Enhanced expression of glial fibrillary acidic protein in the hippocampus one month after whole brain irradiation of adult rats

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ABSTRACT

الأهداف: اختبار التغيرات التي تطرأ على البروتين الدبقي الليفي الحامضي في الحصين بعد تعريض كامل الدماغ إلى الإشعاع.

الطريقة: أُجريت هذه الدراسة التجريبية في المستشفى الثاني التابع لجامعة سوكو، جيانغسو، الصين وذلك خلال الفترة من أكتوبر 2008م إلى نوفمبر 2009م. وشملت هذه الدراسة 41 جرذي ذكر من فصيلة سبراغو دولي (الوزن:180–220 غرام، العمر:6–8 أسابيع) حيث تم تقسيمهم إلى المجموعات التالية: مجموعة الإشعاع والتي تفرعت بدورها إلى 9 مجموعات، ومجموعة الإشعاع الخادع، ومجموعة التحكم. لقد قمنا بتعريض مجموعة الإشعاع إلى جرعة واحدة من الإشعاع وكانت قوتها تبلغ 2، و10، و30 جري، ومن ثم قمنا باللجوء إلى تقنية التفاعل التسلسلي المبلمر ذو النسخ العكسي من أجل تحليل مراسل الرنا في البروتين الدبقي الليفي الحامضي، وقد حدث هذا بعد مرور يوماً، وأسبوعاً، وشهراً واحداً من تعريض الدماغ إلى الإشعاع. وقمنا أيضاً باستخدام تقنية الكيمياء المناعية النسيجية من أجل التقصي عن الخلايا التي ظهر فيها هذا البروتين.

النتائج: أشارت الدراسة إلى عدم اختلاف نتائج التحليل بين مجموعة التحكم والمجموعة التي تعرضت إلى 2 جري من الإشعاع، غير أن معدلات مراسل الرنا في البروتين الدبقي الليفي الحامضي قد زادت بعد يوم واحد، كما ووصلت إلى معدلات أعلى بعد شهر واحد وذلك في المجموعات التي تعرضت إلى 10 جري، و30 جري من الإشعاع. ولقد تم الكشف عن زيادة واضحة في عدد الخلايا النجمية التي ظهر فيها البروتين الدبقي الليفي الحامضي وذلك بعد مرور شهر واحد في المجوعة التي تعرضت إلى 30 جري من الإشعاع.

خاتمة: أثبتت الدراسة مدى أهمية الخلايا النجمية ودورها الفعال في التغيرات الدماغية التي يحفزها الإشعاع في المراحل الأولى.

Objective: To examine the changes of glial fibrillary acidic protein in the hippocampus after whole brain irradiation of adult rats.

Methods: Forty-one male Sprague-Dawley rats (180-220 g weight and 6-8 weeks) were divided into the 9 irradiation subgroups, sham, and control groups. Their whole brain was irradiated by a single dose of 2, 10, and 30 Gy. At one day, one week, and one month after irradiation, reverse transcriptase-polymerase chain reaction assay was used to semiquantitatively analyze the expression of glial fibrillary acidic protein messenger RNA, and immunohistochemistry was performed to detect glial fibrillary acidic protein-positive cells. This study was carried out at The Second Affiliated Hospital of Soochow University, Jiangsu, China between October 2008 and November 2009.

Results: No obvious changes were found between the control and 2 Gy irradiation groups. However, glial fibrillary acidic protein messenger RNA levels were increased after one day and reached a higher level one month after 10 and 30 Gy irradiation. A significant increase in glial fibrillary acidic protein-positive astrocytes was detected one month after 30 Gy irradiation.

Conclusion: Astrocytes may play an important role in the mechanisms underlying radiation-induced brain injuries in the early stages.

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Radiation therapy is an invaluable tool in the treatment of brain tumors. However, it may induce neurotoxic reactions including transient functional deficit, progressive, and irreversible encephalopathy. It has been proposed that these reactions may be due to responses by either vascular endothelial cells or oligodendrocytes. However, neither hypothesis adequately accounts for the fact that most patients with acute or subacute clinical presentations exhibit no signs of overt vasculopathy or demyelination. Increasing evidence has indicated that a decrease in hippocampal neurogenesis provides clues for the pathogenesis of this disease. Moreover, cranial irradiation-induced inflammation, microenvironment alteration, and glial activation are important factors of inhibition of neurogenesis, especially in the early stage. Due to the highly integrated nature of the brain, and its reliance on cell-cell interactions, an understanding of radio responses of different types of glial cells is essential.[1-4] Astrocytes (ACTs) are the most prevalent glial cells in the CNS, as the primary responders to CNS insults, reactive gliosis is a generic reaction in response to damage to a variety of neural and glia cell types. Glial fibrillary acidic protein (GFAP), a cytoskeletal intermediate filament protein in differentiated ACTs, represents the most specific astrocytic marker under normal and pathological conditions. Most types of cerebral insults can promote reactive hypertrophy and proliferation of astroglial cells with the enhanced expression of GFAP.[5,6] Astrocytic response to ionizing irradiation has been previously identified based on morphological changes. Calvo et al[7] reported an increased number and hypertrophy of ACTs in the fimbriae of rats after treatment with 20-25 Gy irradiation. Consequently, Chiang et al,[8] and Hong et al[9] have provided further evidence that astrocytic reactions include hypertrophy, increased GFAP expression, and proliferation. In the past decade, emerging evidence has shown that radiation-induced impairment of neurogenesis results from a disruption of a microvascular niche within the subgranular zone of the dentate gyrus, and it included glial activation.[3,10] Given the manifold effects of ACTs on critical target cell populations, it would be expected that their responses play important roles in determining the brain injury as a whole. Nevertheless, little is known of the cellular and biochemical processes of ACTs in the brain after radiation. The aim of this study was to investigate astrocytic responses to whole brain irradiation (WBI) during the early period, by examining the expression of GFAP at the level of messenger RNA (mRNA) and protein in the hippocampus in adult rats one month after irradiation treated by the single dose of 2, 10, or 30 Gy.

Methods. *Animals and WBI.* Healthy adult male Sprague-Dawley rats (180-220 g weight and 6-8 weeks) were obtained from the Medical Experimental Animal Center of Soochow University, Suzhou, China. The procedures, which were carried out between October 2008 and November 2009, involving animals and their care were conducted in line with Soochow University Experimental Animal Care Guidelines, which were in compliance with the national animal ethical policies. The rats were randomly divided into the irradiation, sham, and control groups. The irradiation group consisted of 9 subgroups according to radiation doses (single dose of 2, 10, and 30 Gy) and experimental time points (one day, one week, and one month after exposure). The WBI procedures and dose identification were performed as described previously.[11-13] The animals in the sham group were handled and positioned the same as those undergoing irradiation, except they were shielded with lead. The control group was neither irradiated nor anesthetized. There were 11 groups in total, and 3 to 5 rats included in each group. The rats were housed with free access to food and water under constant temperature (22-25°C) and relative humidity (approximately 60%). All the rats were weighed weekly, their skin reactions inside the treatment field were examined, and neurological impairment was tested weekly as well, until the animals were sacrificed.

Tissue preparation. At one day, one week, and one month intervals after irradiation, the rats were anesthetized by an overdose injection of chloral hydrate intraperitoneally (3.6%, 2 ml/100 g body weight). Anesthetized animals were perfused through their left ventricles with 500 ml of heparinized 0.9% saline (pH 7.4). Subsequently, the whole brain was immediately removed from the skull and was divided into 2 parts along the midline after decapitation. The hippocampus was then harvested from the midbrain and overlying cerebral cortex based on its distinct morphological appearance. The isolated hippocampus was frozen at -20°C in a refrigerator for GFAP mRNA analyses by reverse transcriptase-polymerase chain reaction (RT-PCR). The other half of the brain was fixed in 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (pH 7.4). The detailed methods were reported,[3,13,14] and the specimens were prepared for GFAP immunohistochemistry and hematoxylin and eosin (HE) staining.

RT-PCR. Total RNA was extracted using Trizol reagent (Invitrogen, Breda, The Netherlands) according to the instructions provided by the manufacturer. Putative contaminating DNA was eliminated by adding 100 U of RNase-free DNase I per 50 μg of total RNA at 37°C for one hour. Purified RNA was then extracted with phenol, precipitated with ethanol, and

resuspended in diethylpyrocarbonate-treated water. The operating process of mRNA qualification and isolation was described formerly.[11,13,14] The mixture was incubated for 60 minutes (min) at 37^{0}C, followed by 5 min at 95^{0}C, and the cDNA product was stored at 4^{0}C. For PCR amplification, specific oligonucleotide primer pairs of GFAP and β-actin (one μl each) were incubated with 5 μl of cDNA template in a 50 μl volume reaction mixture containing 4 μl $Mgcl_2$, one μl dNTPs, 3 μl Taq DNA polymerase (Fermentas, Glen Burnie, MD, USA). According to the following GeneBank accession numbers V01217 (β-actin) and U03700 (GFAP), the forward (F) and reverse (R) primer sequences were as follows: for β-actin, F: TTGTAACCAACTGGGACGATATGG, R: GATCTTGATCTTCATGGTGCTAGG, 764 bp; for GFAP, F: GCTAATGACTATCGCCGCCAACT, R: CTCCTTAATGACCTCGCCATCCC, 426 bp. The primers were synthesized by Shanghai Bioengineering Co, Shanghai, China. The reactions were initially heated at 94^{0}C for 30 seconds (s), 60^{0}C for 30 s, and 72^{0}C for 45 s. The β-actin and GFAP mRNAs were amplified for 23 and 25 cycles. The PCR products were subjected to electrophoresis in 2% agarose gels and observed with ethidium bromide staining, and the signals were quantified densitometrically using an image-analysis system. Individual data were normalized to β-actin level in each sample; the results were expressed as the ratio of GFAP and β-actin optical densities.

Immunohistochemistry and microscopy. Sequential and coronal sections (5 μm thickness) were cut through the dorsal hippocampus with a microtome. The specimens were deparaffinized in xylene and rehydrated, for routine HE staining. With a gross survey of all brain areas, observers performed a detailed evaluation of the hippocampal region. For the GFAP immunohistochemical study, the sections were treated with 0.3% hydrogen peroxide in deionized water for 10 min to block endogenous peroxidase. After 3 washes with phosphate-buffered saline, they were exposed to 10% normal goat serum and then incubated with anti-GFAP (1:100, Neomarker, Fremont, CA, USA) for one hour at room temperature. The biotinylated goat anti-rabbit IgG antibody (1:200, Vector, Burlingame, CA, USA) was added sequentially. Peroxidase was developed with a diaminobenzidine tetrahydrochloride substrate kit (Vector, Burlingame, CA, USA). Alternating sections were stained in a similar manner in the absence of primary antibody, and no immunostaining was observed in such sections. In the GFAP-positive ACTs, the cell body and its processes were immunostained brown, but its nuclei were not stained. The number of positive cells per microscopy field was counted at 200 magnification of light microscopy. There were 3 rats per group, 5 non-overlapping fields per section, and 3 sequential sections of hippocampus were recorded for each rat.

Statistical analysis. The GFAP mRNA relative levels and the number of GFAP-positive ACTs were recorded in each group. The data were presented as the mean ± the standard error of the mean. Statistical evaluations were carried out using unpaired, 2-tailed Student's t-test in all comparisons. A p-value <0.05 was considered to indicate a statistically significant difference.

Results. We found that WBI treated with different doses were well tolerated by most of the rats. Among 41 rats used in this experiment, 4 failed to recover from anesthesia (including one rat in the sham, one rat in the 2 Gy, and 2 rats in the 10 Gy group), they were excluded from this study. All the surviving animals did not develop neurological dysfunctions during a one-month follow-up period. Body weight gain and food intake in both the WBI and the sham animals were not significantly different, compared with those in the control group. Infield alopecia was observed at 2 weeks, and was the most obvious at one month in all of the rats after 30 Gy irradiation. A representative gel of GFAP mRNA expression in the hippocampus by RT-PCR is presented in **Figure 1**. The relative levels of GFAP mRNA with respect to the dosage and time points after irradiation by semi-quantitatively analysis is summarized in **Figure 2**. When normalized to β-actin, GFAP mRNA in the rats treated with 2 Gy showed no significant difference compared with the control and sham rats during the entire experiment period. However, the expression of GFAP mRNA was significantly increased (p=0.022 or 0.000) at one day, one week, and one month after the administration of 10 or 30 Gy WBI. The level of GFAP mRNA was increased one day after irradiation and reached higher levels after one month (p=0.014, one month compared with one day group). However, no significant difference was found in the expression of GFAP mRNA in the

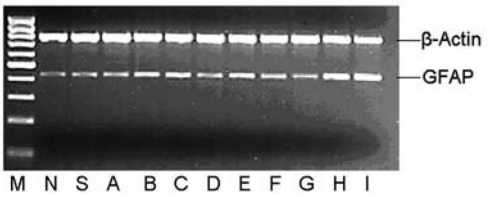

Figure 1 - Detection of the expression of glial fibrillary acidic protein (GFAP) mRNA in rat hippocampus after whole brain irradiation by reverse transcriptase-polymerase chain reaction. M: DNA marker; N: control group; S: sham group; A: one day after 2 Gy of irradiation; B: one day after 10 Gy of irradiation; C: one day after 30 Gy of irradiation; D: one week after 2 Gy of irradiation; E: one week after 10 Gy of irradiation; F: one week after 30 Gy of irradiation; G: one month after 2 Gy of irradiation; H: one month after 10 Gy of irradiation; I: one month after 30 Gy.

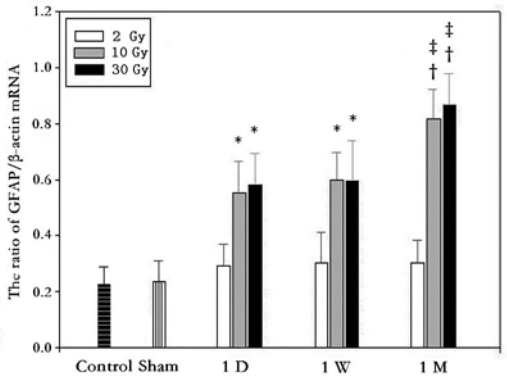

Figure 2 - Semiquantitative analysis of the level of glial fibrillary acidic protein (GFAP) mRNA in rat hippocampus after whole brain irradiation. Each histogram represents mean±standard error of the mean of 3 rats. D: day; W: week; M: month; *p=0.022 versus control and sham group; †p=0.000 versus control and sham group; ‡p=0.014 versus one day or one week at the same irradiation dose (followed by 2-tailed Student's t-test).

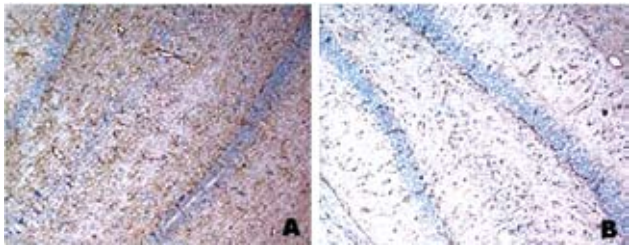

Figure 3 - Photomicrographs showing glial fibrillary acidic protein-positive cells by immunohistochemistry in the hippocampus (x100). **A**) in the rat one month after 30 Gy of whole brain irradiation; **B**) in the control rat.

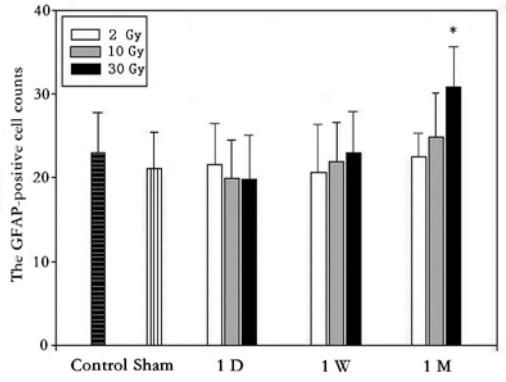

Figure 4 - The number of glial fibrillary acidic protein (GFAP)-positive cells per microscopic field (x 200) in the hippocampus of the rats. Each histogram represents mean±standard error of the mean of 3 rats. D: day; W: week; M: month; *p=0.032 versus control and sham group (followed by 2-tailed Student's t-test).

hippocampus between the rats treated with 10 Gy and 30 Gy groups at one month. For morphological changes in HE sections, no obvious abnormalities were found within one month from all the control, sham, 2, 10, and 30 Gy-irradiated rats. The pathological changes of radiation-induced injury such as edema, vascular dilatation, focal necrosis, and demyelination were not observed in the hippocampus and surrounding tissues. The microphotographs showing immunohistochemical staining and the data of GFAP-positive ACTs in the hippocampus are presented in **Figures 3 & 4**. In the control and sham groups, the average number of GFAP-positive cells per field was approximately 20 to 24 (under 200 magnifications). Relative to the no-radiation groups, the number of ACTs in rats exposed to 2 and 10 Gy WBI showed no difference among all groups at one day, one week, and one month after irradiation. Whereas treated with higher dose of 30 Gy WBI, the GFAP-positive cells appeared still stable at one day and one week. However, at one month after irradiation, an increase in GFAP-positive fibers appeared irregularly in various shapes, and the number of ACTs increased over the whole hippocampus, approaching 31 cells per field, and a significant difference was found when compared with all the other groups (p=0.032). These findings suggest that higher dose (30 Gy) WBI, but not a lower dose (2 Gy) irradiation induced an increased expression of GFAP at levels of both mRNA and protein in the hippocampus after one month. However, un-parallel varieties of mRNA expression and immunoreactivity were observed with 10 Gy. A significant increase in the mRNA was observed from one day to one month after 10 and 30 Gy irradiation, and no significant changes were found in the number of ACTs at the same time points.

Discussion. We have demonstrated astrocytic responses at the early stage of brain radiation injury using RT-PCR assay and immunohistochemistry. We found that in contrast to 2 Gy, GFAP mRNA increased significantly in the hippocampus at one day and one week post WBI, and reached higher level at intervals of one month after 10 and 30 Gy irradiation. However, the change of the level of GFAP protein occurred later than its gene expression. Although few gross histopathological changes were observed, a significant increase in the number of GFAP-positive ACTs was detected in the hippocampus of rats after 30 Gy at one month.

The ACTs involve neuronal functions including homeostatic maintenance, clearance and release of a variety of biological factors, provision of metabolic substrates, and regulating the biology of other cell types in the CNS. In higher vertebrates, following CNS trauma or insults, neurological diseases, or genetic disorders, ACTs become reactive and respond in a typical manner, termed astrogliosis. As reactive ACTs are always present in CNS tissue that is damaged and degenerating, opposing views exist as to whether reactive ACTs have

beneficial or harmful roles. The GFAP is thought to be important in modulating the motility and shape of ACTs by providing structural stability to astrocytic processes. An increase in the number of cells expressing GFAP and the level of its expression is characteristic of reactive gliosis. It has been proposed that GFAP is a sensitive and early biomarker of neurotoxicity.[5,6,9]

For decades, a debate occurred regarding the central roles of the oligodendrocytes versus the vascular endothelial cells. Up to now, with the availability of advanced tools and new insight in neurobiology, the damage mechanism is now increasingly viewed as a continuous, dynamic, and an interacting process. Delineating the pathogenesis will require an understanding not only of the response of the vasculature and oligodendrocyte lineage, but also of the other phenotypes such as ACT as well as critical cell-cell interactions.[1,4] At present, considerable data demonstrate that radiation-associated CNS toxicity is related to alterations in neurogenesis, inflammation, and microenvironmental factors. Strategies aimed at blocking effector molecules, or otherwise reducing oxidative stress is attractive for preventing or mitigating this injury.[10,15,16] Due to its ability to influence many other cell types and subsequent reactive neuro-inflammatory processes, ACTs likely play crucial roles in the brains response to radiation and offers a very interesting research area.[4,12]

Based on morphological criteria, although the hypertrophy of ACTs has been found within irradiated CNS tissues, it was formerly regarded as a secondary response to myelin breakdown or white matter necrosis.[1] Calvo and coworkers[7] found an increased number in the fimbria of the hippocampus in rats 9 months after 20-25 Gy WBI. A concept of "units of tissue injury" was defined based on 4 changes: dilatation of the blood vessel lumen, a thickening of the blood vessel wall, enlargement of endothelial nuclei, and hypertrophy of the adjacent ACTs. They also noted that all the 4 changes became positive before the appearance of white matter necrosis and myelin breakdown in rats x-ray irradiated with 25 Gy. Used as a quantitative marker, the dose-time relationship with radiation injury in the mouse brain of GFAP was first reported by Chiang et al in 1993.[8] The level of GFAP increased after 4-6 months with single doses of 20-45 Gy irradiation, and the number of ACTs increased by 10-20% in a time-dependent manner. The change became significant after a dose greater than 30 Gy, but not with doses of 2 or 8 Gy. In addition to the number of cells, there was also an increase in the intensity of GFAP staining in the corpus callosum and hippocampus.[8,9] After the rats underwent radiosurgical treatment of the caudate-putamen nucleus by a single-fraction maximal dose of 100 Gy, Yang et al[17] found that proliferation was the predominant reaction at earlier time points (3 hours to one day). The expression of GFAP in the proliferating and hypertrophic ACT had an initial peak at 3 days and then peaked between 2 weeks to one month, reactive ACTs appeared not only at the target site but also in the surrounding regions. In addition, a previous study has also shown that an increase in GFAP mRNA and immunoreactivity in the cortex occurred as early as 6 hours (slightly) and one day (significantly) after 15 Gy irradiation.[12] Thus, hypertrophy of astrocytic processes and upregulation of the expression of GFAP was found as a common phenomenon in irradiated CNS tissue.[1]

Nevertheless, in the different quantitative studies, the relationship between the level of GFAP (mRNA, protein, or positive cells) and radiation dose or time-course is considerably variable. In Achanta et al's results,[14] the expression of the GFAP gene in the hippocampus was up-regulated significantly one day after 10 Gy irradiation compared with the 0, 0.3, and 3 Gy WBI in male Fischer 344 rats, whereas a dramatic decrease was found in the 30 Gy dose group compared to the 10 Gy group. Except for the influence of treatment paradigms such as WBI, partial brain exposure, radiosurgical treatment for some special nuclei, and radiation dosage are the main impact factors. Although the biological effects of a prolonged fractionation regimen are anticipated to be more relevant to the clinical situations, WBI with single doses, or a few fractionated doses given over a short period is usually applied in laboratory experiments. Rodents usually tolerate much higher doses than humans, however, WBI doses as low as 2 Gy are sufficient to reduce the rate of proliferation among neuronal progenitors within the hippocampus. Studies by Monje et al[2] have shown that a single dose of 10 Gy is typically used to approximate the biological effects of 2 Gy in humans. A single dose of 8 Gy was equivalent to approximately 18 Gy when delivered in repeated 2-Gy fractions according to biologically effective dose calculation.[4] The reason for 25 Gy WBI chosen in Chiang et al's[8] and Hong et al's[9] initial experiments were that it is sublethal but pathognomonic. Whereas doses used in this study have been widely tested in the most previous experiments.[13] It has also been confirmed that the time-points from one day to one month after irradiation represents the acute to sub-acute phase of the brain injury.[1,16] It is particularly noteworthy that the hippocampus is a neurogenetic region, containing multipotent stem/precursors that divide into cells that migrate away and produce neurons or glial cells within the brain. It has been shown that hippocampal neurogenesis significantly reduced after radiotherapy under both laboratory and clinical conditions. Thus, the hippocampus is considered a prime target in recent years.[2,16]

In addition to ACT responses, microglial reaction and inflammation are thought coincidently important for the regulation of this disease as well. It has been previously found that radiation to the CNS results in glial activation accompanied by the expression of proinflammatory cytokines, associated with gliosis.[8,18] The study by Monje et al[15] has indicated that the increase in GFAP after 2 weeks post-irradiation is associated with proliferating microglial cells, which secrete cytokines altering cell fate in the dentate gyrus. With the same in the rat model, our previous study has shown that interleukin-1β and tumor necrosis factor-α in the hippocampus were significantly increased within 12 hours after 15-30 Gy irradiation.[11] It is believed that persistent neuro-inflammation probably alters the functions of neural progenitors and their interactions with the surrounding glial, neurons, and vascular cells in the local microenvironment. Furthermore, the irradiation-induced inhibition of hippocampal neurogenesis in rodent models is mainly mediated by disruption of the neurogenic microenvironment. Experimental studies suggest that the brain injury can be treated with some drugs in wide clinical use, which exert their effects by blocking pro-inflammatory cytokines and reactive oxygen species. These findings highlight the roles of the microenvironment, in particular, the roles of neuro-inflammation in mediating the damage response.[2,15,18]

In conclusion, our study demonstrated that the up-regulation of GFAP mRNA in the hippocampus occurred as early as 24 hours following 10 and 30 Gy WBI, an increase in the number of GFAP-positive ACTs occurred later than that of mRNA. Our results indicated that gliosis might have already started during the acute phase and continued into the subacute phase, and the changes were time and dose-dependent. Taking together, we postulated that a more active participation of ACT is suggested, and multicellular and multifactorial processes might attribute to this astrogliosis. Neuro-inflammation is likely to be critical in understanding the mechanism underlying irradiation-induced brain damage at an early stage.

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