ABSTRACT

Late effects after radiotherapy for brain tumors can be severe and tend to limit the efficacy of this treatment modality. The mechanisms governing the development of late radiation-induced lesions in the brain are not clear, but they are preceded by cycles of molecular and cellular events including production of cytokines, one of which is tumor necrosis factor (TNF)-α. There is literature to support possible roles for TNF-α as a contributor to edema, gliosis, and demyelination in the brain, all of which are histopathologically associated with radiation-induced brain damage. We have examined the role of TNF-α signaling in the response to brain irradiation using TNFRp55−/− or TNFRp75−/− mice. Seizure activity correlated with the onset of extensive demyelination, and by 6 months, levels of myelin basic protein in irradiated TNFRp75−/− mice were 40% of those seen in the other two strains; the animals were moribund and had to be euthanized. These observations indicate that radiation-induced TNF-α, acting through TNFRp75, protects against the development of late complications of brain irradiation.

INTRODUCTION

Most patients who are currently diagnosed with brain tumors will receive a course of radiotherapy at some point in their treatment. However, even if radiotherapy is combined with other treatment modalities, such as chemotherapy and surgery, the prognosis is generally poor. For example, the mean survival time for patients diagnosed with glioblastoma multiforme is only 11 months, even with surgical resection, chemotherapy, and radiation (1).

The potential consequences of damaging normal tissue within the chosen radiation field can be very severe and limit this form of therapy. Fractionation and careful treatment planning using shaped beams are used in the clinic to spare normal tissue, but some is inevitably still in the field. Understanding the mechanisms of late radiation-induced damage in the CNS at the molecular and cellular level could lead to novel strategies for radioprotection of normal brain tissues that would allow increases in dose and the probability of tumor cure.

The clinical symptoms that may follow brain irradiation can be divided into three major groups according to the time after treatment at which they occur. The acute (during or within the first 24 h after treatment) and subacute effects (a few weeks to a few months later) that include nausea and vomiting tend to be transient and usually resolve on their own. However, late effects (those occurring 6 months to several years after treatment) can be very severe and may seriously decrease the quality of life of the patient. These effects are especially dangerous because they are generally irreversible.

The histopathological features associated with symptoms of brain irradiation are very diverse, although they are almost always confined to the white matter. Vascular damage is reported commonly, and it has been suggested that the cerebral microvasculature might be the primary lesion leading to increased blood-brain barrier permeability, edema, and lymphocyte infiltration in the early phases, as well as telangiectasias, thromboses, and hemorrhage in the late phase (2). Cells of the parenchyma are also affected, as is evidenced by gliosis and demyelination. Despite the apparent diversity of radiation-induced histopathological lesions in the CNS, three cardinal features of late radiation damage can be defined. These are edema, gliosis, and late demyelination.

The molecular and cellular mechanisms governing the development of these responses are not clear but appear to coordinate parenchymal and vascular cells in a response to damage (2). Cytokines and growth factors in large part regulate such interactions. Growth factors such as platelet-derived growth factor, ciliary neurotrophic factor, nerve growth factor, and fibroblast growth factor, as well as cytokines like transforming growth factor-β, are known to be important in the development and homeostasis of normal brain (3–6). Furthermore, studies in other models of brain injury have implicated the proinflammatory cytokines, particularly TNF-α and IL-1, in the pathogenesis of CNS damage. For example, TNF-α and IL-1 have been implicated in edema seen after ischemic and hypoxic injury (7, 8). TNF-α and IL-1 can also regulate proliferation of astrocytes in response to various types of CNS injury (9–11), implicating them in gliosis. TNF-α is also cytotoxic to oligodendrocytes in vitro (12) and is expressed in lesions in multiple sclerosis (13, 14) as well as in EAE (15), implicating this cytokine in demyelination.

Because proinflammatory cytokine production has been associated with edema, gliosis, and demyelination in other models and these are the three cardinal features of radiation-induced brain damage, we examined this aspect of the brain’s response to irradiation in mice. Previously, we reported that radiation induces a coordinated proinflammatory response in the CNS that involves, in particular, the cytokine TNF-α (16–18). mRNA levels for this cytokine peak after 2–8 h and return to baseline by 24 h after irradiation. Also during the acute phase, radiation causes apoptosis of cells in white matter areas such as the corpus callosum, as well as in putative stem cell areas such as the dentate gyrus of the hippocampus and the subventricular zone in the walls of the lateral ventricles (19, 20).

After the acute phase, the TNF-α response in mouse brain follows a cyclic pattern during the subacute and late phases, with peaks occurring at around 2–3 and 5–6 months after irradiation (17, 21). These peaks in TNF-α expression correlate with nadirs in levels of oligodendrocyte markers (myelin basic protein levels and cyclic nucleotide phosphohydrolase activity), increases in expression of proteins associated with reactive gliosis, such as glial fibrillary acidic protein, and, interestingly, with the appearance of observable neuro-
logical side effects (22, 23). To further investigate the role of TNF-α pathways in the development of radiation-induced brain injury, we compared the molecular, cellular, and neurological responses of TNFRp55 and TNFRp75 knockout mice and intact C57BL/6 control mice to brain irradiation. Differences were found that indicate that these dual receptors may mediate mutually antagonistic responses and that the TNFRp75, in particular, performs a radioprotective function after brain irradiation.

MATERIALS AND METHODS

Animals. All mice were bred and housed in the specific pathogen-free colony operated by the Department of Radiation Oncology at University of California at Los Angeles. Mice were given food and water ad libitum and were maintained on a 12-h light/dark schedule. All mice were bred in-house from TNFRp55−/− and TNFRp75−/− breeding pairs obtained from Immunex Corp. (Seattle, WA; courtesy of J. Peschon). The offspring of the breeding pairs were back-crossed for several generations onto in-house C57BL/6 mice. TNFRp75−/− mice from The Jackson Laboratory (Bar Harbor, ME) were used to confirm critical findings. Genotypes were assessed by PCR using 50 ng of tail genomic DNA. TNFRp55−/− mice were genotyped using four primers (Life Technologies, Inc., Rockville, MD): p60-B, 5′-GGGA TTG TCA CGG TGC CGT TGA AG-3′; p60-E, 5′- TGA CCA CGA CGT GTG TGG CCA-3′; p60-spe, 5′-TGC TGA TGG GGA TAC ATC CAT C-3′; and pgk5′-5′-66, 5′-CCG GTG GTG GTG GAA TGT GTG-3′. To identify TNFRp75−/− mice, three primers (Life Technologies, Inc.) were used: p80-Kas, 5′-AGA GCT CCA GGC ACA AGG GC-3′; p80f-1, 5′-AAC GGG CCA GAC CTC GG-3′; and pgk5′-66 (described above). Samples were run in a thermo-cycler for 32–36 cycles at 1 min in 94°C, 1 min at 65°C, and 30 s at 72°C. PCR products were resolved on a 3% agarose gel run in TAE buffer and stained with ethidium bromide for visualization.

All experiments were performed using a minimum of three mice per time point or dose point, with each experiment repeated three times.

Irradiations. The irradiation protocol has been described previously (16). Briefly, C57BL/6, TNFRp55−/−, and TNFRp75−/− mice, 6–8 weeks of age, were anesthetized with Nembutal and placed on their sides into an irradiation jig that allows irradiation of the midbrain while shielding the esophagus, eyes, and the rest of the body. The jig was then placed into a 250 kVp orthovoltage X-ray machine (Phillips). The brains were processed as described above. BrdUrd-labeled cells were detected in five sections/brain using a solution of 30% hydrogen peroxide in methanol. Sections were incubated with trypsin, and cellular DNA was denatured with sodium hydroxide. Sections were blocked and incubated for 45 min with biotinylated anti-BrdUrd antibody, followed by incubation with streptavidin-peroxidase. Diaminobenzidine substrate was used for visualization of BrdUrd-positive cells. BrdUrd-positive cells were counted in the same areas of the brain as were the TUNEL-positive cells, and percentages were calculated using the same technique.

Myelin Staining. To stain for myelin content, tissue sections from mice at 0, 3, and 5.5 months after irradiation were treated with Luxol fast blue and cresyl violet. Sections were stained overnight in Luxol Fast Blue (Sigma Chemical Co.) at 56°C and washed in 95% ethanol and distilled water to remove excess blue stain. The color was then differentiated (until white matter was easily distinguishable from gray matter) in lithium carbonate solution for 15 s, followed by distilled water and three washes of 80% alcohol. Slides were washed in distilled water and stained for 6 min in prewarmed, 56°C cresyl violet solution (Sigma Chemical Co.) to stain nuclei. The cresyl violet stain was differentiated in several changes of 95% ethanol, followed by incubation in butanol for 20 min, a rinse in absolute alcohol, and an incubation in chloroform for 30 min. Slides were washed through fresh xylene twice, mounted with Permount (Fisher), and coverslipped.

Isolation and Assay of MBP in Mouse Brain. MBP was isolated from mouse midbrain tissue by the method of Deibler et al. (25). Three midbrains from each strain were combined to produce pooled extract for each time point (0, 3, and 5.5 months). Tissue was weighed and homogenized in 19 volumes of a chloroform:methanol (2:1) solution. The homogenates were left overnight at 4°C and then centrifuged at 5,000 × g for 10 min. The pellets were homogenized in acidified water (pH 3.0) and left at room temperature for 1 h. After centrifugation at 12,000 × g for 1 h, the supernatants were removed and dialyzed for 4 h against 1 M Tris–HCl (pH 8.8) using a Slide-a-Lyzer 10K dialysis cassette (Pierce). Total protein concentration was measured by the BCA method (Pierce), and MBP was assayed by ELISA. Extracts were placed in NUNC ELISA plates and incubated at 37°C for 1 h and at 4°C overnight. The plates were washed with buffer (PBS containing 1% BSA and 0.05% Tween 20, pH 7) three times and blocked with a solution of 4% BSA in Tris (pH 7.4) at 37°C for 1 h and at 4°C overnight. After washing, MBP monoclonal antibody (1:100) derived from the GB-1 clone (gift from Dr. Cham- pagoni, University of California at Los Angeles) was added, and the plate was incubated at 37°C for 1 h. After washing, a 1:2000 dilution of hors eradish peroxidase-conjugated antirabbit IgG (Chemicon) was added for 45 min at

TUNEL Assays. Apoptosis in tissue sections was visualized in situ using a TUNEL (dUTP-mediated UTP nick-end labeling) assay. Brains from three mice/strains were removed at specified time points (0, 2, 6, and 24 h after irradiation) and processed as above. In situ TUNEL assays were performed on five brain sections from each mouse. The TUNEL labeling reaction was carried out using the In Situ Cell Death Detection kit (Roche) in accord with the manufacturer’s instructions and with appropriate specificity controls. Briefly, sections were incubated with proteinase K (20 μg/ml in 10 mM Tris/HCl; Fisher Biotech) at 37°C for 30 min and washed twice with PBS. Sections were then incubated in TdT/fluorescein-conjugated nucleotide mix for 1 h at 37°C in a humidified chamber and washed three times in PBS. Sections were incubated with alkaline phosphatase-conjugated antifluorescein antibody for 1 h at 37°C washed, and incubated with NaphthFast Red alkaline phosphatase substrate (Sigma Chemical Co.), which stained apoptotic cells red. The sections were then counterstained with hematoxylin and visualized by light microscopy. Apoptotic cells were counted as a percentage of the total number of cells/field for several areas in the brain, including the dentate gyrus of the hippocampus and the corpus callosum.

BrdUrd Incorporation and Detection. In situ proliferation was assessed by BrdUrd uptake and staining. At various time points after irradiations, three mice of each strain were injected i.p. with 0.2 ml of a freshly prepared BrdUrd solution (Calbiochem; 6.6 mg/ml) to pulse-label cells in S-phase. After 1 h, mice were deeply anesthetized and perfused by a transcardiac route with 10% buffered formalin using a peristaltic pump (Bio-Rad). The brains were processed as described above. BrdUrd-labeled cells were detected in five sections/brain using a BrdUrd Staining kit (Oncogene Research Products) according to the protocol provided. Briefly, endogenous peroxidase activity was quenched using a solution of 30% hydrogen peroxide in methanol. Sections were incubated with trypsin, and cellular DNA was denatured with sodium hydroxide. Sections were blocked and incubated for 45 min with biotinylated anti-BrdUrd antibody, followed by incubation with streptavidin-peroxidase. Diaminobenzidine substrate was used for visualization of BrdUrd-positive cells. BrdUrd-positive cells were counted in the same areas of the brain as were the TUNEL-positive cells, and percentages were calculated using the same technique.

Preparation of Brain Tissue for Histology. For apoptosis (TUNEL), proliferation (BrdUrd) assays, denucleinization (Luxol fast blue), and routine staining (H&E), brains were removed and fixed in 10% buffered formalin for 2 weeks. Brains were cut at the level of the bregma using a brain matrix (Harvard Apparatus, Holliston, MA), and the two halves were embedded side-by-side in paraffin. Tissue sections (5-μm thick) were cut for further analysis.

Isolation and RNase Protection Assays. For isolation of total RNA from brain tissue for RNase protection assays, mice were sacrificed by cervical dislocation at various time points after irradiation, as described previously (16). Brains were removed, and the midbrain sections were dissected out and snap frozen in liquid nitrogen. For each time point, three midbrains (still frozen) were homogenized in lysis buffer (4 M guanidine, 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol) in a glass tissue homogenizer. RNA was extracted from these lysates in phenol:chloroform:isoamyl alcohol followed by isopropanol precipitation, as described previously by Chomczynski and Sacchi (24). RNA pellets were then washed in 70% ethanol and resuspended in diethyl pyrocarbonate-treated water at a concentration of 5 μg/μl. Radiolabeling of the probes was carried out using [α-35]SdUTP (ICN Biomedicals, Irvine, CA) and the In Vitro Transcription kit from BD Phar-Mingen (San Diego, CA) according to the manufacturer’s instructions. We used either the mCK-3b (BD PharMingen) or an in-house probe set containing TNFRα, IL-1α, IL-1β, and RPL32 for all assays. After overnight hybridization of probe with 25 μg (5 μl) of total RNA from each sample, RNase protection was carried out using the reagents from the RiboQuant RPA kit (BD PharMingen). Protected species were separated on a 5% polyacrylamide sequencing gel. Bands were imaged using a STORM PhosphorImager (Molecular Dynamics), and densitometry measurements were performed with ImageQuant software (Molecular Dynamics).

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37°C, followed by washing, incubation at room temperature with tetramethylbenzoleate substrate solution (Endogen), and reading in a Tecan plate reader at 450 nm. Bovine MBP was used as a standard (Sigma Chemical Co.).

RESULTS

C57BL/6, TNFRp55−/−, and TNFRp75−/− were irradiated with 25 Gy to the midbrain and individually monitored over a 9-month postirradiation period for any changes in behavior that might signal neurological damage, such as loss of the spread reflex, spontaneous turning, and/or seizure activity. Most strikingly, between 2 and 3 months after irradiation, TNFRp75−/− mice exhibited significant reflex loss as well as violent seizure activity. In general, TNFRp75−/− mice that developed seizures, including the few that developed them early (at 1 and 2 months after irradiation), continued to have them during the later 3–5-month period. The incidence of seizure activity, combined from the three experiments (groups of 10 mice for 30 mice total) from each strain, is depicted in Fig. 1A. Interestingly, the frequency of radiation-induced seizures decreased at 4 to 5 months after irradiation. The percentage of mice that developed seizures after irradiation with different doses was assessed in a separate experiment using 20 mice of each strain. Fig. 1B indicates that the incidence of seizure activity in the TNFRp75−/− mice was dose-dependent between 20 Gy and 45 Gy.

It should be noted that TNFRp75−/− mice that did not develop radiation-induced seizures still showed a loss of spread reflex and decreased activity, although this was harder to quantify, and by 5 and 6 months, all irradiated TNFRp75−/− mice had lost weight, failed to groom, become lethargic, and had to be euthanized. Seizure activity was not observed in irradiated C57BL/6 or TNFRp55−/− animals at any time or any dose after irradiation, nor was it seen in unirradiated animals of any strain. Irradiated mice of other strains did have phases of decreased activity and occasional loss of spread reflex, as we described for C3H mice (23), but these were always less dramatic than in the TNFRp75−/− strain, and only a low incidence of death was recorded over the 9-month observation period.

Previously, we have shown that demyelination occurs in C3H mice during both the subacute and late phases after radiation, and that its extent at 6 months after irradiation correlates well with the delivered dose (22). Because demyelination is a cardinal feature of radiation-induced brain damage and is thought to contribute to many observed neurological side effects, the extent of demyelination was assessed in the TNFR knockout and control mice. Demyelination was quantified using an ELISA-based assay for MBP. The data at 5.5 months after 25 Gy of radiation, pooled from three separate experiments, are presented in Fig. 2. The response of wild-type C57BL/6 mice was similar to that found for C3H/Sed/Kam mice at 6 months after 25 Gy with a 20% decrease in total MBP content that was statistically significant (P < 0.05; Ref. 22). Brains from TNFRp55−/− mice had a similar, although slightly less (14–16%), reduction. In contrast, MBP levels in TNFRp75−/− mice had decreased by 40% by 5.5 months after 25 Gy. Indeed, even after 3 months after irradiation, TNFRp75−/− brains demonstrated MBP levels that had already been reduced to ~80% of the control values (data not shown).

Demyelination was confirmed histologically using Luxol Fast Blue and cresyl violet staining. Unirradiated animals of all three strains exhibited similar CNS vascular and cellular structure and myelination status. At 5.5 months after 25 Gy irradiation, TNFRp75−/− mice showed massive diffuse demyelination predominantly throughout the white matter areas in the brain, particularly in thalamus and hypothalamus (Fig. 3). Demyelination was present in both C57BL/6 and TNFRp55−/− mice, but it was far less marked and more focal. Even at 3 months after irradiation, demyelination in TNFRp75−/− mice was very obvious and much greater than that seen in either TNFRp55−/− mice or the C57BL/6 controls (data not shown). Brains from irradiated TNFRp75−/− mice also displayed considerable axonal loss and neuronal degeneration, which increased between 3 and 5.5 months and could be a result of the widespread demyelination, although defects in the neuronal response to radiation in these animals cannot be ruled out. In none of the strains was there any evidence of marked radiation-induced
hemorrhagic necrosis or other vascular damage. The data appear to correlate with the observed seizures at 3 months and later morbidity in the TNFRp75−/− strain after brain irradiation.

It has been postulated that acute apoptosis of brain cells may contribute to late radiation-induced demyelination (26). In an attempt to clarify this relationship, in situ TUNEL assays were performed soon after brain irradiation in the three mouse strains. It has been shown previously in rats that radiation-induced apoptosis in brain tissue peaks at 6 h (19, 20); therefore, our time course was centered on this point. In unirradiated brains from all strains and by 2 h after 25 Gy irradiation, few or no apoptotic cells were observed. However, by 6 h TUNEL-positive cells were obvious (Table 1). Most were in distinct areas, i.e., the dentate gyrus of the hippocampus, the corpus callosum, and the subventricular zone within the walls of the lateral ventricles. Interestingly, the largest amount of apoptosis in these areas at 6 h was in TNFRp75−/− mice, although TNFRp55−/− mice showed a smaller but statistically significant increase in the corpus callosum, which requires further characterization. Responses subsided by 24 h in both the C57BL/6 controls and the TNFRp55−/− brains, but apoptotic nuclei could still be seen at this time point in the brains of TNFRp75−/− mice (not shown).

To clarify the role of cytokine signaling in the acute response of the murine brain to irradiation, expression of proinflammatory cytokine genes was measured by an RNase protection assay. Samples were taken at 0 and 30 min and 2, 6, and 24 h after a single dose of 25 Gy to the midbrain. A representative gel (Fig. 4A) and the combined quantified data from three separate experiments (Fig. 4B) are presented. During the first 24 h after irradiation, brains from C57BL/6 wild-type mice showed a peak in TNF-α mRNA expression at ~2 h that returned to baseline levels by 24 h. TNFRp55−/− mice showed a similar pattern of TNF-α mRNA expression, both quantitatively and qualitatively. In contrast, TNF-α mRNA in irradiated TNFRp75−/− brains rose to levels that were consistently two to three times higher than the other two strains at 2 h.

Radiation induces a proliferative response by brain cells that peaks ~1 month after 25 Gy (17, 21, 23). To study the potential role of TNF-α signaling in the proliferative response, we irradiated mice from the control and receptor knockout strains and pulse-labeled S-phase cells in the brain at 1 month after irradiation with BrdUrd. There were few proliferative cells in sections from unirradiated brains of all strains. However, 1 month after irradiation an increased number was present, particularly along the blades of the granule cell layer in the dentate gyrus of the hippocampus. Brains from TNFRp75−/− mice showed markedly less radiation-induced proliferation than those from C57BL/6 and TNFRp55−/− mice (Table 2). There was a suggestion of a slight, but not statistically significant, increase in proliferation in the TNFRp55−/− mice. The distribution of the BrdUrd-positive cells in TNFRp75−/− mice was also different from that in the other two strains. Specifically, although the control and TNFRp55−/− animals showed a line of small, BrdUrd-positive cells along the entire length of the granule cell layer, sections from

Table 1 In situ TUNEL assay (percentage of apoptotic cells/field)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dentate gyrus (0 Gy)</th>
<th>Corpus callosum (0 Gy)</th>
<th>Dentate gyrus (25 Gy)</th>
<th>Corpus callosum (25 Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>None</td>
<td>None</td>
<td>0.55 ± 0.08%</td>
<td>None</td>
</tr>
<tr>
<td>TNFRp55−/−</td>
<td>None</td>
<td>None</td>
<td>0.66 ± 0.07%</td>
<td>None</td>
</tr>
<tr>
<td>TNFRp75−/−</td>
<td>None</td>
<td>None</td>
<td>1.74 ± 0.24%</td>
<td>None</td>
</tr>
</tbody>
</table>

* Mean percentage of apoptotic cells ± 1 SE.

b P < 0.05 by Student’s t test.
TNFRp75−/− mice displayed fewer and larger BrdUrd-positive cells situated only at the vertex of the angle formed by the upper and lower blades of the dentate gyrus. The significance of these differences is currently unknown.

The proliferative and demyelination responses were correlated with TNF-α gene expression by evaluating cytokine responses at 48 h, 1 week, 2 weeks, 1 month, 3 months, and 6 months after irradiation (Fig. 5). In C57BL/6 wild-type mice, TNF-α mRNA expression was elevated at 1 and 2 weeks and peaked 1 month after irradiation. It decreased slightly thereafter but remained elevated above baseline levels throughout the 6-month time course study, similar to what we observed in C3H mice (18). TNF-α mRNA responses in TNFRp55−/− mice and TNFRp75−/− mice were similar to controls over the first 2 weeks. In TNFRp55−/− mice, TNF-α mRNA expression levels remain at this elevated level out to 6 months. In contrast, in TNFRp75−/− mice, levels returned to baseline by 1 month after the radiation dose and never increased thereafter.

**DISCUSSION**

In these studies, TNFRp75−/− mice were found to be more susceptible to the neurological effects of brain irradiation than wild-type C57BL/6 mice or TNFRp55−/− mice. The response differed in several important ways. TNFRp75−/− mice developed severe neurological effects, including seizures that peaked by 3 months. These abnormalities correlated with the onset of severe demyelination that could be seen both histologically and by ELISA for MBP. With time, the extent of demyelination increased and, although the incidence of seizures decreased, the condition of the mice got progressively worse, and they had to be euthanized. Radiation-induced demyelination was present in C57BL/6 and TNFRp55−/− brains but was markedly less than that seen in the TNFRp75−/− mice, and although neurological changes were observed, they were generally minor and did not result in seizures or death.

The ability of C57BL/6 mice to survive relatively high doses of radiation to the brain contrasts with C3H/Sed/Kam mice, which die around 6 months after 30–40 Gy brain irradiation (23). The extent of demyelination after 25 Gy is similar in both strains after 5–6 months, but it is more focal in the former strain. No other significant differences were observed, and there was little vascular damage in either strain, but the finding raises the question of whether death after brain irradiation in C3H mice is unrelated to demyelination. Given the close dose-response relationship and histological findings (19), demyelination is obviously a cardinal feature of the response, but death may be attributable to damage to specific critical areas in the brain, and the pattern of demyelination may be important. On the other hand, others have observed differences between C3H and C57BL/6 mice strains in the response of other tissues to radiation, and in particular radiation-induced fibrosis (27). When the molecular basis of these differences is elucidated, they may indicate a mechanism that is shared and involved in brain responses to irradiation.

TNF-α, as well as many other cytokines and growth factors, has varying effects on the different cell types in the CNS, including oligodendrocytes, astrocytes, and even neurons. The responses induced are critical for the development and maintenance of the normal central nervous system, as evidenced by certain gene knockout models (3, 28). These signals must be tightly controlled so that they are expressed and received at the appropriate time and by the appropriate cell type. Radiation-induced proinflammatory gene expression can be considered as a coordinated attempt at wound healing response that involves interactions between parenchymal and vascular cells, as well as infiltrating leukocytes that migrate to the site of injury (18). The repetitive cyclical response observed with time after irradiation may be evidence of dysregulation of this response caused by radiation-induced cell loss and attempts at recovery, leading to inappropriate expression or “out-of-context” reactions. In TNFRp75−/− mice, clearly, several aspects of these apparently coordinated responses are severely affected, arguing in favor of a role for this receptor in linking and controlling acute, subacute, and late molecular and cellular events after irradiation.

It is tempting to think that the increase in acute radiation-induced apoptosis within putative stem cell areas in the brain of TNFRp75−/− mice is linked to the later observed decreased proliferative response in the same areas. Identification of the cells in these areas has been hampered by the lack of markers of stem cell lineage (29), and attempts are being made to better characterize the cells in the apoptotic and proliferative responses. Stem cells in these areas have been shown to proliferate, differentiate, and migrate to sites of injury in the CNS of the rodent, sometimes to areas as distant as the spinal cord (29–31). Hopewell and Cavanaugh (31) were the first to show changes in proliferation in the rat subependyma after irradiation, showing a decrease in mitotic count during the first 24 h after irradiation with subsequent recovery by 3 months if the dose was 20

**Table 2**  
BrdUrd labeling (percentage of labeled cells/field)  

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dentine gyrus</th>
<th>25 Gy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>4.55 ± 1.05%</td>
<td>10.10 ± 0.90%</td>
</tr>
<tr>
<td>TNFRp55−/−</td>
<td>3.75 ± 1.55%</td>
<td>13.45 ± 1.25%</td>
</tr>
<tr>
<td>TNFRp75−/−</td>
<td>3.20 ± 0.80%</td>
<td>4.85 ± 1.05%</td>
</tr>
</tbody>
</table>

*Mean percentage of labeled cells ± 1 SE.  
 P < 0.1 by Student’s t test.  
 P < 0.05.

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**Fig. 5.** Proinflammatory mRNA expression during the subacute and late phases after irradiation to the midbrain in C57BL/6, TNFRp55−/−, and TNFRp75−/− mice. Total RNA was extracted from the midbrains of three mice/strain at the indicated time points. A, representative example of an RPA. *Cont.*, control; *mo*, month; *wk*, week; *R*, pooled data from three separate experiments quantified for TNF-α and normalized for loading controls. As in Fig. 3, both NFSA-TNF mRNA and standard mouse control RNA were used as controls and gel markers. C57BL/6 control mice exhibited a peak in TNF-α mRNA 1 month after irradiation, and levels remained elevated above baseline out to 6 months. TNFRp55−/− and TNFRp75−/− mice also exhibited a peak in TNF-α mRNA during the subacute phase (10–15 days), and in TNFRp55−/− mice the levels remained elevated to 6 months. TNF-α levels in TNFRp75−/− mice, however, returned to baseline levels by 30 days after irradiation and were never re-elevated. *Bars*, SE.
Gy or lower. No recovery was seen at higher doses, even after 6 months. Recently, Tada et al. (30) have shown similar patterns of response in the subependymoma. It has been suggested that the cells in the neural stem cell areas can regenerate functional neurons after injury, especially within the pyramidal layer (32–34). The effect of TNF-α signaling on these specialized stem cell populations requires further investigation, especially because both TNF-α production and radiation-induced apoptosis are elevated in TNFRp75−/− brain at this early time point. In vitro experiments are under way to examine this relationship.

Our data suggest that TNF-α signaling, particularly through TNFRp75, has a central role in regulating the balance, not only of cell death, but also of cell proliferation after radiation exposure. TNF-α expression in TNFRp75−/− brain was abrogated by 1 month after irradiation, as was the proliferative response. There is evidence that TNF-α is protective during other demyelinating diseases, such as EAE (35). Also, recent studies have also suggested a protective role for TNFRp75 during other demyelinating diseases, such as EAE (35).

The exact mechanism of TNFRp75-mediated radioprotection of the brain remains unclear. It is known that TNFRp75, upon binding TNF-α, can activate NF-κB (42, 43), leading to transcription of genes involved in cell survival that can mitigate the effects of various insults, which includes countering TNF-α-induced apoptosis mediated through TNFRp55 (42). One potential NF-κB-dependent mechanism that could prevent cell death or enhance cell survival after irradiation is activation of inhibitor of apoptosis proteins (cIAP1, cIAP2, and XIAP) that bind to TNFRp75/TRA2 complexes and interfere with the apoptotic mechanism, primarily by interfering with caspase-3 and caspase-7 actions (44–46). Although TNFRp55 can also induce NF-κB activation through activating kinase RIP (47, 48), our preliminary data indicate a blunted NF-κB response to irradiation in the TNFRp75−/− mouse brain.4 Because TNFRp75 does not contain a death domain, we hypothesize that this particular pathway to activation of NF-κB counterbalances proapoptotic responses mediated through TNFRp55 pathways and is important in the response of the CNS to irradiation, although other pathways may contribute. Although the TNFRp55−/− mice showed no overt phenotype in this study, in vitro experiments with oligodendrocytes indicate mutual antagonism between TNFRp55 and TNFRp75 pathways.4

The lack of TNF-α expression in the late phases after brain irradiation of TNFRp75−/− mice is of interest. The TNF-α gene itself is a target gene for NF-κB, and the failure of TNFRp75−/− mice to maintain TNF-α production supports its role in a “positive feedback” process that involves TNFRp75−/− and NF-κB activation. TNFRp75 may therefore be required for sustaining a NF-κB response that is required for normal tissue repair and maintenance. There is evidence that TNFRp75 sustains immune responses mediated by TNF-α (49), and a similar mechanism may occur after injury in the CNS.

ACKNOWLEDGMENTS

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Jennifer L. Daigle, Ji-Hong Hong, Chi-Shiun Chiang, et al.


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