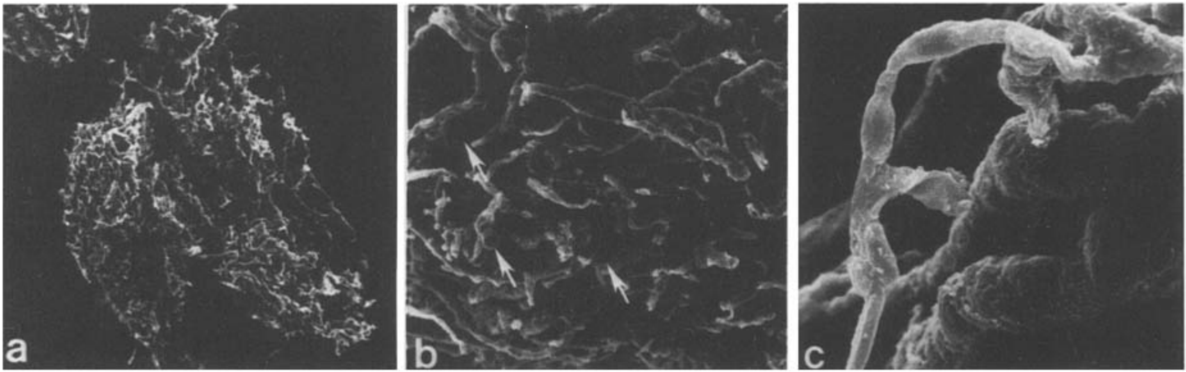

Fig. 3. A. Low power scanning electron micrograph of normal brain MV preparation. B. White arrows indicate red blood cells. C. High power scanning electron micrograph demonstrating the high degree of purity of the MV preparations from normal brain. No contaminating cells or debris can be seen.

MV obtained was approximately 2% of the wet weight of the starting tissue.

Comparison of MV from normal brain and brain tumor

Significant ultrastructural differences between MV from normal brain (and from intracerebrally-grown R175A are clearly demonstrated with TEM (Figs. 4 and 5). Microvilli, prominent on the endothelial cells of R175A MV, are not present on normal brain MV endothelial cells. A distinct, single-layered basement membrane, without pinocytic profiles, can be seen with TEM of normal brain MV preparations. In contrast, MV derived from R175A rat brain tumors have multi-layered irregular basement membranes with evidence of pincoy-

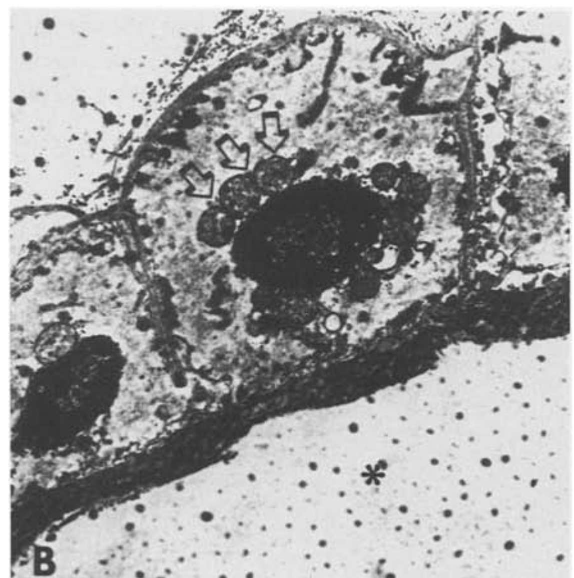

Fig. 4. A. Transmission electron micrograph showing cross-section of a single, normal brain MV: note white blood cell trapped in the lumen (arrow). The endothelial cells are morphologically homogeneous. B. Higher magnification of normal brain MV wall. Open arrows indicate perinuclear mitochondria. The basement membrane is single-layered. No microvilli or pinocytotic profiles are seen (*indicates lumen).

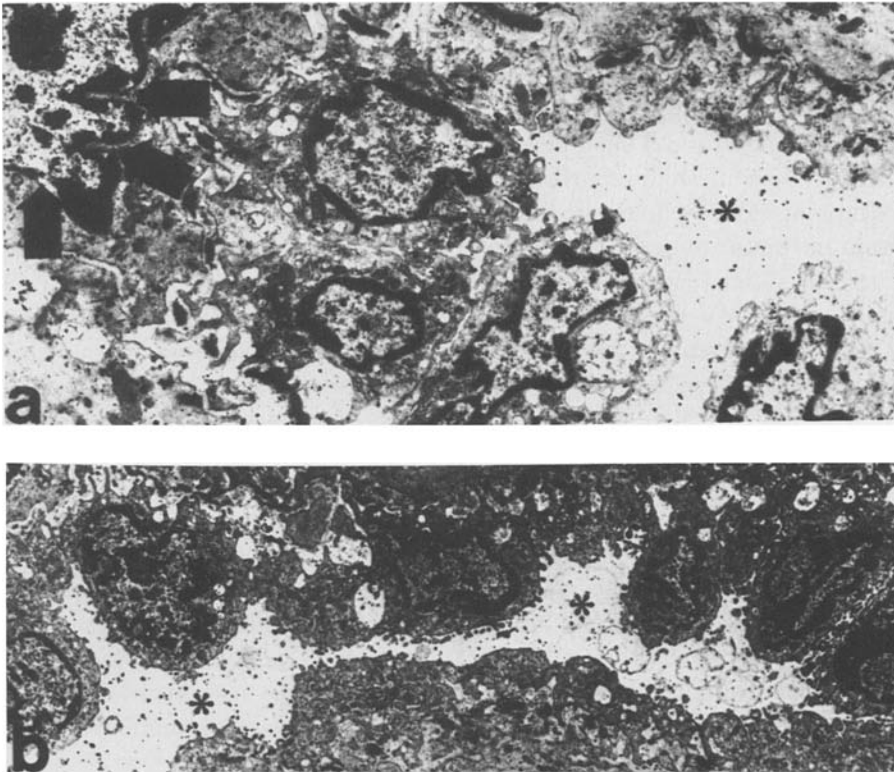

Fig. 5. A. Transmission electron micrograph of brain tumor (R175A) MW. Arrows show a perivascular cell of unknown type. Nuclei are irregularly shaped and surrounded by cytoplasm containing vacuoles, pinocytic vessicles, and a heterogeneous mitochondrial population. The basement membrane is multi-layered in some areas and absent in others. Microvilli can be seen on the luminal surfaces of the cells (*indicates lumen). B. Lower power TEM of a brain tumor MV showing pleomorphic nuclei and pinocytotic activity at luminal surface (*).

tosis. The cytoplasm is vacuolated and heterogeneous in R175A MV, but is homogeneous and finely granular in normal brain MV. Mitochondria are predominantly perinuclear in normal brain MV, but are more sparse and scattered throughout the cytoplasm in R175A MV. The most striking morphologic features of the R175A MV endothelial cells are the pronounced nuclear pleomorphism and the presence of electron dense perichromatin. The nuclei in normal brain MV lack nuclear pleomorphism, have smooth outlines and are uniformly electron dense.

Discussion

The brain microvasculature plays important roles in hemostasis [14], maintenance of the BBB, and response of the brain to toxic and ischemic injury [5]. Alteration of the normal microvascular structure and function in brain tumors has been well described [15]. Highly purified MV preparations derived from normal brain or brain tumors, that can be obtained quickly, inexpensively, and reproducibly, provide a very useful model system with which microvascular and endothelial cell biology in normal brain and in brain tumors can be studied.

Most methods previously described for the isolation of cerebral MV (Table 1) are time-consuming, labor-intensive, yield MV preparations with a considerable degree of impurity, and do not work well when applied to brain tumor tissues. We describe a new rapid and simplified method for isolating highly purified MV from normal rat brain and rat brain

tumors. The critical steps involved in the procedure are: low speed homogenization of minced tissue to release the MV from supporting tissue, a high molecular weight dextran centrifugation gradient to remove myelinated material and cellular debris, and sieving through a 60 μm mesh to remove cells and debris while trapping MV. The initial homogenization of the brain and brain tumor tissue is a very critical step in the procedure. Inadequate disruption results in most of the MV remaining in the tissue fragments and lost in subsequent purification steps. Over-homogenization results in extensive fragmentation of the MV, yielding structurally damaged MV. We found that sieving, when not preceded by a centrifugation gradient step [4, 6, 16–18], allows contaminating cells to remain in the final preparation. Both centrifugation and sieving (with the appropriate mesh size) are especially critical in the isolation of high quality MV from brain tumor tissue. The use of glass bead columns [2, 6, 19] adds technical complexity to the procedure and does not yield pure MV preparations.

Evaluation of MV preparations obtained with the method described, using light microscopy, SEM and TEM (Figs. 2–5), revealed structurally intact MV with little contamination by other cellular brain components. Because structural integrity of the MV is maintained with this technique, the resultant MV are suitable for studies of the normal or tumor blood-brain-barrier. Highly purified MV preparations also provide useful starting material for establishing pure capillary endothelial cell cultures for a variety of studies of endothelial cell biology.

Using the method described, we demonstrated that isolated brain tumor MV differ considerably from those derived from normal brain. Significant ultrastructural changes in MV prepared from R175A included irregular endothelial cell membranes with microvilli, and the presence of pinocytotic vesicles. The endothelial cells of brain tumor MV were heterogeneous with respect to size, shape, and nuclear morphology. The basement membrane was multi-layered in the tumor MV, but not in MV derived from normal brain. These findings are consistent with previously described features of malignant brain tumor neovasculature [20, 21].

Angiogenesis and endothelial cell proliferation is a hallmark of malignant astrocytomas [20]. The abnormal tumor neovasculature deviates significantly from the normal cerebral microvasculature in both structure and function [21]. The ultrastructural differences can be evaluated using MV prepared with the method described here, and include increased microvilli, multilayered basement membranes, and pinocytes [16, 22], all clearly demonstrated by TEM in R175A MV as described earlier. The differences in tumor MV and normal cerebral MV mitochondria may be indicative of altered metabolism in the brain tumor endothelial cells. The marked nuclear pleomorphism in the endothelial cells of R175A (highly anaplastic astrocytoma) MV suggests that these cells may have undergone transformational changes. However, it is unclear whether these changes are a transient or a permanent transformation of the endothelial cells induced by the malignant astrocytoma cells. The method we

Table 1. Methods previously described for isolating MV

Author	Tissue	Method
Bowman *et al.*, 1983 [3]	Bovine brain	Mince, enzyme incubation, dextran gradient
Bowman *et al.*, 1981 [2]	1 month old rat brain	Mince, homogenize, dextran gradient, glass bead column
Spatz *et al.*, 1980 [23]	2 day old rat brain	Mince (scissors), homogenize, sucrose gradient
Drewes *et al.*, 1980 [18]	Dog brain	Flush vessels, mince, homogenize, sieve, sucrose gradient, sieve
DeBault *et al.*, 1979 [17]	Mouse brain	Mince (crossed scalpels), sieve, homogenize, sieve
Panula *et al.*, 1978 [1]	3 day old rat brain	Mince, filter × 2, diffusion gradient, density gradient
Mrsulja *et al.*, 1976 [7]	Rabbit forebrain	Homogenize, sucrose gradient
Goldstein *et al.*, 1975 [6]	1 month old rat brain	Mince, sieve × 3, albumin gradient, glass bead column
Brendel *et al.*, 1974 [4]	Bovine cortex	Homogenize, sieve, homogenize, sieve

describe here may provide an important tool to further investigate this phenomenon and help clarify the nature of the tumor-endothelial cell interaction.

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Address for offprints: D.L. Silbergeld, Department of Neurological Surgery, University of Washington, RI-20, 1959 N.E. Pacific Street, Seattle, WA 98195, USA