

Carbamylated Erythropoietin Reduces Radiosurgically-Induced Brain Injury

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Gamma knife radiosurgery is an attractive noninvasive treatment of brain tumors and vascular malformations that minimizes collateral tissue damage. However, exposure of normal tissue to even low-dose radiation triggers a cascade of acute and chronic injury and potentially significant morbidity and mortality. Because many irradiated patients now survive for years, identifying methods to prevent radiotherapy-induced collateral tissue damage is a major focus of current research. Erythropoietin (EPO), a cytokine produced locally by many tissues in response to injury, antagonizes apoptosis, reduces inflammation, and promotes healing. Systemic administration of recombinant EPO, widely used for treatment of anemia, provides robust protection from numerous insults in a variety of tissues, including the brain. Although irradiation injury is likely sensitive to EPO, the hematopoietic activity of EPO is undesirable in this setting, increasing erythrocyte number and predisposing to thrombosis. To avoid these potential adverse effects, we developed carbamylated EPO (CEPO) which does not stimulate the bone marrow. In this study, we show that CEPO (50 $\mu\text{g kg}^{-1}$ intraperitoneally) improves functional outcome when administered to adult rats just before, and then once daily for 10 d after, a necrotizing dose of radiation (100 Gy) to the right striatum. Immediately following irradiation, use and reflex movements of the contralateral forelimb to vibrissae stimulation were abnormal but rapidly improved in animals receiving CEPO. Moreover, histological examination revealed that the extent of brain necrosis after 90 days was reduced by ~ 50%. These findings further extend the kinds of injury for which administration of a tissue-protective cytokine provides benefit.

Online address: <http://www.molmed.org>

doi: 10.2119/2006-00042.Erbayraktar

INTRODUCTION

Historically, radiation-induced brain injury was thought to exhibit a delayed response (requiring months to years to become evident) as affected cells die. It is now clear, however, that the nervous system responds to radiation in both acute and chronic ways. For example, recent data show that radiation damage is a dynamic process, involving many cell types, similar to other forms of tissue injury (1,2). Moreover, radiation technology that delivers much higher doses in a single treatment (i.e., stereotactic radiosurgery) produces a pronounced acute injury consisting of edema and inflammation and can lead

to the rapid development of necrosis of surrounding normal brain tissue. The presence of a structural lesion may further predispose surrounding tissue to injury by physical distortion, inflammation and the development of fibrosis (2). For example, radiosurgery performed for arteriovenous malformations is associated with an incidence of life-threatening brain necrosis of up to 10% to 20% in treated patients (3,4). Although protracted courses of glucocorticoids and rheological agents such as warfarin, acetylsalicylic acid, and heparin are frequently used as treatment for postradiosurgery injury, these interventions are not primary treatments

and their true efficacy is anecdotal and largely unknown (5-7). Further, incidental damage producing long-term disabilities may occur to important structures such as cranial nerves adjacent to the radiosurgical target, as is frequently seen with the radiosurgical treatment of skull-base tumors such as vestibular schwannomas and meningiomas. With many ongoing improvements in health care, cancer patients and others undergoing radiotherapeutic treatments survive long enough for these effects to become clinically significant and adversely affect quality of life. Therefore, the development of any adjuvant therapy that reduces radiation-related side effects is clearly desirable.

For many years it was unclear how the centrifugal spread of tissue injury away from a damaged region is terminated. Recently, it has been recognized that the cytokine erythropoietin (EPO) is

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Submitted June 12, 2006; accepted for publication July 10, 2006.

produced locally and limits injury in a multifunctional manner by antagonizing the action of proinflammatory cytokines and by preventing apoptosis, reducing edema, maintaining vascular autoregulation, and mobilizing stem cells (reviewed in ref. 8). However, EPO does not appear to directly attenuate necrosis, the primary manner by which radiated lesions are thought to involute following radiosurgical treatment. These characteristics suggest that EPO could limit damage to tissue occurring around the target of a necrotizing dose of radiation, while at the same time not causing radioresistance within the targeted lesion. Because EPO has long been appreciated for its hormonal role in maintaining adequate numbers of erythrocytes—for which recombinant human EPO (rhEPO) is widely used clinically—this molecule has received much interest for use as a general tissue-protective agent (8). The widespread availability of rhEPO has opened up the possibility of exogenous administration for therapy of tissue injuries, including before potential iatrogenic injury, for example, radiosurgery. The beneficial effects of rhEPO have been especially well documented in experimental models of brain and spinal cord injury in which large therapeutic time windows (hours to days) have been documented (8). In preclinical models, however, tissue protection using rhEPO typically requires doses that are higher than those employed in treatment of anemia, especially for nervous tissue. Unfortunately, rhEPO given at a high dose has potential adverse effects, particularly via an interaction between the vascular endothelium and platelets, strongly promoting thrombosis (9). Notably, several cancer clinical trials evaluating rhEPO administration at relatively high doses have encountered serious adverse effects, including increased mortality in the rhEPO treatment arm (10,11). Because radiotherapy is itself well known to be thrombogenic (12), synergistic adverse effects of high-dose rhEPO treatment could negate any beneficial tissue-protective effects.

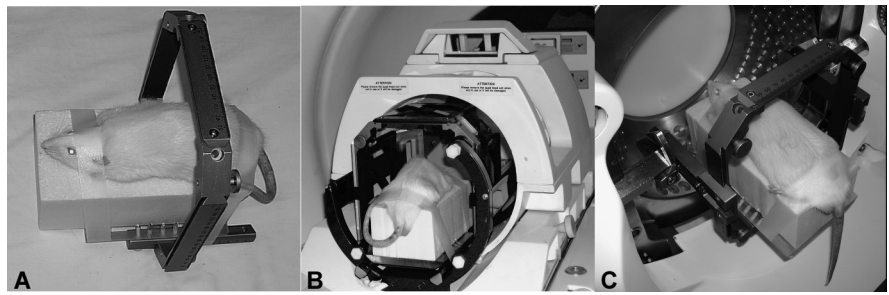


Figure 1. The experimental setup included a modified stereotactic frame to hold and orient the rat (A). This device allowed positioning within the GE Signa MRI scanner (B) and Leskell γ Knife (C) used in this study.

The receptor for EPO expressed by the bone marrow consists of a homodimer [EPOR:EPOR; (EPOR)₂] that until recently was believed to function in both the hematopoietic and tissue-protective pathways. The receptor for EPO expressed by nonhematopoietic tissues [for example, the nervous system (13)], however, possesses a lower binding affinity for EPO than does the hematopoietic receptor and, further, is associated with different proteins, consistent with a distinct receptor isoform. We have recently proposed that the tissue protective receptor is a heteromer comprising the EPOR in association with the common β receptor (β c, also known as CD131) used by the cytokines GM-CSF, IL-3, and IL-5 (14). The binding sites of EPO for the homodimeric receptor are well known, and we have shown how specific chemical or mutational modification of amino acid residues within the binding site produces molecules that do not have affinity for (EPOR)₂, yet are equipotent with EPO for tissue protection (15). For example, carbamylated EPO (CEPO) does not bind to (EPOR)₂ and, therefore, lacks hematopoietic activity yet mediates potent tissue protection through the EPOR: β c receptor (14).

Herein, we demonstrate that CEPO given before and for a short time after necrotizing γ irradiation significantly reduces both the acute behavioral abnormalities and the ultimate extent of necrosis following high-dose radiation injury.

MATERIALS AND METHODS

The animal protocols followed in this study were approved by the Animal Use and Care Committee of the Kenneth S. Warren Institute in accordance with the directives of the Guide for the Care and Use of Laboratory Animals of the National Research Council. Male Sprague-Dawley rats weighing 250 to 275 g were anesthetized using ketamine and immobilized in a MRI-compatible stereotactic device (Figure 1A) as per Kondziolka et al. (16). Noncontrast volumetric imaging using a 1.5 T GE Signa MRI scanner (GE Healthcare Technologies, Waukesha, WI, USA) (Figure 1B) was used to identify a radiosurgical target in the right striatum. A Leskell γ Knife Model B (Elekta, Stockholm, Sweden) and a 4-mm collimator helmet (Figure 1C) were used to stereotactically deliver 100 Gy to the maximal dose point in the right striatum (Figure 2). This dose and site were selected based on a previous study showing that this dose reliably produces necrosis in rat brain by 90 days (16). Before delivery of the γ radiation, the experimental animals were randomized into 2 groups of 6 animals receiving either normal saline or 50 μ g kg⁻¹ intraperitoneal CEPO 24 h before radiation, immediately after, and for an additional 9 days. Researchers were blinded as to which arm of the study the experimental animals were assigned. Follow-up evaluation consisted of 3 neurological tests of motor function performed on the dosing day, day 17, and day 25, as well as histopathological eval-

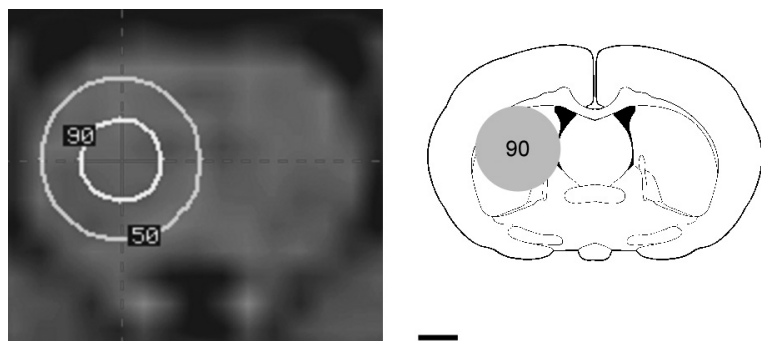


Figure 2. (Left) Concentric circles indicate tissue receiving 90% and 50% of the 100-Gy dose within the targeted region of the right striatum of animal visualized by MRI. (Right) The coronal section indicates the intended target for the 90-Gy dose. Scale bar, 2 mm.

uation of the lesion at 30 days (2 animals each group) and 90 days following injury.

Neurological Testing

Neurological testing included forelimb use asymmetry, vibrissae-elicited limb placement, and forelimb akinesia. To reduce the contribution of learning and adaptation to the behavioral analysis, the animals were habituated to the testing protocols before receiving radiation.

Limb use asymmetry test. Forelimb use in exploratory behavior was assessed according to the protocol of Schallert et al. (17). Briefly, animals were videotaped for 5 min within a transparent cylinder (20 cm diameter, 30 cm height) using a camera capable of single-frame display. A mirror was placed behind the cylinder to permit recording of forelimb movements whenever the animal turned away from the camera. After recording, a single investigator blinded to the treatment evaluated forelimb usage as follows.

(A) A wall exploration score was derived by enumerating: (1) the independent use of the left or right forelimb for initial contact on the wall; (2) use of either forelimb to initiate a weight-shifting movement; (3) use of the left or right forelimb to regain the center of gravity while moving laterally in a vertical posture; and (4) simultaneous use of both forelimbs for contacting the wall or lateral stepping movements along the wall.

(B) A landing score was determined by noting (1) independent use of the left or right forelimb to land after a rearing movement and (2) simultaneous use of both the left and right forelimb for landing after a rearing movement. If the rater could not clearly determine whether a limb was being used independently or simultaneously, that movement was not scored. Additional criteria for exclusion included movements along the ground after landing (stepping) and instances in which an animal performed < 5 landings and < 10 wall movements during a testing session. Additional details for scoring can be found in Schallert et al. (17).

Data analysis of limb asymmetry. Wall exploration and landing scores were determined separately, and each was expressed in terms of (1) the percentage of use of the nonimpaired forelimb relative to the total number of limb-use movements, (2) the percentage of use of the impaired forelimb relative to the total number of limb-use movements, and (3) the percentage of bilateral use of both limbs relative to the total number of limb-use movements. The percentage of use of the impaired forelimb was then subtracted from the percentage of use of the nonimpaired forelimb for exploration and landing. These 2 scores (wall and landing) were averaged together for a single limb-use asymmetry score that corrected for

variability in the number of wall versus landing movements.

Vibrissae-elicited forelimb placement.

Following the protocol of Woodlee et al. (18), animals were held by their torso facing the edge of a table, allowing forelimbs to hang free. Independent testing of each forelimb was induced by gently brushing the respective vibrissae on the edge of a tabletop for a total of 10 trials. In this test, normal animals place the forelimb of both sides quickly onto the countertop. Rats with unilateral damage show varying degrees of impaired limb-placing ability, while still placing the unimpaired limb reliably. The percentage of successful placing responses was determined by counting the number of bilateral limb placements divided by the number of trials.

Forelimb akinesia. Movement initiation for each limb was assessed using a forelimb akinesia test. The hindquarters of the animal were suspended while the animal supported its weight on a forelimb. The animal was allowed to initiate stepping movements in a 10-s period for one forelimb and then the other in a balanced order. Normal animals did not initiate any limb stepping during the 10-s test interval.

Evaluation of brain necrosis

The neuropathological consequences of γ knife irradiation were evaluated at 30 and 90 days after delivery of radiation. The animals were killed by exsanguination under barbiturate anesthesia, and the brains were perfused and fixed with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were then embedded in paraffin, and serial sections were obtained every 5 μ m through the target area and stained with hematoxylin and eosin (H&E). The stained sections were examined microscopically for pathological changes, and the extent of injury was traced using a camera lucida in every 40th section (every 200 μ m) from the beginning of the region of injury by an observer blinded to the experimental group. The images were then digitized, the area of necrosis

was determined by digital planimetry, and the lesion volume was mathematically derived.

Statistical methods

Behavioral data were evaluated by repeated-measures ANOVA. Lesion volume between the groups was assessed using unpaired Student's *t* test.

RESULTS

Both experimental groups successfully underwent delivery of a necrotizing dose of γ irradiation without incident. The day after radiation, animals from both groups exhibited abnormal neurological function, as assessed by the vibrissae and forelimb akinesia tests. These tests, performed serially, also showed large and significant differences between the treatment groups, with a smaller decline and more rapid recovery exhibited by the CEPO group. In contrast, the limb asymmetry test exhibited a significant deterioration much later (> 10 days).

The vibrissae test (Figure 3) exhibited an initial decline from normal for both

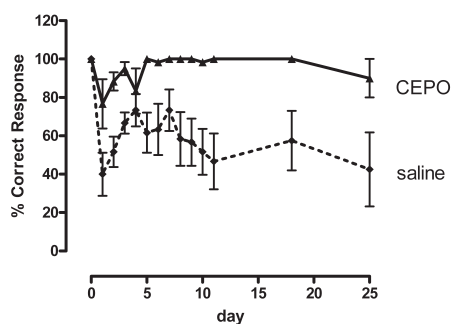


Figure 3. Vibrissae-stimulated limb placing test results indicate that both CEPO- and saline-treated animals exhibited an acute decline in correct responses (both forelimbs extending to table edge after ipsilateral or contralateral vibrissae stimulation) immediately after irradiation. Saline-administered animals recovered only partially, reaching a plateau and then declining. In contrast, this reflex normalized in animals that received CEPO. $P < 0.05$ between groups; 6 animals per group.

experimental groups, but more so for those receiving saline (60% of the time the affected limb was not reflexively extended to the table edge). By the second day, some recovery occurred, reaching a plateau on days 4 to 6 and then falling again after day 7 (Figure 3). Performance on day 25, the last day examined, was at a mean value of only $\sim 50\%$ correct. In contrast, the CEPO group exhibited a transient decrease for 4 days after radiation, with full recovery for the remaining time period (Figure 3).

A similar pattern was observed in the forelimb akinesia test. In this test, recovery was characterized by an acute decline after injury in the saline group, followed by partial nonsustained recovery to day 7, and then a progressive decline through the last data point at day 25 (Figure 4). In contrast, CEPO-treated animals exhibited a smaller acute decline, followed by complete recovery beyond day 5 (Figure 4).

The limb use asymmetry test exhibited a longer latency in onset of abnormal responses (Figure 5). Notably, both groups exhibited a bias for the unaffected limb by day 25, the last time point evaluated. The saline group, however, favored the unaffected forelimb with significantly higher frequency at most time points evaluated.

Thirty days after irradiation, histological examination of H&E-stained sections did not show any discernible differences between the irradiated and nonirradiated basal ganglia (data not shown), even though profound functional deficits were documented. In contrast, 90 days after irradiation, H&E-stained serial brain sections revealed striking differences between treatment groups. As illustrated in Figure 6A, both treatment groups exhibited necrosis within the 90- to 100-Gy target region (inner circle). The group receiving CEPO exhibited extensive tissue salvage within the 50- to 90-Gy region (region between inner and outer circle), distinctly different from the saline group. Upon quantification, the volume of necrotic tissue within the group receiving CEPO was about half that observed

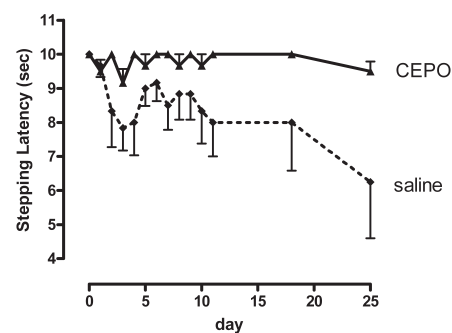


Figure 4. Forelimb akinesia is increased in the saline group, characterized by an acute decline, then partial, nonsustained recovery. The CEPO group differed significantly from the saline group ($P < 0.05$).

in the saline-treated rats (Figure 6B). The periphery surrounding the irradiated region in both treatment groups showed proliferation of reactive glial cells and areas of loose tissue with vacuolation. The central parts of the irradiated region showed varying numbers of necrotic foci with secondary calcification. The necrotic core of saline-treated animals typically contained large masses of calcified granules, in striking contrast to the CEPO group (Figure 6C).

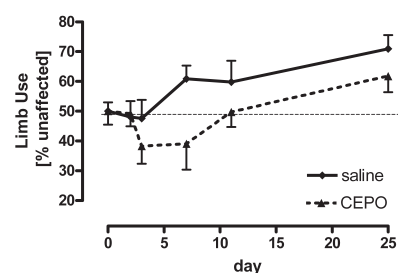


Figure 5. Forelimb use asymmetry test demonstrates a significant difference between saline- and CEPO-treated animals. Saline-treated animals favored the unaffected limb by day 7 after injury. In contrast, CEPO-treated animals deteriorated more slowly, preferentially using the unaffected limb only on the final observation day (25). Points above dashed line indicate favoring the right (unaffected) limb. CEPO significantly differs from saline ($P < 0.05$).

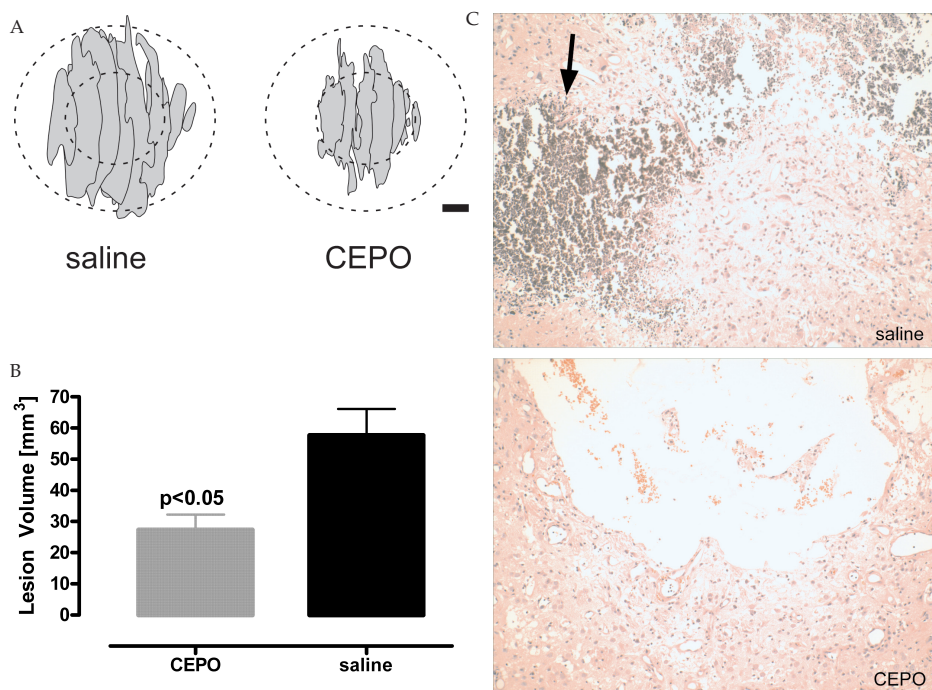


Figure 6. CEPO-treated animals have significantly less necrosis within the targeted brain volume. (A) Lesion size at the center of the necrosis volume in representative animals from the saline and CEPO groups 90 days after the delivery of radiation. Inner dashed circle indicates the delivery of 90 to 100 Gy. Difference between outer and inner circles indicates a region receiving between 50 and 90 Gy. Note that CEPO treatment was associated with a marked reduction in injury within the lower dose range. Distances between slices is 200 μm ; scale bar, 1 mm. (B) Mean volume of necrosis in the CEPO-treated group was half of that observed for the saline treatment group. (C) Representative photomicrographs comparing lesions of the 2 treatment groups. Animals that received saline generally possessed dense calcification (arrow) in contrast to the less affected CEPO group. Original magnification $\times 40$.

DISCUSSION

The observation of more nearly-normal motor function associated with less necrosis at 90 days in the CEPO-treated group supports a potential therapeutic role for nonerythropoietic tissue-protective cytokines in limiting radiation-induced neurological injury. Although the rat brain is well known to be relatively radioresistant compared with that of humans, previous studies have shown that it is a good model for assessing the effects of radiation injury (16,19). However, prior work has focused primarily upon a histological assessment of damage to estimate the severity of injury. Specifically, within hours of a therapeutic dose of γ radiation (6-20 Gy) in a rat model, glia

within the target area became activated and began to proliferate (20). By 14 days, tissue edema was clearly present (16), whereas necrosis of neurons and other cells occurred much later, 30 days or more after injury (20). These effects are directly proportional to the dose employed for a given beam diameter (16,19,20).

In the present study, high-dose radiation (100 Gy) to the striatum produced acute neurological defects as determined by vibrissae and proprioceptive testing of the forelimbs, with a delayed effect on the pattern of use of the forelimbs. Three months after radiation, substantial necrosis was observed in both groups within the 100- to 90-Gy

target region (Figure 6B). The deterioration noted for bilateral limb usage in both groups is presumably secondary to the frank pathological lesion present by 90 days. Of note, a limited histological examination of irradiated brains after 1 month did not show any evidence of injury. Thus the acute behavioral effects following radiation exposure appear to be a sensitive method by which to evaluate the degree of injury in the acute and subacute periods, not requiring the months needed for frank necrosis to occur.

The central nervous system exhibits a large capacity for repair following mild radiation injury [reviewed in Andrantschke et al. (21)]. The mechanisms responsible have not been extensively delineated, but appear to be similar to the underlying responses to other forms of brain injury (1) for which locally produced (endogenous) EPO has been implicated as a key cytoprotective agent.

Stem cell mobilization is thought to be of major importance in the healing phase of radiation-induced injury, and EPO has been shown to recruit stem cells within the brain (22), particularly after ischemic injury (23). However, Fukuda et al. (24) have reported that radiation preferentially damages progenitor cells within the immature rat brain and, further, that this injury could not be attenuated by exogenous rhEPO. This observation is surprising, as neural progenitor cells have been shown to specifically express receptors for EPO and, further, EPO critically modulates apoptosis within this population during brain development (25). Additionally, progenitor cells have been found to generate reactive oxygen species (ROS) and undergo apoptosis after irradiation, cellular responses that can be greatly inhibited by rhEPO in other model systems (8). Notably, Fukuda et al. (24) employed very high doses of rhEPO ($\sim 10,000 \text{ U/kg}^{-1}$ intraperitoneally), which could be problematic, as numerous investigators have shown that EPO protection generally follows an inverted U-shaped curve in vivo and in vitro, in

part potentially owing to ROS generated by high-dose rhEPO (26). On the other hand, caspase inhibition was ineffective in the study by Fukuda et al. (24), suggesting that in this model, injury may be closer to a purely necrotic process that is not sensitive to EPO. Finally, it should be noted that the immature brain is especially sensitive to radiation, which in addition to direct effects on progenitor cells, also alters the microenvironment (27) in ways that potentiate tissue injury. These effects may not occur to the same extent in adult brain.

The clinical presentation of radiation injury can be divided into immediate, subacute, or delayed phenotypes (6). Acute injury is likely mediated by radiation-induced vascular leakiness and the development of vasogenic edema. Potential effects secondary to neuronal dysfunction are certainly possible but have not been specifically demonstrated. One of EPO's prominent tissue-protective effects is on the permeability of the vascular endothelium of the blood-brain barrier. In this role, EPO increases the tightness of the barrier and reduces leakiness in both in vitro (28) and in vivo (29,30) models. Late effects of radiation injury are associated with necrosis, mainly arising from vascular dropout. In a time- and radiation dose-dependent fashion, the endothelium swells, the basement membrane thickens, the microvessels undergo hypertrophy, and ultimately the tissue cellular density diminishes (31). White matter necrosis is especially prominent and depends on oligodendrocyte apoptosis and ischemia-reperfusion injury secondary to perivascular edema (6). The smaller necrosis volume observed in the CEPO group could be explained by a primary effect on protecting the microvascular circulation. If so, the efficacy of CEPO administered for 10 d following injury implies that the evolving pathological processes can be terminated by a brief exposure to tissue-protective cytokines. It is unknown whether other, delayed effects might also respond favorably to longer exposure to CEPO.

The tissue-protective properties of CEPO we have observed in the brain in this report likely also apply for other radiosensitive tissues with microvascular injury as a pathological mechanism. To our knowledge, the only other study evaluating the effects of rhEPO on radiation injury outside of the CNS focused on the adult kidney (21). Curiously, in that study, EPO at 2 doses (500 or 2000 U kg⁻¹) given subcutaneously in 3 doses before and just after irradiation was associated with diminished renal function between 5 and 9 months after exposure. This unexpected finding might be explained by the EPO-mediated acute reduction in renal blood flow that occurs after high doses of EPO (9). The reduced perfusion is similar to that occurring after activation of an organ-specific renin-angiotensin system that has also been hypothesized to play a role in radiation-induced tissue injury [reviewed in Robbins and Diz (32)]. We have recently shown (9) that CEPO, in contrast, increases renal blood flow (both cortical and total). It will be important, therefore, to determine whether lower doses of EPO or use of CEPO will be nephroprotective in the setting of irradiation.

Other interventions with the potential for preservation of normal tissue surrounding the radiated site are currently under investigation and could act synergistically with a tissue-protective cytokine. For example, inhibition of the renin-angiotensin system, growth factors (for example, TGF β), COX-2 inhibitors, and antioxidants, among others (33), have shown some promise, and it will be interesting to evaluate these for possible synergistic effects with tissue-protective cytokines.

The striking improvements in both behavioral and pathological outcomes following short-term CEPO treatment indicate the need for further study. Because both radiotherapy and rhEPO treatment can elicit thrombotic events, we believe that the results observed in this preclinical model foreshadow the promise for nonerythropoietic tissue protection in patients.

ACKNOWLEDGMENTS

Supported in part by the Kenneth S. Warren Institute and Elekta AB to N.d.L. and A.d.L. We thank Dr. Jung Kim for his expert neuropathological assessments, as well as Deborah Diaz and Daniel Gomez for technical support.

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