The Up-Regulation of CXCL12-CXCR4 Axis By Radiotherapy Could Accelerate Glioma Progression

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Abstract

Background:

This study investigated whether the effect of changes in the microenvironment of parenchymal brain tissue caused by radiotherapy for malignant brain tumors affect the recurrence and progression of glioma.

Methods:

3 months after the same 65-Gy irradiation had been applied to the right hemisphere. Irradiated Fisher rats were divided into three groups for in vitro assay as follows. IR/Ipsi-brain; the right-hemisphere tissue was used for experiments. IR/Contra-brain; the left-hemisphere tissue was used. Sham-IR/Brain; sham-irradiation was applied to the brain, and the right-hemisphere tissue was used. The effects of proteins extracted from the brains directly or indirectly affected by irradiation on the growth of F98 cells, the effect on tube formation, the influence on tumor biology, and the influence on cytokine production were investigated. Additionally, irradiated animals were divided into three groups for in vivo assay as follows. IR/Ipsi-tumor; F98 cells (a glioma cell line) were transplanted to the right hemisphere. IR/Contra-tumor; F98 cells were transplanted to the left hemisphere. Sham-IR/Tumor; F98 cells were transplanted to the right hemisphere without irradiation. The median survival time of F98 transplanted rats was also examined.

Results:

X-ray irradiation promoted the secretion of cytokines such as TNF α , TGF- β 1, VEGF-A, and CXCL12 from the irradiated brain. F98 glioma cells implanted in the irradiated brains showed significantly high proliferation and angiogenesis ability, and the post-irradiation F98 tumor-implanted rats showed a shorter median survival time compared to the Sham-irradiation group.

Conclusions:

These results indicate that the up-regulation of CXCL12-CXCR4 axis by radiotherapy could promote tumor proliferation. Radiation therapy is a standard treatment for malignant gliomas including glioblastoma multiforme, but the current study suggests that the microenvironment around the brain tissue in the chronic phase after exposure to X-ray radiation becomes suitable for glioma cell growth and invasion.

Introduction

Initially, the standard treatment of malignant glioma involved surgical removal of as many tumors as possible followed by radiotherapy. Although progression-free survival of glioblastoma is approximately ten months [1], it has been demonstrated that X-ray treatment reduces the recurrence of tumor and increases the life expectancy [2,3]. To prevent the growth of tumor cells invading tumor-surrounding brain tissue, radiotherapy is targeted at the area within 3-4 cm of the tumor resection cavity [4]. Nevertheless,

the recurrence of more than 95% of glioma has been observed in the area within 2-3 cm of the tumor resection cavity at several months or years after radiotherapy [5,6].

It is known that the implantation of tumor cells into the radiation-exposed tissue delays the onset and reduces the growth rate of tumors [7]. The effect, which was later named as the tumor bed effect [8], is considered to be the main cause of reduced neovascularization [9,10]. In general, the radiation-exposed tissue environment, which is characterized by reduced blood perfusion (nasal CPAP) [11], reduced extracellular pH [11], and a hypoxic environment [12], is considered to be the microenvironment that is not suitable for the survival of tumor cells.

Other authors reported that radiation stimulated an infiltration of F98 glioma cells into the brains of tumor-bearing Fischer rats, resulting in a reduction of their MST [13]. This stimulation was associated with pro-inflammatory mediators such as cyclooxygenase-2 (COX-2), interleukin-1 β (IL-1 β), and matrix metalloproteinase-2 (MMP-2). In a related study, the inhibition of inflammatory cytokines by a COX-2 inhibitor prevented F98 glioma progression in the irradiated rat brain and increased the MST [14]. These reports suggested the possibility that tumor growth could paradoxically be stimulated in the acute phase after radiation therapy, but there has been no report discussing the influence of irradiation in the chronic stage on glioma in rodent models.

The tissue microenvironment, in which the regrowth of tumor cells that survived the initial treatment occurs within several months after exposure to the radiation, is the brain tissue different from that of the primary tumor. In this study, we examined the effect of changes in the microenvironment of parenchymal brain tissue (i.e., the tumor bed) in the chronic phase after exposure to X-Ray radiation on F98 glioma cells.

Methods

Cell line

The rat F98 glioma cell line was obtained from ATCC (Manassas, VA) and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin at 37°C in an atmosphere of 5% CO₂. These materials for the culture medium were purchased from Gibco Invitrogen (Grand Island, NY, USA).

Animal experiment A: X-ray irradiation to the rat brain

All of the experimental procedures used in this study were approved by the Animal Review Board and Ethical Committee of Osaka Medical College (Approval No.: 27058). This study was conducted in accordance with the 'Ethics Declaration' for the ARRIVE guidelines' (<https://arriveguidelines.org>). Eight-week-old, male Fischer 344 rats weighting between 270-320 g (Japan SLC, Inc. Shizuoka, Japan) were anesthetized with an intraperitoneal injection of a mixture of medetomidine 0.15 mg/kg, midazolam 2 mg/kg, and butorphanol 2.5 mg/kg.

During X-ray irradiation, the 5 mm-thick lead shield with a 1 cm square window were used to protect the whole body of the rats. For rats in the irradiation groups (IR: n=8), 100-kV X-ray was irradiated to the right cerebral hemisphere through the 1 cm square window, with a single dose of 65 Gy using a linear X-ray accelerator (SOFTEX M-150WE; Kanagawa, Japan). The irradiation area was determined with the bregma as reference points. The sham-irradiation was performed for rats in the control group (Sham-IR: n=7).

RNA and protein extraction from the irradiated rat brain

Three months after the X-ray irradiation, the rats were euthanized to obtain their brain tissue. After the brains were divided along the midline, the tissue protein and whole RNA were separately extracted from the right (IR/Ipsi-brain: n=8) and left cerebral hemispheres (IR/Contra-brain: n=8) of the animals irradiated on the right brain as mentioned in Figure1A. As a control, we prepared proteins and total RNA extracted from the right cerebral hemisphere of the sham-irradiated animals (Sham-IR/Brain: n=7). The RNA was extracted using miRNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA library preparation was performed using a SuperScript VLIO cDNA Synthesis kit (Thermo Fisher Scientific, Cleveland, USA) according to the manufacturer's instructions. The brain tissue protein was extracted with the 3-min total protein extraction kit for animal tissues (101 Bio, Palo Alto, CA). The concentrations of the extracted protein were determined by the Pierce™ BCA Protein Assay Kit (Thermo Scientific-Life Technologies, Waltham, MA).

In vitro cell proliferation assay using F98 glioma cells

The proliferation ability of the tumor cells was analyzed by a WST-8 assay. F98 cells were seeded in 96-well plates at a density of 10,000 cells/well in 100 µl of culture serum-free DMEM. At 24 hours after this pre-incubation, 100 µl of medium containing the extracted brain protein with 1.5, 3.0 or 6.0 µg/ml was added to the wells. 72 hours after the incubation, 10 µl of WST-8 labeling reagent (Cell Counting Kit; Dojindo Laboratories, Kumamoto, Japan) were added to each well and the absorbance at 450 nm was measured with the corona grating microplate reader (Hitachi High-Tech Corporation, Japan).

In vitro tube-formation assay using HUVEC

For the in vitro tube-formation assays, 96-well plates were coated with growth-reduced factor Matrigel (BD Biosciences, catalog number: 354230) on ice and incubated at 37°C for 2 hours until the Matrigel became solid. Human umbilical vein endothelial cells (HUVECs) (Life Technologies, Invitrogen, catalog number: C-003-5C) were seeded in 96-well plates at a density of 20,000 cells/well and cultured in 150 µl of medium containing 3.0 or 6.0 µg/ml of the cerebral proteins extracted the above. After a 3-hour (incubation in 5% CO₂ at 37°C, images were taken with at a magnification of 100× (10 ocular×10 objective), and the lengths of the tubes in four random fields were quantified by Image J software version1.52. (U.S. National Institutes of health, Maryland, USA)

Animal experiment B: glioma cell transplantation into the irradiated rats

Animals groups and study design

Thirty-two male Fischer 344 rats (8-week-old, weighting between 270-320 g) were used.

65-Gy of X-ray was irradiated to the right cerebral hemisphere of 20 rats as described above, and sham-irradiation was performed for 12 rats to prepare a control group.

Three months after the irradiation, the 20 rats were randomly divided into two groups (IR/Ipsi-tumor or IR/Contra-tumor) as mentioned in Figure1B. In “IR/Ipsi-tumor” group (n=12), the F98 glioma cells were implanted to the right cerebral hemisphere of the irradiated animals. On the other hand, in the “IR/Contra-tumor” group (n=8), F98 cells were implanted to the left hemisphere of the irradiated animals. All the sham-irradiated animals (n=12) were implanted with F98 cells into right hemisphere (Sham-IR/Tumor group). The rats were observed daily for body weight and neurological deficits (n=5). They were euthanized by inhalation of Sevoflurane one days before of death, and the brains were removed for the following analyses.

Tumor implantation

At the chronic phase (3 months) after the X-ray irradiation, F98 glioma cells were implanted into the cerebral hemisphere of rats in the above three groups. Under anesthesia, the rat's head were fixed with a stereotactic frame (Model 900; David Kopf Instruments, Tujunga, CA, USA), and a small burr hole was made on the skull with an electric drill at 1 mm anterior and 3 mm lateral of the bregma. 1×10^4 of F98 cells were suspended in 10 μ l of DMEM and injected at a rate of 1 μ l/min into the brain at a depth of 5 mm from the skull burr hole, using a 25- μ l Hamilton microsyringe with a 26-gauge needle (model 1700 RN, Hamilton Bonaduz AG, Bonaduz, Switzerland) and a micro-injector (WPI model UMP3, Sarasota, FL, USA). After the injection was complete, the burr hole was sealed with bone wax.

Immunohistochemical analyses for the tumor

Cell proliferation index: The primary antibody, Ki-67 (clone MIB-1; dilution of 1:100) (Abcam, Cambridge, MA, USA), and a universal secondary antibody (dilution of 1:300) (Roche, Basel, Switzerland) were used. The cell proliferation index was calculated by taking five random pictures of each slide at a magnification of 200 $\times$ (10 ocular $\times$ 20 objective). The index was calculated as the number of MIB-1-stained cells divided by the total number of cells. Hematoxylin was used for the counterstaining for cell nuclei.

Apoptotic index: To detect the cell apoptosis, the terminal deoxynucleotidyl transferase (TdT) -mediated dUTP nick end labeling (TUNEL) assay was performed using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Cosmo Bio, Tokyo, Japan). The apoptotic index was evaluated by counting the number of TUNEL-positive cells over the total number of cells at a magnification of 200 $\times$ (10 ocular $\times$ 20 objective) in five random fields.

Microvascular density (MVD) index: The primary antibody, CD34 (dilution of 1:2500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), was used with a universal secondary antibody (dilution of 1:300) (Roche, Basel, Switzerland). The MVD index was calculated by taking five random pictures of each slide at

a magnification of 200× (10 ocular×20 objective). The index was calculated as the number of CD34-stained cells divided by the total number of cells.

Evaluation of glioma invasiveness

F98 rat glioma model shows tumor cell invasion into normal brain parenchyma at the tumor periphery. Implanted brain tumors were removed from the three groups; IR/Ipsi-tumor, IR/Contra-tumor, and Sham-IR/Tumor groups, fixed with formalin and embedded in paraffin. The FFPE blocks were sliced and stained with hematoxylin and eosin. The invasion index was evaluated by taking pictures of each slide at a magnification of 50× (10 ocular × 5 objective). The index was calculated as the total area of glioma crossing tumor rim divided by the field of tumor regions in serial sections using *Image J* software [53]. The invasive tumor ratio of the Sham-IR/Tumor group was defined as 1.0.

RNA extraction

Total RNA was extracted and prepared cDNA from the F-98 cell implanted tumors in the three groups; IR/Ipsi-tumor, IR/Contra-tumor, and Sham-IR/Tumor as described above.

RNA-sequencing

Whole transcriptome sequencing was applied to the RNA samples of brain tissues in triplicate assay (n=3 for each group), with use of on an Illumina HiSeq 2500 platform in a 75-base single-end mode. Illumina Casava ver.1.8.2 software was used for base calling. Sequenced reads were mapped to the rat reference genome sequences (rn6) using TopHat ver. 2.0.13 in combination with Bowtie2 ver. 2.2.3 and SAMtools ver. 0.1.19. The number of fragments per kilobase of exon per million mapped fragments (FPKMs) was calculated using Cuffnorm ver. 2.2.1. The raw data have been deposited in the Gene Expression Omnibus database of the U.S. National Center for Biotechnology Information (NCBI).

As compared to Sham-IR/Brain, the differentially expressed genes (DEGs) in IR/Ipsi-brain and IR/Contra-brain were obtained using a threshold of 0.05 for statistical significance (p-value) and a log fold change of expression with absolute value of at least 1.5. To identify significantly altered pathways, we used an analysis software, "*iPathwayGuide*" (Advita Corporation, Plymouth, MI, USA; info@advaitabio.com). These DEGs were analyzed with the impact analysis method [54-56], in the context of pathways obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Release 81.0+/01-20, Jan 17) [57,58], and gene ontologies from the Gene Ontology Consortium database (2016-Sep26) [59,60]. (See Supporting information: Methods for pathway analysis)

Real-time quantitative PCR (qPCR)

Total RNAs were reverse-transcribed into cDNA with a SuperScript VLIO cDNA Synthesis kit (Thermo Fisher Scientific, Cleveland, USA). The validation gene for the quantitative polymerase chain reaction (qPCR) was selected based on the results of RNA-sequencing. A quantitative real-time PCR was performed with TaqMan probes and primers using the LightCycler[®] (Roche Applied Science, Penzberg,

Germany). The PCR conditions were as follows: a single denaturation cycle at 95°C for 10 min, followed by 45 amplification cycles of 95°C for 10 seconds and 60°C for 25 seconds. The relative expression ratio was calculated after normalization with reference to the expression of the housekeeping gene 18S RNA. The PCR primers were designed by the Roche Universal Probe Library. The expression of cytokines such as tumor necrosis factor alpha (TNF α), tumor growth factor β 1 (TGF- β 1), vascular endothelial growth factor-A (VEGF-A), C-X-C motif chemokine 12 (CXCL12), C-X-C chemokine receptor type 4 (CXCR4), epidermal growth factor receptor (EGFR), fibroblast growth factor-2 (FGF-2), and extracellular signal-regulated kinase2 (ERK2) was assessed by qPCR.

Statistical Analysis

Data are expressed as the mean $\pm$ standard deviation. Statistical analysis of the groups' MST values was performed using the Wilcoxon log-rank test, and other results were analyzed using Student's t-test. Values of $p < 0.05$ were considered statistically significant.

Data availability

All data generated during the current study are included in this article and are available from the corresponding author on reasonable request.

Results

Proteins extracted from the irradiated brain promoted the proliferation of F98 cells

The proliferation of F98 cells under different concentrations (1.5, 3, 6 and 12 ng/ml) of protein extracted from the brain hemispheres of the IR/Ipsi-brain, IR/Contra-brain, and Sham-IR/Brain groups was measured by WST-8 assay. As shown in Fig. 2A, the cells incubated with medium containing protein extracted from an irradiated hemisphere (here, the IR/Ipsi-brain group) showed a significantly higher cell growth rate compared to the rate of the control group (i.e., Sham-IR/Brain group) under 3, 6 and 12 ng/ml of extracted protein. There was no significance difference in the cell growth rate between the non-irradiated hemisphere (IR/Contra-brain group) and the control group (Sham-IR/Brain group).

Tube-forming activity was increased by brain irradiation

The angiogenic activity was assessed by measuring the length of forming tubes under different concentrations (3 and 6 ng/ml) of protein extracted from the IR/Ipsi-brain, IR/Contra-brain, and Sham-IR/Brain rats. As shown in Fig. 2B, the IR/Ipsi-brain group showed significantly longer tube formation compared to the Sham-IR/Brain group under protein concentrations of both 3 and 6 ng/ml. In the IR/Contra-tumor group, significantly longer tube formation was observed compared to the Sham-IR/Brain group only under the 6 ng/ml protein concentration.

Brain irradiation decreased the MST

As shown in Fig. 3 and Table 1, the rats in the IR/Ipsi-tumor and IR/Contra-tumor groups showed significantly shorter MSTs compared to the rats in the Sham-IR/Tumor group. Interestingly, there was no significant difference in survival between the IR/Ipsi-tumor and IR/Contra-tumor groups. The IR/Ipsi-tumor, IR/Contra-tumor, and Sham-IR/Tumor rats all showed good appetites, and there was no difference in the nutritional state among these groups, just before the tumor implantation. We also confirmed that the X-ray irradiated rats without the tumor cell implantation had been living for more than 12 months.

Changes in tumor biology of rats

As shown in Fig. 4A, the Ki67 labeling index increased significantly in the IR/Ipsi-tumor group compared to the Sham-IR/Tumor group. The Ki67 index indices of the IR/Contra-tumor and Sham-IR/Tumor groups were not significantly different. The apoptotic index of the IR/Contra-tumor group was significantly decreased compared to that of the Sham-IR/Tumor group (Fig. 4B). The MVD index of the IR/Ipsi-tumor group was significantly higher than that of the Sham-IR/Tumor group, but there was no significant difference in the MVD index between the IR/Contra-tumor and Sham-IR/Tumor groups (Fig. 4C).

Increased tumor invasion in the irradiated brain

The tumor surface in the IR/Ipsi-tumor group was irregular and the tumors exhibited strong invasiveness. On the other hand, the Sham-IR/Tumor group showed a relatively clear border around the tumor rim. A significant increase in the number of tumor cells invading normal brain tissues was observed in the IR/Ipsi-tumor group compared to the Sham-IR/Tumor group (Fig. 4D). There was no significant difference between the IR/Contra-tumor and Sham-IR/Tumor groups.

Whole transcriptome analysis (RNA-seq) for the irradiated brain

In comparison with the Sham-IR/Brain (control), 383 differentially expressed genes (DEGs) in the IR/Ipsi-brain group and 336 in the IR/Contra-brain group were identified out of a total of 13,265 and 13,050 genes with measured expression, respectively. Among them, 64 DEGs were commonly up- or down-regulated both in the IR/Ipsi-brain and IR/Contra-brain of the irradiated animals, in comparison with Sham-IR/ Brain, as listed in Supplementary Table 1. 4 out of 52 DEGs up-regulated both in the IR/Ipsi-brain and IR/Contra-brain code the peptides or proteins localizing in the extracellular space; *OSM* (oncostatin M), *LOXL4* (lysyl oxidase like 4), *ADAMTS14* (ADAM metalloproteinase with thrombospondin type 1 motif 14), and *MMP9* (matrix metalloproteinase 9).

The pathway analysis for the DEGs revealed that 41 and 40 pathways were significantly impacted in the IR/Ipsi-brain and IR/Contra-brain groups, respectively, as compared to the Sham-IR/Br (Supplementary Figure 1). Among them, 13 pathways were commonly impacted both in IR/Ipsi-brain and IR/Contra-brain groups, and out of these pathways, four pathways involved tissue inflammation, such as cell adhesion molecules (CAMs), chemokine signaling pathway, leukocyte transendothelial migration, and cytokine-cytokine receptor interaction (Table 2). After the Bonferroni correction, two pathways still showed the significant p-values in IR/Ipsi-brain group, compared to Sham-IR/Brain group (Table 2).

Stimulation of genes and molecules in the brain by irradiation

Based on the results of the above transcriptome analysis (RNA-seq.), we performed validation qPCR for several genes. As shown in Fig. 5, significant increases were observed in the expressions of TNF α , TGF- β 1, VEGF-A, and CXCL12 in the IR/Ipsi-brain group at 3 months after radiation compared to the Sham-IR/Brain group. The expressions of TNF α , TGF- β 1, and CXCL12 in the IR/Contra-brain group were significantly higher than those of the Sham-IR/Brain group.

Stimulation of genes and molecules in the tumor by irradiation

As shown in Fig. 6, significant increases were observed in the expressions of CXCR4, EGFR, FGF-2, ERK2 (MAPK1), and VEGF-A in the IR/Ipsi-tumor group compared to the Sham-IR/Tumor group. The expressions of CXCR4, FGF-2 and ERK2 in the IR/Contra-tumor group were significantly higher than those in the Sham-IR/Tumor group.

Discussion

In this study, we have demonstrated that the production of proinflammatory cytokine increased in the microenvironment of the parenchymal brain tissue (tumor bed) in the chronic phase after exposure to X-ray radiation. This effect is considered to be the microenvironment that is not suitable for the survival of tumor cells. The current study suggest that X-ray radiation made the microenvironment of brain tissue in the chronic phase suitable for the regrowth of glioma cell and invasion of the brain tissue.

First, we applied local X-ray radiation to the right hemisphere of rats. Then, after removing the brain from the rat skull, we extracted the total protein from brain tissue of both hemispheres. Compared with proteins extracted from rats that received sham exposure, proteins extracted from the radiation-exposed hemisphere promoted tumor growth in F98 glioma cells as well as tube formation in HUVECs. Interestingly, proteins extracted from non-exposed parts of the hemisphere in radiation-exposed rats showed similar effects, although they required high concentration of proteins. The results suggest an increase in the expression of proteins that promote the proliferative activity of tumor cells and neovascularity in brain tissue in the chronic phase after exposure to the radiation. In this experiment, X-ray radiation was applied to the right hemisphere using a lead shield with a 10 mm x 10 mm square window. Local X-ray radiation to the hemisphere was confirmed through measuring the area of alopecia of the rat scalp (rats with areas of alopecia that crossed the midline were excluded from this study).

Then, we exposed the right hemisphere to local X-ray radiation and implanted F98 cells in the hemisphere at three months after exposure to the radiation to induce the formation of brain tumors (IR/Ipsi-tumor group). F98 cells were also implanted into two control groups: IR/Contra-tumor group (exposed to the radiation and then implanted with F98 cells in the non-exposed hemisphere) and sham-IR/tumor group (exposed to sham and then implanted with F98 cells in the hemisphere). In this experiment, the implanted tumors did not receive X-ray radiation. Therefore, the difference in the behavior of the implanted tumors may be attributed to the differences in the microenvironment of the tumors. The overall survival (OS) of

IR/Ipsi-tumor group after implantation of F98 glioma cells in the hemisphere previously exposed to X-ray radiation was significantly lower than that of the control (sham-IR/tumor) group (MST: 20.5 vs. 22.5, log-rank test $p=0.002$). Moreover, interestingly, the OS of the group after implantation of F98 cells in the hemisphere that did not receive direct X-ray radiation (the IR/Contra-tumor group) was also significantly lower than that of the control group (MST: 20.5 vs. 22.5, log-rank test $p=0.003$). The results of the analysis of proliferative activity, apoptosis rate (TUNEL staining), and density of tumor vessels (CD34 staining) in tumors harvested from the three groups showed a higher MIB1 index (by about 22%, $p=0.01$) and blood vessel density (by about 40%, $p=0.045$) and lower levels of TUNEL-positive apoptosis (by about 40%, $p=0.03$) in tumors that were formed in the hemisphere after exposure to X-ray radiation (IR/Ipsi-tumor group) than in the control group. Conversely, compared to the control groups, tumors in IR/Contra-tumor group exhibited an increase in MIB-1 index by about 18% ($p=0.04$) and a decrease in TUNEL-positive apoptosis by about 50% ($p=0.01$), but no significant difference in the tissue blood vessel density. Among rodent GBM models, the F98 model is a disease model of tumor formation with extensive invasion of tumor-surrounding brain tissue [15]. The evaluation of the degree of invasion in the surrounding brain tissue showed a significant increase in the invasiveness in IR/Ipsi-tumor group than in sham-IR/tumor group. The above results suggest that X-ray radiation made the microenvironment of brain tissue in the chronic phase suitable for the regrowth of glioma cell and invasion of the brain tissue.

Therefore, to analyze the changes in the microenvironment of the brain tissue in the chronic phase after exposure to X-ray radiation at the molecular level, we performed transcriptomic analysis (RNA sequencing) of RNA extracted from the rat cerebral hemispheres (IR/Ipsi-brain and IR/Contra-brain groups) and the brain tissue harvested from sham group. In the analysis, we identified 52 genes exhibiting significantly high expression in the radiation-exposed hemisphere of rats previously exposed to radiation (IR/Ipsi-brain group) and in the non-exposed hemisphere of rats previously exposed to radiation (IR/Contra-brain group) compared to those in the control group (rats that did not receive radiation). We also identified genes exhibiting twice the expression level among molecules secreted from cells (i.e., Oncostatin M, ADAM metalloproteinase, and Matrix metalloproteinase 9). Additionally, the pathway analysis of differentially expressed genes revealed significant changes in the pathways involved in tissue inflammation, such as cell adhesion molecules (CAMs), chemokine signaling pathway, leukocyte transendothelial migration, and cytokine-cytokine receptor interaction.

Further analysis of TNF α , TGF β , CXCL12, and VEGF-A using RT-PCR revealed a significant increase in the expression of the above-mentioned four genes except for VEGF-A in IR/Ipsi-brain and IR/Contra-brain groups compared to the control group. Conversely, the expression of VEGF-A was significantly high only in IR/Ipsi-brain group compared to the other groups. In previous studies, an increase in the expression of TNF- α , TGF- β 1, CXCL12, and VEGF-A in the rat brain after a single high-dose X-ray radiation has been reported [16,17]. CXCL12 is a factor known to function as a ligand of CXCR4. It is known that CXCL12 is a chemokine that increases its production in the tissue as part of the inflammatory responses, and that the CXCL12/CXCR4 axis promotes the proliferative activity and invasiveness of tumor cells, including malignant glioma and other various cancers, with the expression of CXCR4, a receptor of CXCL12 [18-21]. VEGF-A is a key factor involved in tumor neovascularization. Levin et al. reported that bevacizumab, a

VEGF antibody, prevents radiation necrosis and related cerebral edema through reducing vascular permeability [22,23]. Additionally, bevacizumab has been reported to improve progression free survival in patients with glioma [24]. Furthermore, the activation of CXCR 4 has been reported to induce intratumoral expression of VEGF-A in glioma cells that respond to CXCL12 [25,26]. TGF- β 1 is an important factor involved in tumor growth and metastasis [27]. In vitro and in vivo experiments revealed that TGF- β 1 released from microglial cells increased the invasiveness of glioma cells [28,29]. Moreover, TGF- β 1 is a known factor that is also involved in the neovascularization of malignant tumors [30,31]. TNF α , a proinflammatory cytokine that plays an important role in the microenvironment of tumors has been associated with tumor invasion and metastasis. A previous study showed that TNF α activated MAPK/ERK signals, leading to an increase in the tumor invasiveness in breast cancer [32]. Additionally, TNF α has been reported to increase the invasiveness of C6 glioma as well [33,34]. Moreover, TNF α has been reported to increase CXCR4 expression in glioma cells [35]. A significant increase in the expression of the above-mentioned proinflammatory cytokine in the parenchymal tissue harvested from the hemisphere at three months after exposure to X-ray radiation in this study was consistent with previous studies in humans and animals [36-38]. An increase in the production of proinflammatory cytokine has been suggested to be due to the changes in the microenvironment of the parenchymal brain tissue (tumor bed) in the chronic phase after exposure to X-ray radiation.

Next, to analyze the effect of the microenvironment of the brain tissue (tumor bed) in the chronic phase after exposure to X-ray radiation on the implanted tumor cells at the molecular level, we performed sequencing of RNA extracted from glioma implanted in IR/Ipsi-tumor and IR/Contra-tumor groups as well as sham group. RT-PCR analysis of FGF-2, CXCR4, VEGF-A, EGFR, and ERK2 (MAPK1) revealed significantly higher expression of three genes (i.e., CXCR4, FGF-2, and ERK2) in IR/Ipsi-tumor and IR/Contra-tumor groups compared to the control groups. Conversely, the expression of EGFR and VEGF-A was significantly higher only in IR/Ipsi-tumor group compared to the other groups. As described above, an increase in the expression of CXCL12 in the brain after exposure to X-ray radiation may affect the implanted tumor cells, leading to an increase in the expression of CXCR4 in tumor cells. Additionally, glioma cells also secrete CXCL12, probably leading to the proliferation and neovascularization of glioma through the autocrine/paracrine mechanism involved in the CXCL12/ CXCR4 receptor ligand system. Analysis of the brain tumor model with increased tissue expression of CXCL12 after exposure to the radiation of glioma cells implanted in the brain compared with tumors implanted in the brain that did not receive radiation showed higher MIB1 expression, as a marker of proliferative activity (Fig. 4A), and higher infiltration in the periphery of tumor tissue (Fig. 4D). CXCR4 expression was also detected in vascular endothelial cells, and an increase in CXCL12 expression in the local tissue is known to promote neovascularization [39,40]. Additionally, extracellular CXCL12 promotes tumor angiogenesis through the production of VEGF-A by tumor cells [26]. Analysis of the number of vessels per unit area in tumor tissue also showed high blood vessel density of tumor cells implanted in the brain after exposure to the radiation (Fig. 4C). The expression levels of EGFR, a receptor that exhibits high expression in glioma tissues, have been significantly correlated with the malignancy of glioma [41]. It has been reported that CXCL12 activates EGFR ligands that are located downstream of CXCL12/CXCR4 [42,43]. Our current

study also showed a significant increase in EGFR expression in tumors implanted in the brain with high expression of CXCL12 in the chronic phase after exposure to the radiation (Fig. 6). Analysis of glioma cells implanted in rat brain after exposure to the radiation compared to those in the control groups (i.e., rats that did not receive X-ray radiation) revealed a significant increase in the production of FGF-2 by the tumor cells and a significant decrease in the rate of differentiation of apoptotic cells in the tumor tissue. It is known that an increase in FGF-2 expression induced through autocrine production leads to increased proliferative activity, reduced apoptosis, and increased neovascularization, resulting in the maintenance of glioma stem cells [44,45]. Furthermore, the activation of these growth factors and MAPK (MEK-ERK) pathway, an intercellular signaling pathway involved in cell growth located downstream of their receptors, may lead to an increase in the production of glioma cells [46]. High expression of ERK has been confirmed in glioma cells implanted in rat brain after exposure to the radiation.

The results of this study suggest that the microenvironment (tumor bed) round the parenchymal brain tissue, especially humoral factors (mainly cytokines), at several months after exposure to X-ray radiation, becomes suitable for the replication of tumor cells that survived after X-ray treatment, mainly through modifying the phenotype of the tumors (e.g., proliferative activity of glioma, antiapoptotic effects, invasiveness, and tumor angiogenesis). In this study, glioma cells implanted in the brain did not receive radiation. Therefore, the changes in tumor cells (e.g., proliferative activity, apoptosis, and neovascularization) observed in this study may be attributed to the differences in the microenvironment (tumor bed) of parenchymal brain tissue where glioma cells were implanted. This experiment was able to demonstrate only the effect of radiation-induced late brain injury on tumors. More interestingly, the results of this study revealed a significant increase in the proliferative and antiapoptotic effects on tumors that were also formed in the non-exposed hemisphere of rats previously exposed to X-ray radiation. Such changes in the tissue microenvironment (tumor bed) in the chronic phase after exposure to the radiation may also be observed outside the radiotherapy field. Sequencing of RNA extracted from the brain tissue at three months after exposure to X-ray radiation also ranked high in “the chemokine signaling pathways” as the pathways of differentially expressed genes (Table.2). Therefore, the above-mentioned effects on area outside the radiotherapy field may be due to humoral factors, such as secretory growth factors and cytokines, that are activated through chronic tissue inflammation induced upon exposure to X-ray radiation. In fact, the validation of interstitial expression of TNF α , TGF β 1, CXCL12, and others, using quantitative PCR showed a significant increase in the expression of these humoral factors in the non-exposed hemisphere of rats previously exposed to X-ray radiation (Fig.5). Furthermore, the results suggest that the tissue proteins extracted from the non-exposed hemisphere of rats previously exposed to X-ray radiation increased the proliferative activity of glioma cells in vitro and promoted the tube formation in vascular endothelial cells (Fig.2B).

Currently, among chemotherapeutic agents used for the prevention of the recurrence of tumor after radiotherapy, only alkylating agents (e.g., TMZ, ACNU/BCNU) exhibited evidence of improved prognosis in patients with glioma [47-49]. However, in terms of the prevention of recurrence of glioma, not only the use of above-mentioned agents that directly inhibit the proliferation of tumor cells, but also the development of agents that can potentially delay tumor recurrence through making the microenvironment (tumor bed)

inappropriate for the replication of tumor cells should be considered. The rate of incidence of GBM recurrence outside the radiotherapy field has been reported to be 4% [4]. The incidence may be effectively reduced through inhibiting cytokines using agents, such as CXCL12, and TGF β , among others.

This study has several limitations. First, in this study, we did not assess the volume of implanted tumors using MRI or contrast-enhanced CT. It is because the assessment of the volume of implanted tumors using imaging requires anesthesia for at least 30 minutes that may have affected the prognosis of rats. Second, in this study, the evaluation was conducted only at three months after the exposure to X-ray radiation. If the tumors were implanted at later stage after exposure to X-ray radiation, the proliferative activity and malignancy of glioma must have been higher than those documented in this study due to radiation-induced changes in the secretion of cytokines. Third, although the standard treatment of malignant glioma requires exposure to the radiation to both brain tumors and normal brain tissue; however, in this study, to exclude the direct effect of radiotherapy on tumor cells, glioma cells were implanted in the brain after exposure to X-ray radiation. The generation of a glioma model with stable OS required the implantation of about 10,000 tumor cells. Nevertheless, it was difficult to generate a brain tumor model with glioma recurrence within several months after exposure to the radiation because the OS of the brain tumor model rats was less than four weeks. Lastly, regarding the treatment of malignant glioma, a single radiation dose of 65 Gy is relatively higher than that used for radiotherapy of patients with malignant glioma. Additionally, due to the difference in ischemic tolerance between rats and humans, the effect of radiation on rat cortex is lower than that on human cortex. Studies on radiation-induced late brain injury have reported that a simulation of changes in the chronic stage in a rodent model requires exposure to high-dose X-ray radiation of about 50-60 Gy [50-52]. Although data are not shown in this paper, in our pilot study, we generated a rodent model of radiation-induced late brain injury that exhibited recurrence within 6 months induced through X-ray radiation of 65 Gy. The model exhibited an increase in the expression of chemokines and immunological response-related activation of microglial cells. Therefore, tumors were implanted at three months after a single exposure to the radiation of 65 Gy.

Unlike previous findings on the tumor bed effect (claiming that tumor bed exhibits tumor inhibitory effect), the current study suggests that the microenvironment around the brain tissue in the chronic phase after exposure to X-ray radiation becomes suitable for glioma cell growth and invasion. In the future studies, development of new therapeutic agents to delay the recurrence of glioma should be conducted focusing on the effect of radiation injury on brain tissue after exposure to the radiation (i.e., tumor bed).

Data Availability

All data generated during the current study are included in this article and are available from the corresponding author on reasonable request.

Abbreviations

COX-2: cyclooxygenase-2; **CXCR-4**: C-X-C chemokine receptor type 4; **CXCL-12**: C-X-C motif chemokine 12; **DMEM**: Dulbecco's Modified Eagle Medium; **EGFR**: epidermal growth factor receptor; **ERK2**: extracellular signal-regulated kinase2; **FGF-2**: basic fibroblast growth factor-2 **GBM**: Glioblastoma multiforme; **IL-1 β** : interleukin-1 β ; **MMP-2**: matrix metalloproteinase-2; **MST**: median survival time; **MVD**: Micro vascular density; **qPCR**: quantitative polymerase chain reaction; **TGF- β 1**: tumor growth factor β 1; **TNF α** : tumor necrosis factor alpha; **TUNEL**: terminal deoxynucleotidyl transferase (TdT) -mediated dUTP nick end labeling; **VEGF-A**: vascular endothelial growth factor-A

Declarations

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Author Contributions

NN conceived of the concept of the study and supervised all the experiments. YH, MF and NN designed the experiments and interpreted data. YT performed all in vitro/vivo studies with guidance from YH, TT, MF, SK, NY, YW and RY. DO, DM and SN performed RNA-sequencing. YT and NN independently performed the statistical analysis and analyzed the RNA-sequencing data. YT and NN mainly drafted the manuscript. MF, SK, SM, HN, TK and MW helped to draft the article. All authors read and approved the final manuscript.

Additional information

Competing Interests: The authors declare no competing interests.

Ethics approval and informed consent: All applicable international, national, and institutional guidelines for the care and use of animals were followed. Informed consent was obtained from all individual participants included in the study.

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Tables

Table 1. Median survival times of each group.

Groups	median survival time(days)	n	p value
Sham-IR/Tumor	22.5(22-24)	12	
IR/Contra-tumor	20.5(18-21)	8	0.003
IR/Ipsi-tumor	20.5(18-21)	12	0.002

Table 2. The list of the significantly impacted pathways.

Impacted pathways	IR/Ipsi-brain vs. Sham-IR/Brain		IR/Contra-brain vs. Sham-IR/Brain	
	P-value	Bonferroni correction	P-value	Bonferroni correction
Cell adhesion molecules (CAMs)	1.10E-06	0.00026	0.008	1
Chemokine signaling pathway	9.40E-05	0.024	0.029	1
Leukocyte transendothelial migration	0.00032	0.080	0.0034	0.94
Cytokine-cytokine receptor interaction	0.0012	0.29	0.0071	1

Figures

Figure 1

A. Treatment schema of the three groups of male Fisher rats for in vitro study. X-ray: X-ray irradiation. IR/Ipsi-brain group: the right hemisphere was irradiated and the tissue of the right hemisphere was used. IR/Contra-brain group: the right hemisphere was irradiated and the tissue of the left hemisphere was used. Sham-IR/Brain group: no irradiation was performed and the tissue of the right hemisphere was used. B. Treatment schema of the three groups of Fisher rats implanted tumor for in vivo study. T: implanted tumor. IR/Ipsi-tumor group: F98 cells were transplanted to the right hemisphere after irradiation to the right hemisphere. IR/Contra-tumor group: F98 cells were transplanted to the left hemisphere after irradiation to the right hemisphere. Sham-IR/Tumor group: F98 cells were transplanted to the right hemisphere without irradiation.

Figure 2

A. The cell-viability ratio under each protein concentration extracted from each group by WST-8 assay. Tissue proteins extracted from the IR/Ipsi-brain group contained significantly higher levels of growth factors compared to the Sham-IR/Brain group under the 3, 6, and 12 $\mu\text{g/ml}$ concentrations of extracted protein. B. Measurement of tube lengths by a tube-forming assay under each protein concentration extracted from each group. Tissue proteins extracted from the IR/Ipsi-brain group contained a significantly higher level of angiogenesis factors compared to those of the Sham-IR/Brain group. Under the protein concentration of 6 $\mu\text{g/ml}$, significantly longer tube formation was observed in the IR/Contra-brain group compared to the Sham-IR/Brain group.

Figure 3

Survival curves of Fischer rats implanted with F98 glioma cells. Significantly shorter median survival times were achieved by the IR/Ipsi-tumor and IR/Contra-tumor rats compared to the Sham-IR/Tumor rats (IR/Ipsi-tumor and IR/Contra-tumor = 20.5 days vs Sham-IR/Tumor group = 22.5 days; $p=0.002$ and $p=0.003$, respectively). There was no significant difference in survival between the IR/Ipsi-tumor and IR/Contra-tumor groups ($p=0.8$).

Figure 4

Changes in the biology of the transplanted tumors. Fig. 4A: Ki-67 labeling index. Fig. 4B: Apoptotic index. Fig.4C: Microvascular density (MVD) index. Fig.4D: Tumor invasion index. The tumors of the IR/Ipsi-tumor group exhibited significantly higher proliferation, angiogenesis abilities and invasiveness compared to those of the Sham-IR/Tumor group. Tumors of the IR/Ipsi-tumor and IR/Contra-tumor group showed significantly lower rates of apoptosis compared to the Sham-IR/Tumor group.

Figure 5

Expression of various cytokines in the IR/Ipsi-brain, IR/Contra-brain, and Sham-IR/Brain groups. The expressions of TNF α , TGF- β 1, VEGF-A, and CXCL12 in the IR/Ipsi-brain group were significantly higher compared to those in the Sham-IR/Brain group. The expressions of TNF α , TGF- β 1, and CXCL12 of the IR/Contra-brain group were significantly higher than those of the Sham-IR/Brain group.

Figure 6

Expression of various cytokines in the IR/Ipsi-tumor, IR/Contra-tumor, and Sham-IR/Tumor groups. The expressions of CXCR4, EGFR, FGF-2, ERK2, and VEGF-A in the IR/Ipsi-tumor group were significantly higher than those in the Sham-IR/Tumor group. The expressions of CXCR4, FGF-2 and ERK2 in the IR/Contra-tumor group were significantly higher compared to those in the Sham-IR/Tumor group.

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