Effects of steroids and nonsteroid anti-inflammatory agents on vascular permeability in a rat glioma model

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Cerebral edema produced by brain tumors is clinically and experimentally reduced by steroid therapy. Nonsteroid anti-inflammatory drugs (NSAID’s) which have been used to treat non-neural inflammation and swelling have not been evaluated for their ability to affect edema produced by brain tumors. The authors have used the rat C6 glioma spheroid implantation model to compare the effects of two steroids (dexamethasone and methylprednisolone) and two NSAID’s (ibuprofen and indomethacin) on protein extravasation caused by intracranial gliomas. Evans blue dye was used as a marker for serum albumin extravasation. The concentration of Evans blue dye was measured in the tumor and peritumoral and contralateral brain tissue 1 hour after intravenous injection.

Extravasation of Evans blue dye within the tumor was decreased in all treatment groups when compared to placebo-injected control animals. The differences between the control specimens and those treated with dexamethasone, methylprednisolone, and indomethacin were highly significant (p < 0.005). The Evans blue staining was also decreased in the peritumoral and contralateral brain. These results indicate that NSAID’s compare favorably with steroids in diminishing tumor-induced protein extravasation. It is suggested that NSAID’s may prove to be beneficial in clinical instances used either in conjunction with steroid therapy or alone when steroids are contraindicated.

KEY WORDS • edema • steroid • glioma • blood-brain barrier • rat

Cerebral edema associated with an intracranial tumor is a major cause of morbidity and mortality. The reduction of tumor-associated edema by steroid treatment is well documented. Nonsteroidal anti-inflammatory drugs (NSAID’s) are widely used to treat non-neural inflammation, but their effect on tumor-associated edema in the central nervous system (CNS) is unknown. This paper compares the effects of two commonly used steroids and two NSAID’s on tumor growth and vascular permeability in a new glioma model.

The spheroid implantation glioma model is a simple and reproducible model for inducing primary intracranial gliomas. When C6 astrocytes are cultured in spinner flasks, avascular spheroids of tumor cells are produced. The spheroid can then be surgically implanted into the brain of an adult Sprague-Dawley rat to form a malignant glioma with typical tumor vessels. The permeability of the blood-brain barrier to serum proteins has been evaluated in this model by spectrophotometric measurement of extravasated albumin-bound Evans blue dye.

In this study, we measured the effect of dexamethasone, methylprednisolone, ibuprofen, and indomethacin on vascular permeability associated with intracranial C6 gliomas. The Evans blue dye was quantitated in the tumor and in the peritumoral and contralateral brain to determine the effects of these agents on vascular permeability associated with intracranial C6 gliomas.

Materials and Methods

Tumor Production

Well-developed vascular tumors were produced in Sprague-Dawley rats as described before. Briefly, C6 astrocytes were seeded into spinner flasks and incubated until spheroids 400 to 500 μm in diameter were formed. A single spheroid was placed into a suction-produced defect in the parieto-occipital cortex of
the rat through a burr hole in the cranium. Sham-operated animals received no spheroid. The defect was sealed with bone wax and the overlying skin incision was sutured. The rats were allowed free access to food and water. Forty rats were implanted with spheroids and 12 rats underwent sham operations. Tumor pathology was assessed in formalin-fixed paraffin-embedded specimens stained with hematoxylin and eosin.

Experimental Design

The tumor-implanted rats were randomly allotted to one of five groups. Nine days after implantation (to allow time for tumor growth) each group of rats received one of the following treatments for 2 days by intraperitoneal injection: 1) placebo, every 12 hours (tumor control group: 10 animals); 2) dexamethasone, 1 mg/kg every 8 hours (six animals); 3) methylprednisolone, 30 mg/kg every 8 hours (seven animals); 4) ibuprofen, 8 mg/kg every 8 hours (eight animals); and 5) indomethacin, 7 mg/kg every 12 hours (six animals).*

On Day 11, the rats were sedated with 10 mg intraperitoneal Somnotol, and 0.5 ml of 0.5% lidocaine was infiltrated over the femoral vein. The vein was cannulated and used for injection of Evans blue dye (2 ml/kg of 2% dye in 0.9% saline). The Evans blue dye was allowed to circulate for 1 hour, after which the animal was sacrificed by an injection of Somnotol. Immediately after, a perfusion of 50 ml of 0.9% NaCl was given at a pressure of 100 mm Hg through a transcardiac catheter to clear the intravascular space. Both of the external jugular veins and the right atrium were opened to allow for adequate perfusion. The brain and tumor were removed, examined, and dissected under an operating microscope. The tumor and samples from peritumoral tissue and the contralateral hemisphere were immediately weighed and divided into portions for the measurement of dry weight and of Evans blue dye extravasation into the tissue.

Evans Blue Dye Extravasation

To determine dry weight, the tissue was placed in a vacuum container† at 20°C for 24 hours and then reweighed. The Evans blue staining was measured using a modification of the procedure of Durward, et al., as described by Farrell, et al. The tissue for dye measurement was placed into 1 ml of formamide, extracted for 72 hours, and then centrifuged to remove any particulate matter. The quantity of extracted Evans blue dye in each sample was measured by determining absorbance at 620 nm in a spectrophotometer using formamide as a reference.

* Methylprednisolone and ibuprofen were kindly supplied by the Upjohn Co., Kalamazoo, Michigan. Indomethacin was purchased from Merck Sharp and Dohme Canada, Kirkland, Quebec, Canada. Dexamethasone was purchased from Organon Canada Ltd., Toronto, Ontario, Canada.
† Vacuum container manufactured by Bel-Art Products, Pequannock, New Jersey.

Statistical Analysis

All data quoted in this study are expressed as means ± standard error of the mean (SEM). Tumor weights and peritumoral and contralateral tissue Evans blue dye values were statistically evaluated by the unpaired Student t-test, and p < 0.05 was considered statistically significant. Since tumor size influences extravasation of dye for these values, the p values were obtained from a one-way analysis of variance and covariance and a t-test matrix comparing tumor size and concentration of Evans blue dye between the treatment and control groups.

Results

Figure 1 shows a brain from an untreated rat injected intravenously with Evans blue dye. The tumor is stained blue and is easily removed from the parieto-occipital cortex. The tumor wet weight values were highly variable in all groups. The dry weight of the tumors was not significantly affected by the drug treatments (Fig. 2).

Evans blue dye extravasation was expressed as μg/mg dry weight of tissue. Levels in untreated tumor-bearing rats were significantly higher than in surgical controls (Fig. 3). Extravasation of dye within the tumor was significantly decreased by treatment with dexamethasone, methylprednisolone, and indomethacin when compared to untreated control rats (Fig. 3 left). When ibuprofen was used, the reduction was not statistically significant (p = 0.06). Peritumoral Evans blue dye...
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staining was decreased by methylprednisolone, ibuprofen, and indomethacin. In the contralateral cortex, staining was decreased in all treatment groups (Fig. 3 right).

Discussion

When C6 astrocytoma cells are grown in spinner flasks, uniform spheroids are produced which can be implanted into rat brain to form tumors.8,10 The resulting gliomas have vessels with structural and permeability characteristics similar to those found in primary brain tumors in humans.11,20 The tumors are sharply demarcated from the surrounding cortex and are easily identifiable. This makes removal straightforward, facilitating subsequent analysis of tumor tissue.

Permeability across the endothelial cell and extravasation of protein was quantitated in this model by measuring Evans blue tissue staining. Evans blue dye binds rapidly to albumin upon intravascular injection.24 After the intravascular Evans blue dye is removed by perfusion, residual dye (measured in tissue samples) should quantitate the amount of extravasated protein.6 Other studies have demonstrated a strong correlation between brain edema and protein extravasation14 as well as with the extravasated Evans blue dye-albumin complex.6,24 In this model, animals without tumors had minimal extravasation of dye indicating that the dye-albumin complex is normally maintained in the intravascular compartment. Tumor implantation consistently produced Evans blue dye extravasation which was then quantitated and localized. Leakage of dye into the tumor increases as the tumor grows in size, but does not keep pace with tumor growth. The leakage plateaus

![Graph](image-url)
as necrotic centers develop, and it appears that leakage continues as a function of viable tumor mass.\textsuperscript{10} Since drug treatment at clinical concentrations did not significantly affect glioma growth as measured by dry weight, changes in extravasation of Evans blue dye likely represent drug effects on protein permeability rather than inhibition of tumor growth.

The mechanisms responsible for the production of vasogenic brain edema are poorly understood. Edema generated by intracranial tumors has been shown to occur primarily within the tumor and spread secondarily into the peritumoral area.\textsuperscript{19,26} In this study, Evans blue dye concentrations within the tumor were 10 times those found in peritumoral and contralateral tissue (Fig. 3). This is in accord with findings of other studies carried out in our laboratories.\textsuperscript{10} Dexamethasone is known to decrease peritumoral edema in human and in other experimental models.\textsuperscript{\textsuperscript{17,22,24}} In this experiment, the Evans blue staining in the peritumoral tissue was reduced by dexamethasone but was not significantly different from the control levels. It may be that an extended time course of treatment is required to produce a significant difference.

In this model, the tumor vessels have discontinuous tight junctions between contiguous endothelial cells, and the endothelial cells are fenestrated. However, the density of tubulovesicular profiles is the same as in endothelia of peritumoral, contralateral, and normal brain vessels.\textsuperscript{20} Endothelial cell fenestrae and endothelial discontinuous junctions are presumed to be routes for the passage of serum constituents into the extravascular space which may be modified by drug treatment. Current investigation is under way to compare the structure and frequency of permeability pathways in treated and control animals to determine if there are alterations related to steroid or NSAID treatment.

Biochemical dysfunction may be another mechanism contributing to edema formation and clinical symptoms. Local glucose utilization is abnormal in brains with tumors. These changes are reversible with steroid administration.\textsuperscript{26} In our model, mitochondrial density in tumor endothelial cells is reduced,\textsuperscript{20} which may indicate that metabolic function and the ability of the cell to maintain a blood-brain barrier are compromised. The ability of steroids and NSAID's to restore metabolic function as in cold-induced cerebral lesions\textsuperscript{17} may be one mechanism by which they exert their effect on tumoral edema.

Other studies indicate that metabolic and structural abnormalities are present in areas distant from a pathological focus in the brain (such as tumor\textsuperscript{16,26} and cold lesion\textsuperscript{17}). Some of these abnormalities are reversible with steroid or NSAID treatment.\textsuperscript{17,26} In our model, peritumoral and contralateral brain in rats not treated with drugs had elevated levels of Evans blue staining when compared to surgical control brains. Treatment with steroids and NSAID's decreased the dye concentration in the peritumoral and contralateral cortex. These findings imply that an abnormality is present in the nontumoral brain of tumor-bearing rats which may be reversed following treatment with steroids or NSAID's.

The pathways responsible for increased permeability of the blood-brain barrier have been investigated in other systems.\textsuperscript{4,5} The arachidonic acid prostaglandin system has been implicated in edema production.\textsuperscript{4} Various pathological insults appear to generate arachidonic acid which is associated with disruption of endothelial cell structure and function leading to edema formation.\textsuperscript{4}

Arachidonic acid is an important precursor of prostaglandins,\textsuperscript{15} which are also known to increase vascular permeability\textsuperscript{22} and are postulated to cause metabolic dysfunction in the CNS.\textsuperscript{17} Prostaglandin-induced leakage is reduced by indomethacin in the hamster cheek pouch model.\textsuperscript{21} Indomethacin and ibuprofen also reverse the metabolic depression produced by cerebral cold lesions.\textsuperscript{17} Several types of intracranial tumors\textsuperscript{7} and C6 astrocytoma cells in culture\textsuperscript{12} produce prostaglandins which may contribute to cerebral dysfunction and edema formation. Indomethacin, which is known to inhibit prostaglandin synthesis in the rat brain,\textsuperscript{1} has the potential to minimize or reduce any damage produced by prostaglandins in intracranial tumors. Prior to this study, their effects on CNS tumor-associated edema have not been studied, although ibuprofen and indomethacin have been effective in reducing edema formation associated with intrahepatic carcinomas in rats.\textsuperscript{2}

Corticosteroids remain the standard medical treatment for the management of edema associated with intracranial tumors. Steroids may exert their clinical effects by altering endothelial permeability,\textsuperscript{28} by inhibiting tumor growth,\textsuperscript{28} by improving the metabolic function in parenchymal and endothelial cells,\textsuperscript{17,26} or by modulation of phospholipase activity.\textsuperscript{5,11,13} Steroid therapy is associated with many complications, especially with high-dose long-term administration. The NSAID's are safe and well tolerated by most patients. Oral and intravenous preparations are available, allowing easy administration.

This study has shown that the treatment of tumorbearing rats with steroids for 48 hours prior to sacrifice significantly reduced vascular permeability in the CNS. Indomethacin and ibuprofen also reduced Evans blue dye extravasation, demonstrating that these two NSAID's compare favorably with steroids in reducing vascular permeability. Therefore, NSAID's may be useful in treating intracranial edema either alone or in combination with steroids. Further investigation is necessary to assess the mechanism and usefulness of NSAID's in peritumoral edema, and a human clinical trial appears warranted.

\textbf{Acknowledgments}

We are grateful to the Upjohn Co. for providing the methylprednisolone and ibuprofen and to Dr. P. A. Stewart for reviewing the manuscript.
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References


Manuscript received November 13, 1985.
This work was supported by the National Cancer Institute (Canada) and the Brain Research Fund Foundation.
Dr. Del Maestro is the recipient of an Ontario Ministry of Health Career Scientist Award.
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