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Research Report

Tamoxifen alleviates irradiation-induced brain injury by attenuating microglial inflammatory response in vitro and in vivo

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ABSTRACT

Irradiation-induced brain injury, leading to cognitive impairment several months to years after whole brain irradiation (WBI) therapy, is a common health problem in patients with primary or metastatic brain tumor and greatly impairs quality of life for tumor survivors. Recently, it has been demonstrated that a rapid and sustained increase in activated microglia following WBI led to a chronic inflammatory response and a corresponding decrease in hippocampal neurogenesis. Tamoxifen, serving as a radiosensitizer and a useful agent in combination therapy of glioma, has been found to exert anti-inflammatory response both in cultured microglial cells and in a spinal cord injury model. In the present study, we investigated whether tamoxifen alleviated inflammatory damage seen in the irradiated microglia in vitro and in the irradiated brain. Irradiating BV-2 cells (a murine microglial cell line) with various radiation doses (2–10 Gy) led to the increase in IL-1β and TNF-α expression determined by ELISA, and the conditioned culture medium of irradiated microglia with 10 Gy radiation dose initiated astroglial activation and decreased the number of neuronal cells in vitro. Incubation BV-2 cells with tamoxifen (1 μM) for 45 min significantly inhibited the radiation-induced microglial inflammatory response. In the irradiated brain, WBI induced the breakdown of the blood–brain barrier permeability at day 1 post irradiation and tissue edema formation at day 3 post-radiation. Furthermore, WBI led to microglial activation and reactive astrogliosis in the cerebral cortex and neuronal apoptosis in the CA1 hippocampus at day 3 post-radiation. Tamoxifen administration (i.p., 5 mg/kg) immediately post radiation reduced the irradiation-induced brain damage after WBI. Taken together, these data support that tamoxifen can decrease the irradiation-induced brain damage via attenuating the microglial inflammatory response.

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1. Introduction

Despite advances over the last few decades in the diagnosis and treatment of many forms of cancer, individuals with malignant gliomas still have a poor prognosis because of their location and invasive nature (Hosli et al., 1998). In addition to surgical resection, large field or whole brain irradiation (WBI) therapy with or without adjuvant chemotherapy is one of the most effective treatment modalities of high-grade gliomas (Fisher et al., 2001; Stupp et al., 2002). Although increases in radiation dose improve survival in cancer patients, levels of radiation used for therapy are limited by the risks of radiation necrosis, brain atrophy and other post-irradiation complications in normal brain tissue surrounding the tumor (Bernstein and Gutin, 1981). In the pathological cascades of irradiation-induced brain injury, the inflammatory system and microglia, the intrinsic immune cells of the brain, may be central in mediating many cellular interactions that contribute to dysfunction after whole brain irradiation (Ramanan et al., 2008; Schindler et al., 2008).

Tamoxifen, a synthetic, non-steroidal estrogen receptor modulator which is used extensively in the treatment of breast cancer, has been found to be a radiosensitizer and serves as a useful agent in combination therapy of glioma (Donson et al., 1999; Mastronardi et al., 1998; Zhang et al., 1992). In our recent study and other reports, it has been shown that tamoxifen is a potent neuroprotective agent in both transient and permanent experimental ischemic stroke (Kimelberg et al., 2000; Kimelberg et al., 2003), also in a spinal cord injury model (Tian et al., 2009). The striking neuroprotective effects of tamoxifen are likely exhibited by multi-factorial actions, including inhibiting excitatory amino acid release via swelling activated anion channels in swollen astrocytes (Rutledge et al., 1998), scavenging reactive oxygen species (Wiseman et al., 1993), and decreasing formation of peroxynitrated proteins and nNOS in ischemia (Osuka et al., 2001). In addition, tamoxifen has been found to induce an anti-inflammatory response via modulation of LPS-activated pro-inflammatory signaling cascades in acute models of mouse and rat microglial cells (Suuronen et al., 2005) and reduce microglia reactivity in vivo after peripheral inflammation (Tapia-Gonzalez et al., 2008). In our recent report, we also found that tamoxifen attenuated microglial-induced inflammatory damage in spinal cord injury (Tian et al., 2009). Nonetheless, whether or not tamoxifen can have a similar anti-inflammatory and neuroprotective effect on irradiation-induced brain injury remains to be demonstrated.

In the present study, we have investigated whether tamoxifen would suppress irradiation-induced microglia inflammatory response, attenuated reactive astrogliosis and neuronal cell death, and reduced disruption of the blood–brain barrier and tissue edema formation in the irradiated brains of rats. The results provided evidence that tamoxifen might constitute an effective therapeutic neuroprotectant for WBI by attenuating irradiation-induced inflammatory damage.

2. Results

2.1. Irradiation induces morphological changes in microglial cells

We examined the effect of various radiation doses (0, 2, 4, 6, 8 and 10 Gy) on cell morphology in culture of BV2 murine microglia cells. BV2 murine microglia cells were cultured and irradiated with a single dose of 0, 2, 4, 6, 8 or 10 Gy. After 24 h, cell morphology was observed using light microscopy. Representative figures were from three sets of experiments performed on different occasions using three cultures in each set (excluding controls). Scale bar=100 μm.

Fig. 1 – Irradiation induced morphological changes in microglial cells. BV2 murine microglia cells were cultured and irradiated with a single dose of 0, 2, 4, 6, 8 or 10 Gy. After 24 h, cell morphology was observed using light microscopy. Representative figures were from three sets of experiments performed on different occasions using three cultures in each set (excluding controls). Scale bar=100 μm.

2.2. Tamoxifen decreases production of inflammatory cytokines released from irradiated microglia

To determine the factors released from the irradiated microglia, BV2 murine microglia cells were irradiated with a single dose of 0, 2, 4, 6, 8 or 10 Gy, and the conditioned culture medium was collected 24 h later for ELISA. The expression of cytokines was calculated from three sets of experiments performed on different occasions using three cultures in each set (excluding controls). We found that IL-1β level rose in a radiation dose-dependent manner, a 3-fold increase for 2 Gy, a 7.5-fold increase for 6 Gy and a 8.6-fold increase for 10 Gy irradiation (Fig. 2A). Similarly, irradiating BV-2 cells with various radiation doses led to significant increases in TNF-α expression at 24 h post irradiation (Fig. 2B).

When BV-2 cells were irradiated and then incubated with a clinically therapeutic dose of tamoxifen (1 μM) (Dhandapani and Brann, 2003; Mehta et al., 2003) for 45 min, we observed that the increase in pro-inflammatory cytokines TNF-α and IL-1β was significantly attenuated (Figs. 2A, B), indicating the anti-inflammatory role of tamoxifen after irradiation.

2.3. Irradiated microglia cause astroglial activation in vitro via inflammatory cytokines

Hwang et al. reported that irradiation had little effect on astrocyte morphology in astrocyte cultures, but had significant effects on astrocyte morphology in mixed-cultures of BV2 microglia and astrocytes (Hwang et al., 2006). In the present study, we investigated whether astrocyte phenotype changes were attributable to the factors released from irradiated microglia. BV2 murine microglial cells were exposed to a single dose of 10 Gy and 24 h later the conditioned medium from the irradiated microglia added to astrocyte cultures for 24 h. Results were calculated from three sets of experiments performed on different occasions using three cultures in each set (excluding controls). We found that addition of irradiated microglia conditioned medium activated cultured astrocytes which were characterized by the hypertrophic and extensive changes in the processes, hypertrophy of astrocytic nuclei. Supernatants from irradiated microglia also caused the increased GFAP immunostaining in astrocytes and the increased number of DAPI immunostaining cells (P<0.01, 220±25 per high-power magnification in the irradiation group as compared to 104±12 in the control group) (Fig. 3B1–3). MTT assays indicated that astrocyte cell survival was not affected by addition of the irradiated-conditioned media (data not shown). The altered astrocyte morphology was in a similar manner to that observed in mixed-cultures of microglia and astrocytes as reported by Hwang et al. (2006).

When BV-2 cells were irradiated and then incubated with tamoxifen for 45 min, we observed that addition of irradiated microglia conditioned media also altered astrocyte morphology (Fig. 3C1–3), induced the increase in GFAP immunoreactivity and the number of DAPI cells (147±18), although it was not so evident in comparison with that observed in the irradiation group (220±25).

2.4. Irradiated microglia decrease the number of neuronal cells via inflammatory cytokines

We investigated whether the number of neuronal cells was affected by the inflammatory cytokines released from irradiated microglia. Neuronal cells in vitro were immunostained with the neuronal marker III beta-tubulin. The number of neuronal cells was calculated from three sets of experiments performed on different occasions using three cultures in each set (excluding controls). It was surprising to find that addition of microglia conditioned media significantly decrease the number of beta III tubulin positive cells (P<0.01, shown in Fig. 4) and make the neuronal cells more slender with narrow cytoplasm.

When BV-2 cells were irradiated and then incubated with tamoxifen for 45 min, we observed that addition of irradiated microglia conditioned media also altered the number of
neuronal cells as compared to the control group, the decrease degree of neurons (52±12 cells per 0.25 mm² in control group to 35±8 cells per 0.25 mm² in tamoxifen group) was not evident in that observed in the irradiated group (52±12 cells per 0.25 mm² in control group to 14±3 cells per 0.25 mm² in irradiation group), indicating that tamoxifen partially inverted the decrease in the number of neurons induced by the irradiated microglia.

2.5. Tamoxifen decreases BBB disruption and tissue edema formation induced by whole brain irradiation

It has been shown that the blood–brain barrier was damaged and micro-vascular permeability was disturbed in the irradiation brain, either with the fractioned irradiation (Yuan et al., 2006) or with a large single fraction of radiation (Acker et al., 1998). Disturbances of microvascular permeability will initiate vasogenic edema formation and secondary cell damage following brain injury. In the present study, the amount of Evans blue dye extravasation was measured at day 1 post irradiation (Yuan et al., 2003) (n=5 in each group). As shown in Fig. 5B, whole brain irradiation with a single dose of 15 Gy profoundly caused the increased Evans blue extravasation of 12.4 ±1.9 µg/g of brain tissue. Treatment with tamoxifen significantly attenuated the amount of Evans blue leakage after irradiation, as compared to the irradiation group (P<0.05).

Similarly, measurement of brain water content at day 3 after WBI revealed more than 6% increase as compared to that in the control group (n=5 in each group) (Fig. 5A). Interestingly, administration with tamoxifen significantly attenuated brain edema formation induced by irradiation (P<0.05) and there was no significant difference between the control group and tamoxifen-treated group (P>0.05).

2.6. Tamoxifen attenuates glial activation and decreases neuronal apoptosis induced by whole brain irradiation

Activated microglia, characterized by the round cells with abundant cytoplasm, mononuclear, and OX-42-positive

staining, were sparse in the control group (Fig. 6A1), then became conspicuous and seemed to increase in the number in the cerebral cortex regions of the irradiated brain at day 3 after WBI (n=5 in each group, Fig. 6A2). Similarly, astrocyte, as visualized by GFAP immunostaining, exhibited resting morphology with slender processes and small cell bodies in the control group (Fig. 6B1). After irradiation, several astrocytes became activated at day 3 after WBI in the cerebral cortex regions with reactive, hypertrophied morphology and increase in GFAP immunoreactivity (Fig. 6B2). When treated with tamoxifen, activation in microglia and astrocyte was attenuated significantly after irradiation (Figs. 6A3, B3). In addition, activation in microglia and astrocyte of the cerebral cortex regions at day 3 after WBI in the different groups was determined by Western blot analysis for semi-quantification (n=3 in each group). We found that WBI increased the expression level of OX-42 to 3 fold and GFAP to 5 fold in comparison to the control group; when treated with tamoxifen, the increasing expression of OX-42 and GFAP after WBI was attenuated significantly (Figs. 6D, F, G).

Furthermore, the effects of tamoxifen on neuronal cell death in the CA1 area of rat hippocampus at day 3 after WBI were analyzed by using immunohistochemistry with antibody against NeuN combined with TUNEL staining (n=5 in each group). As shown in Fig. 6C1–3, the TUNEL-positive neurons were sparse in the control group (Fig. 6C1). While in the irradiated brain, many TUNEL-positive cells were observed in the area CA1 hippocampus after WBI (Fig. 6C2), and a large number of these TUNEL-positive cells co-localized with the neuron specific marker NeuN, which was indicative of neuronal apoptosis. There was a significant difference between the number of apoptotic neurons in the control group and that in the irradiation group (Fig. 6E, P<0.01). Then the number of apoptotic neurons was attenuated in the tamoxifen-treated group (P<0.05, as compared to the irradiation group), indicating that tamoxifen attenuated neuronal apoptosis, which might translate as improvement in spatial memory retention deficits after WBI.

### 3. Discussion

The results of this study showed that irradiation induced microglial activation and production of proinflammatory cytokines, which then triggered astrogial activation and decreased in the number of neuronal cells in vitro. These phenomena were attenuated by tamoxifen, a selective estrogen receptor modulator that has been recently served as a radiosensitizer and a useful agent in combination therapy of glioma. Furthermore, tamoxifen significantly alleviated...
disruption of BBB permeability and tissue edema formation, attenuated microglial activation, reactive astrogliosis and neuronal cell death after WBI in rats. Although observation within 1-week is a short period, this drug factually reduced early neuropathological damage following WBI, suggesting the potentially therapeutic effects of this compound in irradiation-induced brain injury. Future studies are planned to assess whether tamoxifen also provides long-term neuroprotection and attenuates the deficits in functions of learning and memory at later stage of irradiation after WBI.

Histological features of the radiation-induced brain injury are diverse in different stages. In the early phases, irradiation induced the tissue damage as vascular endothelial cell injury which may cause the disruption of BBB permeability, edema and lymphocyte infiltration, as well as telangiectasia, thrombosis and hemorrhage. At later stage of irradiation, there are evidences of increasing gliosis and demyelination in primarily parenchyma region (Tofilon and Fike, 2000). In the pathological cascades, the inflammatory system and microglia, the intrinsic immune cells of the brain, may be central in mediating many cellular interactions that contribute to dysfunction after whole brain irradiation (Schindler et al., 2008). In vitro studies have demonstrated that irradiating microglia leads to a marked increase in expression of proinflammatory genes including TNF-α, IL-1β, IL-6, and Cox-2 (Hwang et al., 2006; Kyrkanides et al., 2002, 1999). Radiation-induced increases in microglial TNF-α and IL-1β have been proposed to be responsible for the increase in leukocyte adhesion in the brain via upregulation of ICAM-1 in astrocytes (Kyrkanides et al., 1999). These studies are supported by in vivo experiments in rodents which indicate that brain irradiation leads to the increase in gene expression of TNF-α, IL-1β, and Cox-2 acutely (4–24 h) (Chiang and McBride, 1991; Kyrkanides et al., 2002) and that of TNF-α chronically (6 months) (Chiang et al., 1997). Furthermore, administration of the anti-inflammatory drug indomethacin decreased radiation-induced microglial activation and was associated with an improvement in hippocampal neurogenesis (Monje et al., 2003).

Recently, it has been demonstrated that tamoxifen exerts an anti-inflammatory effect in vivo and in vitro. Tamoxifen attenuates inflammatory responses of mouse N9 microglial cells and rat primary hippocampal microglia to lipopolysaccharide exposure (Suuronen et al., 2005). In vivo, treatment with either raloxifene or tamoxifen, a selective estrogen receptor modulator, can reduce carrageenan-induced acute inflammation in female rats (Esposito et al., 2005; Misiewicz et al., 1996), and also modulate in vivo the activation of microglia induced by the peripheral administration of LPS (Tapia-Gonzalez et al., 2008). In addition, in a recent clinical trial (Cushman et al., 2001), tamoxifen has been shown to

Fig. 5 – Tamoxifen decreases BBB permeability and tissue edema formation induced by total brain irradiation. As shown in panel A, brain water content at day 3 after whole brain irradiation with a single dose of 15 Gy was measured in the control group, irradiation group and tamoxifen group, respectively (*P<0.05, **P<0.01, compared to the irradiation group; n=5 for tissue edema). Similarly, BBB permeability at day 1 post irradiation in each group was determined by Evans blue extravasation of brain tissue (B, *P<0.05, **P<0.01, compared to the irradiation group; n=5 for BBB permeability).
decrease C-reactive protein (an inflammatory marker) by 26%, which suggested that tamoxifen might influence cardiovascular risk through inflammation-related mechanisms. Furthermore, tamoxifen has been found to decrease renal inflammation and alleviate disease severity in autoimmune NZB/W F1 female mice (Wu et al., 2000). Our recent report also demonstrated that tamoxifen significantly attenuated the microglial activation, decreased proinflammatory cytokine IL-1β production, reduced neuronal death and myelin loss, and improved functional outcome in rat spinal cord injury model (Tian et al., 2009). In the present study, we found that the irradiated BV-2 microglial cells were activated morphologically and produced a large number of TNF-α and IL-1β in vitro and in vivo experiment in rats also indicated that irradiation led to microglial activation in the brain. And administration with tamoxifen significantly attenuated these inflammatory responses in vivo and in vitro, suggesting the anti-inflammatory effect of this compound in irradiation-induced brain injury.

Furthermore, we demonstrated that astrocytes were apparently activated which was shown by the increased expression level of GFAP protein and immunoreactivity in the irradiated brain. Surprisingly, addition of irradiated microglia conditioned media into the cultured astrocytes caused astroglial activation, whereas no evident activation was found in the irradiated cultured astrocytes (data not shown), which was in conformity with the report by Hwang et al. that irradiation had little effect on astrocyte morphology in astrocyte cultures, but had significant effects on astrocyte morphology in mixed-cultures (Hwang et al., 2006). Thus, these findings indicate that early activated microglia likely play important roles in initiating and maintaining the later onset reactive astrogliosis after WBI, which is supported by our previous study in spinal cord injury (Tian et al., 2007a), and pro-inflammatory cytokines derived from the activated microglia appear necessary for the conversion of quiescent astrocytes into a reactive state. In the present study, it has also been found that supernatants from irradiated microglia decreased the number of cultured neuronal cells in vitro and irradiation led to neuronal apoptosis in the brain tissue, nevertheless, tamoxifen treatment partially inverted the decrease in the number of neurons induced by the irradiated microglia and reduced the number of apoptotic neurons in the irradiated brain. Several studies demonstrated that brain irradiation resulted in a marked increase in microglial activation associated with both a concomitant decrease in neurogenesis in the subgranular zone of the hippocampus and spatial memory retention deficits (Monje et al., 2002; Rola et al., 2004). These data suggest that the efficacy of tamoxifen to mitigate radiation-induced brain injury may involve inhibition of radiation-induced microglial activation, astrogliosis and neuronal apoptosis.

Evans blue extravasation is used as an indicator of vascular permeability in the CNS (Uyama et al., 1988), and BBB damage and increased microvascular permeability have been shown in the irradiated brain, either with the fractioned irradiation (Yuan et al., 2006) or with a large single fraction of radiation (Acker et al., 1998). It is well known that increased permeability of the BBB will initiate a series of reactions causing alterations in brain homeostasis as well as neuronal, glial and myelin function. In addition, it appears that BBB disruption is the main cause of vasogenic edema formation, a feature seen in this investigation by measuring water content in the irradiated brain. And then brain edema results in increased intracranial pressure, tissue softening, increase in brain volume, and compression of vital centers, thus damaging brain functions; it is also responsible for secondary cell and tissue injury in the brain (Li et al., 2004; Sharma, 2006). Several literatures have confirmed that inflammatory response was involved in BBB permeability (Argaw et al., 2006; McCaffrey et al., 2008) and tissue edema formation in the CNS (Sharma et al., 1993). In the present study, we demonstrated that irradiation induced the BBB breakdown and brain edema formation; however, treatment with tamoxifen alleviated the leakage of Evans blue, decreased tissue water content, and attenuated the acute brain damage characterized by the glial activation and neuronal apoptosis after WBI.

In summary, the data presented here demonstrated that tamoxifen treatment significantly decreased irradiation-induced microglial activation and production of pro-inflammatory cytokines, which attenuated inflammatory damage in the early stage of irradiation, and tamoxifen also attenuated reactive astrogliosis and neuronal apoptosis in the irradiated brain, which might induce delayed brain injury at later stage of irradiation. These findings in the present study support that tamoxifen will be a potentially neuroprotective drug in irradiation-induced brain injury for its known ability to readily cross the blood–brain barrier (Biegon et al., 1996) and the clinical circumstance of its wide use in glioma therapy, combined with irradiation (Perez et al., 2003).

4. Experimental procedures

4.1. Cell cultures

The BV-2 cell line (purchased from the Chinese Academy of Medical Science), which exhibits phenotypic and functional properties of reactive microglia, has been used as a suitable model for in vitro studies of activated microglial cells in our recent study (Zhang et al., 2009). The cultures were maintained at 37 °C and 95% O2/5% CO2 in DMEM (Hyclone, UT), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.5% penicillin-streptomycin (Pen-Strep, Gibco, CA).

Primary astrocytes were prepared from neonatal rat cortex as previously described in our study (Zhu et al., 2007). In brief, the cortices were triturated into single cells in minimal essential medium (MEM) containing 10% FBS, and then plated into culture flasks at a density of 1-3×10⁶/ml and maintained at 37 °C and 95% O2/5% CO2 in DMEM-F12 supplemented with 10% FBS (Hyclone), 10% NBS (Hyclone), and 0.5 mg/ml penicillin/streptomycin. In the second week of in vitro maintenance, non-astroglial cells were removed by shaking and the majority of the remaining cells were astrocytes.

Primary astrocyte cultures were trypsinized and replated at 3×10⁵ cells/cm² onto poly-L-lysine coated glass cover slips. The medium was changed every 3 days until confluence. The purity of astrocytes was confirmed by staining for astrocyte specific glial fibrillary acidic protein (GFAP, Neomarker), resulting usually >99%.
Primary cultures of cortical neurons were prepared from neonatal rat cortex as described by Legutko et al. (2001). In brief, cerebral cortices from E17 rats were isolated in ice-cold calcium and magnesium free Hank's balanced salts solution (HBSS) (Life Technologies). Tissue was digested with trypsin and washed three times with serum free MEM supplemented with L-glutamine (2 mM) and glucose (25 mM). The tissue was dispersed in this medium (10 ml) with a Pasteur pipette at room temperature. Cells were plated at 3 × 10^5 per well on poly-D-lysine coated 6-well plates, and maintained at 37 °C and 95% O2/5% CO2 in MEM supplemented with 10% heat-inactivated horse serum, 10% FBS, 2 mM L-glutamine and glucose (25 mM) without antibiotics. On day 4, 15 μg/ml 5-fluoro-2′-deoxyuridine (FUDU) and 35 μg/ml uridine were added to the cultures to inhibit non-neuronal cell proliferation. Twice a week thereafter, half the medium was exchanged with fresh culture medium lacking FBS. Experiments were performed on neurons grown in culture for eight days.

For cell cultures, all results were calculated from three sets of experiments performed on different occasions using three cultures in each set (excluding controls).

4.2. Animal and cell irradiation

The animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Tongji Medical College. All efforts were made to minimize the number of animals used and their suffering. Adult male Sprague-Dawley rats (n=54; weight 250–300 g) were randomly classified into control group, irradiation group, and irradiation plus tamoxifen group. Before irradiation, rats were anesthetized by isoflurane (i.p.) injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Irradiation was done using a 6 MV Elekta Precise X-ray linear accelerator (Elekta, Stockholm, Sweden). The head of each animal was placed in the center of the 5 x 5 cm treatment field. The beam was directed down onto the head, a single dose of 15 Gy was given at a dose rate of 3 Gy/min and a source to skin distance of 100 cm. During irradiation, the customized lead shielding block covered the bodies of the rats to ensure that only the brains were irradiated. Tissue-equivalent material (1 cm thick) was placed above the head of each animal to establish electronic equilibrium and to ensure that the prescribed dose was delivered uniformly to the tissue.

Immediately after irradiation, rats were randomly and blindly assigned to either irradiation (n=18) or irradiation plus tamoxifen (n=18) groups. Tamoxifen (Sigma, USA) dissolved in 2% DMSO in distilled water (1 mg/ml) was intraperitoneally administered (5 mg/kg) immediately post WBI (Kimelberg et al., 2000, 2003). Vehicle-treated animals received 2% DMSO in distilled water without tamoxifen in the same volumes as in the tamoxifen-treated group. After irradiation, the animals were taken back to a warm environment for 24 h and returned later to their original quarters.

Cells were irradiated with various radiation doses (0, 2, 4, 6, 8 and 10 Gy) at a dose rate of 3 Gy/min. All irradiations were performed at room temperature; the cells in the control group received sham irradiation (0 Gy). After irradiation, the culture dishes were returned to the incubator and maintained at 37 °C and 95% O2/5% CO2 in medium. For experiments with tamoxifen, the cultures were incubated with a clinically therapeutic dose of tamoxifen (1 μM) (Dhandapani and Brann, 2003; Mehta et al., 2003) or vehicle (DMSO) for 45 min immediately after irradiation. Then the cells were rinsed twice with 1×PBS to wash off the compound and prepared for further observations.

4.3. Enzyme-linked immunosorbent assay (ELISA)

To determine the cytokines released from the irradiated microglia, BV2 murine microglial cells were irradiated with a single dose of 0, 2, 4, 6, 8 or 10 Gy, and the conditioned culture medium was collected 24 h later for ELISA. IL-1β and TNF-α secretion was measured using Duoset ELISA development kits (R and D Systems) according to the manufacturer’s instructions. Standards and samples were assayed in triplicate as recommended. The expression of cytokines was calculated from three sets of experiments performed on different occasions using three cultures in each set (excluding controls). The absorbance was measured and results were calculated from the standard curve.

4.4. Evaluation of blood-brain barrier disruption and edema formation

The integrity of the blood-brain barrier (BBB) was investigated with Evans blue dye extravasation according to the method of Uyama et al. (1988) with certain modifications (n=5 in each group). At 24 h after irradiation (Yuan et al., 2003), 4 ml/kg of 2.5% Evans blue dye (Sigma) solution in saline was administered via the femoral vein. One hour later, rats were anesthetized and killed by intra-cardiac perfusion with 250 ml of saline. The total brain was removed and placed in a 50% ethanol, and its fluorescence was quantified using a spectrophotometer at an excitation wavelength of 620 nm and an emission wavelength of 680 nm. Dye in samples was determined as micrograms per gram of tissue from a standard curve plotted using known amounts of dye.

Water content of the irradiated brain was determined to evaluate brain edema formation after WBI (n=5 in each group). The total brains were excised at day 3 post irradiation and immediately weighed (wet weight). Samples were incubated at 80 °C for 24 h and then weighed again (dry weight). The brain water content was measured using the formula (Lin et al., 1993): percentage of water=[1 – (dry weight/wet weight)] × 100.

4.5. Histology and immunohistochemistry

Animals in control, irradiation and tamoxifen-treated groups were decapitated under anesthesia at day 3 after WBI, and then their brains were removed, rapidly frozen in nitrogen-cooled isopentane, and stored at −80 °C (n=5 in each group). The tissue blocks were cut at 10 μm thickness, and sections were mounted on poly-L-lysine-coated glass slides and stored at −20 °C until further analyzed.

For immunofluorescence, the following primary antibodies were used: monoclonal mouse anti-OX-42 (BD Biosciences,
1-50) and rabbit anti-GFAP (Santa Cruz, 1:100). Tissue sections were washed in PBS buffer and blocked in 10% horse serum for 1 h at room temperature. Incubation respectively with the indicated primary antibodies was carried out overnight at 4 °C. After washed in PBS (three times for 10 min), sections were incubated in corresponding secondary antibodies: FITC-conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch, 1:100) or FITC-conjugated goat anti-rabbit IgG antibody (Jackson Immunoresearch, 1:200) for 1 h at room temperature. Finally, the slides were washed twice in PBS and once in distilled water for 5 min each and cover-slipped with anti-fade mounting media. For negative controls, we used nonspecific IgG instead of the primary antibodies. For immunocytochemical staining of cultured astrocytes and neuronal cells, the following primary antibodies were used respectively: rabbit anti-GFAP (Santa Cruz, 1:100) and monoclonal mouse anti-Beta tubulin III antibody (Abcam, 1:200), and nucleuses are stained in blue with DAPI.

For neuronal cell death analysis, cryostat sections were processed according to the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) Apoptag kit protocol (Roche Molecular Biochemicals, USA). The experimental protocol has been described in detail in our previous studies (Tian et al., 2007b; Tian et al., 2006). Tissue sections were briefly overviewed, the markings on the slides were then covered and quantification was carried out blindly. Sections were observed under an Olympus BX-51 light microscope (Olympus, Japan) connected with a computer screen and then analyzed with Micro-image analysis software. The neural cells were stained with indicated antibodies specifically recognizing their cell types, and quantitated stereologically. The OX42+ cell which showed integrated cell body with or without evident processes was regarded as a single microglial cell. The number of GFAP+ astrocytes and OX42+ microglia was counted in the cerebral cortex overlaying the dentate gyrus of the hippocampus. The number of TUNEL positive neurons per 0.25 mm2 of tissue in the hippocampus was counted at high-power magnification (×400). Five contiguous fields were evaluated and the number of microglia, astrocytes and apoptotic neurons was averaged for each group.

### 4.6. Western blot analysis

Western blot analysis was carried out to investigate the changes of OX-42 and GFAP expression in the irradiated brain. Rats in sham, irradiation, and tamoxifen-treated groups were deeply anesthetized and killed at day 3 post WB1 (n = 3 in each group). The experiment protocol in detail was carried out as previously described (Tian et al., 2007b; Tian et al., 2006). The primary antibodies used were as follows: monoclonal mouse anti-OX-42 (BD Biosciences, 1:50) and rabbit anti-GFAP (Santa Cruz, 1:100). To ascertain specific binding of the antibody for the protein, another membrane was stained in a similar way without the primary antibody. The membranes were scanned at 600 dpi, and the resulting digital images were analyzed quantitatively with a Kodak Digital Science 1D system. The integrated optical density (OD) of the signals was semi-quantified and expressed as the ratio of OD from the tested proteins to OD from actin.

### 4.7. Statistical analysis

All data were expressed as means ± SD. Statistical analysis was evaluated by two-way ANOVA followed by Tukey’s post hoc test. The difference was considered significant at P < 0.05.

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