Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma

Alfredo Gorio*†, Necati Gokmen†‡, Serhat Erbayraktar§, Osman Yilmaz¶, Laura Madaschi*, Cinzia Cichetti*, Anna Maria Di Giulio*, Enver Vardar‖, Anthony Cerami, and Michael Brines**††**

*Laboratory of Pharmacology, Department of Medicine, Surgery and Odontoiatry, Faculty of Medicine, University of Milan, Milan 20142, Italy; ‡Anesthesiology and Reanimation, §Neurosurgery, and ¶Animal Research Center, Dokuz Eylül University School of Medicine, Izmir 35340, Turkey; ‖Department of Pathology, SSK Training Hospital, Izmir, Turkey; and **The Kenneth S. Warren Institute, Kitchawan, NY 10562

Contributed by Anthony Cerami, May 14, 2002

Erythropoietin (EPO) functions as a tissue-protective cytokine in addition to its crucial hormonal role in red cell production. In the brain, for example, EPO and its receptor are locally produced, are modulated by metabolic stressors, and provide neuroprotective and antiinflammatory functions. We have previously shown that recombinant human EPO (rhEPO) administered within the systemic circulation enters the brain and is neuroprotective. At present, it is unknown whether rhEPO can also improve recovery after traumatic injury of the spinal cord. To evaluate whether rhEPO improves functional outcome if administered after cord injury, two rodent models were evaluated. First, a moderate compression of 0.6 N was produced by application of an aneursym clip at level T3 for 1 min. RhEPO (1,000 units per kg of body weight i.p.) administered immediately after release of compression was associated with partial recovery of motor function within 12 h after injury, which was nearly complete by 28 days. In contrast, saline-treated animals exhibited only poor recovery. In the second model used, rhEPO administration (5,000 units per kg of body weight i.p. given once 1 h after injury) also produced a superior recovery of function compared with saline-treated controls after a contusion of 1 N at level T9. In this model of more severe spinal cord injury, secondary inflammation was also markedly attenuated by rhEPO administration and associated with reduced cavitation within the cord. These observations suggest that rhEPO provides early recovery of function, especially after spinal cord compression, as well as longer-latency neuroprotective, antiinflammatory and antiapoptotic functions.

Traumatic spinal cord injury (TSCI) occurs frequently and is devastating for the individual patient and costly to society by requiring substantial long-term health care expenditures. Currently, methylprednisolone administered at high dose within 8 h after injury is the only therapy with any recognized benefit (1), which, unfortunately, is relatively minor. Any new treatment of TSCI that allows for major recovery of function would be a significant advance in clinical care.

Injury of the nervous system provokes a complex cascade of proinflammatory cytokines and other molecules that ultimately result in apoptosis and necrosis of neurons, oligodendrocytes, and endothelial cells (2–4). Recent studies have demonstrated that one general response of the brain to injury is the increased local production of the erythropoietin (EPO) and its receptor (5, 6). These proteins are members of the cytokine type I superfamily that provide beneficial effects including inhibition of apoptosis, reduction of inflammation, modulation of excitability (7–11), and mobilization and proliferation of neuronal stem cells (12). Prior study has shown that recombinant human EPO (rhEPO) administered directly into the brain dramatically reduces hypoxic or ischemic injury and conversely, that neutralization of endogenous EPO amplifies injury (8). We have extended these observations by showing that systemically administered rhEPO is not strictly excluded by the blood–brain barrier, as predicted on size considerations, and effectively prevents cellular injury and inflammation when given after ischemic and mechanical trauma (11).

Although a substantial appreciation for the multiple activities of EPO in the brain has accumulated, relatively little is known about its potential role(s) within the spinal cord. Immunohistochemical analyses of the normal spinal cord document abundant expression of EPO and EPO receptor protein (13, 14), especially by motor neurons and myelinated axons. We have recently used a rabbit model to show that rhEPO acts in spinal cord ischemia as it does within the brain, effectively rescuing neurons from apoptosis when administered intravenously as a bolus injection immediately after restoration of blood flow (14).

Physical injuries to the nervous system produce a secondary inflammatory reaction that tends to expand the ultimate size of the lesion. Much of the devastating motor and sensory paralysis after TSCI occurs because of a delayed and widespread oligodendrocyte apoptosis and demyelination of long spinal tracts (reviewed in ref. 4) In this study, we compare the effects of systemically administered rhEPO on spinal cord injury produced by either application of an aneurysm clip or by contusion. We find that even a single dose of rhEPO given 1 h after injury is associated with early improvement of motor function, leading to a near complete functional recovery at 28 days. In contrast, the control animals remained severely affected throughout the study. The dramatic difference in outcome between the two treatment groups is largely explained by the prevention of oligodendrocyte apoptosis and preservation of the white matter tracts involving the site of injury.

Methods and Materials

Experimental Design. Experimental studies were designed to evaluate acute or subacute beneficial effects on behavioral (motor) assessments related to the systemic administration of rhEPO in the setting of TSCI. In the impact model, we also undertook histopathologically analyses of the damaged spinal cord, especially within the cuneate fasciculus. Compressive injury was produced by transient extradural application of an aneurysm clip that produces a moderately severe injury with a distinct vascular component and relatively little hemorrhage (15). In contrast, an impact (concussive) model was used to mechanically disrupt

Abbreviations: EPO, erythropoietin; rhEPO, recombinant human EPO; TSCI, traumatic spinal cord injury; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling; bw, body weight.

†A.G. and N.G. contributed equally to this work.

††To whom reprint requests should be addressed. E-mail: mbrines@kswi.org.

 www.pnas.org/cgi/doi/10.1073/pnas.142287899

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white and gray matter as well as the microvasculature resulting in significantly more intraparenchymal hemorrhage (16).

Aneursym Clip Model. Wistar rats (female) weighing 180–300 g were used in this study. Animals were housed under standard conditions in the Animal Research Laboratory at Dokuz Eylül University. The study protocol was approved by the Animal Research Committee of Dokuz Eylül University. Animals were maintained in a 12-h light/dark cycle with water and food freely available.

The animals were fasted for 12 h before surgery, humanely restrained and anesthetized with an i.p. injection of thiopental sodium [40 mg/kg of body weight (bw)]. Preoperatively, imipenem (10 mg/kg of bw) was administered intramuscularly for prophylaxis of infection. Rectal temperatures were maintained at 37–38°C throughout the operative procedure by exposing the animal to a heat lamp as needed until they completely recovered from anesthesia. The animals were positioned in the prone position and surgery performed under sterile conditions. After infiltration of the skin (bupivacaine 0.25%), a complete single level (T3) laminectomy was performed through a 2-cm incision with the aid of a dissecting microscope. TSCI was induced by the extradural application of a temporary aneurysm clip exerting a 0.6-N closing force on the spinal cord for 1 min. After removal of the clip, the skin incision was closed and the animals allowed to recover fully from anesthesia and returned to their cages. The rats were monitored continuously with bladder palpation at least twice daily until spontaneous voiding resumed.

Forty eight animals were randomly divided into four groups. Animals in the sham group ($n = 6$) underwent the surgical procedure, but their spinal cords were not clipped. In a control group, animals ($n = 14$) received normal saline (via i.p. injection) immediately after the incision was closed. One of the active treatment groups ($n = 14$) received rhEPO (1,000 units/kg of bw i.p.; Eprex, Cilag, Zug, Switzerland) immediately after the incision was closed. A final treatment group ($n = 14$) received three sucessive daily single doses of 1000 units/kg of bw i.p.

Motor neurological function of the rats was evaluated by using the locomotor rating scale of Basso *et al.* (17, 18). In this scale, animals are assigned a score ranging from 0 (no observable hindlimb movements) to 21 (normal gait). The rats were tested for functional deficits at 1, 12, 24, 48, and 72 h, and then at 1, 2, 3, and 4 weeks after injury by the same examiner who was blind to the treatment each animal had received.

Contusion Model. Adult Sprague–Dawley rats (females) weighing 240–260 g were maintained in the animal facilities under standard housing conditions (22 ± 2°C, 65% humidity, artificial lights from 06.00–20.00 h). A standard dry diet and water were available *ad libitum*. All experimental protocols were approved by the Review Committee of the University of Milan, and met the Italian guidelines for laboratory animals that conform to the European Communities Directive of November 1986 (86/609/EEC).

An impaction device was developed initially at the University of Trieste (UTS) by modification of a small materials testing unit (see *Description of UTS-Impactor* and Fig. 5, which are published as supporting information on the PNAS web site, www.pnas.org). The core of the UTS-Impactor is a 2.3-mm end-diameter stainless steel rod that is precisely driven into the spinal cord with a specified velocity and displacement. To accomplish this, the rod is placed at the desired height over the animal's spinal cord, which is immobilized in a frame, and the impact is monitored by means of a miniaturized piezoelectric dynamometer, present within a section of the impacting rod. The device is linked to a computer that records and manages the data.

Fourteen animals were assigned to each experimental group. No animal died during the 4 weeks of evaluation after TSCI. Animals were anesthetized by inhalation of halothane, and a laminectomy was performed at the T9 vertebral level under aseptic conditions. With the aid of an operating microscope, the rat was placed under the impounding piston positioned 1 mm above the exposed cord and set for a 3-mm excursion. A force of 1 N was applied for 1 s, followed by automatic return of the rod. The extent of piston and spinal cord movements were precisely recorded. The animals were administered buprenorphine (0.03 mg/kg) for pain control before awakening and penicillin G (10,000 units/kg) as an antimicrobial after surgery. After TSCI, rats were housed two per cage and had manual bladder evacuation, if required, three times daily. No urine infections were observed in the study.

Animals received rhEPO (epoietin α; Ortho Biotech, Raritan, NJ) via an i.p. injection one hour after impaction. One group received only a single dose of 5,000 units/kg of bw administered 1 h after injury. A second group received daily injections of 5,000 units/kg of bw for 7 consecutive days. A third group was given 500 units/kg of bw daily for 7 days. A fourth group received saline 1 h after injury. Three scorers assessed independently all outcome measures in a blinded fashion. Neurological function was evaluated 24 h after injury and then twice a week (17, 18), as well as by a swimming test (19). In this assessment, a rat was placed in the center of a round tub of water (40 cm diameter) filled to a depth of 30 cm with a wire mesh ladder attached to the side. The animal was rated 0 when neither hind limb was used for swimming and climbing out, 1 when there was a partial use of the hind limbs, 2 when both hind limbs were used normally.

Half of the animals in each treatment group were killed at 7 days for anatomical studies by use of halothane anesthesia followed by transcardial perfusion with 10% paraformaldehyde in isotonic phosphate buffer saline at pH 7.4. The spinal cord encompassing the injury site was further postfixed with the same paraformaldehyde-containing solution for 3 days, and segments of the spinal cord were then embedded in paraffin and 8-μm sections were cut transversely. Every twentieth section obtained was stained with hematoxylin and eosin. Cross-sections containing the lesion epicenter and the extent of total T9 segment cavitation were analyzed with computer-assisted image analysis (Leica DG 100 mounted on a Zeiss microscope). The percent cavitation was calculated as the area of cavitation divided by the total cross-sectional area at the level of the injury.

Apoptosis of oligodendrocytes within the fasciculus cuneatus was determined after 7 days by using the terminal deoxynucleotidyltransferase-mediated dUTP end labeling (TUNEL) methodology using 10-μm-thick sections obtained at a distance of 2.5 mm rostrally to the center of the impact site. Briefly, sections were deparifinized and then treated with proteinase-K (20 μg/ml in 10 mM Tris·Cl, pH 7.6, for 15 min at room temperature), blocked in 3% H_2O_2 in methanol for 10 min, permeabilized for 2 min in 0.1% Triton X-100/sodium citrate at 4°C, and treated with TUNEL reaction mixture according to the manufacturer's protocol (In Situ Cell Death Detection kit, Roche Diagnostics). Positive neurons were identified after development for 15 min in diaminobenzidine, dehydration, and application of cover slips. Terminal transferase was omitted as a negative control. Sections obtained from treated and control animals were examined by using light microscopy, and the total number of TUNEL-positive cells was determined by an observer blinded to the treatment.

Data are expressed as the mean ± SD of at least 6 measurements. Multiple group comparisons of the differences in quantitative measurements were made by ANOVA followed by Dunnett's *t* test. Statistical significance was accepted at $P < 0.05$.

Results

Aneurysm Clip Model. Animals receiving saline injections immediately after aneurysm clip removal suffered a flaccid paraplegia for the first 3 days after injury (mean motor score of 1.2), but

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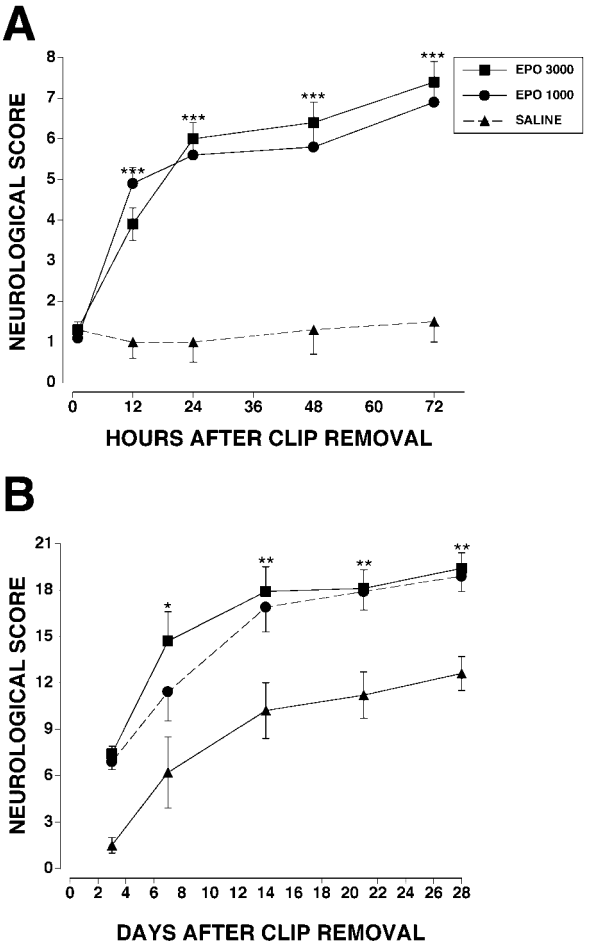

Fig. 1. (*A*) Neurological (motor) scores of animals assessed over 72 h after removal of aneurysm clip in open field testing show significant improvement earlier for animals receiving either a single (1,000 units/kg bw i.p.) or 3 doses of rhEPO (3,000 units/kg of bw total; $n = 14$ each group). (*B*) Neurological scores of animals assessed for 1 month after removal of an aneurysm clip show that animals that received rhEPO exhibited nearly normal motor function. In contrast, saline-treated animals regained much less function ($n = 7$ each group; *, $P < 0.05$; **, $P < 0\ 01$, ***, $P < 0.001$).

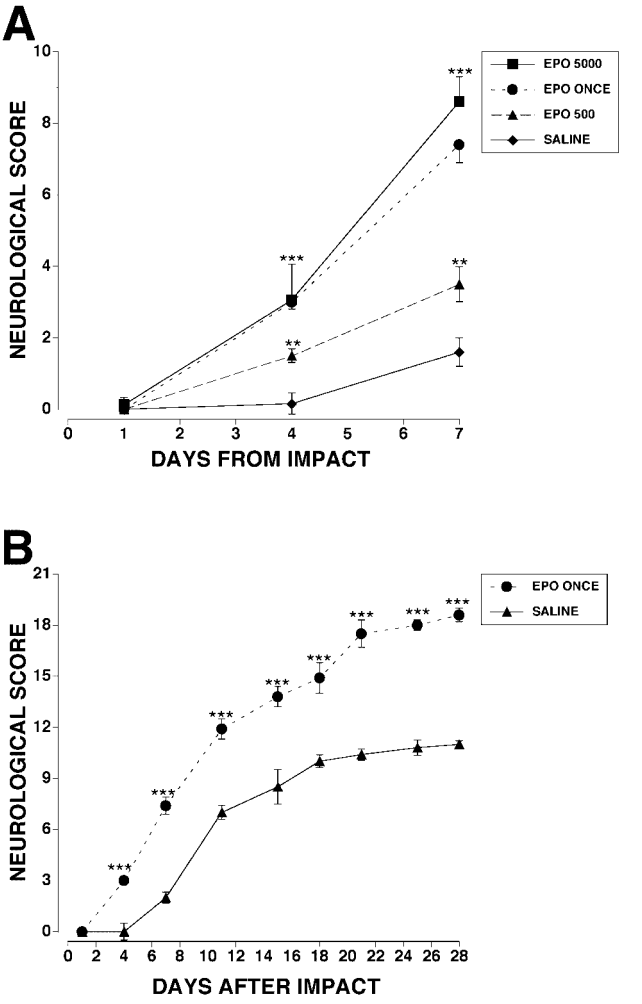

Fig. 2. A. Motor scores of animals assessed for 1 week show that rhEPO given as 5,000 units/kg of bw i.p. once was as effective as 7 doses of rhEPO. Significant recovery of neurological function was observed by 4 days in the rhEPO groups. A dose of 500 units/kg of bw was intermediate in effectiveness. (*B*) Recovery of neurological function steadily continued over 28 days for animals given a single dose of rhEPO (5,000 units/kg of bw i.p.), such that nearly full recovery was obtained. In contrast, saline-treated animals did not materially improve after 14 days following injury. ($n = 6$ each group; **, $P < 0.01$; ***, $P < 0.001$).

recovered some function (mean motor score of 6; slight movement of one or two joints) by day 7 (Fig. 1). In contradistinction, animals receiving either a single dose of rhEPO (1,000 units/kg of bw) or three daily doses of rhEPO followed a superior clinical course despite an equivalent clinical score 1 h after clip removal. RhEPO-treated animals exhibited improvement of motor function by 12 h (mean motor scores of 4.8 and 3.8, respectively, corresponding to movement at all three joints of the hindlimb, $P < 0.001$ compared with control). At 7 days after injury, the rhEPO-treated animals improved greatly, which was only slightly less for the 1,000 units dose compared with the 3,000 units dose (mean motor scores of 11 versus 14 respectively; $P < 0.05$). Clinical recovery continued over the successive observation times. At the termination of the study at 28 days, the final motor scores were quite poor (10) for the saline group, but near normal (18) for both rhEPO-treated groups ($P < 0.001$). As expected, sham-operated animals did not exhibit motor deficits ($n = 6$; score of 21; data not shown).

Contusion Model. Like the aneurysm clip model, all animals subjected to spinal cord contusion were profoundly affected immediately after injury, as assessed by the open field testing (Fig. 2; mean motor score of 0 for all groups). In contrast to the aneurysm clip model, significant recovery was not evident in any group until the 4th day after injury. Between the 4th and 12th postoperative day, rhEPO-treated animals exhibited a marked improvement in motor score that was maintained throughout the entire observational period. Equivalent recovery was observed in the groups receiving a single dose of rhEPO 1 h after injury or multiple doses of rhEPO for 7 days. The group receiving 500 units/kg of bw recovered function to a degree intermediate between the high-dose rhEPO and saline. Motor evaluation obtained by the swim test provided similar results with rhEPO (5,000 units/kg of bw $\times$ 7 days) exhibiting some recovery by 4 days (Fig. 3). In contrast to the open field testing, however, a dose of 500 units of rhEPO administered daily for 7 days was no better than saline treatment.

rhEPO treatment daily for 1 week was also associated with a significant reduction in frank damage to the spinal cord at the lesion epicenter as assessed by the reduction of cavitation volume by about 25% compared with control throughout the

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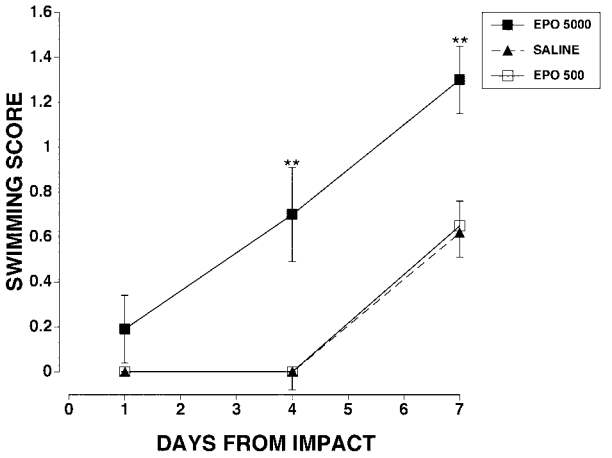

Fig. 3. Motor function as assessed by swimming demonstrated improvement by 4 days after injury for rhEPO at high dose (5,000 units/kg of bw i.p. × 7 days). In contrast, a lower dose of rhEPO (500 units/kg of bw i.p.) was no more effective than saline. ($n = 12$ each group; **, $P < 0.01$).

spinal cord at T9 (Fig. 4 and Table 1; $P < 0.01$). Notably, the sparing could be accounted for by a remarkable preservation of white matter at the light microscopy level. Myelinated axons appeared histologically normal in rhEPO-treated animals, whereas in animals receiving saline, widespread degeneration and swollen myelin sheaths were observed. TUNEL labeling in the fasciculus cuneatus 2.5 mm rostral to the lesion epicenter revealed a mean number of TUNEL-positive cells of 12 ± 3 for saline-treated animals, whereas for animals given rhEPO (5,000 units/kg of bw × 7 days), no TUNEL-positive cells were observed ($n = 12$ each group; $P < 0.001$). Qualitatively, infiltration by inflammatory cells also appeared greatly reduced in animals receiving rhEPO therapy.

Discussion

The results of these experiments demonstrate a major neurological benefit associated with the systemic administration of rhEPO after TSCI produced either by transient compression or blunt trauma. In both models evaluated, a single dose of rhEPO was associated with a markedly superior clinical course of recovery of motor function compared with placebo, characterized by an earlier and more complete normalization of function over a 28-day period of study. Further, injury produced by the aneurysm clip improved significantly within 12 h after injury, a time at which saline-treated animals remained completely paralyzed. After compressive TSCI, a single dose of rhEPO was associated with the same excellent outcome as three rhEPO doses given on successive days. Data obtained from both models suggest that much of the beneficial effect of rhEPO treatment occurs within the first week after injury, as characterized by an earlier recovery of motor function after injury. Thereafter, neurological recovery occurred at the same rate in both saline- and rhEPO-treated animals, despite the dramatic differences observed at the histological level after 7 days.

The observed differences in temporal responses to rhEPO between the models could be explained by the magnitude of delayed injury. Specifically, the aneurysm clip model produces a compressive/occlusive injury characterized by transient vascular occlusion and pressure-related block of axonal conduction, but often with minimal disruption of myelin. Restoration of axonal conduction is likely to occur sooner for an axon possessing an intact myelin sheath. In contrast, the impactor model, being primarily mechanical in nature, differentially injures large myelinated fibers, with the most severe damage occurring at the

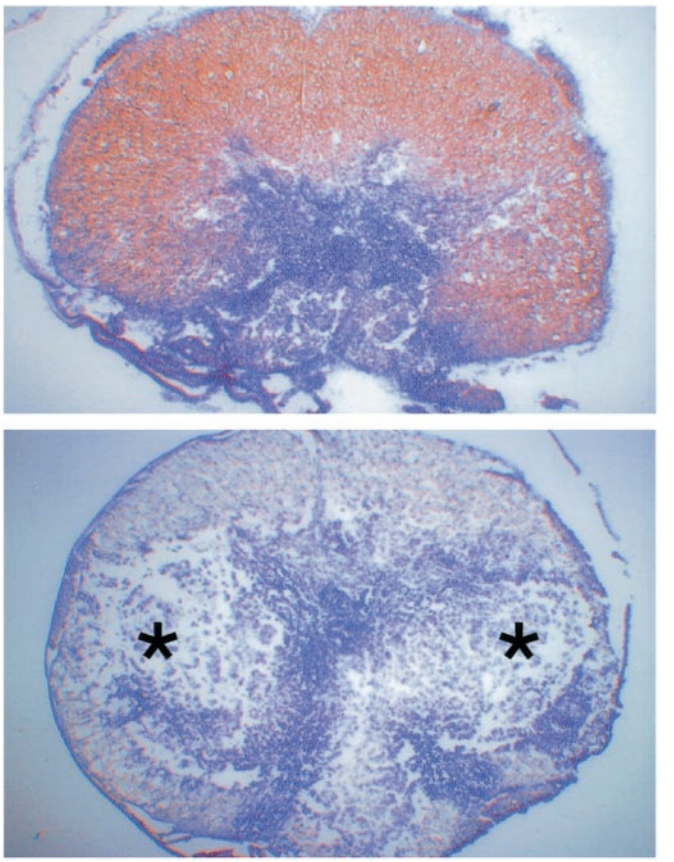

Fig. 4. Representative hemotoxylin-and-eosin-stained rat spinal cord sections obtained at the lesion epicenter 7 days after injury. (*Lower*) Saline-treated. (*Upper*) rhEPO-treated (5,000 units/kg bw i.p. daily for 7 days). Extensive white matter preservation can be observed in the rhEPO-treated group. In contrast, cavitation (asterisks) occupied the central cord of saline-treated animals, surrounded by swollen and fragmented axons. Numerous inflammatory cells were also present throughout (not seen at this magnification). Cavitation volume was reduced in the rhEPO-treated animals by ≈25%. Many TUNEL-positive nuclei corresponding to oligodendrocytes were observed in the fasciculus cuneatus only in the saline-treated animals (see text).

nodes of Ranvier, and tends to spare smaller axons (20). Additionally, significant hemorrhage occurs from the severe parenchymal disruption caused by absorption of kinetic energy. Therefore, in the later stages of response to injury caused by contusion, cellular debris and grossly disrupted axons elicit pronounced inflammatory and degenerative processes that initiate a secondary phase of injury.

The very early recovery observed in the aneurysm clip model could depend on a beneficial effect of rhEPO on the restoration of adequate blood flow after injury. After experimental TSCI, a profound reduction in spinal cord blood flow occurs, which progressively worsens (21) and may last for 24 h (22). In a recent study assessing the effects of EPO on the clinical course after subarachnoid hemorrhage in a rabbit model, rhEPO treatment dramatically attenuated the intense intracerebral arterial spasm

Table 1. Percentage of spared tissue

Treatment	Lesion epicenter	Throughout T9
Saline	41.0 ± 3.7	50.4 ± 3.1
rhEPO	52.1 ± 2.6**	59.6 ± 3.5**

**, $P < 0.01$ compared to saline; $n = 12$ each condition.

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secondary to the irritative effects of blood infused into the subarachnoid space (23). Further, a single dose of rhEPO given peripherally has been shown to preserve autoregulation of cerebral blood flow (24). Thus, rhEPO both actively reduces the cerebral ischemia after hemorrhage by maintaining tissue perfusion, and directly provides neuroprotection for metabolically stressed neurons. The mechanism of this vascular effect has not been directly evaluated, but the potent vascular effects of rhEPO noted within the systemic circulation appears to depend on the modulation of inducible nitric oxide synthase activity (25). In addition to the constrictive effects of compression on circulation within the spinal cord, injury-related neurological dysfunction itself typically produces severe hypotension and bradycardia in both humans (4) and animals (26), further worsening the effects of neuronal ischemia. Nitric oxide has also been implicated in the cardiovascular alterations that occur immediately after spinal cord injury (27). Because one mechanism explaining the neuroprotective effect of rhEPO has been shown to depend on inhibition of nitric oxide production (28), it is reasonable to hypothesize that similar mechanisms may be relevant within the spinal cord.

In the contusion model, the early recovery observed in the rhEPO-treated groups could arise from a primary effect of EPO through prevention of cell death, increasing the rate of recovery, or both. Further studies will be needed to distinguish between these possibilities. Interestingly, a 500 units/kg of bw dose of rhEPO appeared to be of intermediate effectiveness in open field testing but was ineffective in the swim test. This finding differs from our observations obtained from a 3-vessel reversible middle artery cerebral ischemia model in the rat for which 500 units/kg of bw was as effective as higher doses of rhEPO, but lower dosages were ineffective (11). One difference between these studies may be a decreased rate of absorption of rhEPO from the i.p. injection site and mobilization into the injured region of the spinal cord as a result of the profound hemodynamic changes that occur in TSCI.

Secondary injury contributes significantly to spinal cord pathology after TSCI, especially after contusion (29). Results of the histological examination of the spinal cord 7 days after contusion showed a dramatic reduction in the volume of cavitation associated with rhEPO treatment. This decrease was associated with an obvious reduction in the number of inflammatory cells in and around the region of injury. We have previously observed a similar effect in a mouse model of contusive brain injury (11). Interestingly, the relatively small reduction in cavitation volume measured ($\approx$25%) is nonetheless associated with a very large difference in neurological function. Motor score readouts are more sensitive than histological assessment in this model, as white matter may not appear greatly damaged but still may not support transmission of afferent and efferent electrical activity. The presence of apoptotic oligodendrocytes as determined by TUNEL labeling within the region of the fasciculus cuneatus are supportive of this concept. Specifically, no apoptotic nuclei were observed in this region in the rhEPO-treatment group, whereas multiple apoptotic nuclei morphologically consistent with oligodendroglia were observed in the spinal cords of the saline-treated group. Methylpredinisolone and ganglioside GM1, two agents that are partially effective in the contusion model, do not reduce the infiltration of neutrophils immediately after spinal cord injury (30). Inflammatory cells are involved in the late damage that occurs to the oligodendrocytes that provide the myelin for axons within the spinal cord (31). rhEPO appears to reduce the inflammatory infiltrate, and in this manner likely reduces the contribution of late injury to the neurological deficit.

One way the rhEPO could affect inflammation is through modulation of members of the nuclear factor (NF)-κB family that are principal regulators of inflammatory genes (32, 33). Previous work has shown that NFκB itself is strongly upregulated after TSCI, produced by macrophages/microglia, endothelial cells, and neurons (29). One NFκB-dependent gene product is inducible nitric oxide synthase, which has been shown to increase the first day after injury, and peaks by day 7 (34). Recently, EPO has been shown to signal through the NFκB pathway (35) as well as by the janus kinase-2/signal transducers and activators of transcription-5 system (36–38). Further study will be necessary to determine whether the prominent effects on secondary injury depends in part on regulation of NFκB.

To date, the only pharmacotherapeutic with demonstrated effectiveness in TSCI is methylprednisolone. This agent must be given within the first 8 h after spinal cord injury. However, the beneficial effect is frequently only moderate even in animal models (39). In contrast, dramatic and significant effects of rhEPO systemically administered immediately after injury on neurological outcome suggests that this well-tolerated agent may provide a large therapeutic benefit in the setting of TSCI arising from either compression or contusion. The observation that a single dose of rhEPO after injury is highly efficacious suggests that this agent or its analogues may be an ideal immediate treatment after acute injury. Further study will be needed to determine how extensive the time window for effective treatment is and whether there are restorative benefits of rhEPO administered in the setting of chronic spinal cord injury. The outstanding safety record of the use of rhEPO for the treatment of anemia should encourage an early evaluation of the use of this agent in the setting of TSCI.

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