Histological and Elemental Changes in the Rat Brain after Local Irradiation with Carbon Ion Beams

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The left cerebral hemispheres of adult Sprague-Dawley rat brains were irradiated at doses of 30, 50, or 100 Gy with charged carbon particles (290 MeV/nucleon; 5 mm spread-out Bragg peak). The spread-out Bragg peak used here successfully and satisfactorily retained its high-dose localization in the defined region. A histological examination showed that necrotic tissue damage, hemorrhage in the thalamus, and vasodilatations around the necrotic region were induced at 8 weeks after 100 Gy irradiation. The regions with tissue damage correlated well with those expected from the radiation-dose distribution, indicating an advantage of charged carbon particles for irradiating restricted brain regions. An X-ray fluorescent analysis demonstrated a decrease in the concentrations of K and P, and an increase in the concentrations of Cl, Fe, Zn in the damaged region at 8 weeks post-irradiation, though no significant changes were observed before 4 weeks of post-irradiation. This may indicate that even the very high radiation doses used here did not induce acute and immediate neuronal cell death, in contrast with ischemic brain injury where acute neuronal cell death occurred and the elemental concentrations changed within a day after the induction of ischemia.

INTRODUCTION

A number of animal experiments have been carried out in order to elucidate the effects of ionizing radiation on the brain and its underlying mechanisms (as reviewed in ref. 1–3). The major pathological findings in late-delayed radiation injury to the brain have been variably reported as the loss of oligodendrocytes4,5, gliosis6,7, hemorrhage8, vascular changes with hyaline thickening of the vessel wall and endothelial proliferation6,8,9. It has been proposed that higher radiation doses cause glial cell depletion, leading to white-matter necrosis, while lower doses damage endothelial cells and have a longer latent period to occurrence6,10,11.

In these animal experiments, especially in those using rodents, the whole brain, or a relatively large volume of the brain, was irradiated and examined. This is because ordinary irradiation instruments using X-rays or γ-rays cannot concentrate the radiation dose to a small and restricted area of the brain, which means that it is difficult to clarify the radiation sensitivity of a specific brain region individually without a mutual interaction with other brain regions. For example, there is a possibility in whole-brain irradiation that the necrotic degeneration observed in the cerebral cortex may not be attributed to the direct effect of irradiation, but to ischemia caused by a radiation-induced vascular impairment in the basal region.
Recently, a heavy-ion irradiator, which can accelerate ion particles, such as carbon and silicon, has become available for animal experiments at our institute\textsuperscript{12,13}. This prompted us to develop an experimental system for irradiating a small and restricted region of rodent brains, using the feature of heavy-ion beams to administer a large radiation dose in the vicinity of the endpoint in the beam range (Bragg peak). In the present work, we studied the dose distribution, histological observations, and ionic changes after a local irradiation of the rat brain with charged carbon beams. In addition, the change in the ionic levels after irradiation was compared with those after cerebral ischemia induction to examine the possible involvement of immediate neuronal cell death in late (delayed) radiation-induced brain damage.

**MATERIALS and METHODS**

*Experimental animals and irradiation*

Male Sprague-Dawley (SD) rats, aged 10–14 weeks and weighing 255–320 g, were used. The animals were obtained from a domestic breeder (Slc Co. Ltd., Shizuoka, Japan), and maintained under standard animal facility conditions with commercial pellet food and tap water *ad libitum*. In the experiment, the animals were divided into three groups as follows: thirty-six animals were irradiated with carbon beams at 30, 50, or 100 Gy; four were sham-irradiated animals; and three were animals with cerebral ischemia induced by middle cerebral artery occlusion. The animals were anesthetized 10–15 minutes before irradiation with ketamine (40 mg/kg) and xylazine (10 mg/kg), immobilized in a specifically designed jig, and irradiated with 290 MeV/nucleon charged carbon beams in a dorsal-to-ventral direction.

The beam was modulated to have a spread-out Bragg peak (SOBP) of 5 mm width with a range modulator, and collimated such that the left hemisphere, excluding the eyes and ears, was irradiated (Fig. 1). The radiation doses were 30, 50, and 100 Gy equivalent with a linear energy transfer of approximately 50 keV/\(\mu\)m. The position of the SOBP was adjusted to be at 2.5–7.5 mm depth from the surface of the head with a binary filter inserted before the target. The details of the beam adjustment and modulation have been reported elsewhere\textsuperscript{12,13}.

All of the animal experiments were carried out with permission and under regulation of the Institutional Committee for Animal Safety and Welfare of the National Institute of Radiological Sciences, and in accordance with the Regulations on Appropriate Animal Breeding and Treatment, Ministry Office of Japan.
Confirmation of the radiation field and depth

The homogeneity, flatness and depth of the irradiation field were checked by a commercial imaging plate (SR-20, Fuji Photo Film, Co. Ltd, Tokyo, Japan), plastic plates of a tissue culture bottle (T-70, Falcon, Beckton&Dickinson, Co. NJ. USA), and frozen slices of rat brain with different thickness. The imaging plates were covered with a different number of plastic plates or a frozen tissue slice in order to simulate a different position of the brain, placed at the target position of the irradiator, and irradiated (0.1Gy) using the same procedure as that for an animal treatment. After irradiation, the imaging plates were scanned by a Bio-imaging Analyzer (BAS-3000, Fuji Photo Film, Co.

Fig. 2. Depth-dose distribution of a carbon beam with a 5 mm spread-out Bragg peak.

Fig. 3. Shape of the carbon beam at different depths. The beam shapes were obtained with commercial imaging plates, and the depth of tissue was simulated with plastic plates.
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Ltd, Tokyo, Japan), and the dose distributions in the irradiated fields were obtained as two-dimensional images.

**Behavioral and histological observations**

After irradiation, the rats were housed 2–3 to a cage, and their behavior were observed. Twice a week they were removed from their cage, placed on a table, and scored for balance using a subjective scoring scale\(^{14}\). Four levels were used: –, normal; +–, partial loss of balance (showed either an abnormal walking pattern or rotation when suspended by the tail); +, total loss of balance (abnormal both in walking pattern and rotation); ++, severely impaired balance (difficulty in walking, severe rotation when suspended by the tail, and head held in lateral flexion all of the time).

At the scheduled time after irradiation, each animal was anesthetized with diethyl ether and killed by decapitation. The brain was removed quickly, fixed in neutralized 10 % formalin, sectioned at 8 \(\mu\)m, and stained with hematoxylin and eosin. The sections were cut in a coronal direction at the level of the optic chiasma. Since no other neurological histochemistry was performed, the histological examination focused mainly on the necrosis, hemorrhage and vasculization. The results were recorded as follows: –, no visible abnormalities; +–, slight; +, moderate; ++, severely damaged.

**Ischemia induction**

Regional ischemia induced by left-middle cerebral artery occlusion was used as a positive control for histological and ionic changes. The surgical procedures have been described elsewhere\(^{15-17}\). In brief, the animals were anesthetized with ketamine and xylazine, and the left-middle cerebral artery was coagulated between its origin from the carotid artery and the lateral striate branches. The animals were allowed to recover and then treated in the same manner as the irradiated animals.

**Measurement and calibration of XRF**

The change in the constituent elements in the brain after 100 Gy irradiation or ischemia was monitored by X-ray fluorescent analysis (XRF) 1 day, 1 week, 4, 8 and 17 weeks after treatment. The details of the sample preparation and the XRF measurements have been described elsewhere\(^{18}\); after the brain was dissected free and cooled to \(-85^\circ\)C, a 3 mm thick coronal section for XRF was cut about 6 mm posterior to the frontal pole using a tissue slicer. The sections were then dried at \(-20^\circ\)C for approximately 48 hours in a freeze dryer. Energy-dispersive XRF spectrometry was performed with a commercial instrument having a two-dimensional mapping function (Micro Element Monitor SEA2010, Seiko Electronics Ind. Co. Ltd., Chiba, Japan). The brain section in a vacuum chamber was viewed with a light microscope, and the X-ray beam (a round shape of 0.18 mm in diameter) was positioned at different points by moving the specimen holder with stepping motors. A total of 1500 points (50 points vertically \(\times\) 30 points horizontally) were measured at regular intervals over the surface of the brain.

**Fig. 4.** Autoradiograms of the irradiated region with a commercial imaging plate covered with a slice of rat head. A shielding effect of cranial bone was evident in the anterior part of the head, but was negligibly small in the central and posterior parts of heads including the irradiated region (region enclosed with white line). Pr, premaxilla; F, frontal; B, bregma; Pa, parietal.
EFFECTS OF A CARBON BEAM ON RAT BRAIN

Fig. 2 shows the depth-dose distribution measured by changing the thickness of the absorber of the range shifter. A relatively high and uniform dose was delivered between 2.5 and 7.5 mm depth from the surface of the rat head. The relative standard deviation of doses in the region of SOBP (in 9 measured points) was 12%, suggesting a very uniform depth-dose distribution. The dose at the surface of head was estimated to be about 78% of the average dose in the region of SOBP. The beam shapes monitored by imaging plates covered with plastic plates are shown in Fig. 3. The outline of the irradiated field did not become obscure with increasing depth, suggesting that little diffusion of the beam occurred even in deep regions of the brain. The two-dimensional dose distribution in the irradiation field was very uniform, the relative standard deviation of photo stimulated luminescence of the imaging plates being less than 10%. Since the shield effect of the cranium was not well known, we examined the uniformity of the dose distribution using an imaging plate covered with a thin slice of rat head. The shielding effect of the cranial bone on the dose distribution was observed at the region from the nasal

**RESULTS**

The measurement was performed on an approximately 0.3 × 0.3 mm grid, since the average size of the coronal brain sections was 16.1 × 9.2 mm.

<table>
<thead>
<tr>
<th>Dose</th>
<th>6 hrs</th>
<th>24 hrs</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>17 weeks</th>
<th>32 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>30Gy</td>
<td>Behavior(^1)</td>
<td>– (9)(^3)</td>
<td>– (9)</td>
<td>– (9)</td>
<td>– (7)</td>
<td>– (5)</td>
</tr>
<tr>
<td></td>
<td>Histology(^2)</td>
<td>n.e.</td>
<td>n.e.</td>
<td>n.e.</td>
<td>– (2)</td>
<td>+ – (2)</td>
</tr>
<tr>
<td>50Gy</td>
<td>Behavior</td>
<td>– (12)</td>
<td>– (12)</td>
<td>– (10)</td>
<td>+ – (2)</td>
<td>+ (6)</td>
</tr>
<tr>
<td></td>
<td>Histology</td>
<td>n.e.</td>
<td>n.e.</td>
<td>n.e.</td>
<td>– (2)</td>
<td>– (2)</td>
</tr>
<tr>
<td>100Gy</td>
<td>Behavior</td>
<td>– (15)</td>
<td>– (15)</td>
<td>+ – (13)</td>
<td>+ (10)(^4)</td>
<td>+ + (6)(^4)</td>
</tr>
<tr>
<td></td>
<td>Histology</td>
<td>n.e.</td>
<td>n.e.</td>
<td>n.e.</td>
<td>– (2)</td>
<td>– (2)</td>
</tr>
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</table>

\(^1\) Behavior abnormality graded by walking pattern and rotation when held up by their tail: –, normal; + –, slight change either in walking pattern or rotation, +, abnormal both in walking and rotation, + +, severely impaired walking and apparent rotation.

\(^2\) Histological examination: –, normal; + –, slight; +, moderate; + +, severe.

\(^3\) The numbers in parenthesis denote the number of animals examined.

\(^4\) Two animals were lost due to the death during nighttime. These animals did not served for histology.

n.e.: not examined.

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*Fig. 5. Histology of irradiated rat brain 8 weeks after 100 Gy exposure. Vascular dilatation and tissue swelling at the irradiated region were common observations in the early stage of tissue damages. Arrowheads, vascular dilatation; Asterisks, tissue swelling; n, necrosis; N, neocortex; H, hippocampus.*
cavity to the orbital foramen (Fig. 4). However, the interference of the cranial bone was negligible in the irradiated area.

The histological and behavioral changes after irradiation are summarized in Table 1. In group irradiated with 100 Gy, eight of thirteen rats and all of ten rats showed rotation when suspended by the tail at 4 and 8 weeks post irradiation, respectively. Hemiplegic-walking patterns also started to be observed at 8 weeks post irradiation, or thereafter. Although similar behavioral abnormalities were observed in the 50 Gy group, the appearance of any abnormality was delayed and symptoms were milder compared with those of the 100 Gy group. Only a slight and transitional symptom of hemiplegia was observed at 8 weeks post-irradiation in the 30 Gy group.

The most distinctive histological changes after irradiation were vascular dilatation and tissue swelling at the irradiated region (Fig. 5). In the 100 Gy group, these histological changes were observed as early as 8 weeks post-irradiation. At 17 weeks, necrotic rarefaction became dominant at the center of the irradiated region, and telangiectatic changes were observed in the surrounding area (Fig. 6A). Hemorrhaging in the

Fig. 6AB. Histology of irradiated rat brain 17 weeks after 100 Gy irradiation. A, apical region of the left hemisphere. Cortex and hippocampus are severely damaged, and telangiectatic changes are observed in the surroundings. B, contra lateral hemisphere. Although a small part of the cortex just next to the irradiated region is damaged, the cortex and hippocampus seem to be intact. n, necrosis; C, cingulated cortex; N, neocortex; H, hippocampus.

Fig. 7. Gross histology of irradiated rat brain. Severe necrotic changes and hemorrhage in the thalamus are observed. The region with the histological changes is restricted to the irradiated region (2.5 to 7.5 mm from the surface of the head).
thalamus also appeared at this time. In contrast, no obvious histological changes were observed in the contra-lateral hemisphere (Fig. 6B), although regional necrotic changes were observed in the cortex near the left hemisphere. Similar, but milder, histological changes were observed 17 weeks after 50 Gy irradiation. A dose of 30 Gy produced no obvious histological changes for up to 8 weeks, though slight vascular dilatations and tissue swelling were observed at 17 and 32 weeks. The gross histology (Fig. 7) demonstrated that the region with the histological changes described above was well restricted to the irradiated region (2.5 to 7.5 mm from the surface of the head).

The XRF analysis demonstrated a decrease in the concentration of K and P in the region where tissue swelling and necrosis were observed (see Fig. 8A for K). Significant increases in the concentrations of Cl, Fe, Zn were observed in the thalamus and surrounding area of necrosis (see Fig. 8B for Fe). Although 100 Gy carbon particle irradiation produced these ionic changes after 8 weeks post-irradiation, no significant change was observed before 4 weeks after irradiation. In contrast, a sudden decrease in the concentration of K and an increase in Cl were observed as early as 24 hours after ischemia induction in the rats with cerebral ischemia, while the concentrations of P, Fe and Zn did not significantly change (Fig. 9-A,B).

**DISCUSSION**

Physical measurements of the absorbed radiation...
doses showed that the SOBP used here successfully and satisfactorily retained its high dose localization in the defined region (Fig. 2). The deviation of the dose in the SOBP was calculated to be less than 15% of the average dose, suggesting that this system, originally designed for cancer therapy\textsuperscript{19}, is also useful for exposing a small and restricted volume of animal tissue to a high dose of radiation. In contrast to this uniform depth distribution of physical doses, it seemed that the depth distribution of the biological effect was not uniform. The necrotic change was severe in the cortex and hippocampus (approximately 2.5–5.5 mm in depth), but relatively mild in the lateral and posterior thalamus (5.5–7.5 mm in depth), although these regions had been exposed to similar radiation doses. The exact mechanisms for this discrepancy are not readily apparent. Each brain region may have a different susceptibility to carbon beams. Many authors have reported heterogeneous susceptibilities of the brain regions to \( \gamma \)-rays\textsuperscript{6,20,21}. For example, the fimbria and capsula interna were reported to be the most commonly affected regions in rats that had died from radiation-induced brain damage after \( \gamma \)-ray irradiation\textsuperscript{21}. They also found that telangiectatic changes in the neocortex occurred at 12 months, 2 months, and 2 weeks after the irradiation of 50, 75, and 120 Gy, respectively. These effective doses are significantly higher than those observed in the present study. As shown in Table 1, a dose of 50 Gy produced moderate necrotic changes as early as 17 weeks post-irradiation. One possible reason for this discrepancy may be the difference in biological effect between \( \gamma \)-ray and carbon beams. It is well known that high-LET radiation, such as the ion beams used here, has a high relative biological effectiveness. Another explanation is the difference in the dose distribution in the irradiated volume between focal \( \gamma \)-irradiation and the present method. As described above, the carbon beams used here administered relatively uniform radiation doses throughout the target volume. In contrast, focal \( \gamma \)-irradiation (the gamma knife) seems not to administer a uniform dose to the target volume. It should be noted that the dose of 50 Gy in their report indicates the center maximum dose in the target tissue. Although the biological effective doses were different, the results of both studies clearly indicate a dose-latent-period relationship for radiation-induced necrosis in the cerebral cortex after focal (regional) irradiation. Such a relationship was found in rat for radiation-induced necrosis in the cerebral white matter after whole-brain \( \gamma \)-irradiation\textsuperscript{6}. The present study confirmed that the latent period for necrosis after regional irradiation with a carbon ion beam also depends on the radiation dose, as observed in whole brain \( \gamma \)-irradiation.

In some animals, tissue damage was also observed in the right cortex near to the irradiated region. The reason for this phenomenon is not readily apparent at present. There is a possibility that a small part of the cortex in the right hemisphere was accidentally irradiated due to inaccurate positioning of the rat head to the carbon beam. On the other hand, the radiation damage of the irradiated parts, especially the vascular systems, may be responsible for these observations. This point needs to be further investigated.

In positive control rats with cerebral ischemia, the levels of K and Cl altered 24 hours after the induction of ischemia (Fig. 8-A, B). A number of studies have previously demonstrated significant changes in the tissue ionic levels in cerebral ischemia (for review see ref. 22, 23), spinal-cord injury\textsuperscript{24}, and other brain injury models\textsuperscript{25,26}. The decrease in tissue concentration of K and increases in Na, Cl, and Ca are well-known phenomena after sudden neural cell death induced by ischemia and trauma. In contrast, no significant changes in the ionic levels were detected in rat brain at 6 and 24 hours, 1 week and 4 weeks after the 100 Gy irradiation in the present study. This suggests that even the very high radiation doses used here did not induce acute and immediate neuronal cell death, in contrast with ischemic brain injury where acute neuronal cell death occurred and the elemental concentrations changed within one day after the induction of...
ischemia.

The ionic levels in tissue may also be affected by changes in the permeability of blood vessels. Ljubimova et al. measured the endothelial cell population in rat brains after X-ray irradiation, and demonstrated a decrease in the endothelial cell number within 1 day of irradiation. This early loss in cell number was dose-independent and continued for up to 1 month after irradiation. From the results of the present study, it is unlikely that the loss of epithelial cells induced a significant change in the permeability of the blood-brain barrier, because the ionic levels monitored by XRF remained constant before 4 weeks after irradiation. These findings also confirm a study by Rubin et al., who quantified the disruption of the blood-brain barrier after 60 Gy gamma irradiation using magnetic resonance image analysis and horseradish peroxidase perfusion. The blood-brain barrier leakage was not so obvious before 6 weeks post-irradiation, but suddenly became profound after 12 weeks. It should be noted that in the present study, a similar latent period (17 weeks) was necessary to induce significant ionic changes in the irradiated region.

Radiotherapy is a clinical technique that provides good outcomes in the treatment of brain tumors. However, radiation-induced damage to the normal brain, which can occur several months after a treatment, is a limiting factor for the application of this technique. In order to optimize its utility, it is therefore important to know the radiation susceptibility of each region of the brain, and to understand the mechanisms of radiation damage to the brain tissue. Although more experimentation is required before any firm conclusions can be finally determined, the present results clearly indicate that a carbon ion beam is a useful tool to irradiate a small and restricted region of the experimental animal brain, and that the induced damage is similar to that induced by X- and gamma-rays.

REFERENCES


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