Changes of the blood-brain barrier in experimental metastatic brain tumors

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An experimental model for blood-borne cerebral metastases was developed by introducing Walker 256 carcinoma cells selectively into the intracranial internal carotid artery of rats. This model was used to study the regional capillary permeability of rat brain and metastatic brain tumors of various sizes with the aid of $^{14}$C alpha-aminoisobutyric acid (AIB) quantitative autoradiography. The regional capillary permeability varied with the anatomical location and size of the tumor. Intraparenchymal tumors less than 1 mm in diameter showed no increased permeability to AIB. As the tumors enlarged over 1 mm in diameter, the permeability in the intraparenchymal tumors increased proportionally, but remained less than one-third of capillary permeability of subcutaneously transplanted tumors. Capillary permeability in the peripheral invasive part and necrotic center was less than in the viable part of large tumors. Capillary permeability in metastatic tumors of the choroid plexus and meninges was significantly higher than in tumors of the brain parenchyma. The results suggest that the uptake of chemotherapeutic agents that do not cross the blood-brain barrier easily varies with the anatomical location and size of the metastatic tumors.

Key Words • metastatic brain tumor • experimental tumor model • metastasis • blood-brain barrier • autoradiography • chemotherapy

Recent advances in the diagnosis and treatment of cancer have resulted in prolonged survival times of patients with various malignancies. However, there has been a concomitant increase in the incidence of metastases to other organs, including the central nervous system (CNS). In patients with metastatic brain tumors, two major therapeutic modalities are surgery and radiation therapy. In view of the systemic nature of metastatic disease, chemotherapy is the most reasonable therapeutic approach; however, there is little evidence to suggest that chemotherapy is efficacious in the management of cerebral metastases. Effective management of patients with brain tumors necessitates a clear understanding of the pharmacokinetics and delivery of the chemotherapeutic agents to the target site. However, little is known about the pharmacokinetics of metastatic brain tumors. This lack of knowledge is partly attributable to difficulties encountered in clinical studies, such as the heterogeneity of the patient population and the variety of complications, and partly to the lack of adequate experimental models.

We have developed a model involving a blood-borne metastatic brain tumor by injecting tumor cells selectively into the intracranial vessels of rats. With this model, we studied blood-brain barrier changes in metastatic brain tumors, using carbon-14 ($^{14}$C) alpha-aminoisobutyric acid (AIB) quantitative autoradiography.

Materials and Methods

Animal Model

Walker 256 carcinoma was obtained from the Department of Oncologic Surgery of the Research Institute for Microbial Diseases, Osaka University, and maintained in our laboratory by serial subcutaneous transplantation every 2 weeks. Single-cell suspensions were made from solid subcutaneous tumors under aseptic conditions. The tumors were minced with fine scissors in Earle's basic medium and passed first through 40-mesh and then 80-mesh stainless steel screens. The trypan blue exclusion test revealed that the resulting suspension contained 0.5 to 1 x $10^7$ viable cells/ml.

Female Wistar-Imamichi rats, weighing 150 to 200 gm each, were anesthetized intraperitoneally with ketamine (50 mg/kg body weight). The carotid artery
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system was exposed with a microsurgical technique, and the external carotid artery (ECA) and the pterygopalatine artery, which is a large branch of the internal carotid artery, were ligated. A No. 26 needle was introduced from the stump of the ECA into the common carotid artery, and 0.01 to 0.02 ml of the tumor cell suspension containing $1 \times 10^5$ viable cells was injected (Fig. 1). We chose this amount of inoculum because preliminary experiments had shown that, when more than $2 \times 10^5$ cells were injected, some animals developed cerebral infarction; at an inoculum size below $0.5 \times 10^5$ cells, the incidence of tumor take decreased. After the needle was withdrawn, the puncture site on the ECA was coagulated with bipolar forceps for hemostasis.

The rats received food and water ad libitum, and were used for the experiment 8 to 21 days after tumor cell inoculation. By that time they had manifested weight loss and neurological signs of CNS involvement. Sixteen inoculated animals were observed until death, and their tissues were studied histopathologically. Six additional rats were injected subcutaneously with $1 \times 10^5$ cells after carotid injection.

Quantitative Autoradiography

The technique used was essentially the same as that described in preceding reports. Twenty animals with metastatic brain tumors were anesthetized with ketamine, the right femoral artery and right jugular vein were catheterized with Silastic tubes, and their blood pressure and rectal temperature were monitored. An injection of $^{14}$C-AIB (100 μCi/kg of body weight), dissolved in 1 ml of normal saline, was given intravenously over 1 minute at a constant flow rate. Arterial blood was sampled every 2 minutes via an arterial catheter, and plasma radioactivity was measured by a liquid scintillation counter.* The animals were killed by the rapid intravenous injection of saturated potassium chloride solution 10 minutes after radioisotope infusion. They were decapitated and the brain and subcutaneous tumor were rapidly removed, frozen in Freon, and stored at $-40^\circ$C until sectioning. A cryomicrotome† was used to cut 60 μ-thick sections of the brain and subcutaneous tumor at $-10^\circ$C. These were placed on cover slips, and dried rapidly on a warming plate at $60^\circ$C. The cover slips were then mounted on pressed paper boards and attached to single-coated Kodak SB-5 x-ray film with $^{14}$C methyl-methacrylate standards, which had been calibrated to reference 60 μ-thick brain sections with known radioactivity.

After 2 weeks of exposure, the x-ray films were read with a densitometer equipped with a 0.5-mm aperture.‡

* Mark III liquid scintillation counter manufactured by Tracor Analytic, Chicago, Illinois.
‡ Sakura PDA-15 densitometer manufactured by Konishiroku, Tokyo, Japan.

The optical density of a region was determined by averaging the values obtained at no less than three different sites. A calibration curve for the relationship between the optical density and the tissue $^{14}$C concentration for each film was obtained by densitometric measurement of the standards. The radioactivity of the various regions of the brain and tumor was calculated for each densitometric measurement. The calculation of a unidirectional blood-to-brain transfer constant, $K_i$, was based on the equation

$$K_i = \frac{C_b (T)}{J_0 C_p dt}$$

where $C_b$ is the concentration of the labeled material in the brain at the end of the experimental period $T$, and $C_p$ is the plasma concentration of the tracer. According to Blasberg, et al., this equation can be used to calculate $K_i$ if the experimental period is very short and the indicator material is essentially trapped in the tissue by metabolic and/or transport processes. For this purpose, AIB is the most suitable substance because it is a non-metabolized amino acid and is trapped in brain cells by the neutral amino acid transport mechanisms in the cell membrane.

Tissue sections used for autoradiography were stained with hematoxylin and eosin for histological study.
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FIG. 2. Autoradiographic (left) and histological (right) appearance of a small intraparenchymal metastatic tumor (< 1 mm diameter, arrow). There is no increase in capillary permeability in response to alpha-aminoisobutyric acid.

Results

Animal Model

The rats were checked daily. Starting with the 7th day after inoculation, they began to lose weight and became progressively less active. Their fur was raised and pigmentation was present around the eyes. No animals manifested extracranial tumors. Animals not killed for autoradiographic study died 8 to 26 days after tumor inoculation. In 77.3% of the animals receiving carotid tumor cell injection, we noted intracranial tumors. Many of these were intraparenchymal tumors; however, 58.3% of the rats manifested choroid plexus and meningeal tumors. Most of the intraparenchymal tumors were well demarcated but there were some ill-defined invasive tumors. Necrosis was present in only some of the large intraparenchymal tumors.

Quantitative Autoradiography

Among 22 rats, we counted 40 intraparenchymal, 20 choroid plexus, and 11 meningeal tumors. The capillary transfer constants of tumors of various sizes and of regional brain tissues are summarized in Table 1. The capillary permeability of intraparenchymal tumors varied with tumor size. Small tumors demonstrated no increase in permeability to AIB (Fig. 2). However, as tumor size increased, so did capillary permeability; a leveling off of permeability was noted in tumors with a diameter exceeding 4 mm (Figs. 3, 4, and 5). Even the highest capillary permeability observed in large intraparenchymal tumors ($60.2 \times 10^{-3}$ ml/gm/min) was less than one-third of the capillary permeability in subcu-

![Graph showing capillary permeability of intraparenchymal tumors of various sizes in rat brain.](image)

**TABLE 1**

Regional capillary transfer constants (Ki) for AIB in metastatic brain tumors and brain tissues.

<table>
<thead>
<tr>
<th>Region</th>
<th>Transfer Constants $^\dagger$</th>
<th>No. Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>metastatic tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>intraparenchymal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>large tumors (≥ 4 mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>viable portion</td>
<td>$44.7 \pm 3.2$</td>
<td>8</td>
</tr>
<tr>
<td>necrotic center</td>
<td>$35.7 \pm 1.5$</td>
<td>4</td>
</tr>
<tr>
<td>medium tumors (1–4 mm)</td>
<td>$25.7 \pm 2.3$</td>
<td>18</td>
</tr>
<tr>
<td>small tumors (≤ 1 mm)</td>
<td>$3.8 \pm 0.6$</td>
<td>7</td>
</tr>
<tr>
<td>invasive tumors</td>
<td>$15.8 \pm 2.7$</td>
<td>8</td>
</tr>
<tr>
<td>choroid plexus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>large tumors</td>
<td>$178.6 \pm 9.2$</td>
<td>4</td>
</tr>
<tr>
<td>medium tumors</td>
<td>$98.5 \pm 21.9$</td>
<td>8</td>
</tr>
<tr>
<td>small tumors</td>
<td>$53.6 \pm 3.8$</td>
<td>8</td>
</tr>
<tr>
<td>meningeal tumors</td>
<td>$89.4 \pm 15.5$</td>
<td>11</td>
</tr>
<tr>
<td>brain tissue $^\ddagger$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>adjacent to large tumors</td>
<td>$6.3 \pm 0.5$</td>
<td>8</td>
</tr>
<tr>
<td>remote normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cortex</td>
<td>$4.2 \pm 0.5$</td>
<td>22</td>
</tr>
<tr>
<td>white matter</td>
<td>$1.2 \pm 0.2$</td>
<td>22</td>
</tr>
<tr>
<td>choroid plexus</td>
<td>$47.8 \pm 5.4$</td>
<td>22</td>
</tr>
<tr>
<td>pineal body</td>
<td>$52.5 \pm 3.5$</td>
<td>22</td>
</tr>
<tr>
<td>tuber cinereum</td>
<td>$37.8 \pm 5.4$</td>
<td>14</td>
</tr>
<tr>
<td>area postrema</td>
<td>$11.5 \pm 2.1$</td>
<td>4</td>
</tr>
<tr>
<td>subcutaneously transplanted tumors</td>
<td>$212.2 \pm 18.6$</td>
<td>6</td>
</tr>
<tr>
<td>viable portion</td>
<td>$25.9 \pm 3.8$</td>
<td>4</td>
</tr>
<tr>
<td>necrotic center</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^*$ Except in the case of small intraparenchymal tumors, all Ki values of the metastatic tumors were significantly different ($p < 0.01$) from normal rat cortex. AIB = alpha-aminoisobutyric acid.

$^\dagger$ Values are mean ± standard error ($\times 10^{-3}$ ml/gm/min).

$^\ddagger$ Brain tissue not involved by tumor.
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taneously transplanted tumors \( (212.2 \times 10^{-3} \text{ ml/gm/min}) \). Capillary permeability was decreased in the necrotic center of large tumors (Fig. 6), and in the peripheral invasive portion of invasive tumors (Fig. 7). Metastatic lesions in the choroid plexus and meninges showed high capillary permeability even when they were small (Fig. 8). Capillary permeability in large choroid plexus tumors was similar to that seen in subcutaneous tumors (Fig. 6). The choroid plexus, pineal body, tuber cinereum, and area postrema (which were not involved in the tumor) also showed relatively high capillary permeability. A small increase in capillary permeability was observed in brain tissue immediately adjacent to large tumors.

Discussion

**Experimental Model**

For studies on the blood-brain barrier in metastatic brain tumors, especially in the early stages of metastasis, a better physiological tumor model is needed than the conventional ones, which involves direct tumor implantation into the brain. A few models of blood-borne metastatic rat brain tumor have been reported.\(^{1,2,14,18}\) Ushio, et al.,\(^{16}\) developed a model of hematogenously spread metastatic brain tumor by injecting Walker 256 carcinoma cells into the common carotid artery of rats. In their model, cyclophosphamide was used to reduce the incidence of extracranial tumor due to extensive local extracranial disease which developed even after prior ligation of the ECA. The internal carotid artery of rats has a large branch, the pterygopalatine artery, which corresponds to a portion of the internal maxillary branch of the ECA in man.\(^{9}\) The blood flow in the pterygopalatine artery is greater than in the distal internal carotid artery after branching off of the former vessel which supplies blood widely to the skull base, orbit, and pharynx.\(^{19}\) By ligating the pterygopalatine artery as well as the ECA, all tumor cells injected into the common carotid artery can be introduced selectively into the intracranial internal carotid artery. In our model, not a single extracranial tumor was detected clinically or pathologically, and intracranial metastatic tumors (most of which were intraparenchymal) developed consistently. The ease of preparing our model and its reliability make it a valuable research tool for the study of blood-borne metastatic rat brain tumors.

**Blood-Brain Barrier and Chemotherapy of Metastatic Brain Tumors**

The development of effective chemotherapy in patients with brain tumors largely depends on a better understanding of the pharmacokinetics of the chemotherapeutic agents used. The blood-brain barrier is a major factor affecting drug delivery to the CNS. Vick
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Figure 6. Autoradiographic (upper) and histological (lower) appearance of two large metastatic tumors in the choroid plexus and cortex. Note the difference in capillary permeability between the two tumors of approximately the same size. The intraparenchymal tumor contains necrotic foci (arrows) with relatively low capillary permeability.

Figure 7. Autoradiographic (upper) and histological (lower) appearance of an infiltrative metastatic tumor. Note the slight increase in capillary permeability in the infiltrative area (arrowheads). The solid portion (arrow) of the tumor shows high capillary permeability.

and Bigner20 reported that the blood-brain barrier is not a factor in the chemotherapy of brain tumors, as they observed the free passage of peroxidase through the discontinuous capillary endothelium. Other workers11,13 observed fenestrated and discontinuous endothelium in human metastatic and primary brain tumors. Those morphological studies, however, lack quantitative data regarding the frequency and distribution of defects.

Recently, Blasberg, et al.,5 developed a quantitative autoradiographic technique that facilitates measuring the local capillary permeability in brain regions with a diameter as small as 100 to 200 μ. Using their method and our model, we investigated the capillary permeability in and around metastatic tumors in the CNS. We found that capillary permeability of intraparenchymal tumors varied with tumor size. Small tumors (less than 1 mm) showed no increase in capillary permeability compared to the surrounding brain tissue; however, as tumor size increased, so did capillary permeability. This change may be due to endothelial alterations brought about by tumor cells in the pericapillary space,1 but may be mainly due to newly formed abnormal capillaries with disrupted endothelium.11,13 Folkman8 also observed the formation of new vessels in tumors exceeding 2 to 3 mm in diameter. Blasberg, et al.,5 also demonstrated an increase in capillary permeability with increase in tumor size where metastatic brain tumors were induced in rats treated with cyclophosphamide, and Yamada, et al.,23 observed a similar phenomenon in rat glioma. The linear increase in capillary permeability with size of intraparenchymal metastatic brain tumors was not without limitation, however. The greatest capillary permeability we observed in large intraparenchymal tumors was less than one-third of the capillary permeability of subcutaneously transplanted tumors. On the other hand, the capillary permeability of large tumors in the choroid plexus was almost as high as in subcutaneously transplanted tumors. Therefore, even in large intraparenchymal tumors, the blood-brain barrier seems to be partially functioning. Irrespective of whether a tumor was located in the parenchyma or in the choroid plexus, capillary permeability was lower in the invasive portion than in the center of the solid part. These findings indicate that the state of the blood-brain
Our findings and those of others indicate that there is no single effective agent for the chemotherapeutic treatment of metastatic brain tumors. The drug or combination of drugs must be chosen individually, based on tumor sensitivity and size, and the nature and anatomical location of the metastatic brain tumor.

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References


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