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Similarity between the Effects of Carbon-ion Irradiation and X-irradiation on the Development of Rat Brain

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The effects of carbon-ion irradiation and X-irradiation on the development of rat brain were compared. Twenty pregnant rats were injected with bromodeoxyuridine (BrdU) at 9 pm on day 18 of pregnancy and divided into five groups. Three hours after injection (day 19.0) one group was exposed to 290 MeV/u carbon-ion radiation by a single dose of 1.5 Gy. Other groups were exposed to X-radiation by 1.5, 2.0 or 2.5 Gy, or sham-treated, respectively. Fetuses were removed from one dam in each group 8 h after exposure and examined histologically. Extensive cell death was observed in the brain mantle from the irradiated groups. The cell death after 1.5 Gy carbon-ion irradiation was remarkably more extensive than that after 1.5 Gy X-irradiation, but comparable to that after 2.0 Gy or 2.5 Gy X-irradiation. The remaining rats were allowed to give birth and the offspring were sacrificed at 6 weeks of age. All of the irradiated offspring manifested microcephaly. The size of the brain mantle exposed to 1.5 Gy carbon-ion radiation was significantly smaller than that exposed to 1.5 Gy X-radiation and larger than that exposed to 2.5 Gy X-radiation. A histological examination of the cerebral cortex revealed that cortical layers II-IV were malformed. The defect by 1.5 Gy carbon-ion irradiation was more severe than that by the same dose of X-irradiation. Although the BrdU-incorporated neurons were greatly reduced in number in all irradiated groups, these cells reached the superficial area of the cortex. These findings indicated that the effects of both carbon-ion irradiation and X-irradiation on the development of rat brain are similar in character, and the effect of 1.5 Gy carbon-ion irradiation compares to that of 2.0–2.5 Gy X-irradiation.

INTRODUCTION

A Shuttle flight in outer space results in exposure to cosmic rays. These rays are composed of high-energy charged particles, such as protons, as well as helium, carbon, silicon and

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iron ions. Although the flux of high-Z and energy (HZE) particles in cosmic rays is rather low when compared with light particles, like protons, it accounts for about half of the total dose-equivalent for space flights with a low-earth orbit, because of their high energy deposit[1]. In the 9th Shuttle-Mir Mission (STS-91), organ/tissue absorbed doses of radiation were measured using a phantom torso. The absorbed doses at internal organs were found to be comparable to, even higher than, the skin dose in space[2]. This contrasts with the fact that the skin-absorbed dose of low-energy radiation is high. This form of ionizing radiation is not usually found on earth, and is assumed to affect human reproduction in outer space[3,4].

The developing fetal brain is particularly susceptible to low linear-energy transfer (LET) radiation, such as X-rays and gamma-rays[5–7]. However, the effects of HZE radiation on the fetal brain have not been studied. The present experiment compared the effect of 290 MeV/u carbon-ion radiation, which is practically the only species of HZE radiation available for animal experiments in Japan[8], with that of X-radiation in characteristics and strength on the development of fetal brain using rats. Since knowledge of the biological effects of X-irradiation has been accumulated, their comparison can help to predict the reproductive risk in the outer-space environment.

MATERIALS AND METHODS

The animals employed were Slc:Wistar rats kept in a specific pathogen-free environment. All animals were used in accordance with the Guidelines for Animal Experimentation, Nagoya University. Sexually mature females were mated with males overnight. Copulation was determined by expelled vaginal plugs the following morning, and the last midnight was regarded as day 0.0 of pregnancy.

Twenty pregnant rats were intraperitoneally injected with 25 mg/kg of bromodeoxyuridine (BrdU) at 9 pm on day 18 of pregnancy. This procedure allowed us to label fetal brain cells in the S-phase, which begin to differentiate into neurons at this stage and to migrate to the superficial layers of the cerebral cortex[9–11] (rat embryonic day 19 corresponds to mouse day 17)[12]. The animals were then divided into 5 groups of 4 rats each. Three hours after injection (midnight, day 19.0 of pregnancy), a group of rats was restrained individually in perforated plastic tubes and exposed to 290 MeV/u energy carbon-12 ion beams (LET 50 keV/μm) of the 6-cm spread-out Bragg peak (SOBP), delivered from the Heavy Ion Medical Accelerator in Chiba (HIMAC) synchrotron, at a single dose of 1.5 Gy. The other three groups of pregnant rats were exposed to X-rays (140 kVp, 5 mA, 0.5 mm Al + 0.5 mm Cu added filtration) at a single dose of 1.5, 2.0 or 2.5 Gy at 3 h after BrdU injection. During exposure, the rats were put in individual plastic cages which were rotated at 4 rpm on a turntable to achieve homogeneous dose distribution. The last group was restrained and sham-exposed, serving as a control.

One rat from each group was sacrificed by an overdose of diethyl ether 8 h after exposure, and the fetuses were removed and fixed in 4% formaldehyde. The fetal brain was embedded in paraffin, sectioned in the frontal plane at 5 μm thickness, and stained with hematoxylin and

eosin. The remaining animals, three rats in each group, were allowed to give birth and rear their litters. The offspring at 6 weeks of age were deeply anesthetized with diethyl ether and perfused via the heart with 4% formaldehyde; the brain was then removed and weighed. The size of brain mantle (rostro-caudal length and length at the medial line, Fig. 2) of male rats was measured to compare the severity of microcephaly among groups.

The cerebrum was then embedded in paraffin, frontally sectioned at 10 μm thickness for Klüver-Barrera's stain, or at 3 μm thickness for immunostain for BrdU. The parietal cerebral cortex, area 3 of Krieg[13], was chosen for an observation and comparison of the cortical architecture. For immunohistochemical staining of BrdU, sections were processed using monoclonal antibody against BrdU (1:50, Becton Dickinson) and Vecstain Universal Quick Kit (Vector).

RESULTS

Acute effects

The brain mantle of rat fetus on day 19 of pregnancy is composed of four zones: the marginal zone, cortical plate, intermediate zone and ventricular zone (Fig. 1). The ventricular zone consists of proliferating immature cells, which are known to be particularly vulnerable to radiation-induced cell death[6,7]. Many dead cells were observed in the brain mantle 8 h after

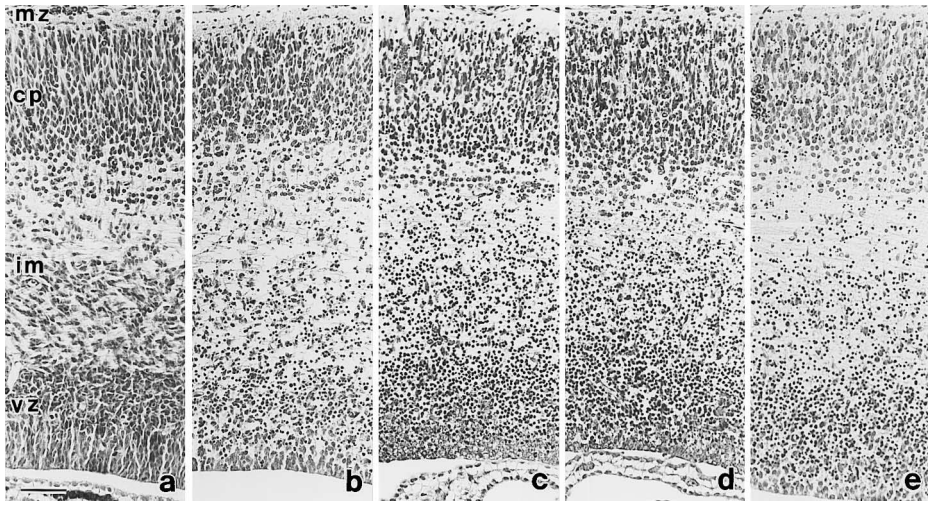

Fig. 1. Fetal brain mantles 8 h after (a) sham-irradiation, (b) 1.5 Gy, (c) 2.0 Gy and (d) 2.5 Gy X-irradiation or (e) 1.5 Gy carbon-ion irradiation. mz, marginal zone; cp, cortical plate; im, intermediate zone; vz, ventricular zone. Intensely stained nuclei of dead cells are observed mainly in the ventricular zone and in the inner area of the intermediate zone after 1.5 Gy X-irradiation. Following 2.0 Gy and 2.5 Gy X-irradiation, as well as 1.5 Gy carbon-ion irradiation, dead cells are abundant throughout the ventricular zone, intermediate zone and cortical plate. Hematoxylin-eosine stain. Scale bar = 50 μm.

exposure to either X-radiation or carbon-ion radiation. The amount of dead cells and the area they were observed to vary, depending on the doses and type of radiation. Following exposure to 1.5 Gy X-radiation, dead cells were observed mainly in the ventricular zone and in the inner area of the intermediate zone. In the outer area of the intermediate zone and in the cortical plate, a small number of dead cells were scattered. Following exposure to 2.0 Gy and 2.5 Gy X-radiation, dead cells were abundant throughout the ventricular zone, intermediate zone and cortical plate. After exposure to 1.5 Gy carbon-ion radiation, dead cells were abundant in the ventricular zone and in the inner area of the intermediate zone; many dead cells were also observed in the outer area of the intermediate zone and in the cortical plate. The cell death in the fetal brain exposed to 1.5 Gy carbon-ion radiation was remarkably more extensive than that exposed to the same dose of X-radiation, being comparable to those exposed to 2.0 Gy and 2.5 Gy X-radiation, at the light-microscopy level (Fig. 1).

Long-term consequences

The gross appearance of the brain of 6-week-old rats following prenatal exposure to either X-radiation or carbon-ion radiation is shown in Fig. 2. Prenatally irradiated brains showed microcephaly, with small cerebral hemispheres exposing the superior colliculi, which were covered by the cerebral hemispheres in the control. The size of brain mantle, i.e., rostro-caudal length and length at the medial line (Fig. 2), prenatally exposed to 1.5 Gy carbon-ion radiation, was significantly smaller than that exposed to 1.5 Gy X-radiation and larger than that exposed to 2.5 Gy X-radiation; the brain mantle exposed to 2.0 Gy X-radiation was not significantly different from those exposed either to 1.5 Gy and 2.5 Gy X-radiation or 1.5 Gy carbon-ion radiation (Table 1). The weight of the whole brain, from the olfactory bulb to the medulla oblongata, was significantly decreased in animals exposed to either form of radiation and any applied doses. However, among groups exposed to radiation there were no statistically significant differences in the brain weight (Table 1).

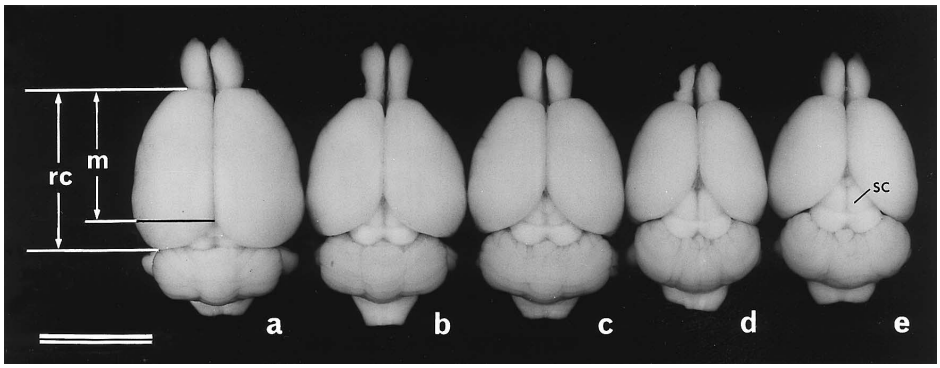

Fig. 2. Gross appearance of the brains of 6-week-old rats following prenatal exposure to (a) sham-irradiation, (b) 1.5 Gy, (c) 2.0 Gy and (d) 2.5 Gy X-irradiation or (e) 1.5 Gy carbon-ion irradiation. sc, superior colliculus. The locations of brain mantle measured are also indicated: (rc) rostro-caudal length and (m) length at the medial line. Prenatally irradiated brains show microcephaly with small cerebral hemispheres, exposing the superior colliculi which are covered by the cerebral hemispheres in the sham-irradiate control. Scale bar = 10 mm.

Table 1. Mean body weight, weight of brain, and size of brain mantle at 6 weeks of age. See Fig. 2 for measurement of length (rc) and (m). (n = 5 males for each group, figures in parentheses are range: smallest - largest)

	Sham	X-rays			Carbon-ions
	0 Gy	1.5 Gy	2.0 Gy	2.5 Gy	1.5 Gy
Body weight [g][a]	143	120	98	107	102
	(131 – 154)	(90 – 137)	(78 – 123)	(86 – 130)	(98 – 107)
Brain weight [g][a]	1.48	1.16	0.99	1.00	1.05
	(1.43 – 1.53)	(1.00 – 1.26)	(0.83 – 1.20)	(0.86 – 1.11)	(0.95 – 1.11)
Length rc [mm][b]	13.5	12.0	11.6	10.6	11.0
	(13.2 – 13.6)	(11.7 – 12.4)	(10.1 – 12.2)	(10.4 – 10.9)	(10.9 – 11.1)
Length m [mm][b]	11.0	8.3	6.3	5.2	6.2
	(10.8 – 11.4)	(8.0 – 9.0)	(4.7 – 8.4)	(4.7 – 6.1)	(5.8 – 6.8)

[a] Sham > 1.5 Gy X-rays; No significant difference among irradiated groups.

[b] Sham > 1.5 Gy X-rays > 1.5 Gy Carbon-ions > 2.5 Gy X-rays ($p < 0.05$ by Mann-Whitney U test)

Klüver-Barrera-stained and BrdU-immunostained sections of cerebral cortices are presented in Fig. 3 and Fig. 4, respectively. The cerebral cortices of prenatally irradiated brains were thinner than that of the control. Following exposure to 1.5 Gy X-radiation, the cortical layers II, III and IV could not be differentiated (Fig. 3b), although BrdU-incorporated neurons had reached layers II-IV (Fig. 4b). In brains exposed to 2.0 and 2.5 Gy X-radiation, as well as

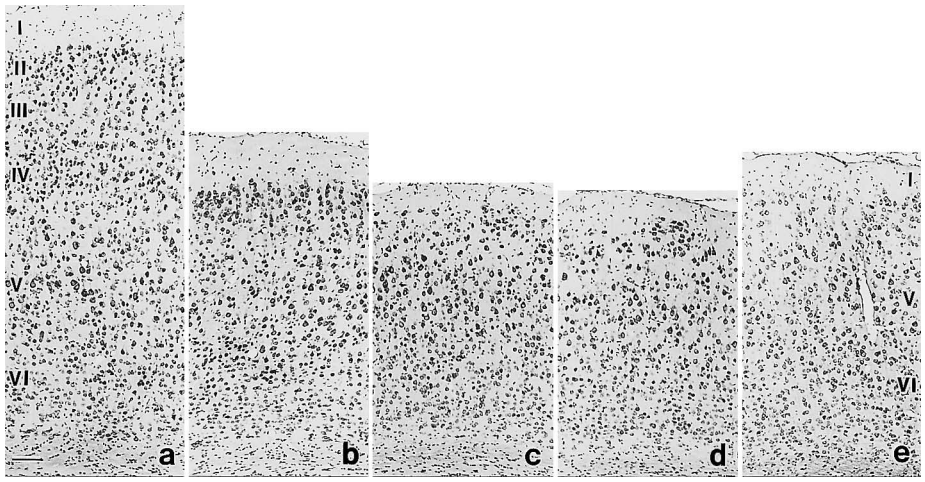

Fig. 3. Cerebral cortices of 6-week-old rats following prenatal exposure to (a) sham-irradiation, (b) 1.5 Gy, (c) 2.0 Gy and (d) 2.5 Gy X-irradiation or (e) 1.5 Gy carbon-ion irradiation. I-VI indicate the cortical layers. Prenatally irradiated cerebral cortices are thinner than that of the control. The cortical layers II, III and IV cannot be differentiated in the brain exposed to 1.5 Gy X-radiation (b). In brains exposed to 2.0 and 2.5 Gy X-radiation, as well as 1.5 Gy carbon-ion radiation, the cortical layers II-IV are deficient (c,d,e). Klüver-Barrera's stain. Scale bar = 100 μm.

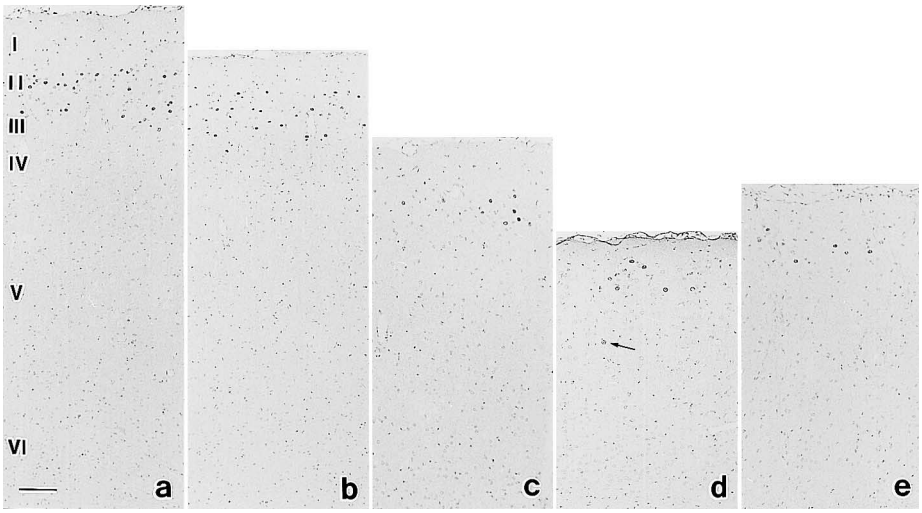

Fig. 4. BrdU-labeled neurons in the cerebral cortices of 6-week-old rats following prenatal exposure to (a) sham-irradiation, (b) 1.5 Gy, (c) 2.0 Gy and (d) 2.5 Gy X-irradiation or (e) 1.5 Gy carbon-ion irradiation. I-VI indicate the cortical layers. BrdU-labeled neurons in prenatally irradiated brains are fewer than those in the control, but they have migrated to the superficial area of the cerebral cortex. A labeled neuron is seen in the deeper area (d, arrow). BrdU-immunostain. Scale bar = 100 μm.

1.5 Gy carbon-ion radiation, the cortical layers II-IV were deficient (Fig. 3c,d,e), and BrdU-labeled neurons were few, though the majority of them had reached the superficial area of the cortex (Fig. 4c,d,e). Occasionally a few BrdU-labeled neurons were found in the deeper area of the cerebral cortex (Fig. 4d).

DISCUSSION

The acute effect of both carbon-ion irradiation and X-irradiation on the fetal brain of the rat was cell death in the brain mantle. The most radiosensitive cells are the ventricular cells[7]. Young neurons migrating in the intermediate zone are also radiosensitive[6]. By 1.5 Gy X-irradiation, the ventricular cells and young migrating neurons were destroyed. When the X-ray doses were increased, the death of neuronal cells of the cortical plate was also observed. The extent and area of dead cells observed after 1.5 Gy carbon-ion irradiation were similar to those after 2.0 Gy and 2.5 Gy X-irradiation. This suggests that the effect of carbon-ion irradiation on cells in the fetal brain is more lethal than that of X-irradiation.

The relationship between the RBE (relative biological effectiveness) and LET (linear energy transfer) for several biological events, such as cell lethality and mutagenicity, has been studied, and the highest RBE is shown at the LET of around 100 keV/μm in these events[14–18]. For killing of cultured Syrian hamster embryo cells, the RBE values of carbon-ion irradiation with LETs of 50 and 100 keV/μm were reportedly 1.8 and 2.6, respectively[19]. In two human hepatoma cell lines, RBEs of carbon-ion irradiation with an LET of 76 keV /μm for cell

lethality were 2.57 and 2.59[20]. Similar to these reports from in vitro experiments, the present in vivo experiment showed the effect of carbon-ion irradiation with a LET of 50 keV/μm on cell lethality in the fetal brain was relatively stronger than X-irradiation.

The common long-term consequence from damage to the fetal brain caused by either type of radiation was microcephaly. The size of the brain mantle prenatally exposed to 1.5 Gy carbon-ion radiation was significantly smaller than that exposed to 1.5 Gy X-radiation and larger than that exposed to 2.5 Gy X-radiation; it was not statistically different from that exposed to 2.0 Gy X-radiation. This result suggests that the effect of carbon-ion irradiation is somewhat stronger than X-irradiation in induction of microcephaly.

Histologically, layers II-IV of the cerebral cortex were deficient in brains prenatally exposed to 1.5 Gy carbon-ion radiation, and to 2.0 and 2.5 Gy X-radiation. In a brain exposed to 1.5 Gy X-radiation, neurons in layers II-IV were larger in number than those exposed to 1.5 Gy carbon-ion radiation; however, clear differentiation of the layers II-IV was difficult. These architectural malformations of the superficial layers of the cerebral cortex were already reported in rat brains exposed to X-radiation on day 18–19 of pregnancy[21]. Thus, the characteristics of brain anomaly induced by carbon-ion irradiation were similar to those caused by X-irradiation. The abnormal cortical architecture could be due to cell death during histogenesis of the cerebral cortex[7,22]. As mentioned above, the effect of carbon-ion irradiation on cell lethality was stronger than that of X-irradiation at the fetal stage. As a consequence, the cerebral architecture anomaly produced by carbon-ion irradiation was more severe than that caused by the same dose of X-irradiation.

Ionizing radiation also disturbs neuronal migration of the cerebral cortex. This has been reported from experiments using low-dose or low-dose-rate radiation, which induced less extensive cell death in the brain mantle[11,23,24]. In the present study, extensive cell death was observed not only in the ventricular zone but also in the intermediate (migratory) zone after irradiation, resulting in a marked decrease of BrdU-labeled neurons. This made it difficult to compare the disturbance of neuronal migration among irradiated groups. However, the majority of BrdU-labeled neurons had migrated to the superficial area of the cerebral cortex, mimicking the normal migration behavior, though the cortical layers II-IV were deficient. This finding is consistent with reports that even in the microcephaly caused by X-irradiation, the principle pattern of neuronal migration to the cerebral cortex remained unaffected[25,26]. It was also found that disturbed migration of neurons to the cerebral cortex following prenatal irradiation was remarkable in young mice, but dislocation of neurons became minimal in mature animals[24].

The results of the present study indicate that the effect of carbon-ion irradiation is similar in character to the effect of X-irradiation on the development of rat brain, but the effect of carbon-ion irradiation is somewhat stronger than that of X-irradiation. Although the present experiment used only one type of HZE radiation, one can estimate from the results that the effects of HZE irradiation on development are in fact stronger, but not vastly stronger, than those of low LET irradiation. Our results indicate that it is possible to predict the reproductive risk in an outer-space environment from the data accumulated on low-LET irradiation on earth.

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