Erythropoietin as a Tissue-Protective Cytokine in Brain Injury: What Do We Know and Where Do We Go?

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In the 10 years since neurotrophic activity was first reported for erythropoietin (EPO), a broad understanding of its multiple paracrine/autocrine functions has emerged. Recent studies firmly establish EPO as a multifunctional molecule, typical of the pliotrophic cytokine superfamily of which it is a member. The realization that EPO activates neuroprotection by multiple mechanisms has identified a generalized system of local tissue protection with EPO as a critical component. Here, the authors characterize the biology of the local tissue-protective system, review data that support this concept, and suggest why nonhematopoietic analogues of EPO may be better choices as therapeutics. NEUROSCIENTIST 10(2): 93–98, 2004. DOI: 10.1177/1073858403259187

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Many diseases lacking effective treatments are triggered by focal tissue injury complicated by reactionary biological responses such as inflammation. These mechanisms are ancient in an evolutionary sense and evolved as an "innate immune response" to infection (reviewed by Rivest 2003). The initial response to the presence of microbes consists of vigorous reaction orchestrated by an influx of inflammatory cells to sterilize the immediate microenvironment and wall-off infectious agents. In the process, normal cells adjacent to the microbial nidus are killed, ensuring a more complete containment. The components of the innate response system are also activated in focal noninfectious injuries, such as ischemia. However, unlike the situation of infection, creation of a dead zone by apoptosis is biologically inappropriate.

It has become evident over the past decade that in addition to the inflammatory-tissue-damaging component of this innate response to injury, a counteracting arm simultaneously limits the centrifugal spread of injury and prevents generalization. Recent work has shown that erythropoietin (EPO) is a major component of this tissue-protective system. An understanding of the biology of this system provides a rationale for using EPO or its nonhematopoietic analogues currently under development as tissue-protective therapeutics.

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Regulation of the EPO Tissue-Protective System

For EPO to function in a paracrine-autocrine (localized) system, tissues must express both the EPO receptor (EPOR) as well as EPO, and regulation of these proteins must occur independently of the circulating (hormonal) system. In the normal adult brain, both EPO and EPOR are not highly expressed in most regions but are rapidly inducible by metabolic stress, for example, ischemia (Chin and others 2000). The character of this response is clearly shown by pathological examination of ischemic brain using immunohistochemical methodology. These studies show that a marked increase in neuronal EPOR occurs first (< 12 hours) around the lesion, followed later by EPO expression by glia and the vascular endothelium (Bernaudin and others 1999).

EPO is one of the genes induced by activation of hypoxia-inducible factor-1 and thus by hypoxia in the brain (Zaman and others 1999). However, a variety of other stressors, for example, hypoglycemia, also has been shown to up-regulate EPO. The common theme is generation of reactive oxygen species (Chandel and others 2000). Although EPO can up-regulate its own receptor in hematopoietic cells (Ogilvie and others 2000), EPO is not the primary inducer in the brain as it lags EPOR expression. Important inducers of brain EPOR are proinflammatory cytokines (e.g. TNFα), which markedly up-regulate expression by neurons.

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EPO and EPOR Mediate the Phenomenon of Preconditioning

The latency of the changes of the EPO tissue-protective system following ischemia could explain the phenomenon of "delayed preconditioning" in which mild, noninjurious stress increases resistance to a subsequent toxic stress (reviewed by Dawson 2002). This has been recently confirmed for the brain by several groups (Ruscher and others 2002; Prass and others 2003), requiring ~24 hours to become significant and lasting for ~72 hours. The importance of the tissue-protective system for preconditioning can be confirmed by antagonizing the action of EPO (e.g., by addition of soluble EPOR that complexes and neutralizes EPO), which abolishes preconditioning.

For many injuries, a variable window of time exists over which the tissue damage gradually becomes permanent. In the case of ischemic stroke, for example, a penumbra of neurons at risk for undergoing programmed cell death surrounds the dead and dying core of infarction. This relic of the response to infectious agents conceptually corresponds to a planned backfire burn to contain a forest fire—available fuel to feed the fire (inflammatory antigens) is eliminated. The apoptosis that is triggered greatly diminishes the subsequent inflammatory response but does not eliminate it completely as microglia and other inflammatory cells respond to specific cell surface signals (e.g., phosphatidyl serine) and are mobilized to remove the dead cells (reviewed in Savill and others 1993; Henson and others 2001).

Although the volume of the penumbra can be reduced by EPO, two factors act to reduce the probability that sufficient endogenous EPO reaches cells at risk before they become irreversibly committed to apoptosis. First, the long latency for EPO production (hours) implies that EPO may be produced too late for optimal tissue protection. Second, proinflammatory cytokines directly inhibit EPO production (Nagai and others 2001). Thus, although EPOR-expressing cells surround the lesion, little EPO is available and the cells die (Fig. 1). Administration of exogenous recombinant human EPO (rhEPO) circumvents these two problems, penetrating the penumbra deeply within a short period of time. Local administration into the brain obviously presents a clinical problem in general. Fortunately, rhEPO can be administered systemically with the same effects (see below).

Abundant data have accumulated confirming this concept. One prediction is that the character of the penumbra—for example, whether it is large or small, static or changing with time—will determine the degree to which and the time during which rhEPO will be effective. For example, for models of experimental stroke with reperfusion characterized by a large penumbra, rhEPO markedly reduces infarct (by 50%–75%) (Brines and others 2000). In contrast, permanent occlusion models may have virtually no penumbra, for which rhEPO is minimally effective (unpublished observations).

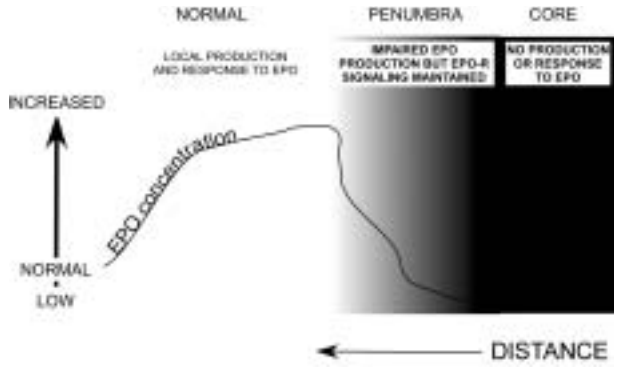

Fig. 1. Proposed mechanism by which lesion extension is minimized. Simplified illustration of endogenous erythropoietin (EPO) concentration as a function of distance from a lesion a few hours old. Within the core (right), cells are undergoing necrosis and neither make nor respond to EPO. Within the penumbra (middle), many cells are still viable but may be triggered for apoptosis. Although these express the EPO receptor and are capable of response (and rescue), nearby endogenous EPO production is impaired. The high EPO concentration within tissue at a distance from the lesion (left) protects cells within this region from undergoing apoptosis and thus limits the extent of the lesion. Use of exogenous EPO increases the local concentrations near the necrotic core, rescuing cells.

With these principles in mind, one would predict that spinal cord injury is particularly suited for treatment with EPO. Specifically, although the trauma that occurs at one site within the cord may kill many resident neurons and glia outright, the initial clinical severity is dictated primarily by the involvement of fibers passing in the vicinity of the lesion. This damage does not necessarily kill the affected neurons immediately, which may remain viable for days. Notably, in a rodent contusion model, injury finally becomes permanent only 8 to 14 days later when oligodendrocytes die (Liu and others 1997). In other words, the penumbra spreads out along the spinal cord away from the lesion epicenter as a function of time, effectively widening the therapeutic time window. Therefore, even very late treatment by rhEPO in spinal cord injury (e.g., 24 hours) is almost as effective as treatment given at the time of injury (Fig. 2).

How Does Exogenous EPO Reach Injured Tissues?

EPO is a large molecule (~34 kD) that is extensively glycosylated and therefore possesses a long-circulating half-life. Organs with tight endothelial barriers (e.g., the brain) generally exclude such molecules. Although the blood-brain barrier may break down in the setting of injury, permeability develops with delays of a few hours, occurring long after neuronal injury has occurred (Albayrak and others 1997). Systemically administered rhEPO appears within the CSF of the rat, sheep, and monkey with a delay of only about 60 minutes at concentrations known to be neuroprotective in vitro

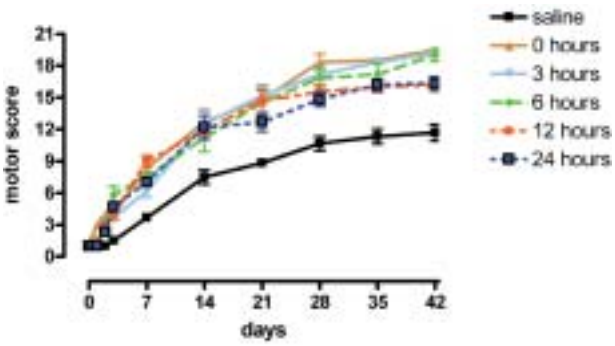

Fig. 2. The therapeutic time window for recombinant human erythropoietin (rhEPO) in experimental spinal cord injury is very wide. Spinal cords of adult rats were compressed for 1 minute with an aneurysm clip at level T3, administered rhEPO (1000 U/kg-bw) at the times indicated as a single intraperitoneal dose, and followed for 6 weeks. All rhEPO-treated animals are different from saline at the $P < 0.001$ level at the end of the observation period (n = 6 animals each group, repeated measures ANOVA). Error bars are SEM. Motor score 0 = complete paralysis, 21 = normal. Previously unpublished data. Methodology as described in Erbayraktar and others (2003).

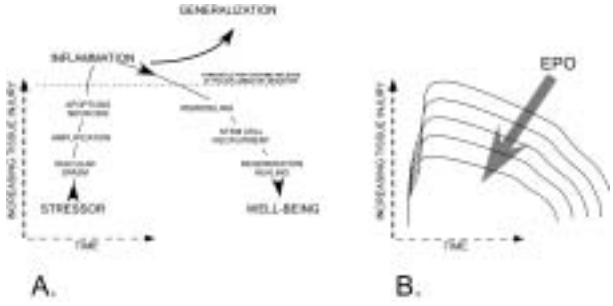

Fig. 3. Multiple mechanisms and temporal domains over which erythropoietin (EPO) acts to preserve tissues following injury. *A*, Beginning with a stress-induced injury (e.g., ischemia), a cascade of injury develops. Using animal models, recombinant human EPO (rhEPO) has been demonstrated to have effects at all levels listed. If a sufficiently large lesion develops, inflammation can generalize. *B*, The effect of exposure to rhEPO is a reduction of peak of injury, preventing generalization and leading to faster recovery times.

(reviewed in Juul 2002). The mechanism is consistent with receptor-mediated transcytosis, as has been established for a number of other proteins, including transferrin, insulin, and leptin. In this process, capillary endothelial cells express the EPOR, and on the level of the electron microscope, EPOR-positive pits and vesicles are observed within the endothelial cells. On the abluminal side, astrocytes highly positive for the EPOR completely envelop the capillary cells. rhEPO labeled with biotin and injected parenterally can be observed to be localized outside the capillary in the brain a few hours after administration (Brines and others 2000).

Mechanisms Underlying EPO's Tissue-Protective Activities

Mechanistically, EPO acts at multiple levels and over many time domains, as illustrated in Figure 3. As one example, experimental subarachnoid hemorrhage is characterized by a loss of vascular autoregulation, leading to symptomatic cerebral vasospasm and consequent brain ischemia in 70% and 36% of patients, respectively. The resulting ischemia then leads to unregulated release of excitatory neurotransmitters, amplifying injury. Apoptosis/necrosis then supervene. A single dose of rhEPO has been shown to be very effective in reversing this abnormality in a rabbit model, almost completely eliminating residual neurological deficits (Grasso and others 2002). Furthermore, we definitively investigated the ability of exogenous rhEPO to exert a vascular effect and, in particular, to counteract the spastic response of the cerebral arteries to extravasated blood. By morphometric analyses of the basilar artery, we observed that rhEPO administration significantly reduced the vasospasm in treated animals.

The Protective Effects of EPO Are Not Limited to the Nervous System

The paradigm developed above suggests that EPO may also provide protection in other tissues. We have evaluated the efficacy of rhEPO in experimental myocardial infarction. Contrary to the long-standing view that cell death in myocardial infarction occurs via necrosis, recent studies have established that much of the injury actually occurs via apoptosis (Nadal-Ginard and others 2003). We have shown that primary myocardiocytes maintained in vitro are protected from hypoxia-induced apoptosis by rhEPO (Calvillo and others 2003). Furthermore, in a rat model with 30 minutes of occlusion of the left main coronary artery followed by reperfusion, myocardial cell loss is greatly attenuated by the administration of rhEPO (beginning at reperfusion) when evaluated after 7 days. As predicted by these findings, normal cardiac function was maintained in the rhEPO-treated animals as assessed invasively at study termination.

Is EPO the Right Molecule?

Ordinarily, the hematopoietic activity of EPO is distinct from its tissue-protective roles because of the large differences in concentrations required for each function, and the separate tissue compartments limit the possibility of cross-talk. For example, the affinity of EPO for the EPOR expressed by hematopoietic cells is ~100- to ~1000-fold higher than for the receptor expressed by neural cells (Masuda and others 1993). All animal models studied thus far have required high doses of rhEPO for tissue protection, above those conventionally used

for the treatment of anemia, which will activate hematopoiesis. Hematopoietic activity is undesirable in the setting of injury because increases in hematocrit (rheological abnormalities) and prothrombotic activities act in concert to reduce effective tissue perfusion. For example, an animal model with increased expression of endogenous EPO exhibits increased cerebral infarct size following arterial occlusion in spite of high levels of EPO within the brain (Wiessner and others 2001). In addition to adverse effects at the level of injury, serious systemic complications are possible, as, for example, the well-publicized fatal outcomes observed following "blood doping" by athletes (Fisher 2003). Furthermore, new data reveal that many malignancies express receptors for and respond to EPO by an increased mitotic rate (Acs and others 2001). It is particularly worrisome that a large clinical trial evaluating the use of EPO in patients with metastatic breast cancer was recently halted after an increase in mortality within the EPO treatment arm due to tumor progression and/or thrombotic events (Leyland-Jones 2003).

Development of a molecule that is devoid of hematopoietic activity but is still active as a tissue protectant is theoretically possible. Although only a single gene for the EPOR has been described, EPORs obtained from tissues differ in molecular weights and affinity for EPO (Masuda and others 1993; Yamaji and others 1996). Likewise, receptor-signaling pathways have not been completely defined but include both the Jak2 STAT5 system used in erythrocyte maturation (Siren and others 2001) as well as the NFkB system so important for cell survival (Digicaylioglu and Lipton 2001). It is unclear at present whether different signaling themes are expressed by different tissues. In spite of current limitations of knowledge, promising work to define new non-EPO analogues is currently in advanced stages of development in our laboratories.

One alternative strategy to differentiate the hematopoietic and tissue-protective activities of EPO is based on the observation that neuroprotection by EPO occurs via a gene expression program requiring only 5 minutes of exposure (Morishita and others 1997). In contrast, during hematopoiesis, a continuous population of new cells appears, which require a continued presence of EPO. Thus, EPO with a reduced half-life will preferentially target tissue injury rather than the bone marrow. In fact, it is straightforward to reduce the serum half-life of EPO (to < 2 min) by removing the sialic acid moieties terminating the oligosaccharides of the molecule. We have produced asialoEPO by use of sialidase, and as previously known, this molecule is not erythropoietic in vivo even up to doses of 500 ug/kg body weight in mice (equivalent to 50,000 erythropoietic units) (Erbayraktar and others 2003). In models of nervous system injury, however, including stroke, spinal cord compression, and peripheral nerve crush, asialorhEPO is as effective as rhEPO itself (Fig. 4).

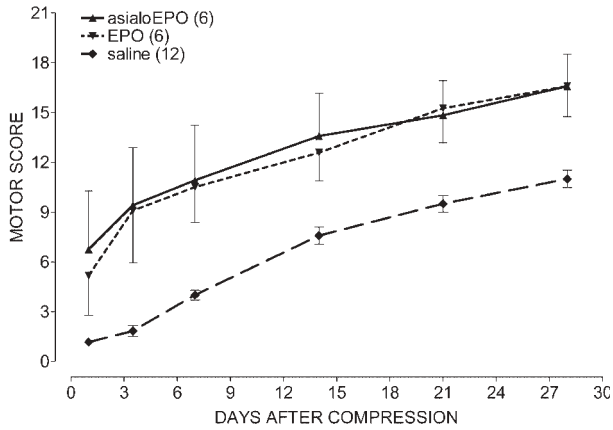

Fig. 4. Asialoerythropoietin (asialoEPO) is as effective as recombinant human EPO in treatment of experimental spinal cord injury. Three daily intravenous doses (10 ug/kg-bw), then biweekly beginning immediately following a 1-minute compression of the spinal cord at level T3 with a vascular aneurysm clip. Numbers in parentheses indicate animals used. Error bars SEM. Reproduced with permission from Erbayraktar and others (2003).

Will Successful Preclinical Studies Using Tissue-Protective Cytokines Translate Well into Human Studies?

Many neuroprotective therapies with excellent preclinical efficacy have failed in human trials for a variety of reasons, including species' differences or unacceptable side effects. The same fate may not befall EPO analogues as these molecules target an endogenous tissue-protective system with multiple targets. Encouraging results have been obtained in the first human trial using rhEPO to treat ischemic stroke (Ehrenreich and others 2002). In this study, patients with MRI-confirmed non-hemorrhagic lesions within the distribution of the middle cerebral artery were enrolled and treated within 8 hours of onset of symptoms (mean of ~5 hours). Patients were well matched for other treatments, for example, use of anticoagulation. Patients in the rhEPO arm received a total of 100,000 units intravenously in three daily divided doses. In spite of the small number of patients enrolled, those who received rhEPO improved earlier in the stroke scores and had significantly better functional outcomes at termination of the study at 30 days (Fig. 5). Whether these improvements are maintained after longer follow-up will be answered by additional clinical trials currently underway.

Conclusions and Prospects

Identification of a local tissue response tempering the biological reaction to metabolic stress and the realization that this system explains the powerful protective mechanism of delayed preconditioning suggest a new therapeutic target to limit tissue injury. By systemic adminis-

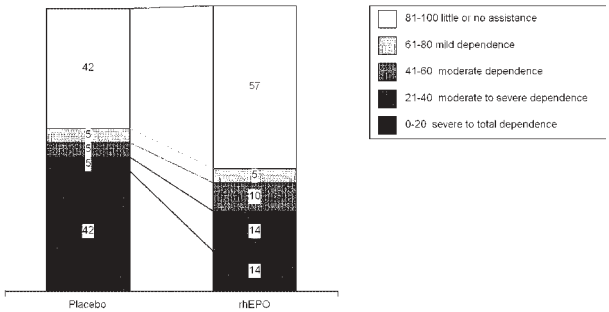

Fig. 5. Clinical outcome (Barthel's Index) in a phase II human stroke trial illustrates that patients who received recombinant human erythropoietin (rhEPO) (100,000 units in three divided doses over 3 days intravenously) exhibited significantly less disability 30 days following an ischemic stroke in the territory of the middle cerebral artery ($P < 0.05$ between the groups). Reproduced with permission from Ehrenreich and others (2002).

tration of a paracrine/autocrine concentration of rhEPO (> ~5 times the hematopoietic dose), multiple preclinical models have shown impressively positive outcomes. The multiple mechanisms by which EPO is active, as well as the successful phase II trial in human stroke, suggest that the use of rhEPO will likely translate successfully into human trials. However, the hematopoietic activities of rhEPO could lead to serious hematopoietic and neoplastic complications, particularly following multiple doses. The nonhematopoietic analogues that are currently under development offer a promise of novel therapy for a wide variety of tissue injuries by specifically targeting an endogenous protective system that is a component of the innate immune response.

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