Radiation-induced Diffuse Brain Injury in the Neonatal Rat Model

—Radiation-induced Apoptosis of Oligodendrocytes—

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Abstract

The mechanism of radiation-induced diffuse brain injury was investigated using a model of delayed myelination in the irradiated neonatal rat brain in which the number of oligodendrocytes decreases without associated necrosis of the cerebral white matter. Immunohistochemical analysis using antibody against the large myelin-associated glycoprotein, a specific marker of oligodendrocytes at an early stage of development, showed that the number of the oligodendrocytes associated with myelination decreased in the irradiated hemisphere 1 day after irradiation and remained low until 5 days after irradiation. In situ terminal deoxynucleotidyl transferase-mediated nick end-labeling assay revealed that apoptosis mainly occurred in the cerebral white matter of the irradiated hemisphere. Three hours after irradiation, apoptotic cells were found in the subcortical white matter and the periventricular white matter. Six hours after irradiation, apoptotic cells were found in the internal capsule, and the numbers of apoptotic cells in the periventricular white matter and subcortical white matter increased. One day after irradiation, the number of apoptotic cells in the periventricular white matter decreased. Three days after irradiation, apoptotic cells were not observed in the cerebral white matter. These results suggest that the oligodendrocytes associated with myelination may be damaged via radiation-induced apoptosis, and depletion of the oligodendrocytes may cause delay of myelination.

Key words: radiation, apoptosis, large myelin-associated glycoprotein, oligodendrocyte

Introduction

Radiation therapy is universally employed in the treatment of intracranial malignant tumors. However, radiation therapy may cause progressive mental deterioration or diffuse brain atrophy in adult patients.1,2,5,17,18 Our histopathological study of patients with radiation-induced diffuse brain atrophy revealed extensive and diffuse demyelination in the cerebral white matter associated with minor damage in the axons and neurons of the cerebral cortex.19 Moreover, a decrease in the number of oligodendrocytes without associated necrosis and delay of myelination was observed in the white matter of the our irradiated neonatal rat model.40 Based on these findings, we hypothesized that apoptosis may be involved in the depletion of oligodendrocytes and subsequent delay in myelination in radiation-induced brain injury.

Myelin-associated glycoprotein (MAG) is localized in periaxonal myelin membranes, and may be involved in maintaining the junction between the myelin sheath and the axon.20 MAG consists of two polypeptide isoforms, large MAG (L-MAG) and small MAG. L-MAG-positive oligodendrocytes appear in the cerebral white matter at an early stage of development, and gradually increase in number with the development of the normal rat brain.21,22 To test this hypothesis, the occurrence of radiation-induced apoptosis in oligodendrocytes was investigated in the irradiated neonatal rat brain model by immunohistochemistry using antibodies

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495
against the L-MAG as a marker of oligodendrocytes and by in situ terminal deoxynucleotidyl transferase (TdT)-mediated nick end-labeling assay for assessing apoptosis.

Materials and Methods

I. Irradiated brain model using neonatal rat

Our initial attempt to develop an irradiated adult rat model to study radiation-induced diffuse brain atrophy did not succeed, so we developed a neonatal rat model to examine the effects of irradiation on myelination in the cerebral white matter.

Neonatal rats at 1–2 weeks of age with body weight of 10–17 g were used in the experiments. The unanesthetized rats were immobilized with tape. The unilateral (right) cerebral hemisphere was irradiated with a single dose of 15 Gy (400 rads/min) using a Linear Accelerator (LMR-15C; Toshiba Corporation, Tokyo). The unirradiated unilateral (left) cerebral hemisphere was used as a control.

Our previous histological examination using hematoxylin eosin (HE) and Klüver-Barrera staining revealed that the number of glial cells, including astrocytes and oligodendrocytes, decreased in the irradiated hemisphere of this neonatal rat brain model, whereas neurons and vascular endothelial cells exhibited no abnormal changes.

Apoptotic cells were detected in the early stages between 3 hours and 3 days after irradiation in our pilot study, so the animals were sacrificed in the earlier stages, i.e., 2, 3, and 6 hours and 1, 3, and 5 days after irradiation.

II. Immunohistochemistry

Antibodies against L-MAG were provided by Dr. Shuzo Sato and Dr. Takashi Inuzuka (Department of Neurology, Niigata University).7 Cerebral specimens were embedded in paraffin and serial coronal sections of 6-μm thickness were prepared. Brain sections were deparaffinized and incubated in 0.3% hydrogen peroxide in absolute methanol to inactivate endogenous peroxidase. The sections were then washed with 0.01 M phosphate buffer (pH 7.2), containing 0.85% sodium chloride (PBS), and treated with 10% normal horse serum. After washing with PBS, the anti-L-MAG antibody diluted 1000-fold with PBS was applied to the sections. The sections were washed with PBS and incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif., U.S.A.). Peroxidase activity was detected by immersing the sections in diaminobenzidine (DAB) solution. The sections were stained with HE.

III. Detection of apoptotic cells

Brain sections were deparaffinized, incubated with 20 μg/ml protein kinase K, and thoroughly washed in sterile distilled water. The slides were rinsed in TdT buffer before immersion in a mixture of TdT and biotin deoxyuridine triphosphate in TdT buffer for 60 minutes. The sections were rinsed in sterile distilled water, immersed in a 2% aqueous solution of bovine serum albumin for 10 minutes at room temperature, rinsed in 0.05 M Tris-buffered saline, pH 7.4, and covered with alkaline-phosphatase-conjugated streptavidin (1:30). After visualization of the reaction using DAB, the slides were lightly counterstained with HE and mounted.

Fig. 1 Photomicrographs showing immunostaining for the large isoform of myelin-associated glycoprotein (L-MAG) 3 days after irradiation. upper: Subcortical white matter in the irradiated hemisphere contains a decreased number of L-MAG-positive cells. Original magnification × 400. lower: Subcortical white matter in the unirradiated hemisphere. Original magnification × 400.
Fig. 2 Distribution of apoptotic cells 3 hours (upper left), 6 hours (upper right), 1 day (lower left), and 3 days (lower right) after irradiation. In the irradiated hemisphere, apoptotic cells are scattered in the subcortical white matter, the hippocampal dentate gyrus, periventricular white matter, and the subependymal layer of the lateral ventricle (5.5–6.9%) at 3 hours; densely distributed in the periventricular white matter (90.6%) and the hippocampal dentate gyrus (60.8%) and moderately distributed in the subcortical white matter (29.7%), subependymal layer of the lateral ventricle (23.7%), and the internal capsule (26.8%) at 6 hours; moderately distributed in the periventricular white matter (27.8%), subependymal layer (21.6%), and the hippocampal dentate gyrus (24.5%) at 1 day; and scattered in the subependymal layer (5.3%) at 3 days. In the unirradiated hemisphere, apoptotic cells are scattered in the subcortical white matter, hippocampal dentate gyrus, periventricular white matter, internal capsule, and the subependymal layer (6.4–8.6%) at 6 hours; and scattered in the periventricular white matter, subependymal layer, and the hippocampal dentate gyrus (5.7–7.1%) at 1 day. Values in parentheses indicate the ratio of the number of apoptotic cells to the sum of normal and apoptotic cells. IR: irradiated hemisphere, C: control (unirradiated) hemisphere. Vertical shading indicates areas with a dense distribution of apoptotic cells, horizontal shading indicates areas with a moderate distribution of apoptotic cells, and slanted shading indicates areas with a scattered distribution of apoptotic cells.

Results

I. Expression of L-MAG
The number of L-MAG-positive cells in the subcortical white matter, periventricular white matter, and internal capsule of the irradiated hemisphere decreased 1 day after irradiation and then did not change until 5 days after irradiation (Fig. 1).

II. Distribution and time course of apoptosis
Three hours after irradiation, apoptotic cells were found in the subcortical white matter and periventricular white matter of the irradiated hemisphere.

Six hours after irradiation, apoptotic cells were observed in the internal capsule, and the numbers of apoptotic cells in the periventricular white matter and subcortical white matter of the irradiated hemisphere increased. One day after irradiation, the number of apoptotic cells decreased in the periventricular white matter. Three days after irradiation, apoptotic cells were not observed in the cerebral white matter. Apoptotic cells were also detected in the subcortical white matter, periventricular white matter, and internal capsule of the unirradiated hemisphere 6 hours after irradiation, and in the periventricular white matter of the unirradiated
hemisphere 1 day after irradiation, although the number of apoptotic cells in the unirradiated hemisphere was less than that in the irradiated hemisphere. Apoptosis also occurred in the hippocampal dentate gyrus and subependymal layer, but not in the cerebral cortex (Figs. 2 and 3).

Discussion

Radiation-induced diffuse brain atrophy is a serious adverse effect of radiation therapy. Our investigation of the mechanism of radiation-induced diffuse brain injury used a neonatal rat model in which a delay in myelination concomitant with decreased number of oligodendrocytes was observed in the white matter of the irradiated cerebral hemisphere. In the present study, L-MAG, which is produced in the cytoplasm of oligodendrocytes at an early stage of development and may be important in myelination during development, was used as a marker of oligodendrocytes. The number of L-MAG-positive cells decreased in the cerebral white matter 1 day after irradiation and remained low thereafter, indicating the delay of myelination in the irradiated hemisphere.

Our previous study demonstrated a decrease in the number of oligodendrocytes without associated necrosis in the irradiated cerebral white matter. Therefore, we postulated that apoptosis may be involved in the decrease in the number of oligodendrocytes. In the present study, apoptotic cells appeared in the subcortical white matter and periventricular white matter of the irradiated hemisphere by 3 hours after irradiation, and a significant increase in the number of these apoptotic cells was observed by 6 hours after irradiation. One day after irradiation, the number of apoptotic cells decreased. Three days after irradiation, no apoptotic cells were found in the cerebral white matter. In contrast to the decrease in the number of oligodendrocytes, the number of glial fibrillary acidic protein-positive astrocytic glial cells increased by 5 days after irradiation and vascular endothelial cells in the irradiated cerebral white matter showed no abnormal changes (data not shown). These results suggest that the cells falling into apoptosis in the cerebral white matter are oligodendrocytes. Apoptosis after irradiation of the central nervous system has recently been observed in various parts, such as the granular layers of the cerebellum, the hippocampus, the subependyma, and the spinal cord. Oligodendrocytes, and not astrocytes, neurons, or vascular endothelial cells, underwent apoptosis in the irradiated rat spinal cord, and so oligodendrocytes may be the target cells for radiation-induced apoptosis and apoptosis in the oligodendrocytes may be a major cause of subacute demyelination. Radiation-induced apoptosis of oligodendrocytes in the irradiated neonatal rat brain, such as that found in the irradiated rat spinal cord, may be involved in the delay of myelination. Our study found apoptotic cells in the unirradiated hemisphere in the same regions as in the irradiated hemisphere at time points corresponding to 6 hours–1 day after irradiation. The reason for the presence of apoptotic cells in the unirradiated hemisphere is as yet unknown.

In conclusion, oligodendrocytes associated with myelination may be damaged via radiation-induced apoptosis, and the decrease in the number of oligodendrocytes may cause delay of myelination.
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References


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Commentary

The authors investigate the mechanism of radiation-induced diffuse brain injury using a model of delayed myelination in the irradiated neonatal rat brain. Immunohistochemical analysis using a specific marker of oligodendrocytes at an early stage of development (L-MAG) showed that the number of oligodendrocytes associated with myelination decreased one day after irradiation and remained low until five days after the irradiation. Apoptosis was mainly revealed in the cerebral white matter of the irradiated hemisphere. The number of apoptotic cells showed a peak six hours after the irradiation and decreased to zero by three days after. The authors concluded that oligodendrocytes associated with myelination may be damaged via radiation-induced apoptosis, and the decrease in the number of oligodendrocytes may cause delay of myelination.

Radiation injury to the central nervous system (CNS) can result in devastating neurological deficits or cognitive impairment. These neurotoxicities are usually manifested one to several years after completion of treatment. Consequently, they tend to affect the quality of life of the patients. The dramatic nature of radiation-induced neurotoxicity causes clinicians to avoid this complication, sometimes even by compromising tumor control probability. As a result of that practice, clinical material on radiation my-
elopathy is limited. During the last two decades, radiation biologists have contributed data relevant for clinical practice. Experimental studies have helped to establish realistic estimates of the dose-incidence relationship, age effects, time-dose fraction and repair parameters, volume effects, drug-radiation interactions, and recovery from occult radiation injury. Concepts and parameters gained from such laboratory studies have been useful for the development of radiotherapy strategies to maximize tumor control probability.

Further advancement in therapeutic strategies, however, must wait for elucidation of the pathogenesis of radiation-induced CNS injury because such knowledge is essential for developing rational approaches to modulate the radiation damage to increase CNS tolerance.

The authors have performed an interesting study in investigating the pathogenesis of radiation neurotoxicity. Regarding the study protocol, we think it is rather questionable to use the unirradiated contralateral cerebral hemisphere as a control. In view of the complex tissue organization and the cell-cell interactions, it is no longer logical to preserve the traditional thought that radiation-induced CNS injury is simply the result of killing of parenchymal stem cells. Rather it should be viewed as an injury that produces cell killing that in turn induces complex pathophysiological reactions in which the response of surviving cells may contribute to determine the fate of the irradiated tissue. The observation that radiation myelopathy accompanied by significant inflammatory reactions has shorter latencies is consistent with that theory. Obviously the validity of this idea should be tested rigorously. If confirmed, it may open possibilities for prevention, interruption, and reversal of pathways that contribute, in concert with cell killing, to the production of radiation-induced end-stage lesions. With the potential for involvement of substances such as cytokines in the pathogenesis, innovative methods such as suppressing production of these compounds or blocking, at the appropriate time, their presumed effects on augmentation of radiation injury, or both, may hold prospect of modulating radiation effects on tissues.

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The authors have previously reported that radiation induces diffuse and extensive demyelination of the cerebral white matter. In this current article, the authors have clarified that apoptosis is involved in the deletion of oligodendrocytes, which subsequently induces delays in myelination in radiation-induced brain injuries. Radiation therapy is essential for the treatment of malignant brain tumors. However, radiation therapy may bring about brain atrophy as a long-term side effect, even in adults. This adverse effect of radiation therapy was observed not only in respect of cerebral function but also in relation to the morphology of the developing brain.

In this experiment, the brains of neonatal rats were radiated with 15 Gy. Unfortunately, this experimental model might not be appropriate for clinical applications. Fritsch et al. (ref. 5 of this article) have studied the radiation-induced apoptosis in the cerebellum of 14-day-old rats after whole-body irradiation of 0.25–3 Gy. Further study about the relationships between the age of animals, dose of radiation, the frequency of apoptosis, the distribution of apoptosis, and the kind of target cells would make for a more interesting and original article.

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