Capillary permeability factor secreted by malignant brain tumor

Role in peritumoral brain edema and possible mechanism for anti-edema effect of glucocorticoids

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Conditioned media from two human malignant gliomas, C6 rat glioma, Walker 256 carcinosarcoma, and normal human glia were concentrated 50-fold to create a culture supernatant (SUP-C). The effect of SUP-C on rat brain capillary permeability was investigated by measuring the entry of 14C-aminoisobutyric acid (14C-AIB) by means of quantitative autoradiography. The SUP-C contained proteins with a molecular weight of 10 kD or greater. The SUP-C from all tumor cells markedly increased brain capillary permeability, indicating the presence of a permeability factor, whereas that from normal glial cells did not. Glioma cells produced more factor after incubation for 20 hours than 4 hours. The activity of capillary permeability factor in the SUP-C was inhibited by pretreatment of animals with BW755C (lipoxygenase inhibitor), but not with indomethacin (cyclo-oxygenase inhibitor). Pretreatment of animals with dexamethasone prior to intracerebral infusion of tumor SUP-C significantly reduced the factor-induced increase in capillary permeability. On the other hand, coincubating glioma cells with dexamethasone produced SUP-C with a permeability activity that was about one and a half times greater than that without dexamethasone. These results indicate that glucocorticoids produce their anti-edema effects by directly acting on capillary endothelial cells, possibly through the inhibition of phospholipase A2 activity, resulting in a decrease of lipoxygenase rather than cyclo-oxygenase products. The production of capillary permeability factor by tumor cells was not inhibited, but rather enhanced, by administration of glucocorticoids.

Key Words • brain neoplasm • capillary permeability factor • edema • glucocorticoid • malignant glioma • rat

Increased capillary permeability in malignant brain tumors and in peritumoral tissues produces cerebral edema which substantially contributes to neurological morbidity and mortality. The mechanism by which brain tumors increase capillary permeability of brain is poorly understood. Previous investigations of human and experimental brain tumors suggest that structural alterations of tumor capillaries (including endothelial fenestration, increased number of pinocytotic vesicles, and widened intercellular junctions) are responsible for the increased capillary permeability within brain tumor tissues. 6,13 However, quantitative autoradiographic (QAR) studies performed at this laboratory on experimental brain tumors have also demonstrated increased capillary permeability in brain tissue adjacent to tumors and brain tissue surrounding tumors, where there are few or no tumor cells and no apparent tumor neovessels. 10 It was hypothesized that the tumor must manufacture a soluble material that can diffuse into the surrounding brain tissue and alter the capillaries. We have recently reported the production of such capillary permeability factors by rat C6 glioma cells grown in culture; 15 these factors were present in the culture supernatants. One factor is a protein with a molecular weight (MW) greater than 10 kD, the other is a water-dispersible lipid.

In the present report, cultured cells from human malignant gliomas and from rat Walker 256 carcinosarcoma (which produces marked cerebral edema when implanted in rat brain) 16 are shown to secrete the same
kind of protein factor as produced by the C6 glioma, whereas normal glial cells in humans do not. Also identified are possible mechanisms for the anti-edema action of glucocorticoids.

Materials and Methods

Cells and Tissue Culture

Five different cell types were grown in tissue culture and used for the present experiments. Two human malignant glioma cell lines (EI and HFA) and normal human glial cells (kindly supplied by Dr. Joan Rankin Shapiro) were established and maintained in tissue culture with Waymouth's MAB medium supplemented with 25% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO2 and 95% air. Tumor EI was obtained from a 46-year-old woman with bifrontal glioblastoma multiiforme; the modal chromosome number of the primary tumor was 67,XXX and at passage 9 was 75,XXX. Tumor HFA was obtained from a 71-year-old woman with a left parietal glioblastoma; the modal chromosome number of the primary tumor was 92,XX, and at passage 3 was the same. The normal human glial cells were obtained from a patient undergoing neurosurgery for brain trauma; the cells were characterized as previously described. Briefly, the primary resected tissue was dissociated and cloned into multiwell plates. All clones were stained for glial fibrillary acidic protein (GFAP); GFAP-positive cells from 300 to 400 wells were combined into a cell line and frozen. After thawing, the cells usually survive through 20 passages. For these experiments, the cells were used at passage 3. The C6 gliomas* were cultured in McCoy's 5A medium supplemented with 10% FBS. Walker 256 carcinosarcoma was maintained in tissue culture in Ham's F10 medium with 25% FBS. The normal human glial cells were used at passage 3. The C6 gliomas* were cultured in McCoy's 5A medium supplemented with 10% FBS. Walker 256 carcinosarcoma was maintained in tissue culture in Ham's F10 medium with 25% FBS.

Preparation of Supernatants

Cells from confluent flasks were harvested with 0.05% trypsin and 0.02% ethylenediaminetetra-acetic acid in Hanks' balanced salt solution, washed twice with phosphate-buffered saline (PBS), and resuspended in Eagle's minimum essential medium (EMEM) without serum. Tissue culture flasks (150 sq cm) containing 2.5 × 107 cells in 10 ml of serum-free EMEM were incubated at 37°C in humidified atmosphere with 5% CO2 and 95% air. Four hours after incubation, the culture supernatants were collected, centrifuged at 5000 G for 20 minutes, and passed through a 0.22-μm filter. All cell types, except Walker 256, were further incubated in serum-free EMEM for 20 hours under the same conditions, and supernatants were obtained as from the 4-hour incubations. All supernatants were concentrated 50-fold by a negative pressure dialysis-concentrator, using a membrane with a 10,000-MW cut-off against PBS with Ca++ and Mg++ (pH 7.4). The concentrated supernatants (named "SUP-C") were assayed for their ability to alter brain capillary permeability. The protein content of SUP-C was measured by the method of Bradford. Samples of EMEM were concentrated in the same way as the supernatants and used as a control solution.

Assay of Capillary Permeability

To determine the effect of the supernatants on capillary permeability, SUP-C solutions were infused into normal rat brains, then the rats were given 14C-aminoisobutyric acid (14C-AIB). Later, the brains were removed and assayed by QAR. The techniques have been detailed elsewhere (T Ohnishi, et al.: unpublished data). Briefly, male Wistar rats, weighing 300 to 325 gm, were anesthetized with N2O-O2-ethrane and placed in a stereotactic apparatus. Two burr holes were made symmetrically on the frontal bone 1 mm anterior to the bregma and 3 mm bilaterally from the midline. Number 30 needles were inserted into the brain to a depth of 6 mm from the skull surface through the pre-bored holes. The tip of the needles corresponded to the right and left caudate nucleus-putamen. With a Harvard pump, SUP-C was infused through the right needle, and the control EMEM solution through the left needle at a rate of 1 μl/min for 50 minutes. After infusion, the animals were allowed to awaken from anesthesia. The rats were reanesthetized and the right femoral artery and vein were cannulated; the animals were then mounted on a warming block and permitted to recover from anesthesia. Six hours after the intracerebral infusions began, 1 ml of 14C-AIB (100 μCi/ml), adjusted to a pH of 7.4, was injected into the femoral vein as a bolus. Timed blood samples were rapidly collected from the femoral artery and centrifuged, and plasma radioactivity was measured with a liquid scintillation counter. Throughout the experiment, arterial blood pressure and body temperature were monitored and maintained at 125 ± 0.8 mm Hg and 37.1 ± 0.1°C, respectively. Arterial pO2, pCO2, and pH were measured just before and during the experiment, and their values remained within a normal physiological range. Fifteen minutes after 14C-AIB administration, the rats were decapitated, and the brains were processed for QAR and histological examination as described previously.

Calculations and Measurements

Regional measurements of 14C-AIB uptake were performed by QAR using an image digitizer§ and VAX

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* C6 glioma cells purchased from American Type Culture Collection, Rockville, Maryland.

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† 14C-aminoisobutyric acid (50 mCi/mmol) obtained from New England Nuclear, Boston, Massachusetts.
‡ Liquid scintillation counter, Model LS3801, manufactured by Beckman Instruments, Fullerton, California.
§ Image digitizer, Model 785, manufactured by Eikonix Corp., Bedford, Massachusetts.
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750 computer as described previously (T Ohnishi, et al.: unpublished data). Capillary permeability, expressed as a unidirectional blood-to-brain transfer constant, $K$ (µl/gm/min), was calculated from the experimental data according to the following equation:

$$K = \frac{(C_i(T) - V_p \cdot C_p(T))}{\int_0^T C_p(t) \, dt},$$

where $C_i$ is the tissue concentration of $^{14}$C-AIB at time $T$ (nCi/gm of brain tissue), $C_p$ is the arterial plasma concentration (nCi/ml), and $V_p$ is the plasma volume in the region of brain (ml/gm). The value of $V_p$ for capillary permeability was assumed to be 0.005 ml/gm. This relationship assumes no brain-to-blood backflux of AIB during the experimental period.

Treatment Studies with Dexamethasone, Indomethacin, and BW755C

To determine the effects of pretreatment of the animals with glucocorticoid, cyclo-oxygenase inhibitor, and lipoxygenase inhibitor on SUP-C, dexamethasone (10 mg/kg), indomethacin (6 mg/kg) in trihydrate, and 3-amino-1-(m-trifluoromethyl)-phenyl-2-pyrazoline (BW755C, 40 mg/kg), respectively, were administered intraperitoneally 1 hour before intracerebral infusion of SUP-C from EI cells or C6 cells.

To determine the effect of glucocorticoid on the production of capillary permeability factor by tumor cells, SUP-C from EI cells was assayed for capillary permeability activity after EI cells were coincubated with $2 \times 10^{-5}$ M dexamethasone for 4 hours. As a control preparation, SUP-C from the same number of EI cells that were not exposed to dexamethasone was used.

Results

Figure 1 depicts computer-generated pseudocolor images of typical coronal sections of autoradiograms from normal rat brains that were infused with SUP-C (right side of each brain) or EMEM control solution (left side) from four different cell types: two human gliomas, EI (Fig. 1 upper left) and HFA (Fig. 1 upper right); C6 glioma (Fig. 1 lower left); and normal human glia (Fig. 1 lower right). Data from infusions of SUP-C solutions from 4- and 20-hour incubations are shown. In addition, SUP-C from a 4-hour incubation of Walker 256 carcinosarcoma, W256, was also investigated (not shown). The images are expressed in terms of the $^{14}$C-AIB transfer constant, $K$. Supernatants from all tumor cells markedly increased capillary permeability around the inserted needles, whereas that from normal glia produced little increased capillary permeability. Capillary permeability in the contralateral hemispheres, into which control solutions had been infused simultaneously, showed no change. Capillary permeability was increased to a greater degree by SUP-C from 20-hour...
incubations than from SUP-C from 4-hour incubations, especially with respect to tumors EI and HFA (see below).

To quantify the effect of the infusion materials on capillary permeability, mean K values were determined in sequential 0.2-mm (approximate) "rings" around the needle track and these mean K values were plotted as a function of distance from the needle track. Figure 2 illustrates these sequential rings around the needle track (inset) and the K-distribution curves of the five different cell types. There were differences in the distribution curves among the five cell types; the curve from W256 was highest and the curve from the normal human glial cells was almost flat. From these curves, two parameters, K\(_{\text{max}}\) and D\(_{\text{max}}\), were defined as follows: K\(_{\text{max}}\) was the average of the three highest mean K values among the sequence of rings, and D\(_{\text{max}}\) was the maximum distance (in mm) from the needle track at which the mean K values associated with the infused test materials were greater than two standard deviations from the mean of the controls in the contralateral cerebral hemisphere. K\(_{\text{max}}\) indicates the maximum effect of the infusion materials, and D\(_{\text{max}}\) indicates the spatial extent of the effect (that is, diffusion) of the infusion materials.

Values of K\(_{\text{max}}\) and D\(_{\text{max}}\) along with the protein concentration of each SUP-C after 4 hours' incubation of cells are presented in Table 1. The SUP-C from EI cells caused the most intense and the most extensive change of capillary permeability among the three glioma cells, even though the number of plated EI cells (5 \(\times\) 10\(^7\)) was half of that of the others. The Walker 256 carcinosarcoma has been employed as a model of metastatic brain tumor and has been shown to produce a greater increase in the capillary permeability of adjacent tissue than does C\(_6\) glioma when implanted in rat brains.\(^3\) The W256 tumor increased capillary permeability twice as much as did the C\(_6\) glioma. The SUP-C from normal human glial cells did not significantly increase capillary permeability. The SUP-C from W256 contained very large amounts of protein. This may be due to the relatively low viability of the cells when plated (80%), compared to that of other cells (> 95%).

The effect of the SUP-C from 20-hour incubations of glioma cells (EI, HFA, and C\(_6\)) was much more prominent than that of the SUP-C from 4-hour incubations, as illustrated in Fig. 1 and quantified in Fig. 3. Particularly, the SUP-C from human malignant gliomas EI and HFA incubated for 20 hours increased capillary permeability two to three times as much as did the SUP-C from the shorter incubation. The SUP-C from the normal human glia, however, showed no difference in activity between the two incubation times. The protein content in the SUP-C after 20 hours of incubation increased no more than 20% over that obtained after 4 hours of incubation; in fact, the protein content of EI cells after 20 hours of incubation was less than that...
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obtained after 4 hours (data not shown). On the other hand, cell viability (measured by the trypan blue exclusion method) after 20 hours of incubation was not different from that after 4 hours. Thus, the capillary permeability factor activity did not relate to the total amount of protein in SUP-C.

Pretreatment of animals with dexamethasone or BW755C (lipoxigenase inhibitor) significantly inhibited the capillary permeability activity of SUP-C from tumor EI (p < 0.0001 and p < 0.05, respectively; Fig. 4). Indomethacin, which inhibits cyclo-oxygenase activity when it is given at a relatively low dose, had no effect against the SUP-C from C6 glioma. On the other hand, the SUP-C obtained from EI culture, in which the cells were coincubated with dexamethasone, produced capillary permeability activity greater than that without dexamethasone (Fig. 5).

**Discussion**

Previous QAR studies have demonstrated that C6 glioma cells secrete diffusible factors that increase capillary permeability of normal rat brain. One factor, a protein from the conditioned medium of C6 glioma cells, was very potent and its activity was inhibited by glucocorticoids. The protein factor had an MW of more than 10 kD and was sensitive to heat treatment (70°C, 40 minutes). We now report that human malignant gliomas and rat Walker 256 carcinosarcoma also secrete a protein factor with the same characteristics as that of C6 gliomas, while normal glial cells do not secrete the factor.

The activity of the protein factor in the SUP-C depended on the incubation time of tumor cells in serum-free medium. The measurement of cell viability with trypan blue showed that there was no difference in viability between the two incubation times (4 and 20 hours).

**TABLE 1**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Infusion Materials</th>
<th>No. Cells</th>
<th>Protein Concentration SUP-C (μg/ml)</th>
<th>Capillary Permeability</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Kmax (μl/gm/min)</td>
<td>Dmax (mm)</td>
</tr>
<tr>
<td>EI (n = 4)</td>
<td>SUP-C</td>
<td></td>
<td>0.5 x 10^6</td>
<td>1370</td>
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<td></td>
<td>control</td>
<td></td>
<td></td>
<td>5.20 ± 0.27</td>
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<td></td>
<td></td>
<td>3.50 ± 0.23</td>
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<td></td>
<td></td>
<td></td>
<td>2.33 ± 0.17</td>
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<tr>
<td>HFA (n = 3)</td>
<td>SUP-C</td>
<td></td>
<td>1.0 x 10^6</td>
<td>915</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td></td>
<td></td>
<td>2.72 ± 0.33</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.23 ± 0.33</td>
</tr>
<tr>
<td>C6 (n = 2)</td>
<td>SUP-C</td>
<td></td>
<td>1.0 x 10^6</td>
<td>750</td>
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<tr>
<td></td>
<td>control</td>
<td></td>
<td></td>
<td>4.74 ± 0.13</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>3.00 ± 0.10</td>
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<td></td>
<td></td>
<td>2.43 ± 0.30</td>
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</tr>
<tr>
<td>W256 (n = 3)</td>
<td>SUP-C</td>
<td></td>
<td>1.0 x 10^6</td>
<td>4550</td>
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<tr>
<td></td>
<td>control</td>
<td></td>
<td></td>
<td>6.23 ± 0.12</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt; 4.0</td>
</tr>
<tr>
<td>HNG (n = 3)</td>
<td>SUP-C</td>
<td></td>
<td>1.0 x 10^6</td>
<td>525</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td></td>
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<td>2.91 ± 0.31</td>
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<td></td>
<td></td>
<td>0.57 ± 0.07</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2.29 ± 0.32</td>
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</table>

*Capillary permeability is expressed as Kmax and Dmax (means ± standard error of the means), see text. Dmax could not be measured at distances greater than 4 mm because of the geometry of the rat brain. EI and HFA are human malignant cell lines; C6 = C6 glioma; W256 = Walker 256 carcinosarcoma; HNG = normal human glia; n = number of experiments; SUP-C = concentrated supernatant. Significance of difference: † = p < 0.05, ‡ = p < 0.001 compared with control values (t-test analysis).

The activity of the protein factor in the SUP-C depended on the incubation time of tumor cells in serum-free medium. The measurement of cell viability with trypan blue showed that there was no difference in viability between the two incubation times (4 and 20 hours).
hours), while incubation for more than 48 hours in serum-free medium decreased cell viability and increased the number of floating cells. Moreover, the protein contents of supernatants after 20 hours of incubation differed little from those after 4 hours of incubation. These results indicate that the higher activity observed in SUP-C after longer incubation was not due to the release of intracellular components by cell lysis from damaged cells; instead, tumor cells actively secreted more factor with time. It is interesting, however, that conditioned medium from confluent cells (matched for the cell number) showed only a slight increase of capillary permeability (data not shown). On the other hand, such a time-dependent increase in activity of SUP-C as seen in glioma cells was not observed in the supernatant of normal glial cells.

It is well known that glucocorticoids can reduce peritumoral brain edema in both human brain tumor and experimental brain tumor. Studies of experimental brain tumors have indicated that glucocorticoids reduce increased capillary permeability in the tumor tissue and the adjacent brain tissues; however, the exact mechanism of the hormone's action is not known. The results from our dexamethasone pretreatment study suggested that glucocorticoids can reduce capillary permeability by directly acting on the capillary endothelial cells. The fact that pretreatment with a cyclo-oxygenase inhibitor (indomethacin) did not inhibit the activity of the factor, while a lipoxygenase inhibitor (BW755C) suppressed the activity leads to the following hypothesis. The anti-edema effect of glucocorticoids in peritumoral brain edema functions through the inhibition of membrane phospholipase A2 by the hormone. Further, lipoxygenase products (including leukotrienes) may be responsible for the effect of the protein factor on brain capillary permeability. The observation by Black, et al., that the level of leukotriene C4 is increased in human glioblastomas supports this hypothesis. Coincubation of tumor cells with dexamethasone enhanced the capillary permeability activity of the SUP-C, but the mechanism of action of the hormone on the production of the capillary permeability factor is unknown.

It has been reported that some tumor tissues produce high-MW substances that increase capillary permeability. These include: tumor plasminogen activator, proteases such as trypsin, pronase, and collagenase, and vascular permeability factor (VPF). The capillary permeability factor reported here in malignant gliomas and Walker 256 carcinosarcoma differed in several ways from these substances. Our factor demonstrated a delayed onset of activity of 90 minutes with reversibility by 24 hours. The activity was parallel to the intensity of chemotactic response which was observed at the infused area (data not shown). The production of the factor was not inhibited by coincubation of tumor cells with dexamethasone, but rather enhanced by such treatment.

Recently, Bruce, et al., and Criscuolo, et al., have reported the presence of a VPF in serum-free conditioned medium from human malignant glioma cultures. The effect of the VPF was measured using a Miles assay in guinea-pig skin. Cycloheximide or dexamethasone inhibited the expression of this activity in vitro. The VPF was an acid-stable heat-labile macromolecule that was inactivated by trypsin and pepsin but unaffected by ribonuclease-A. Further studies have suggested that it was a cationic polypeptide with a MW of 41 to 56 kD. Pretreatment of guinea pigs with systemic dexamethasone prior to VPF injection markedly diminished the microvascular permeability. The VPF activity was not inhibited by coinjection of VPF with antihistamine (anti-H1 with or without anti-H2). It appears that VPF binds to heparin, and its endothelial cell binding is inhibited when cell-surface ganglioside (sialic acid) residues are destroyed by enzymes or blocked by the β-subunit of cholera toxin. While differing somewhat from our permeability factor (which is not inhibited in vitro by dexamethasone), the two substances may be closely related. It would be valuable to test the VPF in a brain system in addition to the intradermal permeability test in guinea-pig skin.

These studies clearly indicate that brain tumors produce factors that can alter capillary permeability in the surrounding brain tissue. Such alterations are likely to be the source of peritumoral cerebral edema. The factors may also be essential for tumor growth, particularly for tumor invasion into the normal brain tissue where
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there are no leaky tumor vessels, because generation of tumor stroma always requires host plasma proteins which usually cannot cross normal blood-brain barrier. Increased capillary permeability may also produce an expanded extracellular space where tumor cells can move more easily away from the tumor mass. Further investigation and identification of capillary permeability factors in brain tumors may permit the development of new therapeutic strategies, not only for peritumoral brain edema but also for eradication of malignant brain tumors.

References

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