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Growth Cone Collapse and Neurite Retractions: An Approach to Examine X-irradiation Affects on Neuron Cells

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Growth cone collapse/Neurite retractions/X-rays/DRG/Sympathetic neurons.

The growth cone is a structure at the terminal of a neurite that plays an important role in the growth of the neurite. The growth cone collapse assay is considered to be a useful method to quantify the effects of various factors on nerve tissue. Here, we investigated the effect of x-irradiation on growth cones and neurites and also the comparative radiosensitivity of different neurons. Dorsal root ganglia and sympathetic chain ganglion were isolated from day-8 and -16 chick embryos and cultured for 20 h. Neurons were then exposed to x-irradiation and morphological changes were quantitatively evaluated by growth cone collapse assay. Cell viability was examined using TUNEL and WST-1 assays. The results showed that radiation induced growth cone collapse and neurite retraction in a time- and exposure-responsive manner. Growth cone collapse, apoptosis and WST-1 assays showed that no significant difference between the neurons throughout the study period ($p \geq 0.5$) after irradiation. Both types of day-8 neurons were more radiosensitive than day-16 neurons ($p \leq 0.05$). The time course of the growth cone collapse was significantly correlated with the apoptotic and cell viability responses at different irradiation doses. Growth cone collapse may represent a useful marker for assaying the effect of x-irradiation on normal cell neurons.

INTRODUCTION

Prediction of delayed adverse effects after radiotherapy is an important issue in the optimization of a treatment plan. Exposure of the nervous system to irradiation primarily effects three different cell types: neurons, vascular endothelial cells and glial cells, thus, these cells in turn will be involved in the complications which arise after irradiation.[1–4)] Neurons are believed to be the most radio-resistant cells because most of them stop proliferating before birth. In another hand, *in vitro*, it is well known that culturing neuron alone is rather difficult which make their radiosensitivity has not been well investigated.

Here, we used the growth cone collapse (GCC) assay which has been reported to be a useful means of quantifying the effects of various biochemical and physical factors on cultured explants of nervous tissue.[5,6)] During neuronal development, the establishment of specific neuronal networks requires the guided extension of the developing axons to their appropriate targets via a variety of diffusible and surface-bound extracellular cues. The motile growth cones of elongating neurites are responsible for sensing spatially and temporally distributed guidance signals that direct the growth cone to steer toward (attraction) or away from (repulsion) the guidance source to reach their targets.[7,8)] To our knowledge, however, the merit of this assay in evaluating the effect of irradiation on neurons has not been investigated yet. Our purpose in utilizing this assay is to evaluate the morphological events leading from the initial insult (irradiation) to neuronal dysfunction and death.

In the present study we investigated the effects of x-irradiation (X-rays) on neurite extension from different neurons by examining the morphological changes which take place in growing neurons exposed to X-rays. For quantitative assessment, we used a GCC assay that has been established to examine the effect of irradiation on growing neurons.[9,10)] To examine which neuronal subtype is most susceptible to the X-ray effect, two different neuronal tissues were isolated; the dorsal root ganglion (DRG) as an example of peripheral sensory neurons , and sympathetic ganglion chain (SYMP) as autonomic neurons. Further, we also investigated the difference in radiosensitivity between day-8 neurons which correspond to immature neurons, and day-16 neurons which correspond to the highest density of func-

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tional mature neurons.[11]

MATERIALS AND METHODS

Explant cultures

Following the approval by the Institutional Animal Care Committee of Gunma University, chick neural tissues were isolated from day-8 and day-16 embryos. For preparation of the neurons, DRG were dissected from lumbar paravertebral sites and SYMP from lumbar sympathetic chains. After the trimming of cell clusters, the tissues were plated on laminin-coated cover slips and cultured in F-12 medium supplemented as described by Bottenstein *et al.*[12] The medium contained 100 μg/ml bovine pituitary extract, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 ng/ml mouse 7S nerve growth factor. Cell cultures were incubated at 37°C in a humidified air atmosphere containing 5% CO_2.

Dissociated neurons culture

After the dissection of DRG and SYMP from the day-8 and day-16 chick neurons, dissociated neurons were prepared according to Marusich *et al.*[13] The ganglia were moved separately into a centrifuge tube containing 0.25% trypsin in warm HBSS followed by incubation for 30min. The trypsin effect was stopped by adding an equal volume of F-12 medium containing 10% fetal bovine serum (FBS). The resultant cell suspension was gently mechanically dissociated with a flame-constricted Pasteur pipette. Neuron cells were seeded on poly-L-lysine-coated coverslips ($0.1–0.5 \times 10^4$ cells/cm^2) in a similar way as the explant culture.

Irradiation

After confirming the growth of neurites and growth cones after 20 h culture, the cells were then irradiated (200kV X-ray: Siemens-Asahi Medical Technologies, Tokyo, Japan) with doses of 1, 3, 5, 10, 20 and 30 Gy at room temperature, with non-irradiated culture cells (control) which handled in parallel with the irradiated samples.

Growth cone collapse assay

Dose- and time-response relationships of the neurons were examined at 2, 4, 8, 12, 24, 36 and 48 h after irradiation. Observation of living cells was completed within 5 minutes and confirmed on digitally stored microscopic images using CoolSNAP (Photometrics; Roper Scientific, Inc., USA). In time-course studies, the tissue culture was considered intact when neurites were extending without detachment from the plate and the tissues were kept incubated for a further 48 h after exposure. The tissues were viewed with a 40x phase objective using phase-contrast microscopy (IX70: Olympus Optical Co. Ltd. Tokyo, Japan). Growth cones at the periphery of explants were scored for collapse if they were not in contact with the plate or in close proximity to other growth cones or neurites. One hundred growth cones were viewed and scored per cover slip, and GCC assay was performed as previously described.[9,10] In brief, the scoring method was as follows: an intact growth cone which had filopodia and extending lamellipodia was counted as zero, a growth cone having no filopodia and shrunken lamellipodia was counted as 0.5, and a growth cone without filopodia or lamellipodia was counted as one. Neurites were also morphologically examined and the lengths were measured.

Apoptotic assay

To evaluate radiation-induced apoptosis, the explant cultures after 12, 24 and 48 h exposed to irradiation were processed and subjected to terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay,[14] using an ApopTag Plus Fluorescein in Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. Briefly, fixed cells were permeabilized with ethanol acetic acid 2:1 for 15 min at –20°C. They were then washed twice with phosphate-buffered saline (pH 7.4) for 5 min and incubated with ApopTag equilibration buffer for 5 min prior to terminal deoxynucleotidyl transferase enzyme linkage of dUTP-digoxigenin to the 3'-OH DNA ends at 37°C for 60 min. The reaction was then terminated in a stop/wash buffer at 37°C for 30 min. After washing, the slides were treated with antidigoxigenin peroxidase for 30 min, and mounted with Vectorshield (Vector Laboratories, Burlingame, CA, USA) containing DAPI. TUNEL-positive cells with pyknotic nuclei were considered to be undergoing apoptosis. The percentage of apoptotic cells was determined from the total cell number by DAPI staining. Cells found to be TUNEL-positive and DAPI-negative were excluded. After staining, all fluorescent images were captured with a Zeiss Axioplan microscope (Zeiss, Jena, Germany) equipped with (a) several objective lens and a photometric series (CoolSnap fx) cooled CCD camera, the Coolsnap fx. (Photometrics, Tucson, Az, USA) and (operated with) Metamorph software (Universal Imaging, West Chester, PA, USA).

Cell viability assay

The cell viability of neurons at the end of morphological assessment was examined using a colorimetric assay in which cultured cells were exposed to 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzenedisulfonate (WST-1). In this assay, the amount of WST-1 formazan product takes place only when mitochondrial reductase enzymes are active, and therefore conversion is directly related to the number of viable cells. The absorbance of this colored solution can be quantified at a wavelength of 450 nm using a plate-reading spectrophotometer. The assay kit was used as recommended by the manufacturer (Roche Diagnostics Gmbh, Mannheim, Germany).

Statistical analysis

The dose- and time-response curves for GCC and apoptosis were analyzed by two-way ANOVA analysis of variance for repeated measurements. Mean values were compared by two-way ANOVA. Post hoc analysis was performed by the Bonferoni test. The correlation coefficient between GCC and cell viability was analyzed using Pearson test. Statistical analyses were performed using Origin 7.0 software (Origin Lab Co., Northampton, MA, USA). p values less than 0.05 were considered significant. Values are shown as the mean ± SD.

RESULTS

Morphological Observation

Prior to exposure to X-rays, more than 90% of neurites

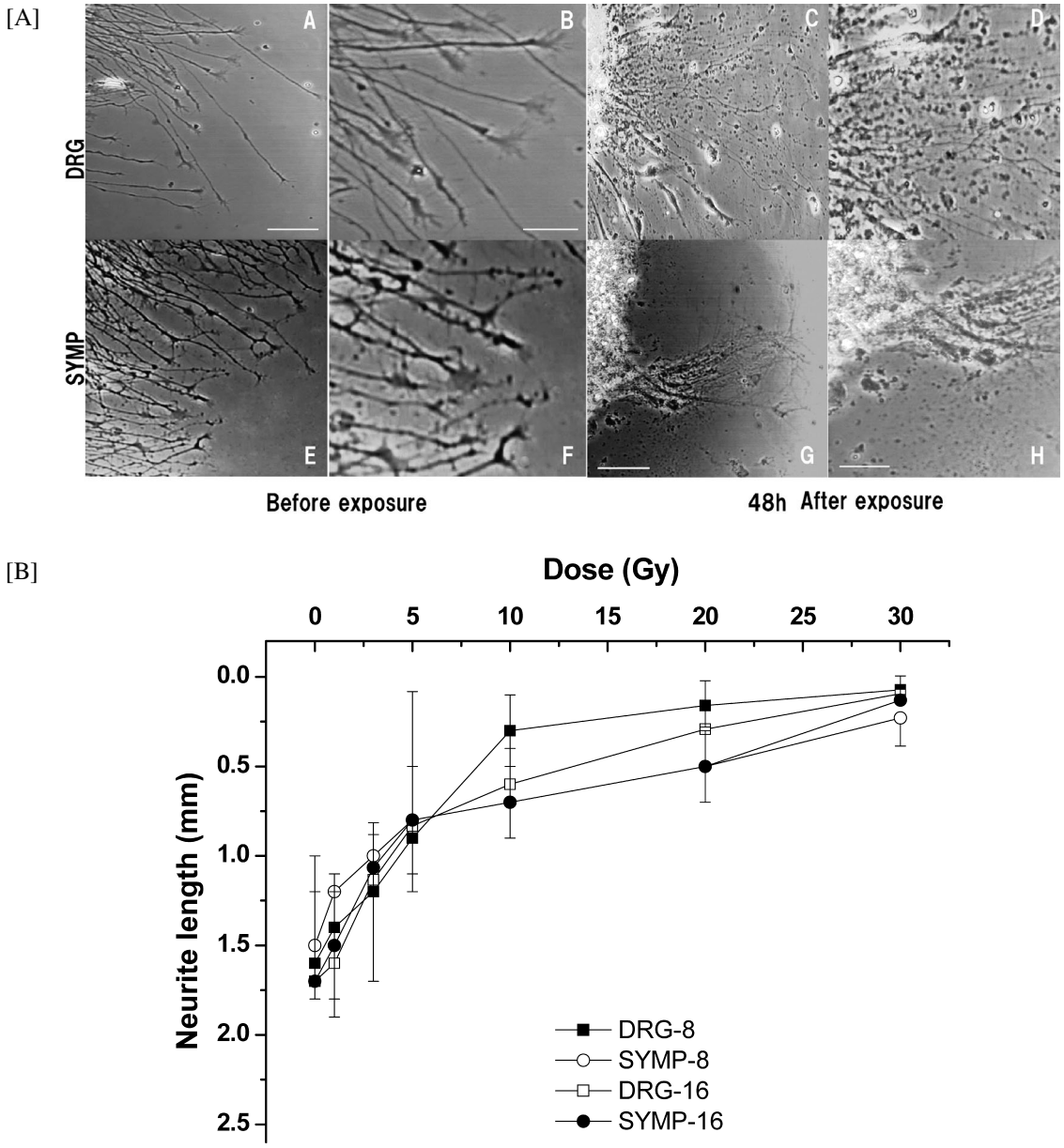

Fig. 1. Typical GCC and neurite retraction induced by 10 Gy of X-rays. [A] DRG (A–B) and SYMP (E–F) neurons after being cultured for 20 h, before exposure to irradiation. Both the growth cones and neurites display normal growth cones and neurite integrity. Growth cone collapse and retraction of neurites in DRG (C–D) and SYMP (G–H) occurred at 48 h after exposure to 10 Gy irradiation. The bleb formations alongside the neurite shifts are a sign of necrosis. [B] This Fig. showed exposure-response effect on neurite lengths for both DRG and SYMP neurite lengths. *One way analysis of variances (ANOVA) ($p > 0.09$). The size bar is 60 μm in A, C, E, G while the size bar is 10 μm in B, D, F, H.

displayed growth cones with lamellipodia and filopodia at their leading edges (Fig. 1). The neurites and growth cones of Day-8 neurons exhibited rapid growth and increase in number and length (Fig. 1A and 1B). Following exposure to irradiation, the growth cones and neurites remained intact during the first hour after irradiation, followed subsequently by morphological changes in the growth cones and neurites as mentioned below.

The data shown here are the difference between the total GCC and that observed in the control.

Morphological observation in Day-8 neurons

At 2–4 h following exposure to a high dose of X-rays (≥ 10 Gy), the filopodia of the growth cones exhibited retraction and the lamellipodia displayed a decrease in number and size (Fig. 1). The neurites exhibited a bleb-like formation alongside the neurites and growth cones. After GCC, the neurite shaft narrowed and some of the clusters of cell bodies became detached from the bottom of the well due to the loss of adhesion to the culture plate. The length of these neurites was proportional to the exposure dose (Fig. 1B).

Morphological observation of day-16 neurons

Following exposure to a high dose of irradiation (≥ 10 Gy), the day-16 neurons displayed similar GCC and neurite retraction to that seen in day-8 neurons, however, exhibited greater resistance requiring more time to collapse and retract in DRG ($p < 0.05$) and SYMP ($p = 0.01$). The neurite length at 48 h after exposure was proportional to the exposure dose (Fig. 1B).

Exposure-response relationship

Following X-rays, the day-8 and -16 explants showed significant differences in the GCC dose-response relationships throughout the observed period in DRG ($p < 0.05$) and SYMP ($p = 0.01$) (Fig. 2). At 4 h, compared to the non-irra-

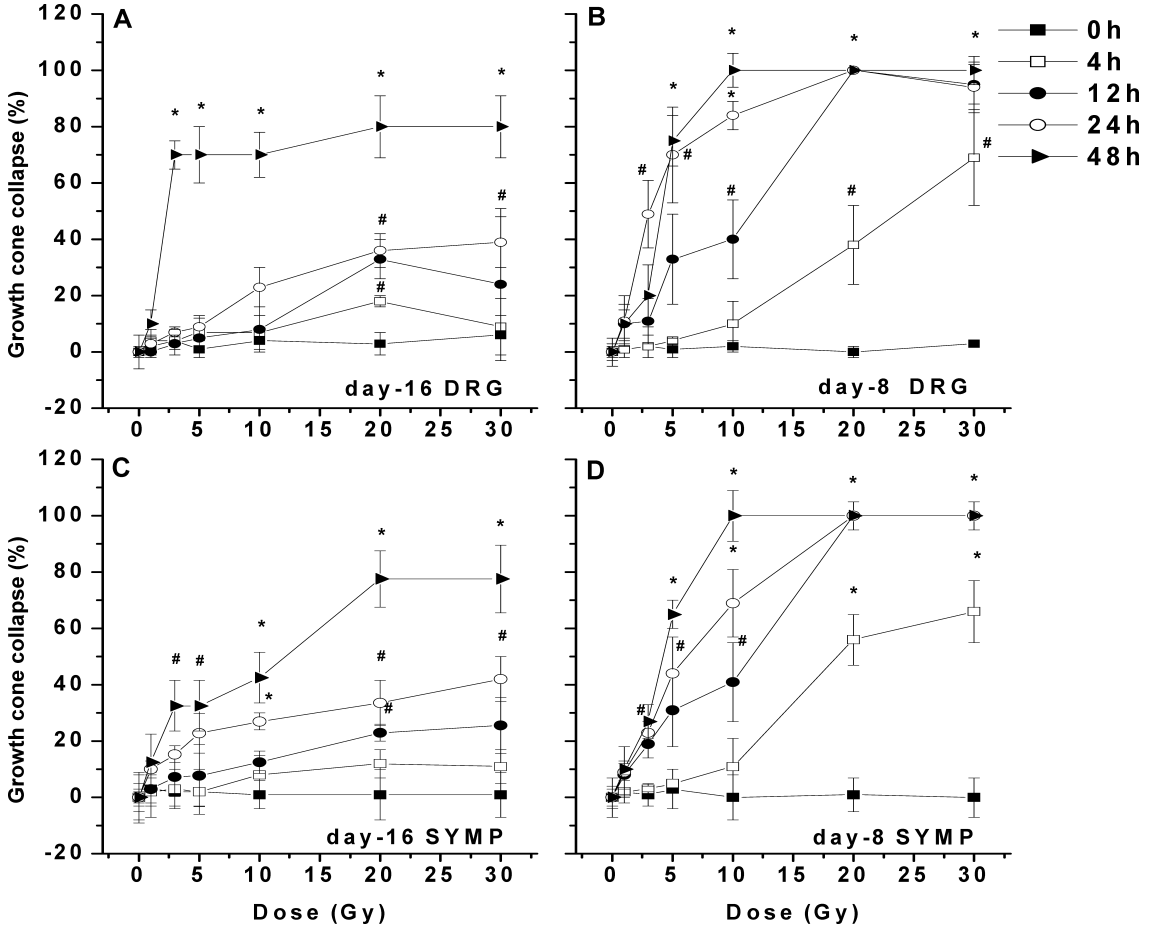

Fig. 2. Exposure-response curve of GCC induced by X-rays in DRG and SYMP. The percentage of GCC shown at 0, 4, 12, 24, and 48 h after X-rays in day-16 and day-8 neurons; day-16 DRG (A), day-8 DRG (B), day-16 SYMP (C), and day-8 SYMP (D). The slop increases with the doses, and it is steeper in day-8 neurons while gradually increasing in day-16 neurons. The two-way ANOVA analysis indicated that day-16 and day-8 neurons exposure- response curves were significantly different in DRG and SYMP ($p < 0.05$ and 0.01, respectively). They were also significantly different from non-irradiated neurons (*$p < 0.01$ and #$p < 0.05$). Number of ganglia used: $n = 16$. Values are the mean ± SD.

diated cells, significantly higher percentages of GCC were obtained in both day-8 DRG and SYMP neurons following irradiation at higher than 10 Gy ($p < 0.05$ and $p < 0.01$, respectively). However, neurons exposed to doses ≤ 3 Gy exhibited no more than 30% GCC compared to the non-irradiated cells, with this difference there was no statistically significant difference ($p = 0.09$).

Time course of growth cone collapse

In day-8 DRG and SYMP cultures, the percentage of GCC was higher than the corresponding value for non-irradiated cells, at 4 h following the exposure to 20 and 30 Gy ($p < 0.05$ and $p < 0.01$, respectively). The respective values in day-16 cultures, in contrast, were similar after 12 h ($p < 0.05$). Compared to the non-irradiated cells, significantly higher percentage collapse was obtained in day-8 DRG and SYMP cells at 24 h and 12 h following exposure to 3 Gy, respectively, ($p < 0.05$). In day-16 cells, significant GCC was obtained on exposure to doses greater than 5 Gy at 24 h in DRG ($p < 0.05$), and by 3 Gy in SYMP ($p < 0.05$).

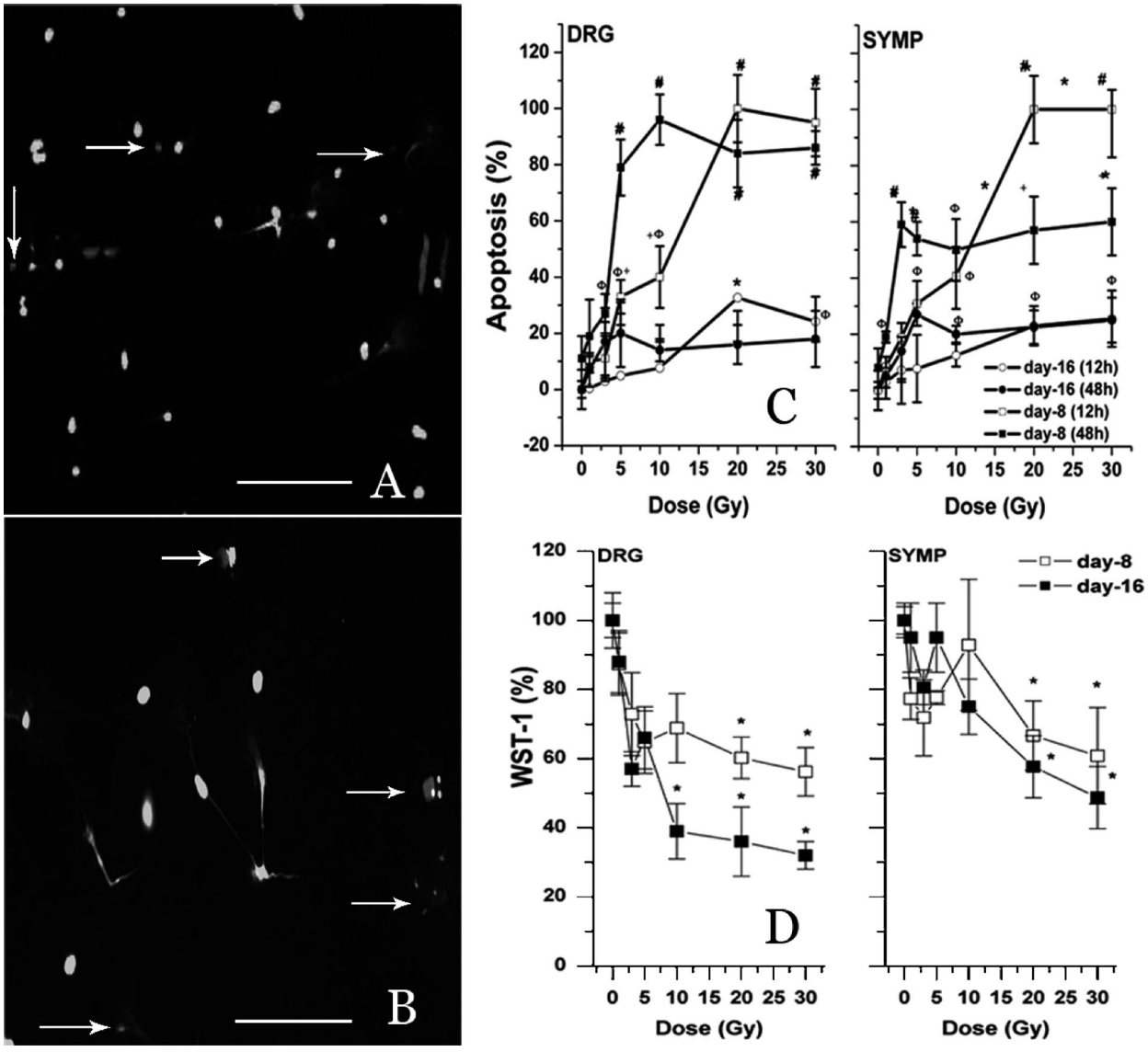

Fig. 3. TUNEL and WST-1 assays to examine cell viability after X-ray exposure. (A) Day-8 DRG and (B) SYMP explant culture after exposure to 10 Gy. Apoptotic cells (TUNEL-positive) are indicated by the arrows. (C) Plot curves in day-16 DRG and SYMP neurons were significantly different from day-8 neurons ($^{\#}p < 0.01$, $^{+}p < 0.05$, respectively). They were also significantly different from non-irradiated neurons ($^{*}p < 0.01$, $^{\phi}p < 0.05$). (D) Cell viability of neurons indicated by WST-1 assay, showing that neither the day-8 nor day-16 in both types of neurons reaches 0%. The two-way ANOVA analysis indicated that WST-1 assay in DRG and SYMP were not significantly different ($p = 0.5$), as well as day-16 and day-8 neurons ($p = 0.5$). *: Significantly different from non-irradiated neurons ($p < 0.05$). (D). Number of ganglia used $n = 16$. Scale bar = 80 μm.

At 48 h after exposure to 1 Gy, in contrast, the percentage of collapse was not significant in day-8 and -16 cells (10–16%) in either DRG or SYMP.

Apoptosis

The time course of apoptosis in the explant cultures was observed at 12, 24 and 48 h after X-rays. The two-way ANOVA analysis of variance revealed no significant differences in the apoptotic ratio at 12, 24 or 48 h ($p = 0.7$) after exposure (Fig. 3A, 3B and 3C). After 12h, the slope increased proportionally with doses up to 20 Gy in day-8 and -16 neurons, thereafter, the slope was steady up to 30 Gy. At 24 and 48h, the apoptotic slope increased abruptly with doses up to ≥ 5 Gy in day-8 and -16 neurons, thereafter, the curves were steady up to 30 Gy (Fig. 3C). To address the radiosensitivity of the neurons, we compared the time course

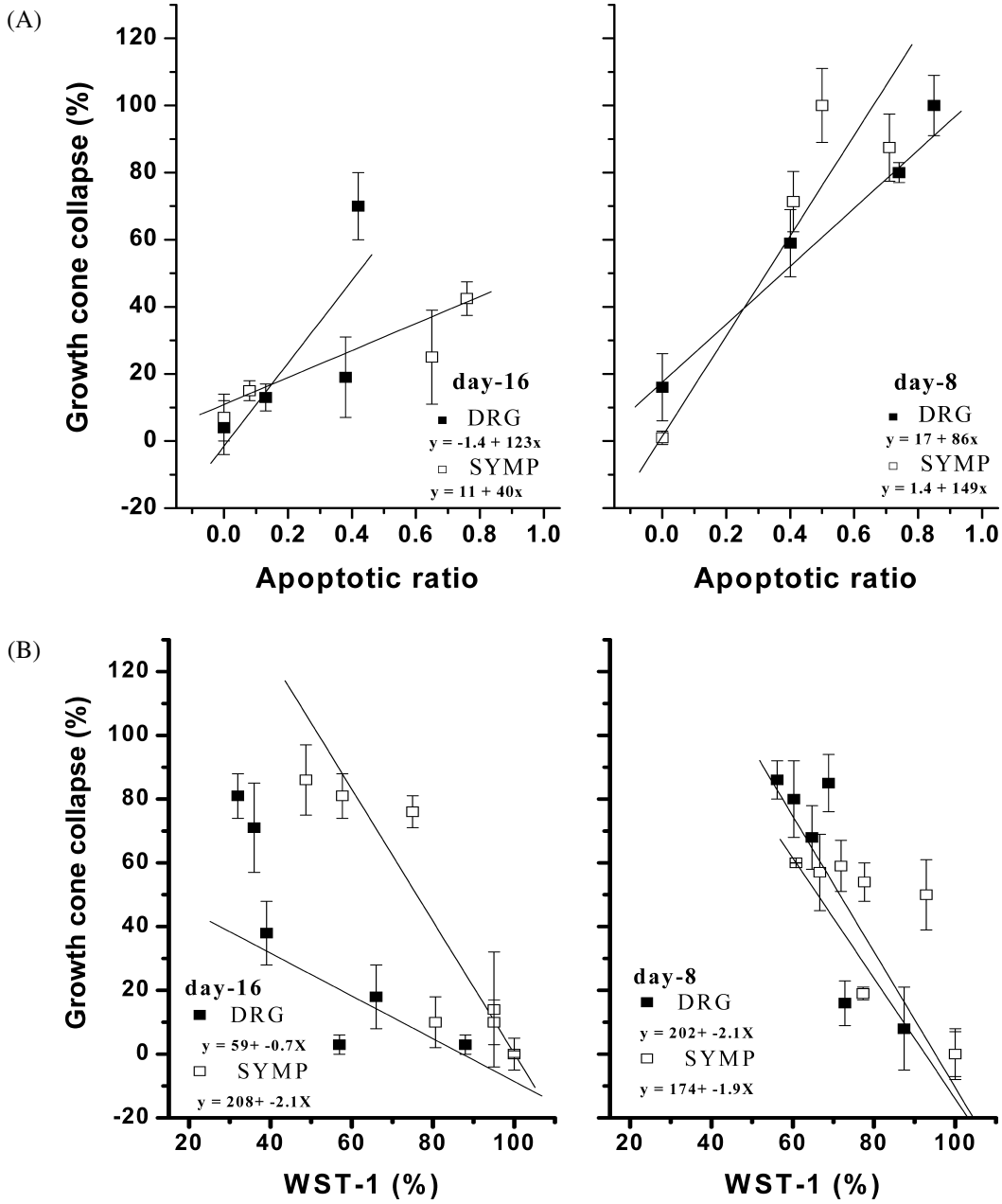

Fig. 4. Time courses of the GCC with Apoptosis and WST-1 with 10 Gy X-rays. The data show a significant correlation between GCC, TUNEL (4A) ($p = 0.0485$) and WST-1 assays (4B) ($p \leq 0.01$). The slop of the correlation was steeper in case of day-8 compare to that in day-16, though, the correlation was significant in both days. The correlation at day-16 DRG and SYMP were, $r = 0.83$ and 0.98, respectively. Meanwhile, the correlation of the day-8 DRG and SYMP neurons were, $r = 0.98$ and 0.97, respectively. The linear regression was fitted individually with the intercepts which shown in the legend.

of the GCC and apoptotic response at different X-rays doses (Fig. 4A). Subsequently, the correlation coefficients were analyzed. The coefficients in day-16 DRG and SYMP were r = 0.83 and 0.98, respectively, at 10 Gy irradiation, while in day-8 DRG and SYMP they were r = 0.98 and 0.97, respectively, ($p < 0.05$). This correlation was significant with doses higher or equal to 10 Gy in day-16 neurons, and was significant at doses higher or equal to 3 Gy in day-8 neurons (data not shown). The two-way ANOVA showed that the apoptosis in day-8 neurons was statistically significant compared with that in day-16 neurons ($p = 0.0485$), but there was no significant difference between DRG and SYMP ($p = 0.7$).

Cell viability (WST-1)

Since there was no significant difference between apoptotic data at any time point examined in this study, we performed the WST-1 assay after 48 h. Cell viability ratio never reached 0% (Fig. 3D) despite exposure to high irradiation doses. The WST-1 ratio was approximately 57 and 60% in both day-8 DRG and SYMP, respectively. On the contrary, WST-1 was 44% and 38% in day-16 SYMP and DRG, respectively. Neither day-8 nor -16 neurons exhibited any statistically significant difference either in DRG or in SYMP ($p = 0.5$). To confirm the neuronal radiosensitivity, we compared the time course of the GCC and cell viability at different X-rays doses (Fig. 4B). The correlation coefficients in day-16 DRG and SYMP were r = –0.65 and –0.95, respectively. In contrast, day-8 DRG and SYMP neurons were r = –0.87 and –0.92, respectively, ($p \leq 0.01$).

DISCUSSION

The findings showed that the growth cones and neurites in day-8 neuronal cells were more radiosensitive than those in day-16 neurons. Furthermore, the radiosensitivity of the DRG and SYMP neurons was comparable based on GCC, TUNEL and WST-1 assays. It is reported that[15,16] central neurons are more radiosensitive than peripheral neurons, but no such difference between peripheral and autonomic neurons has been established. This study reveals that the radiosensitivity in DRG (peripheral) and SYMP (autonomic) neurons to be comparable. The neuronal radiosensitivity observed here might depend on the neuronal precursor cells and their maturation process, which have been shown to be extremely radiosensitive, even to low doses of X-rays.[17] It might also be related to the cell cycle status. Thus, this study is consistent with the previously reported data.[14,18] However, we were not able to determine whether the neuronal cell cultures were in a mitotic or post-mitotic phase, notwithstanding the previous findings that mitosis in cultures of adult sympathetic ganglia occur as early as 24 h after explantation,[19,20] and that peak mitotic activity in chick embryo neurons *in ovo* occurs on the seventh to eighth day.[21]

Among the additional findings, apoptosis was also seen in explant and dissociated cell cultures, as evidenced by the presence of nuclear pyknosis. This study is in support of other reported data[14,22] which indicated that apoptosis is a characteristic feature of neurons undergoing irradiation. Although the time course findings at 12, 24 and 48 h of the apoptotic ratio demonstrated no significant difference in this study, the time course of the GCC in correlation with apoptotic responses showed that day-16 neurons required high doses (≥ 10 Gy) to induce significant GCC and apoptosis, while a dose of approximately 3 Gy was required to exert an effect in day-8 neurons. This correlation is indicative regarding the examined neuron radiosensitivity and suggests the value of the GCC assay as a tool in radiobiological investigation.

It is evident that cell death or disturbance of the cellular environment,[23] on the other hand, causes GCC and neurite retraction. However, in the present study, the WST-1 and TUNEL assays data yielded different percentages of cell viability, even though the cells displayed 100% GCC and neurites retraction. This phenomenon is consistent with that cellular morphological severity does not necessarily reflect morbidity. Morbidity is more often related to the extant size and location of the lesion than to specific morphology. The limitation of the explant culture in detecting the effect of X-rays on the neuronal soma and its neurite individually, let us use a dissociated neurons culture. The dissociated neurons were utilized for long-time observation, at up to 120 h after exposure, viable cells with complete retracted neurites could be observed more at day-16 neurons than day-8 neurons using TUNEL assay (data not shown). In this study, though, we did not investigate the mechanism of the irradiation effect on growth cones and neurites. It is known that axonal growth and synaptic plasticity are controlled by both global and local protein translations. Both types of protein translation are restrained by the activation of dsRNA-dependent protein kinase (PKR).[24] Therefore, the hydrogen peroxide induced as a result of irradiation can affect neurite integrity through the activation of PKR signaling.[25] This inhibits the protein synthesis that is needed for the cross links to form between RNA and specific ribosomal proteins under the base of dendrites spines,[26,27] which is necessary for maintaining the cytoskeleton, signaling, and synaptic plasticity.[28] In addition, irradiation can damage proteins and lipids directly.[29] All of those mechanisms might cause direct GCC and neurites retraction. Thereafter, the cell will either continue with its death processes or reinstate its viability (with respect to the severity of the disturbances incurred).

The differing susceptibility of cells to irradiation damage is also likely to be reflected clinically. For example, radiation-induced leukoencephalopathy is a form of diffuse destruction of the myelin sheaths in the white matter and considered to be a major complication of radiation therapy to the brain especially in leukemia patients.[30] The patients of leukoen-

ceopalopathy usually suffer from seizures, mental dysfunction, and ataxia following the exposure to irradiation and/or chemotherapy. it was reported that cerebral radio-necrosis occurs in the white rather than gray matter of patients with brain tumors.[2–4] Further, young brain tumor patients treated with radiotherapy display an insufficient development of white matter during maturation, which is associated with cognitive impairment and a suppression of the development as well as functional disturbance of normal tissues.[1,31] The cause of these radio-necrosis complications has yet to be confirmed. Although our present results cannot be applied clinically, they are consistent with the possibility that nerve fibers (growth cones and neurites) are more sensitive to irradiation than the cell body with respect to the sensitive dose distribution.

In conclusion, radiation-induced growth cone collapse and neurite retraction which can be used as an early sign for cell environmental disturbance or/and cell death. Day-16 neurons were more radio-resistant than day-8 neurons. Growth cone collapse assay is potentially beneficial for radio-biological investigations.

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