Neoplastic and pharmacological influence on the permeability of an in vitro blood-brain barrier

PAUL A. GRABB, M.D., AND MARK R. GILBERT, M.D.

Departments of Neurology, Medicine, and Neurological Surgery, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

The authors investigated the effects of glioma cells and pharmacological agents on the permeability of an in vitro blood-brain barrier (BBB) to determine the following: 1) whether malignant glia increase endothelial cell permeability; 2) how glucocorticoids affect endothelial cell permeability in the presence and absence of malignant glia; and 3) whether inhibiting phospholipase A₂, the enzyme that releases arachidonic acid from membrane phospholipids, would reduce any malignant glioma–induced increase in endothelial cell permeability.

Primary cultures of rat brain capillary endothelium were grown on porous membranes; below the membrane, C6, 9L rat glioma, T98G human glioblastoma, or no cells (control) were cocultured. Dexamethasone (0.1 μM), bromophenacyl bromide (1.0 μM), a phospholipase A₂ inhibitor, or nothing was added to culture media 72 hours prior to assaying the rat brain capillary endothelium permeability. Permeability was measured as the flux of radiolabeled sucrose across the rat brain capillary endothelium monolayer and then calculated as an effective permeability coefficient (Pₑ). When neither dexamethasone nor bromophenacyl bromide was present, C6 cells reduced the Pₑ significantly (p < 0.05), whereas 9L and T98G cells increased Pₑ significantly (p < 0.05) relative to rat brain capillary endothelium only (control). Dexamethasone reduced Pₑ significantly for all cell preparations (p < 0.05). The 9L and T98G cell preparations coincubated with dexamethasone had the lowest Pₑ of all cell preparations. The Pₑ was not affected in any cell preparation by coincubation with bromophenacyl bromide (p > 0.45).

These in vitro BBB experiments showed that: 1) malignant glia, such as 9L and T98G cells, increase Pₑ whereas C6 cells probably provide an astrocytic influence by reducing Pₑ; 2) dexamethasone provided significant BBB “tightening” effects both in the presence and absence of glioma cells; 3) the in vivo BBB is actively made more permeable by malignant glia and not simply because of a lack of astrocytic induction; 4) tumor or endothelial phospholipase A₂ activity is probably not responsible for glioma-induced increased in BBB permeability; and 5) this model is useful for testing potential agents for BBB protection and for studying the pathophysiology of tumor-induced BBB disruption.

KEY WORDS • blood-brain barrier • glioma • permeability • dexamethasone • phospholipase A₂ • rat

Brain edema complicates many intracranial diseases. Malignant brain tumors, in particular, are commonly associated with substantial brain edema. This tumor-associated brain edema is often responsible for more clinical symptoms and signs than the tumor mass itself. Fortunately, glucocorticoids can reduce this type of brain edema and improve many associated signs and symptoms. The mechanisms by which brain tumors cause brain edema and how glucocorticoids reduce it however, remain unclear. Proposed mechanisms have primarily centered on changes in the permeability of the blood-brain barrier (BBB). Although astrocytes induce the BBB characteristic of reduced permeability in capillary endothelium, malignant astrocytes are often associated with increased BBB permeability as demonstrated by contrast-enhanced computerized tomography images and brain edema as demonstrated by T₂-weighted magnetic resonance images.

Proposed mechanisms by which malignant gliomas cause brain edema include an increase in phospholipase A₂ (PLA₂) activity (increased arachidonic acid release);²,³,⁸ release of leukotrienes or prostaglandins;²,⁷,⁸,¹²,¹⁶ secretion of a “vascular permeability factor” or “capillary permeability factor”;²⁶ increased endothelial enzyme activity;¹ decreased endothelial enzyme activity;² or proliferation of “leaky” neovasculature.²,³ Proposed mechanisms by which glucocorticoids may reduce tumor-associated brain edema include a messenger ribonucleic acid–dependent inhibition of PLA₂ activity;²,³,¹³,³¹ inhibition of cytokine activity;²⁰ reduction in endothelial adenine triphosphatase activity;² and a poorly defined “membrane stabilization” quality. Whether these mechanisms
are dependent on specific glucocorticoid activity or on nonglucocorticoid properties of the steroid molecule is unknown. The possibility that a nonglucocorticoid property could reduce BBB permeability has not been excluded. Phospholipase A₂ activity has been particularly suspect in the pathophysiology of tumor-associated brain edema and serves as the rate-limiting step for the production of arachidonic acid by cleaving membrane phospholipids into a lysophospholipid and arachidonic acid, the precursor to leukotrienes and prostaglandins by the lipoxygenase and cyclooxygenase pathways, respectively. Leukotrienes, in particular, have been linked to tumor-associated and cyclooxygenase pathways, respectively. Leukotrienes to leukotrienes and prostaglandins by the lipoxygenase arachidonic acid by cleaving membrane phospholipids and serves as the rate-limiting step for the production of in the pathophysiology of tumor-associated brain edema.

Thus, tumor-associated preoperative PLA₂ activity. Thus, tumor-associated preoperative PLA₂ activity can reduce any malignant glioma-induced increase in endothelial cell permeability. The purpose of this study was to develop an in vitro BBB model in which the permeability of a brain capillary endothelial cell monolayer could be quantified precisely in the presence and absence of various types of glia, glucocorticoids, and bromophenacyl bromide. The in vitro model also allowed precise control of cell numbers and drug concentrations without in vivo model variables such as trauma, immunological response, infection, nutrition, oxygenation, and cardiovascular stability.

The purpose of this study was to develop an in vitro BBB model in which the permeability of a brain capillary endothelial cell monolayer could be quantified before and after exposure to glioma cells, glucocorticoids, and a PLA₂ inhibitor, bromophenacyl bromide. This would allow us to determine the following: 1) whether malignant glial cells increase in vitro endothelial cell permeability; 2) whether glucocorticoids can inhibit any malignant glioma–induced increase in endothelial cell permeability; and 3) whether inhibition of PLA₂ activity can reduce any malignant glioma–induced increase in endothelial cell permeability.

Materials and Methods

Glioma Cell Lines

Glioma cells from the C6 rat line* were used at passages 43 and 45 and grown in Dulbecco’s modified essential medium (DMEM) and 10% fetal calf serum (FCS). The C6 cells were demonstrated to be positive for glial fibrillary acidic protein (GFAP) by immunoperoxidase staining, had a doubling time of 19 hours, and were relatively uniform in appearance with minimal pleomorphism. The C6 cells served as “nonneoplastic” astrocytes for our purposes. As shown by other investigators, C6 cells behave as nonneoplastic astrocytes in in vitro BBB induction of increased tight junction, reduced permeability, enhanced BBB-specific enzyme activity, and endothelial cell polarity.13,14,17,19,30

---

* Rat glioma cells obtained from American Tissue Culture Collection, Rockville, Maryland.

---

Gliosarcoma cells from the 9L rat line† were used at passages 19 through 21 and grown in DMEM and 10% FCS. These cells had a doubling time of 17 hours. Microscopically they were much more pleomorphic with a higher cell density than the C6 cells. Glioblastoma cells from the human T98G line‡ were used at passages 22 through 25 and grown in DMEM and 10% FCS. The T98G cells exhibited very pleomorphic features, stained poorly for GFAP, and had a doubling time of 16 hours. All of these cells were tested and found to be free of Mycoplasma contamination.

Cell Preparation Technique

Brain capillary endothelial cells were isolated in primary culture from 21-day-old Sprague-Dawley rats. Using the technique described by Bowman and colleagues with some modification, rats were exposed to lethal amounts of inhaled methoxyfluorane and their brains were quickly removed under sterile conditions and placed in ice-cold Medium 199. Cortical gray matter was separated sharply from the remaining brain and minced into 1-mm³ pieces using a No. 10 scalpel blade. The minced cortical gray matter was placed in an equal volume of Medium 199 and homogenized with 15 to 20 strokes of a hand-held Teflon pestle (0.25-mm clearance). The homogenate was placed in an equal volume of 30% dextran solution and centrifuged at 5800 G for 10 minutes. The pellet was resuspended in 15% dextran solution and recentrifuged. The resulting pellet was washed in Medium 199, resuspended in 0.1 mg/ml collagenase/dispease, and placed in a gyrating shaker at 37°C for 5 to 16 hours. The solution then was centrifuged at 1500 G for 5 minutes. The pellet was resuspended in Medium 199, and 2-ml aliquots were placed on a previously formed Percoll gradient and centrifuged at 1500 G for 10 minutes. Fractions were collected in 24-well multilplates and observed under phase-contrast microscopy. Wells containing clusters of refractile cells were pooled and centrifuged twice after dilution with Medium 199 and 5% horse serum. The resulting pellet was then resuspended in DMEM/F12 with 100 U/ml heparin, 2.5 μM hydrocortisone to inhibit growth of any contaminating fibroblasts, 10% FCS, and 1% antibiotic solution, and then plated on collagen-coated dishes or membranes.

The purity of the primary endothelial cell culture was monitored with phase-contrast microscopy (Fig. 1) for the “cobblestone” appearance of proliferating endothelial cells and by immunocytochemical analysis with an antibody to von Willebrand factor, which is an antigen unique to vascular endothelial cells. Media were changed in all culture preparations every other day during the 1st week, and then daily beginning the 7th day of endothelial cell culture, so that accumulation of metabolic waste products would not occur in the more proliferative cell preparations such as 9L and T98G.

In Vitro Blood-Brain Barrier

Endothelial cells were plated at 100,000 cells/ml on the membrane in the upper compartment (Fig. 2) by means of a dual chamber model separated by a porous collagen-coated membrane. A near-confluent monolayer of endothelial cells was present on the collagen-coated porous membrane after 7 days; media could be sampled and changed from either compartment via ports without disturbing the system. All three cell lines were plated in the lower compartment at a concentration of 50,000 cells/ml. The C6 cells were plated on the 3rd day, the other two cell lines, 9L, and T98G, on the 4th day, and all were cultured under 7% humidified 7th day of endothelial cell culture. Each cell system was coincubated with and without 0.1 μM dexamethasone, or 1.0 μM bromophenacyl bromide, a PLA₂ inhibitor, beginning on the 7th day of endothelial cell culture. This specific dexamethasone concentration was chosen because of its demonstrated ability to inhibit tumor-induced increas-
Permeability of an *in vitro* blood-brain barrier

![Photograph taken through a phase-contrast microscope of rat brain capillary endothelial cells after 3 days in culture showing the “cobblestone” appearance of the cells as they form a monolayer.](Image)

Permeability Assays

The transendothelial flux of radiolabeled sucrose across the *in vitro* endothelial cell monolayer was measured in all the coculture systems described above on the 10th day of endothelial cell culture. Performing the assays on the 10th day allowed for the confluent endothelial cell layer to be exposed to any added agent, such as dexamethasone or bromophenacyl bromide, for 72 hours, and near-confluent tumor cells for 72 hours. Sucrose, which is not taken up by the endothelial cells by either an active or facilitated transport mechanism,9,19,25,28,30 is a hydrophilic molecule that does not readily cross cell membranes. Therefore, it serves as a relatively inert polar substance of low molecular weight (M, 342) to measure the permeability of the endothelial cell monolayer.

There were four different cell preparations: endothelial cells only (control), endothelial and C6 cells, endothelial and 9L cells, and endothelial and T98G cells. Each of these four cell preparations was incubated with no added agent, 0.1 μM dexamethasone, or 1.0 μM bromophenacyl bromide for 72 hours before measuring the Pe. To determine the transendothelial flux, the media were aspirated and replaced with serum-free media in both compartments. To the upper compartment, 1600 μl of serum-free media containing 0.67 μCi/ml 3H-sucrose was added at time zero. The lower compartment contained 2500 μl serum-free media. Cluster plates containing the wells were placed in a shaking gyrorator at 37°C. At 15, 30, 60, and 90 minutes after addition of radioactivity, 20 μl of media was withdrawn from the upper and lower compartments and the radioactivity contained in each aliquot was counted with a liquid scintillation beta counter. Volumes withdrawn were immediately replaced with equivalent volumes of serum-free media so that volume relations remained constant. Aliquots removed and replaced represented approximately 1% of chamber volume and did not contribute to significant dilutional effects over time.

An equation has been described by Sill, et al,31 defining an “effective permeability coefficient,” Pe = (V/A)(Tl/Tu)/t in centimeters per second, where V is the volume of media in the upper chamber, A is the area of the endothelial cell–covered membrane, Tl is the concentration of tracer (3H-sucrose) in the lower compartment, Tu is the concentration of tracer in the upper compartment, and t is the time in seconds after addition of tracer. Thus a larger Pe value reflects a more permeable endothelial cell layer. Phase-contrast microscopic examination was performed to ensure that no visible interendothelial gaps existed before performing the transendothelial flux assays.

Statistical Analysis

Experiments were performed in quintuplicate for each of the cell preparations described. An overall mean Pe and standard deviation were calculated for each cell preparation. First, a mean Pe was calculated for each of the five samples of a given cell preparation by averaging the Pe s obtained from the four points in time (15, 30, 60, and 90 minutes). Over this time range the transendothelial flux of tracer maintained a linear relationship with time. Measurements of transendothelial flux of tracer much earlier (0 to 5 minutes) and much later (120 minutes) did not maintain a linear relationship with time and were therefore excluded from analysis. This provided five mean Pe s for each of the 12 specific cell preparations, which were averaged to obtain an overall mean Pe and standard deviation. A paired t-test was used to compare mean values and standard deviation of Pe of different combinations of cell types and added agent. Significant statistical analysis was achieved when p was less than 0.05.

Results

At the time of the transendothelial flux assays, all cell cultures showed confluence of endothelial and glial cell cultures by phase-contrast microscopy. Doubling times for all cell types were unchanged by the presence of 0.1 μM dexamethasone or 1.0 μM bromophenacyl bromide (our
unpublished data). The mean effective permeability coefficients and standard deviation for each of the 12 cell culture preparations are shown in Fig. 3.

When dexamethasone and bromophenacyl bromide were not present in the media (“no added agent”) the permeability of the endothelial cell monolayer was influenced by C6 cells differently from T98G and 9L cells. The C6 cells reduced the $P_e$ significantly ($p < 0.05$), whereas 9L and T98G cells increased the $P_e$ significantly ($p < 0.05$) compared to endothelial cells only (control).

The addition of dexamethasone caused a significant reduction in $P_e$ for all cell culture preparations. However, there were differences in the mean $P_e$ among the four cell culture preparations coincubated with dexamethasone. Although the control preparation had a significant reduction ($p < 0.05$) in $P_e$ with the addition of dexamethasone, it remained the most permeable of all preparations coincubated with dexamethasone. The $P_e$ of the C6 preparations with dexamethasone was less than the control preparation with dexamethasone, but not significantly so. The $P_e$ of the 9L and T98G preparation with dexamethasone was significantly lower ($p < 0.05$) compared to the control with dexamethasone. In fact the $P_e$ for 9L and for T98G with dexamethasone were the lowest of all preparations.

The addition of bromophenacyl bromide, a PLA$_2$ inhibitor, resulted in $P_e$'s very similar to $P_e$'s with no added agent. Although each of the four preparations coincubated with bromophenacyl bromide revealed a slight reduction in the mean $P_e$ relative to those with no added agent, those changes were minimal ($p < 0.45$).

**Discussion**

The in vitro BBB model used in this experiment reliably reflected changes in endothelial cell monolayer permeability after varying pharmacological and cellular exposures. Induction of reduced permeability or “tightening” of the in vitro BBB by coculture with C6 rat glioma cells demonstrated the dependence of brain capillary endothelial cells on an astrocytic influence to induce the BBB characteristic of reduced permeability. As shown by other investigators, C6 glioma cells paradoxically behaved as nonneoplastic astrocytes in inducing BBB characteristics in endothelial cells in vitro.$^{3,17,29}$ Our results also support the concept of a soluble factor released by glia and not a dependence on glial–endothelial contact for the induction
Permeability of an in vitro blood-brain barrier

of BBB characteristics. Therefore, the C6-endothelial cell coculture served as the best in vitro model of the native BBB in our experiments. This was reflected in our data in which the lowest permeability of any culture preparation before the addition of pharmacological agents was in the C6 cell culture preparation. Although the C6 cells may have behaved as normal astrocytes in reducing endothelial cell permeability, the malignant cell lines of 9L and T98G significantly increased the endothelial cell monolayer permeability compared to both the C6 cell preparation and the control preparation. This in vitro observation paralleled the clinical observation that malignant gliomas are associated with a leaky BBB leading to enhancement on intravenous contrast-enhanced imaging studies as well as tumor-associated brain edema. Thus, the increased permeability of capillaries associated with malignant gliomas appears secondary to a BBB disrupting factor and not simply secondary to a lack of a nonneoplastic astrocytic influence.

Dexamethasone, a potent glucocorticoid, significantly reduced 9L- and T98G-induced increases in permeability of the in vitro BBB. This observation paralleled the clinical observation of reduction in tumor-associated brain edema after the administration of glucocorticoid. It is interesting to note that the lowest permeabilities were seen when the malignant T98G and 9L cell lines were cocultured with dexamethasone. Because of the evidence of higher metabolic activity displayed by these two cell lines, such as shorter doubling times and more frequent media administration requirements, more BBB “tightening” factors as well as more BBB “disrupting” factors may be released by these more metabolically active malignant cells. Although the glucocorticoids appear to negate any effect by the BBB “disrupting” factors, the glucocorticoid may unmask the influence on endothelial cells on relatively higher amounts of BBB “tightening” or trophic factors released by malignant glia in vitro such as fibroblastic growth factor. This could explain the observation of the lowest permeability in our experiments occurring in the presence of dexamethasone and the more highly malignant cell lines. This tightening factor appears specific to tumor cells of astrocytic lineage, as coculture with fibroblasts or smooth-muscle cells does not influence the endothelial monolayer. Malignant gliomas are known to overexpress a number of factors, such as fibroblastic growth factor, which do influence endothelial cells. It may be that whatever factor provided by normal astrocytes and C6 that induces the BBB phenotype in brain capillary endothelial cells is also produced by malignant astrocytes, but remains overshadowed by BBB “disrupting” factors from these malignant cells. These results underscore the concern of reduced chemotherapeutic or cytokine delivery in glioma patients on steroids because of steroid-associated reductions in BBB permeability.

The primary cultures of rat brain capillary endothelium were grown in media containing hydrocortisone, because it helps to retard the growth of any contaminating fibroblasts. Physiological levels of hydrocortisone were achieved when the top chamber media were diluted by the hydrocortisone-free media from the bottom chamber. However, the endothelial cells were initially exposed to hydrocortisone concentrations containing glucocorticoid activity equivalent to that of 0.1 μM dexamethasone before equilibration with the bottom chamber. Removal of hydrocortisone from the media did not influence the P_e of any preparation (our unpublished data). Therefore, it would appear that dexamethasone: 1) has an effect on endothelial monolayer permeability that is independent of its glucocorticoid activity; or 2) is able to deliver its glucocorticoid activity to the endothelium more efficiently than a physiologically similar dose of hydrocortisone. An in vivo study found BBB permeability to Evans blue dye in animals that underwent adrenalectomy to be increased over that of animals with an intact adrenal cortex, which suggested a BBB dependence on cortisol. The possibility, however, that a nonglucocorticoid action may provide BBB protection or edema reduction gives impetus to develop nonglucocorticoid therapies for tumor-associated brain edema.

An effect on capillary permeability has been suspected to be the mechanism by which synthetic glucocorticoids reduce tumor-associated brain edema. Our data would support this hypothesis as dexamethasone provided significant reduction in the P_e even in the absence of glial cells and in the presence of physiological concentrations of hydrocortisone. One theory based on the ability of glucocorticoids to inhibit PLA_2 via a mechanism dependent on protein synthesis has been proposed as a mechanism of steroid-induced tumor-associated brain edema. The enzyme PLA_2 cleaves arachidonic acid from membrane phospholipids and thus serves as the rate-limiting step for the production of arachidonic acid and its inflammatory metabolites, such as prostaglandins and leukotrienes, that have also been associated with this form of brain edema. Although PLA_2, inhibitor to prevent malignant glioma–induced increases in permeability speaks against abnormally elevated PLA_2 activity as a major cause of BBB disruption and tumor-associated brain edema. Assays in all the above cell lines have failed to detect elevated PLA_2 activity, further casting doubt on PLA_2 as a major mechanism of this brain edema. Although arachidonic acid and its metabolites can cause vasogenic brain edema and strong evidence exists for leukotrienes as a causative factor in tumor-associated brain edema, how arachidonic acid is made available for subsequent metabolism into leukotrienes does not appear to depend on tumor or endothelial PLA_2 activity.

Further studies will seek to correlate the in vitro endothelial permeability influencing effects of malignant glial cells and dexamethasone with changes at the electron microscopic level (tight junction density and competence) and alterations in specific gene expression (vascular permeability/growth factor) associated with central nervous system neoplastic angiogenesis. Also, nonglucocorticoid agents such as the α1-aminosteroids will be tested to determine their effect on the in vitro BBB in the presence of malignant glial cells.

Conclusions

The in vitro BBB model employed in these experiments displayed the following features: 1) astrocytic (C6) induction of reduced endothelial cell permeability; 2) malignant glioma–induced increases in endothelial cell permeability; 3) the endothelial barrier “tightening” effects of dexamethasone before equilibration with the bottom chamber. Removal of hydrocortisone from the media did not influence the P_e of any preparation (our unpublished data). Therefore, it would appear that dexamethasone: 1) has an effect on endothelial monolayer permeability that is independent of its glucocorticoid activity; or 2) is able to deliver its glucocorticoid activity to the endothelium more efficiently than a physiologically similar dose of hydrocortisone. An in vivo study found BBB permeability to Evans blue dye in animals that underwent adrenalectomy to be increased over that of animals with an intact adrenal cortex, which suggested a BBB dependence on cortisol. The possibility, however, that a nonglucocorticoid action may provide BBB protection or edema reduction gives impetus to develop nonglucocorticoid therapies for tumor-associated brain edema.
methasone in the presence and absence of glioma cells; and 4) the unlikely role for PLA₂ in tumor-associated increases in BBB permeability.

The *in vitro* BBB was more permeable in the presence of 9L and T98G cells because of a malignant glia–associate
ded effect, not because of a lack of astrocytic induction of reduced permeability. The permeability-reducing or “tightening” effect of dexamethasone may not be dependent entirely on its glucocorticoid activity. This implies that an agent may have BBB “tightening” effects without the unwanted side effects of glucocorticoids.

This model will allow for the testing of other agents such as 21-aminosteroids that may reduce tumor-associated
brain edema so that both safer therapies for edema reduction and a better understanding of the pathogenesis of tumor-induced BBB disruption may be developed.

References

34. TAO-ChENG HJ, Nagy Z, Brightman MW: Tight junctions of brain endothelium in *vitro* are enhanced by astroglia. *J Neurosci* 7:3293–3299, 1987

Manuscript received March 15, 1994.
Accepted in final form September 22, 1994.
Address reprint requests to: Paul A. Grabb, M.D., Suite B-400 Presbyterian-University Hospital, Department of Neurological Surgery, 200 Lothrop Street, Pittsburgh, Pennsylvania 15213–2582.