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Expression of TNF-α and TGF-β1 in the Rat Brain After a Single High-Dose Irradiation

Cytokines and growth factors are important regulatory proteins controlling the growth and differentiation of normal and malignant glial cells. In this study, we investigated the expression and origin of tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) in the subacute brain injury after a single high-dose irradiation using 60 Sprague-Dawley rats. The right cerebral hemispheres of rats were exposed to a single 10 Gy dose of gamma rays using Ir-192. The radiation effect was assessed at 1 week, 2 weeks, 4 weeks, 6 weeks, and 8 weeks after irradiation, and the results were compared with those in sham operation group. Histological changes characteristic of radiation injury were correlated with the duration after the single dose irradiation. The loss of cortical thickness also increased with the lapse of time after irradiation. The TNF-α expression in the irradiated cerebral hemispheres was significantly increased compared with that in the sham operation group. TGF-β1 expression was also increased in the irradiated hemispheres. Immunohistochemical study revealed that TGF-β1 was expressed predominantly by infiltrating macrophages and astrocytes around the necrotic areas. These findings indicate that TNF-α and TGF-β1 may play prominent roles in the radiation injuries after a single high-dose irradiation.

Key Words : *Cranial Irradiation; Radiation Injuries; Cytokines; Tumor Necrosis Factor-α; Transforming Growth Factor-β1*

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INTRODUCTION

Radiation injuries occurring after a single high-dose irradiation include apoptosis as well as radiation necrosis and approximately 4% of patients who have received a single high-dose irradiation suffer from radiation necrosis (1). It has been assumed that a compromise in cerebral microcirculation due to vascular endothelial cell injury may play a role in radiation necrosis (2, 3), however, precise mechanisms for radiation necrosis are not clearly known. Apoptosis or the programmed cell death means the natural death of cells, which can be found as a physiological phenomenon in many growing tissues (4). It was reported that even the cells of healthy tissues may undergo apoptosis when they are exposed to DNA-destructive factors, such as radiation, anticancer medicine, and cytotoxins (5).

Cytokine is one of the important regulatory proteins controlling the growth and differentiation of normal cerebral cells and cancer cells exposed to radiation (6-8). Tumor necrosis factor-α (TNF-α), typical of cytokine, serves as an intermediator of the immune system, protecting the host from the attacks of various infections and cancer cells, and induces the death of cancer cells by causing apoptosis (9). Recent studies have reported that radiation increases the expression of specific proteins including TNF-α (2, 10). Transforming growth factor-β (TGF-β), which is produced mainly from astrocytes and macrophages, is a polypeptide with various biological actions in the human body and the expression of TGF-β is also increased after irradiation (3). If we can clarify the roles of cytokine in relation to radiation injury in normal cerebral cells after a single high-dose irradiation, we can study the methods of restricting or accelerating the expression of the cytokines. And those methods are believed to serve as an important tool to prevent complications after radiotherapy. This study was designed to clarify the mechanisms of radiation necrosis and apoptosis taking place after a single high-dose irradiation, by using the animal model to identify whether TNF-α and TGF-β1 are expressed and from what they are originated.

MATERIALS AND METHODS

Sixty Sprague-Dawley rats weighing 350-400 g were used irrespective of their sex. To avoid empty stomach and dehydration, the rats were given water and food ad libitum till the experiment was conducted.

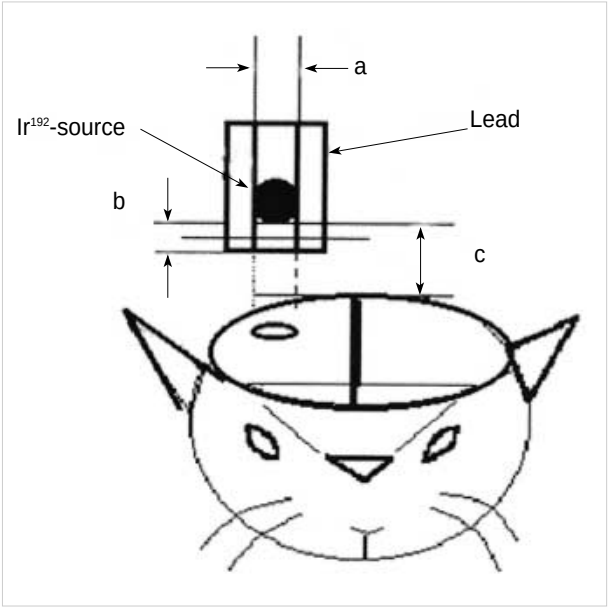

Fig. 1. Schematic drawing of irradiation. (a: diameter of radiation tube; 0.6 cm, b: distance from the radiation source to the distal cone; 0.5 cm, c: distance from source to the brain surface; 2 cm).

Single high-dose irradiation and forebrain extirpation

One percent ketamine (50 mg/kg), known to have no radioprotective effect (11), was injected to the rat intraperitoneally and then the head was fixed to a head holder (SN-8N Semi-Chronic Head Holder, Narishige Co., Tokyo, Japan) in a ventral decubitus position. A longitudinal incision was made on the scalp along the median line. Under an operative microscopy, a hole of an 8-mm diameter was made on the right parietal area using a small drill to expose the dura mater. Because it was technically impossible to conduct intraoperative irradiation using electron beams on a tiny rat brain, iridium-192 (GammaMed 12i, MDS Nordion, Canada), which has the same characteristics as the electron beam and is commonly used for high dose rate brachytherapy, was used as a source of radiation. A shield was made of Lipowitz metal (Cerrobend®, MED-TEC, Orange, IA, U.S.A.), and then a hole of 0.6 cm was made at an appropriate place in the middle to ensure that the iridium source was located 0.5 cm above the hole. The source was kept away appropriately to ensure that the impact of diffused beams on the irradiation site could be minimized and a portion of 1 cm diameter on the surface of the rat brain could be exposed to radiation, and that 10 Gy could be irradiated by a single irradiation on the brain surface 2 cm away from the source (Fig. 1). The 10 Gy dose was selected because it is known as the lowest dose to have a radiation effect during intraoperative irradiation (12). After irradiation, the scalp was sutured in an aseptic condition. Sixty rats were divided into 6 groups (10 in each group), and their forebrains were extirpated at 1 week (group A), 2 weeks (group B), 4 weeks (group C), 6 weeks (group D), and 8 weeks (group E) after irradiation. Ten non-irradiated rats also underwent forebrain extirpation at 1 week as a sham operation group.

Histopathological examination

Extirpated brains were fixed in 10% buffered formalin for 6 hr to maintain their antigenicity. Then a coronal section slice of a 5-mm anteroposterior diameter was made centering around the irradiated portion. Non-irradiated left cerebral hemispheres were used as the control group. According to the usual tissue processing procedure, tissues were passed through ethanol for dehydration and embedded in paraffin. Then, a thin section of a 5-μm thickness was made and H-E stained. The cortical thicknesses of irradiated right cerebral hemispheres and non-irradiated left hemispheres were measured to compare the cortical injury. That is, the loss of irradiated cerebral cortex at various time points was compared, and the morphological changes with the lapse of time after irradiation were observed using an optic microscope.

Quantitative analysis of TNF-α and TGF-β1 using enzyme-linked immunosorbent assay (ELISA)

Using the quantitative sandwich ELISA method with an ELISA kit (Quantikine®, R&D Systems, Minneapolis, MN), we quantified the expressions of TNF-α and TGF-β in the irradiated right hemispheres and the non-irradiated left hemispheres. As a control group, the brains of the non-irradiated sham operation group were also used. To extract the protein, the paraffin-embedded tissues were sectioned in a 10-μm thickness, and then processed in 100% xylene for 5-10 min at room temperature for deparaffinization. Then they were centrifuged at 13,000 rpm for 3 min twice, and left in 95% acetone at room temperature for 5 min. Again, they were centrifuged at 13,000 rpm for 3 min twice and washed in distilled water for 10 min. With 100 μL of protein lysis buffer put into each tube, they were left at 4˚C for 1 hr, and then centrifuged at 1,000 rpm for 30 min. Then, the upper layer was separated for the quantitation of protein according to the Bradford method (13). The specimen was diluted with a coating buffer to make 2 μg/mL, and then 100 μL/well was put into a 96-well plate. The plate was covered, incubated at 37˚C for 2 hr, and then washed with 200 μL/well phosphate-buffered saline Tween-20 (PBST) 3 times at intervals of 3-4 min. With an addition of 3% bovine serum albumin (BSA) 200 μL/well, the plate was further incubated for 2 hr at 37˚C, and again washed 3 times with PBST. The plate was incubated with primary antibody diluted at 1:200 with PBST (100 μL/well) at 37˚C for 2 hr, and washed 3 times with PBST. Then, it was incubated with the secondary antibody diluted at 1:1,500 with PBST (100 μL/well) at 37˚C for 2 hr, and then washed 3 times with PBST. Finally with an addition of substrate peroxidase (POD) buffer (100 μL/well), it

was left at room temperature for 20 min for a reaction, and then 50 μL/well of 5N H_2SO_4 was added to stop the reaction. By measuring the optical density with a precision microplate reader with an ultraviolet length of 450 nm (Molecular Devices, Sunnyvale, CA, U.S.A.), TNF-α and TGF-β1 were quantified. The expression of TNF-α and TGF-β1 with the lapse of time after irradiation was measured in each experimental group (irradiated right cerebral hemisphere and non-irradiated left hemisphere), and then the values were compared with those of the sham operation group.

Immunohistochemical staining of TNF-α and TGF-β1

A paraffin section was heated in an incubator at 56-58˚C for 30 min, and then deparaffinized and dehydrated at room temperature. To restrict the endogenous peroxydase activity, the section was incubated for 10 min with methanol and 0.3% hydrogen peroxide, and then washed with phosphate-buffered saline (PBS, pH 7.4) for 5 min 2-3 times. Then, it was processed with a blocking solution (non-immune sheep serum, DAKO kit 1:5, Carpinteria, CA, U.S.A.) at room temperature for 20 min. The specimen was incubated with monoclonal anti-TNF-α (Genetech, San Francisco, CA, U.S.A.) and TGFβ1 (V) (Rabbit Polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) diluted at 1:100 in PBS at room temperature for 1 hr. Goat anti-mouse antibody, biotin-labeled secondary antibody, and avidin-biotin complex were administered for 1 hr each. After a 10-min incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB) as a substrate for a color reaction, the specimen was stained with Mayer's hematoxylin, sealed up with a glycerol mounting medium (DAKO-PATTS), and observed under an optic microscope. As a positive control for the expression of cytokine, the spleen and tonsillar tissues were used. With the help of a pathologist, the cells with their nuclei stained dark brown over the whole scope of the coronal section on the irradiated cerebral cortex were checked using an optic microscope at a 200 magnification. The expression of TNF-α and TGF-β1 with the lapse of time after irradiation was compared between experimental groups.

Statistical processing

The expression of cytokine with the lapse of time after irradiation was statistically processed using the Sigmastat for Windows version 1.0 (Jandel Corp., San Rafael, CA, U.S.A.). One-way ANOVA was used to verify the measurements between experimental groups, and paired t-test was used to verify the difference in measurements between right and left cerebral hemispheres.

RESULTS

Histopathology with the lapse of time after irradiation

The loss of the irradiated cerebral cortex was 2.8±2.1% (1 week), 7.9±1.5% (2 weeks), 35.3±6.4% (4 weeks), 53.1±2.3% (6 weeks), and 71.4±5.1% (8 weeks), which showed a statistically significant increase with the lapse of time (one-way ANOVA, $p<0.05$) (Fig. 2). A gradual loss of the outermost molecular layer was also shown with the lapse

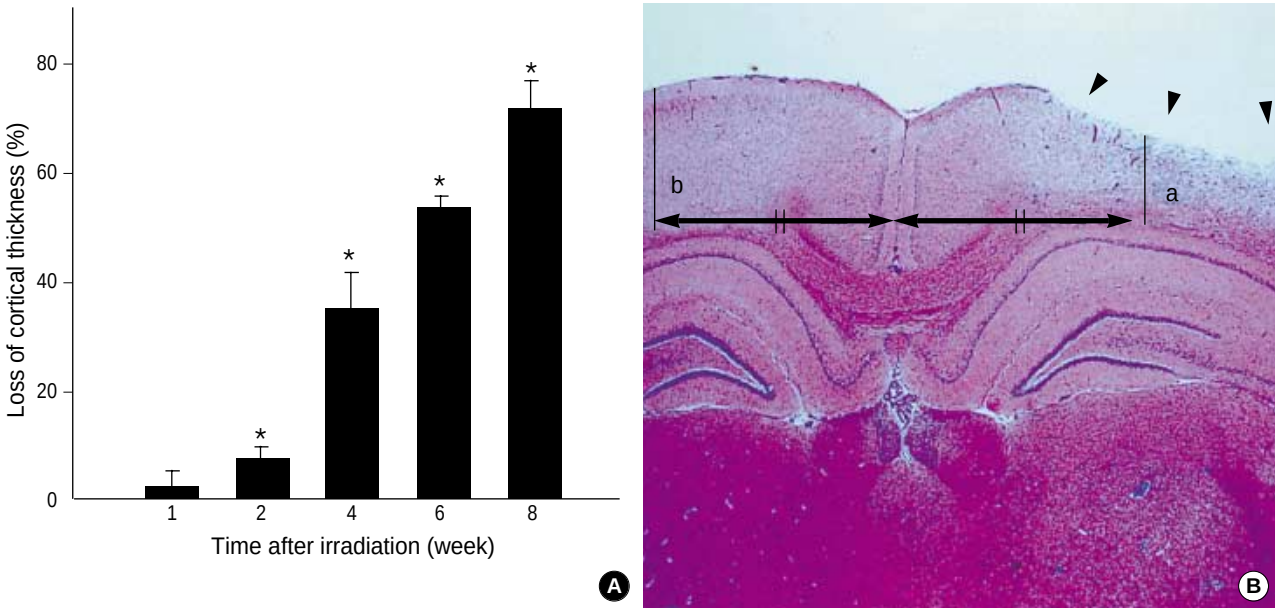

Fig. 2. Loss of cortical thickness according to the time in the irradiated hemisphere. *$p<0.05$ by one-way ANOVA. (A). Measurement of loss of cortical thickness in the irradiated hemisphere (arrowheads), (b-a)/b × 100 (%). (H-E stain, × 12.5) (B).

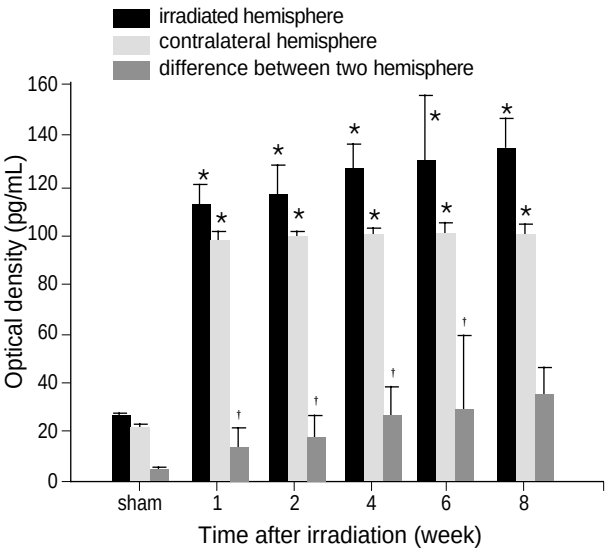

Fig. 3. Expressions of TNF-α according to the time after irradiation. *p<0.05 by one-way ANOVA, †p<0.05 by paired t-test.

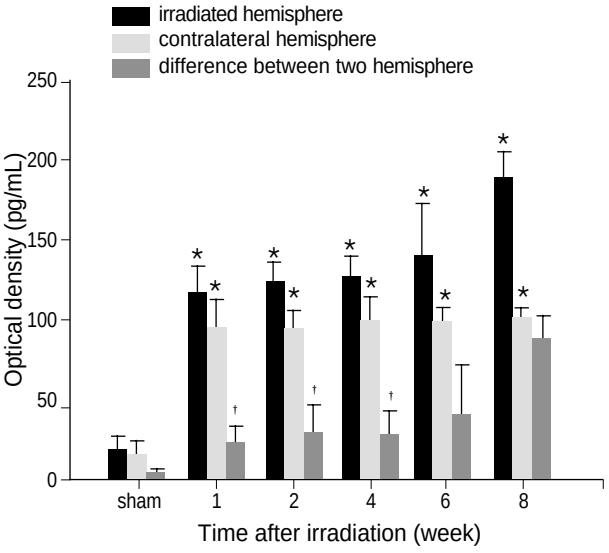

Fig. 4. Expression of TGF-β1 according to the time after irradiation. *p<0.05 by one-way ANOVA, †p<0.05 by paired t-test.

of time after irradiation, and other characteristics including fibrous gliosis, appearance of inflammation cells, proliferation of vascular endothelial cell, and necrosis increased.

Quantitative analyses of cytokines with the lapse of time after irradiation

The expressions of the TNF-α and TGF-β1 in the irradiated right cerebral hemispheres and non-irradiated left hemispheres were quantified as follows. The expression of TNF-α in the irradiated right cerebral hemispheres was 111.4±8.0 pg/mL (1 week), 115.7±12.6 pg/mL (2 weeks), 125.6±10.1 pg/mL (4 weeks), 128.1±27.3 pg/mL (6 weeks), and 133.4±11.0 pg/mL (8 weeks), which showed a statistically significant increase as compared with 26.5±0.5 pg/mL in the sham operation group (one- way ANOVA, p<0.05), but there was no significant difference among the measurements through 1 week and 8 weeks (Fig. 3). The expression of TNF-α in the non-irradiated left cerebral hemispheres was 97.8±3.4 pg/mL (1 week), 98.4±3.5 pg/mL (2 weeks), 99.1±3.0 pg/mL (4 weeks), 99.2±5.4 pg/mL (6 weeks), and 99.6±2.8 pg/mL (8 weeks), which showed statistically significant increase when compared with 22±1.1 pg/mL in the sham operation group, although the extent was smaller than in the right cerebral hemispheres (one-way ANOVA, p<0.05). The difference in the expression of TNF-α between the right and left cerebral hemispheres was 13.6±8.5 pg/mL (1 week), 17.3±11.5 pg/mL (2 weeks), 26.5±11.7 pg/mL (4 weeks), and 28.9±29.5 pg/mL (6 weeks), which showed statistically significant differences (paired t-test, p<0.05).

The expression of TGF-β1 in the irradiated right cerebral hemispheres was 117.5±16.3 pg/mL (1 week), 123.3±11.1 pg/mL (2 weeks), 127.1±12.6 pg/mL (4 weeks), 139.9±

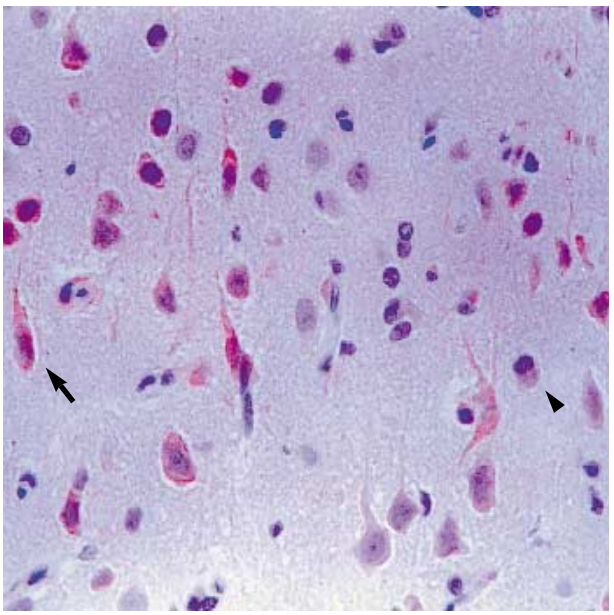

Fig. 5. Immunohistochemical findings demonstrating the expression of TGF-β1 after irradiation. TGF-β1 is expressed predominantly by infiltrating macrophages (arrowhead) and astrocytes (arrow) (6 weeks, ×200).

32.4 pg/mL (6 weeks), and 189.3±14.6 pg/mL (8 weeks), which showed a statistically significant increase with the lapse of time when compared with 19.5±9.1 pg/mL in the sham operation group (one-way ANOVA, p<0.05) and this increase was more prominent at 8 weeks after irradiation (Fig. 4). The expression of TGF-β1 in the non-irradiated left cerebral hemispheres was 95.3±17.1 pg/ mL (1 week), 94.9±12.6 pg/mL (2 weeks), 99.4±15.4 pg/ mL (4 weeks), 99.1±7.6 pg/

mL (6 weeks), and 101.5±6.5 pg/mL (8 weeks), which showed a significant increase when compared with 15.5± 8.8 pg/mL in the sham operation group, although the extent was smaller than in the right cerebral hemispheres (one-way ANOVA, $p<0.05$). The difference in the expression of TGF-β1 between the right and left cerebral hemispheres was 22.2± 10.0 pg/mL (1 week), 28.4±17.9 pg/mL (2 weeks), 27.7± 13.2 pg/mL (4 weeks), and 40.8±29.2 pg/mL (6 weeks), which showed statistically significant differences (paired t-test, $p<0.05$).

Immunohistochemical analyses of the expression of TNF-α and TGF-β1

Immunohistochemical staining for TNF-α showed severe cross reactivities, so that the results could not be interpretated. Immunohistochemical staining for TGF-β1 clearly showed the positive expression of TGF-β1 in necrosis-neighboring areas and the origins of the expression were macrophages and proliferating astrocytes (Fig. 5).

DISCUSSION

A number of studies on malignant brain tumor have been made to date and revealed that a higher dose of irradiation would be more effective in killing the remaining brain tumor cells in most cases (14, 15). The development of neuroimaging technology enabled the replacement of the standard external beam radiation (teletherapy) with a relatively wide range of irradiation with the interstitial brachytherapy or the focused beam teletherapy using a gamma knife or linear accelerator (3, 7, 16, 17). Recently, intraoprative radiation therapy (IORT) is clinically used for a more effective radiation and many researchers are employing this method. IORT enables the intraoperative direct examination of tumors and delivery of a great amount of radiation on the tumor site, which can maximize the radiation effect and prevent the recurrence of tumors effectively (14). It is believed, however, that even with a shield in place during irradiation to protect normal tissues at a maximum efficacy, irradiation may cause a considerable damage to normal cerebral tissues (18). It is well assumed that such damage may be different from that with a relatively low radiation so-called conventional radiotherapy, however, few studies have been made on radiation injuries after a single high-dose irradiation, such as radiation necrosis and apoptosis. Cerebral injury due to irradiation has been widely known to vary from a temporary malfunction to overall changes in quality, but the precise physiopathological mechanisms have not been clarified yet. There are hypotheses that a number of cytokines and immunoregulatory molecules expressed in the irradiated brain cells may be involved in enhancing the vascular permeability and causing cytotoxicity, astrocytosis, or glial proliferation (19-23). According to Calvo et al. (24), the incidence of necrosis in the cerebral white matter of the rat after a high-dose irradiation is closely related to the amount of radiation and the lapse of time after irradiation. The higher dose of radiation and the more lapse of time after irradiation were associated with the more serious injury of cerebral tissues because of necrosis due to the proliferation of vascular endothelial cells. Our study also found that over an 8-week period (subacute stage) after irradiation of 10 Gy on normal cerebral tissues of the rats, the outermost molecular layer and the overall cortical thickness of the irradiated site were gradually lost with the lapse of time, accompanied with gradual increases in fibrous gliosis, appearance of inflammation cells, proliferation of vascular endothelial cell, and necrosis.

Various cytokines and growth factors play an important role in controlling the growth and differentiation of normal cerebral and cancer cells exposed to radiation (25), and focus is now placed upon the roles of the cytokines in normal or troubled cerebral tissues (8, 26-28). Recent studies have found that the radiation accelerates the expression of mRNA of TNF-α (2, 10). Kureshi et al. reported that cerebral biopsy after radiation treatment for patients with brain tumors revealed a considerable increase of TNF-α, interleukin-6 (IL-6), and TGF-β (3). Our study also found that TGF-β1 was strongly expressed in macrophages and proliferating astrocytes around the site of necrosis. TNF-α is a 17 kDa polypeptide, which is typical cytokine produced mainly by macrophages and monocytes (29, 30).

TNF-α is an intermediator of the immune system protecting the host from attacks of various infections and cancer cells, and notably, induces death of cancer cells by causing apoptosis (9). It has also been known that TNF-α induces the production of various cytokines in human cells, e.g., interleukin-1 (IL-1) in vascular endothelial cells and tumor cells, and IL-6 in fibroblasts (31-34). Helseth et al. reported that TNF-α accelerated the cytotoxity of TGF-β in human glioblastoma cells (35). Chiang et al. reported the in vitro findings that a low-dose irradiation caused the production of TNF-α in microglial cells and astrocytes (2). In addition, there has been a report that the production of TNF-α was increased in cultured monocytes taken from patients who had underwent a radiation treatment (36). Based on these study results, the expression of TNF-α caused by radiation was assumed to play an important role in relation to several complications after irradiation. Neta et al. reported that the injection of TNF-α into the rat before irradiation caused a radioprotective effect in the hematopoietic system while the pretreatment of an antibody against TNF-α decreased the survival of experimental animals, demonstrating that TNF-α served as the innate defense mechanism against radiation by restricting oxidative damage and inducing *bcl-2* genes, and thereby decreasing apoptosis (38, 39). Based on these findings, Kureshi et al. asserted that TNF-α might be involved in the physiological radioresistance and recovery process (3). However, the exact roles of TNF-α in brain and malignant cells after a single hige-

dose radiation are not clearly established up to now.

TGF-β produced mainly from astrocytes and macrophages is a polypeptide with various biological actions in the human body and composed of 5 subtypes (39-42). TGF-β1, a typical subtype, is 25 kDa homodimer protein made up of 112 amino acid polypeptides (39). TGF-β has been reported to work in two directions, i.e., to restrict or accelerate the growth of cancer (43). It serves as a defensive molecule restricting the proliferation of cancer cells at least during the initial development (44, 45). There was a report that a high dose of irradiation increased the expression of TGF-β in the rat liver (3). Neta et al. asserted that TGF-β should decrease the production of TNF-α in the hematopoietic system, thereby serving as a radiosensitizer (37). Using cerebral biopsy specimen in patients with brain tumor who had underwent a radiation treatment, Kureshi et al. reported that TNF-α and IL-6 were expressed mainly by macrophages and TGF-β by astrocytes and macrophages (3). The present study also shows consistent results, finding that TGF-β1 was expressed mainly by macrophages and astrocytes after a single high-dose irradiation.

In the present study, we found that the expressions of TNF-α and TGF-β1 in the irradiated right cerebral hemispheres were significantly increased with the lapse of time during an 8-week post-radiation period (subacute stage) when compared with those in the sham operation group. These results are consistent with the report that the functions of TNF-α and TGF-β1 are not fixed but have a multidirectional nature depending on given cells, tissues, and their conditions, through a cascade and interaction with other cytokines. In this manner, they relate to the proliferation of astrocytes, vascular endothelial cells, and the destruction of blood-brain barriers usually shown after irradiation and contribute to the radiation injury including gliosis, vascular injury, and necrosis (3). Further studies are needed to clarify the mechanisms for the interactions among various cytokines, including TNF-α and TGF-β1, in irradiated cerebral tissues. Of note, our study showed that the expressions of TNF-α and TGF-β1 were also significantly increased in the non-irradiated left cerebral hemispheres, although such increase was smaller than that in the irradiated hemispheres. Although the mechanism is unclear, these results suggest the possibilities of inflammation caused by burrhole procedure or migration of macrophages to the opposite hemispheres from the irradiated hemisphere or inflow of cytokines through the tissue fluid.

In summary, we demonstrated that the radiation injuries after a single high-dose irradiation that is used for various malignant tumors in recent days consist of radiation necrosis and apoptosis, which were associated with the expression of TNF-α and TGF-β1. If we can clarify the roles of cytokines in relation to radiation injury, we can develop methods of restricting or accelerating the expression of the cytokines, which will contribute to the prevention of complications after radiation therapy.

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