Radiation-induced Apoptosis of Endothelial Cells in the Murine Central Nervous System: Protection by Fibroblast Growth Factor and Sphingomyelinase Deficiency

Louis A. Peña, Zvi Fuxs, and Richard N. Kolesnick

ABSTRACT

Injury to the central nervous system (CNS) by ionizing radiation may be a consequence of damage to the vascular endothelium. Recent studies showed that radiation-induced apoptosis of endothelial cells in vitro and in the lung-in vivo is mediated by the lipid second messenger ceramide via activation of acid sphingomyelinase (ASM). This apoptotic response to radiation can be inhibited by basic fibroblast growth factor or by genetic mutation of ASM. In the CNS, single-dose radiation has been shown to result in a 15% loss of endothelial cells within 24 h, but whether or not this loss is associated with apoptosis remains unknown. In the present studies, dose- and time-dependent inhibition of apoptosis was observed in the C57BL/6 mouse CNS. Apoptosis was quantified by terminal deoxynucleotidyl transferase-mediated nick end labeling, and specific endothelial apoptosis was determined by histochemical double labeling with terminal deoxynucleotidyl transferase-mediated nick end labeling, and specific endothelial apoptosis was determined by histochemical double labeling with terminal deoxynucleotidyl transferase-mediated nick end labeling and Lycopersicon esculentum lectin. Beginning at 4 h after single-dose radiation, apoptosis was ongoing for 24 h and peaked at 12 h at an incidence of 0.7–1.4% of the total cells in spinal cord sections. Up to 20% of the apoptotic cells were endothelial. This effect was also seen in multiple regions of the brain (medulla, pons, and hippocampus). A significant reduction of radiation-induced apoptosis was observed after i.v. basic fibroblast growth factor treatment (0.45–4.5 µg/mouse). Identical results were noted in C3H/HeJ mice. Furthermore, irradiated ASM knockout mice displayed as much as a 70% reduction in endothelial apoptosis. This study demonstrates that ionizing radiation induces early endothelial cell apoptosis throughout the CNS. These data are consistent with recent evidence linking radiation-induced stress with ceramide and suggest approaches to modify the apoptotic response in control of radiation toxicity in the CNS.

INTRODUCTION

Considerable effort has been made over the years to quantify the tolerance of normal tissues to provide a baseline for therapeutic irradiation at maximum biologically effective doses (1, 2). Normal tissue injury by ionizing radiation differs depending on the target organ and cell types. Acute or early reactions primarily reflect the balance between the rates of cell killing by irradiation and regeneration by surviving stem cells, whereas late reactions occur when radiation depletes the tissue stem cell compartment, leading to tissue necrosis, fibrosis, and organ dysfunction.

In the CNS, there is a high degree of cellular heterogeneity, and both early and late injuries occur (3, 4). Whereas symptoms of acute and subacute injury can be reversed to some extent with steroid medication, the late forms of CNS injury are irreversible. The TDo/50–TD50/5 for single-dose exposure are 15–20 Gy and are similar for spinal cord myelopathy, brain white matter necrosis (leukoencephalopathy), and microvascular damage (1). The pathogenesis of myelin degeneration and leukoencephalopathy is not fully understood. Whereas one hypothesis suggests that oligodendrocyte injury mediates this response, it is also possible that microvascular damage is a primary culprit (5–7). Acute vascular changes within 24 h after irradiation include increased endothelial cell swelling, vascular permeability and edema, lymphocyte adhesion and infiltration, and apoptosis (1, 8). Late vascular effects occur weeks to months after irradiation and include capillary collapse, thickening of basement membranes, scarring and fibrosis, teleangiectasias, and a loss of clonogenic capacity (1, 8).

Despite the importance of radiation effects on the vascular endothelium in the CNS, there is little current in vivo data on endothelial cell death, and that which does exist is contradictory. A study of irradiated rodent brain showed that up to 15% of endothelial cells are lost within 24 h of irradiation (9), the time frame in which apoptosis is most likely to occur. However, another study failed to detect any endothelial cell apoptosis within the same time frame (10, 11).

The present work was undertaken to determine whether endothelial apoptosis occurs after CNS irradiation in a rodent model and to determine the mechanism of this response.

Recent studies have demonstrated that microvascular endothelial apoptosis constitutes an early and critical element in radiation pneumonitis and in the LPS-induced septic shock syndrome (12, 13). The proapoptotic effects of radiation and of LPS were shown to be mediated via the ASM and the generation of ceramide (13, 14). Genetic mutations that inactivate ASM or treatment with i.v. injections of bFGF abrogated ceramide generation and apoptosis. The same mechanism of radiation-induced apoptosis via ceramide generation was also observed in endothelial cells in vitro (15), and the antia apoptotic action of bFGF (16–18) was shown to be mediated, at least in part, via activation of the α isoform of PKC (17). A similar interaction between radiation, ceramide, PKC, and apoptosis was reported in the SQ-20B squamous carcinoma cell line, in which radiation failed to elicit an apoptotic response unless PKC was blocked, leading to increased apoptosis and clonogenic cell death in vitro and in vivo (19, 20). Radiation-induced apoptosis in lymphoid cells is similarly associated with a balance between ceramide and PKC activity, although in this system, Bcl-2 also regulated ceramide- and radiation-induced apoptosis (21, 22). Similarly, a loss of neutral sphingomyelinase has been shown to confer radiation resistance in WEHI-231 and TF-1 lymphoid cells, in which a reduction in both clonogenic death and apoptotic death was associated with deficient ceramide production (19, 20, 23). Zundel and Giacca (24) have shown that stress signals apoptosis via ceramide-induced down-regulation of phosphatidylinositol 3’-kinase and inhibition of the kinase Akt/PKB. Inhibition of...
this pathway resulted in decreased phosphorylation of Bad, a death effector in the Bcl-2 family. Signaling via the kinase suppressor of Ras/mitogen-activated protein kinase pathway may also cause the inactivation of Akt/PI3K and the dephosphorylation of Bad, although direct data on ionizing radiation as the apoptotic stimulus for this pathway are currently lacking (25). Hence, radiation-induced apoptotic signaling through ceramide appears to involve multiple, co-ordinated pathways, and the apoptotic outcome in vitro and in vivo depends on the balance between the activities of pro- and antiapoptotic signaling systems.

In this report, we examined in vivo the extent and kinetics of CNS endothelial cell responses to radiation in the mature CNS of mice. Dose- and time-dependent induction of apoptosis was observed in the endothelium of the brain and spinal cord. A significant reduction of this apoptotic response was observed after i.v. bFGF treatment or in ASM KO mice. These radioprotective effects are consistent with an emerging body of evidence linking radiation-induced stress responses in endothelium with ceramide and suggest new approaches to modify the apoptotic response in the control of radiation toxicity in the CNS.

MATERIALS AND METHODS

Animals. Male C57BL/6, C3H/HeJ, and 129/Sv mice were purchased from Taconic (Germantown, NY) and housed in the Sloan-Kettering animal facility until reaching a specific age for each experiment (16 weeks old, unless stated otherwise). A colony of transgenic mice harboring a KO of the ASM gene (ASM KO) was established at our animal facility, having originally been obtained from Dr. Edward Schuchman (Department of Genetics, Mount Sinai School of Medicine, New York, NY; Ref. 26). These mice have a 129/Sv × C57BL/6 genetic background, therefore wild-type individuals of this cross were used as controls in experiments involving ASM KO mice. It should be noted that whereas ASM KO mice begin to exhibit Niemann-Pick disease-like symptoms at approximately 20 weeks of age, experiments in this study were carried out using ASM KO animals at 8 or 16 weeks of age.

Irradiation. Mice were lightly sedated with ketamine (0.1 mg/g), placed prone in a 137Cs irradiator (Shepherd Mark-I, Model 68), and subjected to total body irradiation at a dose rate of 220 cGy/min. At various time points, animals were euthanized and perfusion fixed intracardially with fresh 4% paraformaldehyde in PBS. Brains and spinal cords were dissected and post-fixed overnight and then placed in an automated vacuum tissue processor for paraffin embedding over a 16-h period. In some experiments, mice were also given i.v. injections of recombinant bFGF or other cytokines (R&D Systems, Minneapolis, MN) in a vehicle of 0.1% gelatin under metaphane anesthesia into the peritoneum. Brains and spinal cords were harvested 12 h after irradiation. A total of 10–18 cross sections of spinal cord per animal (representing cervical, thoracic, and lumbar regions without bias) was stained using the TUNEL method and counterstained with hematoxylin. The highest rate of apoptosis (1.1 ± 0.2% per spinal cord section) was observed at the earliest time point tested (8 weeks of age) and gradually decreased with time, reaching a plateau at 16 weeks and beyond (0.5 ± 0.1% per spinal cord section). Therefore, all experiments in this series were carried out with mice at 16 weeks of age.

To further characterize the apoptotic response to radiation in the CNS, time course and radiation dose-response studies were carried out. Fig. 1 depicts the dose response in spinal cord sections of duplicate mice at several time points within a 24-h period. Previous work on the radiation response of the mature rat CNS reported peak levels of apoptosis in the subependymal zone of the brain that occurred at 6–8 h after irradiation (38), and peak levels of apoptosis occurred at 10–12 h in the spinal cord (10). Our analysis of the adult mouse CNS confirmed this time course. The apoptotic response was dose dependent and peaked at 12 h after irradiation (Fig. 1). Similar results could be observed in various regions of the brain, e.g., hippocampus and pons (data not shown). For subsequent experiments, the dose of 50 Gy was preferred to increase the statistical significance of cell counting of low frequency events. This dose is likely to have relevance to biological phenomena because previous studies showed that single-dose radiation of less than 10 Gy produced no morphological changes in the CNS, whereas significant early or latent effects were seen with doses between 20 and 40 Gy (39). Furthermore, fractionated radiation in the range of 45–60 Gy represents the lower tolerance threshold in humans (40). Thus a 50 Gy single dose to the rodent CNS would be expected to produce sublethal, subacute effects near the lower tolerance threshold for the CNS (41).

In the experiments quoted above, the absolute numbers of apoptotic cells, rather than the percentage, were used to quantify the response to radiation. However, it should be noted that previous studies reported that whereas the numbers of neurons may vary considerably among different spinal cord levels, the total numbers of cells, including neurons, glial cells, oligodendrocytes, and endothelial cells, remained somewhat constant (42, 43). The present studies confirmed these findings. Image analysis of hematoxylin-stained spinal cord sections

RESULTS

Whereas the radiation sensitivity of rodent tissue is age dependent (36, 37), initial experiments were carried out to determine the age at which the apoptotic sensitivity of C57BL/6 mice represents the sensitivity of adult CNS. Three strains of mice (C57BL/6, C3H/HeJ, and 129/Sv) were irradiated at different ages with a single dose of 50 Gy, and their spinal cords were harvested 12 h after irradiation. A total of 10–18 cross sections of spinal cord per animal (representing cervical, thoracic, and lumbar regions without bias) was stained using the TUNEL method and counterstained with hematoxylin. The highest rate of apoptosis (1.1 ± 0.2% per spinal cord section) was observed at the earliest time point tested (8 weeks of age) and gradually decreased with time, reaching a plateau at 16 weeks and beyond (0.5 ± 0.1% per spinal cord section). Therefore, all experiments in this series were carried out with mice at 16 weeks of age.
of 16 week old C57/BL6 mice revealed that the total number of cells was consistent across the total length of the cord, averaging 1667 ± 318 cells per cross section. It should also be noted that no significant variations between individual mice of the same or different strains were discerned (data not shown). For example, the average cross section of 16-week-old ASM KO mice had a mean of 1600 ± 294 cells. These data indicate that in evaluating apoptotic responses to radiation in the spinal cord, it is legitimate to report the absolute number of apoptotic cells rather than a percentage of apoptotic cells per section. Furthermore, in irradiated spinal cord specimens, the total number of apoptotic cells [range, 0.7–1.4%, in agreement with the studies of Li et al. on rat spinal cord (10, 11)] was also found to be constant across different cord levels and across strains (see controls in Fig. 3A).

To identify which specific cell types were undergoing apoptosis, we used double staining procedures with cell phenotype-specific reagents. In this study, we describe results pertaining to endothelialial cells. Widely used antibodies directed against specific adhesion molecules [e.g., PECAM-1 (CD31) and E-selectin (CD62e)] are effective in specifically identifying endothelial cells in several tissues, but not in the CNS, where these cell surface markers are down-regulated coincident with the development of the blood brain barrier (34, 35). Consistent with this observation, we found that staining with an antibody against CD31 failed to stain microvessel endothelium within the brain (data not shown). In contrast, lectin staining, such as staining with LEL and RCA-1, has been shown to reliably label endothelium in the CNS (28–33, 44). The LEL staining method used in the present study is a single-step procedure and preferentially enhances endothelial staining while reducing microglial staining. Fig. 2 shows double labeling of cells with LEL and TUNEL at high magnification (×1000). An endothelial cell exhibiting intensely fluorescent LEL staining (Fig. 2A, top) is the same cell whose apoptotic nucleus is TUNEL positive (Fig. 2A, middle). These images were digitally superimposed to demonstrate the colocalization of these two markers (Fig. 2A, bottom). Lectin stains can also label microglia (30–33), the resident tissue macrophages of the CNS. The distinct morphology of microglia is demonstrated in Fig. 2B, whose significantly weaker staining has been enhanced in this exposure only for the purposes of reproduction for publication. The double stain studies revealed that the fraction of endothelial cells in the total population of cells undergoing apoptosis at 12 h after exposure to 50 Gy was 16–20%. The majority of apoptotic cells that were nonendothelial are likely to be glial, based on morphological criteria, in agreement with data reported by Li et al. (10).

To explore the mechanism of radiation-induced apoptosis in the CNS endothelium of the adult mouse, we studied the effects of i.v. injection of aFGF or bFGF. In previous studies, we demonstrated that bFGF serves as an antiapoptotic survival factor for endothelial cells irradiated in vitro and in a lung model in vivo (12, 16, 17, 45). The effect of FGF in the murine CNS was first assessed by treating animals with three equal doses of aFGF or bFGF, which were administered i.v. immediately before and after, and at 1 h after 50 Gy of irradiation (total dose delivered, 0.45–4.5 μg/mouse). TUNEL-positive apoptotic cells were counted in CNS specimens obtained 12 h after irradiation. Both bFGF and aFGF reduced the total level of apoptosis by approximately 50% (Fig. 3A). All treated animals were significantly different from sham-injected controls (P < 0.001). Similar significant reductions in apoptosis were measured in two strains of
mice tested, C3H/HeJ and C57BL/6 (P < 0.001 each; Fig. 3B). Differences between strains were not significant (P = 0.08). To explore whether the antiprotective effect of FGF was specific to the endothelial cell subpopulation of the irradiated CNS, the double labeling technique with TUNEL and LEL was used. Table 1 shows that similar to the effect of bFGF on the total cell population, treatment with bFGF resulted in an approximately 50% reduction in endothelial cell apoptosis. These ratios remained approximately the same in separate experiments with varying doses of radiation (1, 5, 10, and 20 Gy) and a constant dose of bFGF (data not shown). These data indicated that whereas FGF may protect more than one of the subpopulations of CNS cells, endothelial cells in vivo are indeed definitely radioprotected, as has been observed previously in vitro and in other organs in vivo (12, 16, 46–50).

Previous studies of cultured endothelial cells showed that bFGF blocks radiation-induced apoptosis (17, 18, 45), likely via inhibition of ASM-mediated hydrolytic degradation of membrane sphingomyelin (14, 15). Furthermore, ASM KO mice that have a defect in ceramide generation (26) exhibit resistance to the apoptotic effects of radiation in vivo (14). A comparison of irradiated ASM KO mice with wild-type controls in the present studies showed that the total number of apoptotic cells was decreased 50–75% in the spinal cords of ASM KO mice (Fig. 4). Quantitative differences between wild-type and ASM KO mice were also evident in different regions of the brain, such as the hippocampus (Fig. 5), midbrain, and hindbrain (data not shown). Differences between wild-type and ASM KO mice were significant (Table 2). TUNEL/LEL double staining to identify the endothelial cell subpopulation showed a 3-fold difference between the wild-type and KO strains (Table 3). These data suggest that the effect of radiation to induce apoptosis in the cells of the CNS microvasculature is mediated via the ASM/ceramide pathway.

**DISCUSSION**

In the present studies, we demonstrated that radiation induces an apoptotic response in the CNS at times and doses ranges relevant to...
Acute radiation toxicity. After single-dose irradiation, there was evidence of ongoing apoptosis between 4 and 24 h after exposure, which peaked at 12 h. The peak incidence of apoptosis was 0.7–1.4% of the cells in spinal cord sections. These time frames and the incidence are consistent with the observation of Li et al. (11), who showed in rat spinal cord that apoptosis occurred throughout the first 24 h after single-dose irradiation and peaked at 12 h at an incidence of 3%. Furthermore, that study also demonstrated that this level of ongoing apoptosis translated into an 18% loss of spinal cord cells by 24 h. This magnitude of apoptosis is likely to have functional consequences for the generation of radiation toxicity.

Our present studies also showed that 16–20% of the cells undergoing apoptosis were endothelial cells, whereas the rest involved glial subpopulations of the CNS parenchyma. It has been estimated that the number of endothelial cells in the adult mouse spinal cord is 2.50 × 10^6 of a total population of 13.08 × 10^6 cells (42), representing approximately 20% of the total. Hence, the incidence of endothelial apoptosis was proportional to the endothelial cell fraction of the total CNS cell population. Whereas the peak incidence of endothelial apoptosis appears to be low, the overall apoptotic response over the first 24-h period after radiation may be considerable and significant for the generation of tissue responses. Consistent with this notion is the study by Ljubimova et al. (9), which reported that 15% of the CNS endothelium is lost by 24 h after exposure to 25–100 Gy.

The fact that both endothelial cells and other parenchymal CNS cells undergo concomitant apoptotic lesions opens the question of which component is critical to the evolution of acute radiation toxicity in the CNS. In previous studies, we demonstrated that microvascular endothelial apoptosis was the primary and critical response in the generation of tissue damage by radiation and other stresses. The microvascular endothelium was the earliest tissue component to undergo apoptosis in the irradiated lung of C3H/HeJ mice (12, 16, 17) and in the small intestine, lung, and fat tissue of C57BL/6 mice during experimental septic shock (13). The critical role of this response in the evolution of tissue damage was suggested by the fact that both endothelial apoptosis and the eventual tissue damage were abrogated by i.v. bFGF therapy. Furthermore, ASM KO mice were found to be refractory to lung irradiation- and LPS-induced microvascular endothelial apoptosis, tissue damage, and animal death in response to these stresses (13, 14). The present studies showed that in the CNS, both FGF and ASM deficiency reduced radiation-induced apoptosis in the endothelial compartment. However, because endothelial apoptosis and nonendothelial apoptosis occur simultaneously, whether apoptotic damage to the microvasculature is critical to the pathogenesis of acute radiation damage to the adult mouse CNS remains unclear. The finding that bFGF and ASM deficiency reduces the apoptotic response to radiation provides a possible approach to further explore whether this acute apoptosis, over the long term, has relevance to early or late CNS injury.

Whereas the present studies provided evidence for radiation-induced endothelial apoptosis, prior investigations by Li et al. (10, 11) failed to detect endothelial cell apoptosis in the irradiated CNS. Differences in the histochemical assays to identify the cell types undergoing apoptosis may have accounted for this apparent discrepancy. Whereas we used the TUNEL/LEL double labeling technique to distinguish between endothelial and nonendothelial apoptosis, Li et al. used single-agent staining with either the Leu-7 antibody, anti-GFAP antibody, or the RCA-1 lectin (10, 11). In the Li et al. studies, apoptotic cells were stained only by the Leu-7 antibody, leading to the conclusion that only oligodendrocytes undergo apoptosis in response to CNS irradiation. Leu-7 labels the myelin-associated glycoprotein of oligodendrocytes and oligodendrogliomas because the carbohydrate residues of this protein contain the HNK-1 epitope (51–53). However, the HNK-1 epitope also occurs in a class of glycolipids known as sulfoglucuronosyl paraglobosides, which are present in brain microvascular endothelial cells (54–56). These sulfo-glycolipids are thought to mediate activated lymphocyte attachment to endothelial cells and subsequent infiltration into the parenchyma of the CNS. The HNK-1 epitope has also been found on neurons (57, 58) and immature astrocytes (52), in addition to the population of natural killer cells and T-lymphocytes from which it was originally described (59). Hence,

**Table 2. Total apoptosis in brain regions of wild-type and ASM KO mice**

<table>
<thead>
<tr>
<th></th>
<th>WT mean ± SE</th>
<th>ASM KO mean ± SE</th>
<th>P test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampus</td>
<td>13.4 ± 1.1</td>
<td>2.7 ± 0.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mid-pons</td>
<td>8.5 ± 1.4</td>
<td>2.5 ± 0.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Medulla</td>
<td>4.8 ± 0.6</td>
<td>1.9 ± 0.6</td>
<td>&lt;0.0003</td>
</tr>
</tbody>
</table>

**Table 3. Apoptosis of endothelial cell subpopulation in ceramide generation-impaired ASM KO mice**

<table>
<thead>
<tr>
<th></th>
<th>Endothelial apoptosis per section mean ± SE</th>
<th>t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>No irradiation, wt</td>
<td>0.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>No irradiation, ASM KO</td>
<td>0.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>50 Gy, wt</td>
<td>5.9 ± 1.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>50 Gy, ASM KO</td>
<td>2.0 ± 0.7</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*Compared with unirradiated control.
*Compared with wild-type strain after 50 Gy.
Leu-7/HNK-1 staining appears to lack specificity and may stain CNS endothelial cells in addition to oligodendrocytes. The present observations are also consistent with a large body of data demonstrating survival effects for FGF on several CNS cell types in vivo after a variety of injuries or stresses (for review, see Refs. 60–66). Furthermore, FGF has been shown to exert radioprotective effects in several non-CNS tissue systems in vivo. Ding et al. (46–48) and Okunieff et al. (49, 50) have reported that in C3H mice, both bFGF and, to a lesser extent, aFGF exhibit radioprotective effects in lymphoid cells and intestinal crypt cells, and our group has previously observed protection by bFGF in the lung (12, 13, 16). Although the mechanisms involved in the radioprotective effects of FGF are not fully known, evidence suggests that PKC, which serves as the effector system mediating the radioprotective effect of bFGF in primary cultures of bovine endothelium, may act by blocking sphingomyelin hydrolysis to ceramide (15, 17). Oligodendrocytes and other glial cells (61, 62, 67, 68) express FGF receptors, and oligodendrocyte apoptosis has been linked to ceramide generation in some instances (for a review, see Ref. 69). Whether bFGF protects glial elements from radiation-induced apoptosis directly or indirectly by preserving microvessels cannot be answered by the present data. Derivatized forms of bFGF, either biotinylated or radiolabeled with 111In or 125I, failed to gain access to the parenchyma of the brain and remained in the intima of capillaries (16, 70), and lacked protective effects, whereas metabolically labeled [14C]bFGF freely crossed the blood brain barrier and exerted protective effects (71). Further experimentation is necessary to determine whether FGF is effective in abrogating the deleterious tissue effects of radiation on the CNS and whether it is mediated via an antiapoptotic effect on oligodendrocytes, endothelial cells, or both. The study of ASM KO mice may be useful for this distinction.

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