Sequential Development of Hematogenous Cerebral Metastatic Tumors in Rats

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Abstract

To study the sequential growth patterns of hematogenous cerebral metastatic tumors, syngenetic RG2 glioma cells were injected into the right common carotid artery of 61 adult CDF rats.

Electron microscopic study 1 hr and 24 hrs after injection revealed arrested tumor cells in the capillaries, some with faint glial filaments in their cytoplasm. Perivascular edema of the right hemisphere which developed following occlusion of the right common carotid artery during injection was noteworthy concerning the attachment of the tumor cells to the capillary walls and their penetration into the perivascular spaces. Luminescence microscopic observation with acridine orange 1 hr and 24 hrs after injection showed circulating or embolized tumor cells in the capillaries, and about 5 days after injection, the cells began to penetrate the perivascular space. For light microscopic examination, 51 rats were sacrificed from 1 hour to 20 days after injection. From 9 days after injection on, microtumors began to appear and metastatic tumors developed in 76% of the right hemispheres, and in 57% of the left hemispheres. Considering all sections of the 21 rat brains from 9 to 20 days, the difference of tumor frequency between the right (42%) and left (19%) hemispheres became even more significant. Morphometrical evaluation using a planimeter showed the growth curve of the tumor area to have a low pitch from 12 to 18 days which became steeper from 18 to 20 days.

Synthesizing the results sequentially, the following five stages could be defined: 1) a silent period; 2) a penetrating and mitotic period; 3) a microtumor period; 4) a multiple proliferating period; and 5) an expanding period.

Key words: cerebral metastasis, experimental metastatic model, glioma, ultrastructure, syngenetic cells

Introduction

According to recent reports, the incidence of metastatic tumors in all brain tumors is very high, from 13 to 37%. However, current methods for prevention and treatment of metastatic brain tumors have not yet been well established and prognosis is extremely poor at present. Though animal experiments on cerebral metastatic tumors have recently been reported using various models, very few detailed studies have described the sequential development of hematogenous cerebral metastasis.

The aim of the present study was to establish a metastatic model in rat brains by injection of syngenetic cloned RG2 glioma cells into the right common carotid artery using an operative microscope. Using this model, hematogenous cerebral metastatic tumors were consistently produced, and 61 rats were sacrificed at...
various intervals from an early stage (1 hour) to the terminal stage (20 days) after injection. Then the brains were examined histologically, including luminescence and electron microscopic observations, to determine the sequential growth pattern of the injected tumor cells.

Materials and Methods

I. Tumor model

Primary brain tumors were induced by a single transplacental application of 50 mg/kg ethylnitrosourea into CDF-Fischer rat fetuses. One of these brain tumors was propagated in cell culture and the cell line (RG₂) was cloned. It was recently characterized as a glioma in tissue culture.¹⁰

To prepare the tumor cells for injection, the cells were removed with 2 ml 0.05% trypsin and 0.02% EDTA (Ethylenediamine Tetraacetic Acid) solution. To activate the trypsin, fresh Earle MEM (Minimum Essential Medium) was added. The cells were counted using a cell counter and then 1 x 10⁶ cells were placed in 0.3 ml MEM. These cell suspensions were kept in disposable syringes with small plastic catheters for injection.

Sixty-one adult female CDF-Fischer rats weighing 200 to 250 g were used. After anesthesia with 0.5 ml/kg Hypnorm (Beerse, Belgium), the rats were laid out in a supine position. The right common carotid artery was exposed and the right external carotid artery was isolated and ligated. A thin plastic catheter (Ø 0.7 mm) was inserted into a small incision in the right common carotid artery in the cephalic direction. 1 x 10⁶ cells in 0.3 ml MEM were slowly injected by hand through the common carotid artery into the internal carotid artery. The common carotid artery was ligated while withdrawing the catheter. It should be stressed that the right common carotid artery was occluded completely so that the tumor cells were directed to the brain through the internal carotid artery, diminishing the number of cells entering the external carotid artery which reduced the incidence of scalp, face, and neck tumors. All operations were performed under a Zeiss operating microscope (FRG).

II. Light microscopic and morphometric evaluation experiment

A total of 51 rats were inoculated. Three rats were sacrificed at: 1 hr, 6 hrs and 1–10, 12, 14, 16, 18 and 20 days after the tumor injection. Whole brains except spinal cords were removed and fixed in 6% formalin.

For the histological observations, the brains were cut coronally at every 2 mm and embedded in paraffin. Seven blocks were usually obtained and seven sections from each rat were stained with hematoxilin-eosin. These seven sections approximately corresponded to the frontal pole, frontal, central, parietal, occipital with midbrain, cerebellum with pons, and medulla.

Photographs of the seven sections of tumor recognized brains were taken with a Leitz Focusing Bellows microscope (FRG) and the area of tumor nodules was measured with a Videoplan planimeter (Kontron, FRG). The ratio of the area of tumor nodules to the right hemisphere was calculated. A growth curve of the tumor area was made.

III. Luminescence microscopic experiment with acridine orange

Tumor cells were exposed to 0.01% acridine orange in tissue culture. Tumor cells (1 x 10⁶) including acridine orange in 0.3 ml MEM were prepared and injected into six rats by the same method as described above. A seventh control rat was injected with RG₂ cells without acridine orange and sacrificed 1 hour after injection. Two rats were sacrificed at each 1 hr, 24 hrs, and 5 days after injection. The parietal brains were quickly frozen in 2-methyl-butan at −70°C and sectioned by cryostat (Slee Medical Equipment Co., Ltd., England) at 4 μm, and then mounted with 15% glycerin. Photographs were taken under a Leitz Orthomat-W fluorescein microscope (FRG) to determine the distribution of tumor cells in the capillaries and brain parenchymal tissues.

IV. Electron microscopic experiment

Two rats in each group were sacrificed 1 hr and 24 hrs after injection. A fifth control rat was injected with medium without cells and killed 15 minutes after injection. Parts of cortex, subcortex, and basal ganglia of both hemispheres of each brain were fixed with 2.5% glutaraldehyde for 3 hours and post-fixed for 2 hours with 1% osmium tetroxide. After dehydration with graded alcohol, they were embedded in Epon and thin sections were cut with an ultramicrotome. The ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM9 electron microscope (FRG). To accurately identify the injected tumor cells growing within the brain tissues, tissue culture cells were also studied under electron microscope. A well grown monolayer of cells were removed from a petri dish by trypsinisation and centrifuged at 2,000 rpm for 15 minutes. The supernatant was removed and the tumor cells of the sediment were prepared, i.e., fixed, embedded, and impregnated, in quite the same manner as described above.

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Results

1. Light microscopic examinations

In the relatively early stage from 1 hour to 8 days after injection, some cells were suspected to be injected tumor cells, but absolute identification was difficult. Starting on the 9th day post-injection, microtumors composed of several cells were found under light microscope (Fig. 1). Clusters of several tumor cells including mitotic cells apparently expanded the surrounding brain tissues. From 12 days after injection, small multifocal tumor nodules (Fig. 2) could be distinctly observed not only in the right, but also in the left hemisphere, although with less frequency. These tumor nodules increased gradually in number and extent and at the terminal stage, 20 days after injection, huge multiple tumor nodules enlarged the right hemisphere leading to compression of the left hemisphere (Fig. 3). In these metastatic nodules, tumor cells had round or ovoid nuclei with abundant chromatin and showed polygonal or fusiform shapes. Atypia, polymorphism, and mitoses of tumor cells were seen, which indicated their malignant character (Fig. 3B). Neovascularization, endothelial proliferation, necrosis, and cyst formation were minimal, even in specimens taken 20 days after injection.

In the right hemisphere the metastatic tumors were frequently located in both the central (76%) and parietal (62%) areas, followed by the occipital with midbrain (57%), frontal (52%), frontopolar (29%), and cerebellar (14%) regions (Table 1). No tumor was seen in the medulla. This order was also the same in the left hemisphere. From 9 days after injection, when the microtumor was first found, to 20 days after injection, the frequency of tumor bearing rats was 76% in the right hemisphere and 57% in the left (Table 2). Considering the incidence in all sections (n = 147) of 21 rat brains from 9 to 20 days after injection, the difference between the right (42%) and left (19%) hemispheres became very significant (Table 1).
in the lung and liver, although systemic examination of these organs was not performed.

### II. Morphometric evaluation of the tumor areas

The ratio of the tumor areas to the right hemispheres from 9 to 20 days after injection were measured with a planimeter (Table 3). Then the percentages of each group were calculated assuming that the ratio of the areas 20 days after injection was 100%, and a growth curve of tumor area was made (Fig. 4).

#### Table 1 Incidence of metastatic tumors in seven sections of each group from 9 to 20 days after injection

<table>
<thead>
<tr>
<th>Days after injection*</th>
<th>Hemisphere</th>
<th>Sections of rat brains</th>
<th>Total (%)</th>
<th>(n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>fronto-polar</td>
<td>frontal</td>
<td>central</td>
</tr>
<tr>
<td>9</td>
<td>rt.</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>lt.</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>rt.</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>lt.</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>rt.</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>lt.</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>14</td>
<td>rt.</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>lt.</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>rt.</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>lt.</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>18</td>
<td>rt.</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>lt.</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>20</td>
<td>rt.</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>lt.</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total (%)</td>
<td>rt.</td>
<td>6 (29)</td>
<td>11 (52)</td>
<td>16 (76)</td>
</tr>
<tr>
<td></td>
<td>lt.</td>
<td>2 (10)</td>
<td>6 (29)</td>
<td>11 (52)</td>
</tr>
</tbody>
</table>

*: 3 rats were used in each group.

### Table 2 Numbers of cerebral metastatic tumor bearing rats from 9 to 20 days after injection

<table>
<thead>
<tr>
<th>Days after injection</th>
<th>Hemisphere</th>
<th>Numbers of rats with tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>rt./lt.</td>
<td>2/1</td>
</tr>
<tr>
<td>10</td>
<td>rt./lt.</td>
<td>2/0</td>
</tr>
<tr>
<td>12</td>
<td>rt./lt.</td>
<td>2/2</td>
</tr>
<tr>
<td>14</td>
<td>rt./lt.</td>
<td>2/2</td>
</tr>
<tr>
<td>16</td>
<td>rt./lt.</td>
<td>3/2</td>
</tr>
<tr>
<td>18</td>
<td>rt./lt.</td>
<td>3/3</td>
</tr>
<tr>
<td>20</td>
<td>rt./lt.</td>
<td>2/2</td>
</tr>
<tr>
<td>Total (%)</td>
<td>rt./lt.</td>
<td>16 (76)/12 (57)</td>
</tr>
</tbody>
</table>

*: 3 rats were used in each group.

in the lung and liver, although systemic examination of these organs was not performed.

### III. Luminescence microscopic examinations with acridine orange

In a preliminary experiment, the activity of acridine orange with the tumor cells in tissue culture could be recognized under fluorescence microscope up to 6 days after application. So it seemed that acridine orange could be used for observation *in vivo* within nearly the same period.

Round tumor cells were scattered or embolized in the capillaries of the rat brain 1 hour after injection (Fig. 5). Tumor cells circulated not only in the right, but also...
in the left hemisphere, probably through the collateral circulation. Nearly the same findings were seen in the brain 24 hours after injection, although the numbers of tumor cells had decreased.

Five days after injection, scattered tumor cells were found not only in the capillaries, but also in the brain parenchymal tissues. Moreover, in some cells their round shape observed within 24 hours after injection had become uneven, showing similarities to the processes and bodies of cells (Fig. 6).

IV. Electron microscopic examinations
Arrested cells in the capillaries were often found, but it was difficult to differentiate the tumor cells from the usual blood cells such as leucocytes, lymphoid cells, and macrophages. To determine the ultrastructural characteristics of the tumor cells, electron microscopic observation of the sediment from tumor tissue culture was performed. The nuclei of the tumor cells in the sediment showed smooth round or ovoid forms with nucleoli and heterochromatin, but few indentations (Fig. 7A). Their cytoplasm contained abundant mitochondria, ribosomes, and rough surfaced endoplasmic reticula, but faint glial filaments were found only in restricted cells (Fig. 7A). Various stages of mitotic cells were also seen. Comparison between the ultrastructural features of tumor cells in sediment and of arrested cells in the capillaries was performed to identify the arterial injected tumor cells. Some cells in the capillaries which showed similar ultrastructural features as the tumor cells were considered injected tumor cells (Figs. 8 and 9A). Some had faint glial filaments in their cytoplasm (Fig. 8). Moreover, remarkable swelling of the perivascular spaces and astrocytic feet 1 hr and 24 hrs after injection was noteworthy (Figs. 8 and 9A, B), being minimal in the left hemisphere (Fig. 7B). Twenty-four hours after injection, heterochromatin and chromosome-like substances were seen in the embolized cells (Fig. 9C, D). Tumor cell penetration into the
brain parenchymal tissues from the capillary lumens could not be found.

Discussion

Various kinds of blood cells are usually seen in the capillaries of rat brains. To identify the injected tumor cells under the electron microscope, morphological comparison between arrested cells in the capillaries and tumor cells of the sediment from tissue culture was performed. Few of the tumor cells in tissue culture, however, had the very faint glial filaments specific for glial cells, although this cell line had been recently characterized as glioma in tissue culture. So glial filaments could not be used as a marker to identify the cells in rat brain tissues.

Nevertheless, the authors could identify cells in the rat brain tissues 1 hr and 24 hrs after injection which had nearly similar ultrastructural features as the tumor cells. These injected cells had mitochondria, ribosomes, and faint glial filaments in their cytoplasm. In addition, their lysosomes and cytoplasmic dense bodies were more prominent than those of the tumor cells in tissue culture. It seemed reasonable to assume that these cells were undergoing degeneration. According to both Machinami and Ballinger et al., such degenerative tumor cells are often found in the capillaries also. They indicated that most of the arrested tumor cells would degenerate and that only a few tumor cells were able to proliferate. Ballinger et al. found platelet aggregates and fibrin in the cytoplasm, which are considered to have an important role in the attachment of tumor cells to the vascular wall. In the present study, however, such substances were not seen.

Another interesting finding was a remarkable perivascular edema in the right hemisphere which was minimal in the left hemisphere. This could be the result of ischemia by occlusion of the right common carotid.
artery during surgery, although clinical and light microscopic evidence was not manifest. To proliferate and form metastatic nodules in the brain, first, injected tumor cells should attach to the capillary wall and then penetrate into the perivascular spaces. We have not yet found this mechanism and many factors may be involved. The perivascular edema following ischemia and its effects may be one such factor.

Penetration of tumor cells through the capillary wall into brain parenchymal tissues could not be found. Despite screening enormous numbers of brain sections, Ballinger et al. were not able to document this event and so assumed that this process was relatively rapid. Autoradiographic and histochemical techniques may be necessary for such examination.

Acridine orange is an efficient metachromatic fluorochrome that binds with both deoxyribonucleic acid and ribonucleic acid. Acridine orange has been used as a vital stain in dilute solution. Luminescence microscopic observation with acridine orange in the specimen sacrificed 1 hour after injection revealed the tumor cells circulating in the capillaries, and some of them seemed to be embolized. Tumor cells were also found in the left hemisphere, and this is reasonable in light of the collateral circulation. Twenty-four hours after injection, the numbers of cells decreased which might be due to cell death or to their circulation into other organs.

Five days after injection, the shape of the tumor cells had changed from evenly round in the early stages into irregular shaper which seemed similar to the processes and bodies of cells. At the same time, moreover, tumor cells had scattered out of the capillary lumens into the brain parenchymal tissues. From these findings, it was suspected that tumor cells attached to the capillary walls began to penetrate into the perivascular space at around 5 days post-injection.

Microtumors composed of several cells were first seen under light microscope 9 days after injection. Thus tumor cells in the perivascular space might begin to mitose and proliferate during 5 to 8 days after injection. From 12 days on, many small tumors were recognized in cortex, subcortex, and basal ganglia of the cerebral hemisphere. The incidence of tumor nodules was quite low in the cerebellum and brain stem, which is reasonable in view of the territory of the internal carotid artery. Metastatic nodules gradually increased both in number and extent, and 20 days after injection they were nearly fatal for the rats.

According to the morphometrical evaluation, the growth curve of the ratio of the area of tumor nodules to the right hemisphere showed no direct relationship to time. From 12 to 18 days after injection, it showed a relatively low pitched curve which became steep from 18 to 20 days after injection; a rather quick extension leading quickly to death.

In conclusion, the following hypothesis for the sequential growth pattern of metastatic brain tumors from arterial injection of syngenetic tumor cells in this model is put forward (Fig. 10). Up to around 5 days

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**Fig. 10** Hypothetic five stages of growth patterns of hematogenous cerebral metastatic tumors from the present model.

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after injection, the injected tumor cells remained in the capillary lumens. This first stage was named the silent period. From 5 to 8 days after injection, tumor cells began to penetrate into the perivascular space and to mitose. This second stage was named the penetrating and mitotic period. Thirdly, from 9 to 12 days after injection microtumors were found. This third stage was named the period of microtumors. Next, multiple tumors gradually proliferated in both number and extent from 12 to 18 days after injection. This fourth stage can be called the multiple proliferating period. And last, from 18 days after injection to death, tumor nodules expanded quickly and compressed the opposite hemisphere within a relatively short time. This fifth stage can be said to be the expanding period.

Further analysis of the mechanism of growth of metastatic tumors are worthwhile for progress in the prevention and treatment of cerebral metastatic tumors.

Acknowledgment

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