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Acceleration of astrocytic differentiation in neural stem cells surviving X-irradiation

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Abstract

Neural stem cells (NSCs) are highly susceptible to DNA double strand breaks (DSBs), however, little is known about the effects of radiation in cells surviving radiation. While the nestin-positive NSCs predominantly became glial fibrillary acidic protein (GFAP)-positive in differentiation-permissive medium (DM), little or no cells were GFAP-positive in proliferation-permissive medium (PM). We found that more than half of the cells surviving X-rays became GFAP-positive in PM. Moreover, localized irradiation stimulated differentiation of cells outside the irradiated area. These results indicate for the first time that ionizing radiation is able to stimulate astrocyte-specific differentiation of surviving NSCs, whose process is mediated by both direct activation of NF-κB and indirect bystander effect induced by X-irradiation.

Keywords;
X-ray, glial fibrillary acidic protein, NF-κB, bystander signal
**Introduction**

Cranial irradiation is one of the most effective tools in the treatment against various primary or metastatic tumors that occur in the central nervous system. However, a number of observations indicated that this treatment causes significant injury to normal brain functions [1-4]. As these are deficits in hippocampal-dependent function, it is implicated that detrimental effects of therapeutic radiation are resulted from an impairment of radiosensitive regions of the brain tissues [5, 6].

NSCs have a prominent proliferative capacity and can differentiate into astrocytes, oligodendrocytes, and neurons [6, 7], but they are particularly susceptible to ionizing radiation (IR)-induced DNA damage in vitro. Furthermore, in the animal model of cranial irradiation, neural stem/precursor cells in the hippocampus undergo apoptosis and neurogenesis is suppressed [5, 8]. Thus, it is likely that cognitive impairment caused by IR may be resulted from apoptosis induction in NSCs [9-11].

Recently, it has been demonstrated that IR causes various delayed effects in survived cells [12]. Moreover, indirect effects, which collectively called non-targeted effects, are identified in cells that have never been exposed to radiation [13]. Therefore, comprehensive understandings of radiation affects on neurogenesis needs to investigate neurogenic differentiation of NSCs surviving radiation exposure. In this study, we examined the effects of X-irradiation on NSCs derived from the cerebral cortex of embryonic rat.
**Methods**

**Cell culture**

Rat primary cortical stem cells were isolated from the cerebral cortex of embryonic E14.5 Sprague-Dawley rats (R&D Systems Inc., Minneapolis, MN). Cells were grown on poly-L-ornithine- (15 µg/ml, R&D) and fibronectin- (1 µg/ml, R&D) coated flasks or dishes in PM containing DMEM/F-12 medium supplemented with 2.5 mM L-glutamine and N-2 (GIBCO). Cells were passaged every 2 to 3 days to keep exponential growth. For differentiation studies, cells were cultured on poly-L-ornithine- and fibronectin-coated cover slips in DM supplemented with insulin-like growth Factor I and 1% of fetal bovine serum (R&D). The cells were cultured at 37°C in a 5% CO₂ incubator. Cells were pre-treated with inhibitors for 30 minutes before X-irradiation, and cultured in PM for 7 days.

**Radiation treatment**

For conventional X-irradiation, cells were exposed to X-rays generated by an X-ray generator (Toshiba, Tokyo) at 200 kVp and 15 mA with filters combined 0.5 mm copper and 5.5 mm aluminum. The dose rate was 0.5531 Gy/ min. Localized X-irradiation was carried out using Nagasaki University focused ultrasoft X-ray microprobe as described previously [14]. Briefly, the cells were grown on poly-L-ornithine- and fibronectin-coated 0.9 µm-thick Mylar film (Good fellow Ltd., UK). Cells were identified under visible light, and cells in circle area, whose diameter was 200 ~ 300 µm, were locally irradiated with carbon K-shell characteristic X-rays.
Immunocytochemistry

Cells were fixed with cold methanol for 10 minutes on ice, washed with phosphate-buffered saline (PBS) for three times, and stored at 4°C. The primary antibodies (see Table S1, Supplemental Digital Content 1, which demonstrates the primary antibodies) were prepared and treated as described previously [12]. Briefly, the samples were incubated for 2 hours in a humidified CO₂ incubator at 37°C. Then, the secondary antibodies (see Table S2, Supplemental Digital Content 1, which demonstrates the secondary antibodies) were added and incubated for another 1 hour in a humidified CO₂ incubator at 37°C. The nuclei were counterstained with 0.1 mg/ml of DAPI. The samples were examined with a widefield microscopy equipped with motorized stages (F3000B fluorescence microscope, Leica, Tokyo). Digital images were captured and the images were analyzed by FW4000 software (Leica).

Measurement of NF-κB activation

The fixed samples were stained with rabbit anti-phosphorylated NF-κB at serine 536 antibody (Clone 93H1, Cell Signaling Technology Japan) and Alexa488-labeled anti-rabbit IgG. Randomly captured 20 digital images, each containing approximately 50 cells, were analyzed by FW4000 software, and total green and blue fluorescence per image was measured. Relative fluorescence was calculated by dividing total green fluorescence by total blue fluorescence.
Statistical analysis

Data are represented as the means ± SEM of at least three experiments. A Student's $t$ test was used to evaluate significant difference between the control and irradiated cells. $P$ values of less than 0.05 were considered significant difference.
**Results**

**Residual DNA damage and apoptosis-induction by irradiation**

Persistence of DSBs was examined in NSCs exposed to 5 Gy of X-rays. An average number of foci was approximately 23 per nucleus at 4 hours, and disappearance of the foci was most apparent during the first 24 hours (see Fig. S1, Supplemental Digital Content 1, which demonstrates DSB repair). While 100% of cells showed foci induction during 12 hours post-irradiation, the number of foci-positive cells was decreasing thereafter, and approximately 25% of cells still retained foci at day 5. Apoptosis-related DNA fragmentation was assessed by homogeneous signals of H2AX phosphorylation. Apoptotic nuclei are continuously induced in about 10% of cells over time (see Fig. S2, Supplemental Digital Content 1, which demonstrates apoptotic cell death), which results in time-dependent cell death, and only about 20% of the initial cells were survived 7 days after X-irradiation.

**Induction of astrocyte-specific differentiation by IR**

As shown in Fig. 1A, we confirmed little or no expression of differentiation-specific cytoskeletal proteins in NSCs maintained in PM. In contrast, NSCs cultured in DM for 7 days predominantly induced GFAP expression. We found that astrocytic differentiation was tremendously stimulated by 5 Gy of X-rays even in PM (Fig. 1 and see Fig. S3, Supplemental Digital Content 1, which demonstrates neuronal differentiation).

We could not detect STAT3 activation in NSCs in response to X-irradiation, and neither 0.3 µM Stat3 inhibitor VII nor 1 µM WP1066 affected the number of GFAP-positive cells.
In contrast, 20 nM NF-κB activation inhibitor, which inhibited activation of NF-κB significantly (see Fig. S4, Supplemental Digital Content 1, which demonstrates NF-κB activation and inhibition), showed 50% suppression of GFAP-positive cells (Fig. 1C). Using a focused ultrasoft X-ray microprobe, we targeted NSCs in a circle area whose diameter was 200 ~ 300 µm (see Fig. S5, Supplemental Digital Content 1, which demonstrates effects of local irradiation). About 50 ~ 100 cells within the targeted area were exposed to X-rays, and the frequency of GFAP-positive cells was determined in non-targeted area, which was at least 1000 µm apart from the targeted area. According to the residual foci in the targeted area 24 hours after irradiation (see Table S3, Supplemental Digital Content 1, which demonstrates foci numbers), the cells were expected to receive 4 ~ 5 Gy of X-rays. We found that 25% of cells in non-targeted area became GFAP-positive and concurrent treatment of NF-κB activation inhibitor compromised the astrocytic differentiation (Fig. 1C).
**Discussion**

NSCs used in this study showed sufficient proliferative activity in PM and predominantly became GFAP-positive in DM. While GFAP expression was also observed in neural stem cells [15], we did not detect GFAP signal in NSCs used in this study. Therefore, we concluded that they predominantly differentiated into astrocytes. Interestingly, we found that 5 Gy of X-rays significantly increased the number of GFAP-positive cells cultured in PM. Although a study demonstrated that radiation exposure to whole rat brains increased mRNA and protein levels of GFAP through microglia activation [16], our result clearly demonstrated that astrocytic differentiation was directly accelerated by X-irradiation in survived NSCs. Although several previous studies have indicated that STAT3 activation is involved in astrocytic differentiation of NSCs [17-19], we failed to detect STAT3 activation. Instead, we observed that X-irradiation significantly up-regulated phosphorylation of NF-κB. The result was in agreement with the previous studies, which reported transcriptional activation of NF-κB [20]. Furthermore, we confirmed that a specific inhibitor of NF-κB suppressed the X-irradiation-induced increment of GFAP-positive cells. Thus, it was indicated that activation of NF-κB by X-irradiation in survived cells could be one pathway accelerating astrocyte differentiation.

Using a focused ultrasoft X-ray microprobe, we targeted NSCs within a localized area, and cells might receive 4 ~ 5 Gy of X-rays. We observed significant increase of GFAP-positive cells in non-irradiated areas, indicating that factor(s) secreted from irradiated NSCs accelerated astrocytic differentiation in bystander cells. Interestingly, the NF-κB activation inhibitor reduced astrocytic differentiation. Therefore, NF-κB signal
might also be involved in the secretion of differentiation factors, like IL-6, which has been reported as an IR-induced bystander factor [13].

It has been well recognized that radiation exposure causes defect in brain function via apoptosis induction. However, the present study demonstrated that X-irradiation significantly stimulated neural differentiation of survived NCSs. These unexpected findings should provide new insights into the effects of cranial radiation, which should contribute to develop strategies to ameliorate cognitive impairment caused radiation exposure.

**Conclusion**

While radiation exposure induces cell death, our results demonstrate that it also stimulates astrocyte differentiation in surviving NSCs, which is mediated by NF-κB activation in surviving cells and by secreted factors induced by bystander cells exposed to radiation.
Acknowledgements

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References


stem cells via the c-Jun NH2-terminal kinase (JNK) pathway. Oncogene. 2006;25:3638-3648.


**Figure legend**

Fig. 1. Effect of IR on neuronal differentiation of NSCs. **A,** Immunofluorescent detection of astrocytic (red) and neural (green) differentiation. NSCs were incubated in proliferation-permissive medium (PM) or differentiation-permissive medium (DM) for 7 days. NSCs were also incubated in PM after 5 Gy of X-rays (5 Gy) or localized IR. The nuclei are counter stained with DAPI (Blue). **B,** Astrocytic differentiation determined by GFAP expression. **C,** Inhibition of astrocytic differentiation by NF-κB activation inhibitor. NSCs were exposed to radiation with or without 20 nM NF-κB activation inhibitor and incubated for 7 days. In case of localized irradiation, GFAP-positive cells were counted in unirradiated area. The data represent the mean ± SEM. **p < 0.01. Bar indicates 5 µm.

**List of Supplemental Digital Content**

Supplemental Digital Content 1. ppt
Figure 1
Induction and repair of DSBs in NSCs receiving 5 Gy of X-rays. **A**, Immunofluorescent detection of DNA double strand breaks by the foci of phosphorylated histone H2AX at serine 139 (red). Closed arrow heads indicate fragmented nuclei and phosphorylated H2AX-positive intact nuclei, respectively. Bar indicates 5 µm. **B**, Time-dependent change in the foci number per cell. **C**, Time-dependent change in the foci-positive cells. The data represent the mean ± SEM. Significance was evaluated between the control and irradiated cells. **p < 0.01, *p < 0.05.**
A apoptotic cell death caused by 5 Gy of X-rays. A, Time-dependent increase in apoptotic nuclei, which were detected by homogeneous nuclear staining by anti-phosphorylated histone H2AX antibody. B, Time-dependent change in the cell number, which was determined as described in Materials and Methods. The data represent the mean ± SEM. Significance was evaluated between the control and irradiated cells. **p < 0.01.
Effect of IR on neuronal differentiation of NSCs. NSCs were incubated in proliferation-permissive medium (PM) or differentiation-permissive medium (DM) for 7 days. NSCs were also incubated in PM after 5 Gy of X-rays (PM + 5 Gy). **A**, Neuronal differentiation. **B**, Oligodendrocytic differentiation. The data represent the mean ± SEM. **p < 0.01. Bar indicates 50 µm.
Involvement of NF-kB activation in astrocytic differentiation in NSCs after 5 Gy of X-rays, **A**, Immunofluorescent detection of NF-kB activation (green) using anti-phosphorylated NF-kB at serine 536. Cells were fixed 1 hour after X-irradiation. The nuclei are counter stained with DAPI (Blue). **B**, Inhibition of NF-kB activation by 20 nM NF-kB activation inhibitor. NSCs were exposed to 5 Gy of X-rays with or without 20 nM NF-kB activation inhibitor and incubated in PM for 7 days. Astrocytic differentiation was determined by anti-GFAP antibody. The data represent the mean ± SEM. **p < 0.01. Bar indicates 5 µm.
Figure S5

Effect of localized X-irradiation on astrocytic differentiation of NSCs. **A**, Immunofluorescent detection of DSBs visualized 53BP1 foci (red). White circle indicates the targeted area. **B**, Higher magnification picture indicates localized DSBs within the targeted area. The nuclei are counter stained with DAPI (Blue). **C**, Astrocytic differentiation of NSCs determined by anti-GFAP antibody. **D**, Neural differentiation of NSCs determined by anti-b-III tubulin antibody. IR; localized X-irradiation. The data represent the mean ± SEM. **p < 0.01. Bar indicates 50 µm.
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Table S2  The secondary antibodies used in this study

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Table S3  Foci induction of localized irradiation

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<tr>
<td>Non-targeted</td>
<td>0.05 ± 0.04</td>
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Foci numbers in cells in the targeted and non-targeted areas were counted 24 hours after targeted irradiation. Non-targeted area was set about 1000 µm apart from the targeted area.