Low-Dose Therapy With the Long-Acting Erythropoietin Analogue Darbepoetin Alpha Persistently Activates Endothelial Akt and Attenuates Progressive Organ Failure

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Background—The hematopoietic cytokine erythropoietin has cytoprotective effects in endothelial cells in vitro that are mediated through direct activation of the pro-survival Akt tyrosine kinase signaling pathway. We tested the hypothesis that low-dose therapy with the long-acting recombinant human erythropoietin analogue darbepoetin alpha protects vascular endothelium in vivo in a classic remnant kidney rat model characterized by severe endothelial damage, progressive vascular sclerosis, and ischemia-induced tissue fibrosis.

Methods and Results—Using a parallel group study design, we randomly assigned animals after 5/6 nephrectomy to treatment with either saline (n=36) or 0.1 μg/kg body wt darbepoetin (n=24) subcutaneously once weekly. We monitored hematocrit, blood pressure, and serum creatinine regularly and obtained renal tissue 6 weeks after nephrectomy for morphological and immunohistochemical analysis. Darbepoetin-treated animals had significantly improved survival compared with saline-treated controls (63% versus 33%; \(P<0.05\)), although hematocrit levels were similar in both groups. Darbepoetin treatment ameliorated endothelial damage; attenuated the composite tissue injury score (saline 1.9±0.4; darbepoetin 0.4±0.2; \(P<0.001\)), which included vascular sclerosis, glomerulosclerosis, and tubulointerstitial damage; and preserved renal function. We found persistent activation of the pro-survival Akt signaling pathway in endothelial and epithelial glomerular cells in darbepoetin-treated animals, accompanied by a significant reduction of apoptotic cell death in renal tissue.

Conclusions—Low-dose darbepoetin treatment confers vascular and tissue protection that is associated with persistent stimulation of the pro-survival Akt signaling pathway. The use of recombinant human erythropoietin or analogues may have utility in preventing ischemia-related progressive vascular injury and organ failure. (Circulation. 2004;110:1006-1012.)

Key Words: apoptosis • erythropoietin • tissue • vasculature • endothelium

Recent experimental studies on the progression of chronic renal diseases have revealed that the process features progressive glomerular capillary sclerosis, loss of peritubular capillaries, and ischemia-induced tubulointerstitial fibrosis.1,2 The integrity of the endothelium forming the glomerular capillary tufts and the peritubular capillaries is vital for the preservation of renal function, and proangiogenic growth factors such as vascular endothelial growth factor (VEGF) play a central role in maintaining renal morphology and function after injury.3,4

Another proangiogenic growth factor currently receiving attention in cardiovascular research is the hematopoietic cytokine erythropoietin (EPO). EPO has pleiotropic effects well beyond the maintenance of red blood cell mass.5-7 In the embryo, EPO is a major regulator of vascular formation and organ growth; EPO receptors are found in almost every embryonic tissue.8 EPO receptors also exist in many adult tissues, and the notion of autocrine or paracrine EPO systems has been raised.9-11 EPO also acts on endothelial cells.12,13 Human endothelial cells respond to EPO by differentiating into vascular structures.14,15 In addition, EPO has important cytoprotective effects, including protection from ischemic injury and inhibition of apoptotic death–related pathways.16,17 We speculated that EPO might preserve its role as an endothelial cell regulator to preserve or even repair capillaries in the adult organism.18 To test this possibility, we studied

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chronic renal disease progression in the established 5/6 nephrectomy rat remnant kidney (RK) model. The model features progressive injury to the renal microvascular endothelium, leading to glomerular sclerosis accompanied by ischemia-induced tubulointerstitial damage. The course of the disease is inexorable and leads to death. We chose low-dose treatment with the long-acting recombinant human EPO (rHuEPO) analogue darbepoetin alpha because we recently found that darbepoetin stimulates human bone marrow–derived endothelial progenitor cells at doses that do not increase the hematocrit level. An increased hematocrit level could possibly contribute to elevated blood pressure and/or vascular thrombosis.

Methods

Studies on Survival After Induction of 5/6 Nephrectomy

The Animal Care Committee of Lower Saxony approved the study. We measured baseline blood pressure and renal function in 60 male Sprague-Dawley rats (Charles River Wiga GmbH, Sutzelfeld, Germany) weighing between 250 and 300 g by a tail-cuff method. We performed selective ligation of the extrarenal renal artery branches of the left renal artery under ketamine and xylazine anesthesia to obtain a two-thirds renal infarction. The opposite kidney was then removed.

We used a parallel group study design, and all animals were randomly assigned to receive either saline (0.9% NaCl) or 0.1 µg/kg body wt darbepoetin alpha (Aranesp, Amgen Co) subcutaneously once weekly after 5/6 nephrectomy starting after surgery. Animals were randomized in a 3:2 manner to saline (n = 24). We chose this protocol because in a pilot study exploring the effect of darbepoetin on hematocrit levels in RK rats, we observed fewer deaths in darbepoetin-treated RK animals than in saline-treated animals. All animals had free access to water and were given standard chow. We measured body weight and hematocrit before nephrectomy and weekly thereafter. Systolic blood pressure was measured in conscious animals with the use of an occlusive tail-cuff plethysmograph attached to a pneumatic pulse transducer (TSE blood pressure system); for the measurements we kept animals in a humidified chamber at room temperature with the following primary antibodies: polyclonal anti-gout heat shock protein 27 (HSP27) 1:100; polyclonal anti-rabbit VEGF (A-20, Santa Cruz Biotechnology) 1:200; polyclonal anti-rabbit heme oxygenase-1 (HO-1; Stressgen Biotechnologies) 1:1000; and monoclonal anti-rat CD31 (clone TLD-3A12; Pharmingen) 1:500. For fluorescence detection of bound primary antibodies, sections were subsequently incubated with Cy3-conjugated respective secondary antibodies (Jackson ImmunoResearch) for 1 hour. In addition, terminal deoxyribonucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit) for detection of apoptotic nuclei was performed on paraffin sections according to the manufacturer’s protocol (Chemicon International). The pathologist examining the renal tissue had no knowledge of the group assignment.

Studies on Time Course of Akt Activation and Apoptotic Activity

To elucidate in more detail the time course of Akt activation and apoptotic activity in renal tissue of RK rats, we induced 5/6 nephrectomy in 24 animals as described above. Thereafter, animals were randomly assigned either saline (n = 12) or 0.1 µg/kg body wt darbepoetin alpha (n = 12) subcutaneously once weekly. The animals were euthanized after 4 days (saline: n = 5; darbepoetin: n = 6) and 14 days (saline: n = 5; darbepoetin: n = 6) of treatment (2 saline-treated RK rats died during the follow-up), and we obtained renal tissue for analysis of Akt activation and apoptotic activity as described above. These 2 time points were chosen to detect early changes after nephrectomy (4 days) and to assess Akt activation and apoptotic activity in the critical phase of vascular and tissue damage in the RK model (14 days).

Statistical Analysis

We used the SPSS package (SPSS 11.51 for Windows). We analyzed survival with a log-rank analysis and created Kaplan-Meier survival curves. In addition, we compared baseline and end point characteristics with ANOVA and appropriately corrected t tests for random data. We analyzed data on the time course of Akt activation and apoptosis with ANOVA and appropriately corrected t tests. Differences were considered significant at P < 0.05. Data are mean ± SEM unless otherwise stated.

Results

Animal Survival

Fifteen of 24 darbepoetin-treated RK rats (63%) survived 6 weeks, compared with 12 of 36 saline-treated RK controls (33%; P < 0.05). The Kaplan-Meier survival curve is shown in Figure 1. Both serum creatinine concentrations and urinary protein excretion in saline-treated RK rats increased significantly during the observation period and were significantly higher at 6 weeks compared with darbepoetin-treated RK animals (Table). Systolic blood pressure in saline-treated RK animals increased markedly, whereas in darbepoetin-treated RK animals systolic blood pressure was slightly but not significantly higher at 6 weeks than at baseline (Table). Hematocrit levels in both groups increased slightly postoperatively, probably because of reduced water and food intake. Thereafter, hematocrit levels decreased steadily, as shown in...
Figure 2. The hematocrit levels were not different between the groups.

Renal Morphology
Photomicrographs of the histopathology are presented in Figure 3. Severe vascular and glomerular damage occurred in saline-treated RK rats. Onionlike vascular wall proliferation occurred in larger renal vessels, reminiscent of human malignant nephrosclerosis. Partial or complete glomerulosclerosis with hyalinosis and fibrinoid necrosis of the adjacent afferent arterioles were prominent features. Furthermore, as shown in Figure 4, focal rarefaction of peritubular capillaries was present. The vascular changes were accompanied by tubulointerstitial inflammation and fibrosis. All these typical RK model changes were attenuated with darbepoetin treatment. The composite tissue injury score that encompassed vascular sclerosis, glomerulosclerosis, and tubulointerstitial damage was significantly reduced in darbepoetin-treated RK rats compared with saline-treated RK rats (0.4±0.2 versus 1.9±0.4; P<0.001). In addition, rarefaction of peritubular capillaries was prevented by darbepoetin treatment (Figure 4).

Renal Immunohistology
Renal tissue staining for activated Akt (p-Akt [Ser473]) is shown in Figure 5. We found activation of the Akt pathway in glomerular endothelial cells and epithelial cells at day 4 after nephrectomy in both treatment groups. Akt activation was more pronounced in darbepoetin-treated animals. After 2 weeks of treatment, marked Akt activation was detectable in tissue of darbepoetin-treated animals, but it remained modest in RK rats treated with saline. In darbepoetin-treated animals surviving 6 weeks after nephrectomy, p-Akt staining in tissue of darbepoetin-treated animals was more prominent than in saline-treated RK rats (Figure 5). We did not observe differences with respect to HO-1, HSP27, and VEGF expression between the groups (data not shown). Staining for apoptotic cell nuclei (TUNEL) revealed a large increase of apoptotic activity in renal tissue of nephrectomized animals at treatment days 4 and 14 (Figure 6). Apoptosis was significantly reduced in darbepoetin-treated RK rats compared with saline-treated animals. In animals surviving 6 weeks after nephrectomy, we found only low-grade apoptotic activity in renal tissue of both saline- and darbepoetin-treated RK rats.

Clinical Parameters in Darbepoetin Alpha– and Saline-Treated RK Animals 6 Weeks After 5/6 Nephrectomy

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<tr>
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<th>Darbepoetin Alpha</th>
<th>Saline</th>
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<tr>
<td>Body weight, g</td>
<td>287±4</td>
<td>279±6</td>
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<tr>
<td>Serum creatinine, μmol/L</td>
<td>32±2</td>
<td>30±3</td>
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<tr>
<td>Urinary protein excretion, mg/d</td>
<td>109±7</td>
<td>129±6</td>
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<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>146±8</td>
<td>125±6</td>
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*P<0.05, end vs baseline.
†P<0.05, darbepoetin alpha– vs saline-treated animals.

Discussion
We found that chronic treatment with the long-acting EPO analogue darbepoetin alpha prevented endothelial injury in an experimental model of progressive chronic renal disease. One important feature of renal injury in this particular model is hypertension, which alone perpetuates endothelial activation, inflammation, and proliferation, followed by vascular obliteration and glomerulosclerosis. As a consequence, peritubular capillary disappearance accompanied by ischemia-induced tubulointerstitial fibrosis finally leads to renal failure and to death of the animals. The process involves chemokine...
and cytokine release, adhesion molecule expression, platelet and macrophage infiltration, cell activation, proliferation, matrix protein deposition, and eventually sclerosis and obliteration. Darbepoetin treatment significantly reduced glomerulosclerosis and prevented destruction of arterial integrity, maintaining the peritubular capillary network and the architecture of the renal interstitium, a particularly vulnerable part of the kidney to ischemic injury. Its integrity critically depends on blood flow in peritubular capillaries, which evolve from the efferent arteriole. These effects were accompanied by improved renal function and significantly better survival. The preserved renal function probably contributed to a lesser blood pressure elevation in the darbepoetin-treated animals.

EPO may also protect other organs from various sorts of injury. In a recent study, rHuEPO was given to animals with experimentally induced myocardial ischemia/reperfusion injury. Treated animals exhibited improved cardiomyocyte survival and preserved myocardial function. In a similar study, Parsa et al performed myocardial preconditioning experiments with a single high dose of rHuEPO in a rabbit model of acute myocardial infarction. Pretreatment with rHuEPO protected cardiomyocytes against ischemic injury, mitigated myocyte apoptosis, and led to improved myocardial function within 3 days after infarction. Both studies were relatively small, and therefore a beneficial effect on survival could not be tested. Because rHuEPO treatment may have adverse effects, improved survival would be an important

Figure 3. Renal sections from RK rats receiving saline or darbepoetin alpha are shown. Normal tissue was obtained from the preserved right kidney and is displayed in the left panel (A, D, and G). Saline treatment is shown in the middle panel (B, E, and H), and darbepoetin treatment is shown in the right panel (C, F, and I). Darbepoetin attenuated proliferation and sclerosis of small and medium-sized arteries (A through C) and collapsing glomerulosclerosis (D through F) compared with saline. Arteries of darbepoetin-treated rats showed only mild morphological changes with edema of the media, but no intimal or medial proliferation. Tubulointerstitial damage and fibrosis were reduced (G through I). The composite tissue damage score was significantly lower ($P<0.001$) in darbepoetin-treated (n=15) than in saline-treated (n=12) RK rats (J).
In our study, a clear-cut beneficial effect on survival was evident.

The effect of rHuEPO on cell and whole organ function has been examined in earlier studies. The rHuEPO doses used in many of these studies were extremely high. For instance, Parsa et al gave 5000 U/kg body wt in their preconditioning experiment. The dose corresponds to \( \approx 350 \times 10^3 \) U in a 70-kg man. Such a dose is not conceivable clinically. RHuEPO overdose may lead to hypertension, seizures, vascular thrombosis, and death, perhaps related to abruptly increased hematocrit values. Parsa et al reported a 20% increase in hematocrit by day 4 in their rabbit study.

**Figure 4.** CD31 staining of peritubular capillaries in renal tubulointerstitium of RK rats treated with saline or darbepoetin alpha. The CD31 stain revealed regular peritubular capillary network in darbepoetin-treated animals (left), whereas focal rarefaction of peritubular capillaries was seen in saline-treated rats (right).

**Figure 5.** Immunohistochemical analysis for Akt phosphorylation (p-Akt [Ser473]) in glomeruli of RK rats treated with saline or darbepoetin alpha. Akt activation was detectable at day 4 after nephrectomy in both treatment groups; it was more pronounced in darbepoetin-treated animals (B and C). After 2 weeks of treatment we found marked Akt activation in darbepoetin-treated animals, whereas in RK rats treated with saline, Akt activation remained modest (D and E). Finally, in darbepoetin-treated animals surviving 6 weeks after nephrectomy, p-Akt staining in glomerular endothelial cells and epithelial cells was prominent, whereas in saline-treated RK rats it was almost absent (F and G). Normal tissue is shown as control (A).
We chose a low-dose darbepoetin treatment to avoid changes in blood rheology that could have confounded our results. We did not observe significant differences in hematocrit levels in our RK rats treated with non-iron-supplemented saline and darbepoetin. Because long-term standard-dose rHuEPO therapy has been used safely for more than a decade, treatment with low-dose rHuEPO or analogues could be a promising therapeutic strategy for tissue and organ protection in cardiovascular, reconstructive, and regenerative medicine.

EPO exerted a cytoprotective effect and protected from ischemic injury in earlier studies. We selected low-dose darbepoetin for our study because of our earlier observation that darbepoetin at low doses markedly stimulated regenerative endothelial progenitor cell recruitment in vitro and in vivo. This effect was related to activation of the Akt/protein kinase B pathway. Akt activation served to maintain mitochondrial membrane potential in that study. In accord with these results, we were able to demonstrate persistent activation of the pro-survival Akt pathway in glomerular endothelium of darbepoetin-treated RK rats. Other potentially protective mechanisms such as HO-1 or HSP27 activation were not involved in our model. Activation of the Akt pathway is a powerful mechanism to protect cells from ischemic injury. VEGF is also an important Akt pathway stimulator. Recently, mesenchymal stem cells genetically enhanced with Akt1 were found to be ischemia resistant. In the heart, the genetically engineered cells prevented cardiac remodeling and restored performance of hearts with infarction, compared with stem cells not overexpressing Akt1. These findings suggest that Akt may have a central role in cell protection from ischemia. Our results in RK rats are in agreement with this assumption. They also support the notion that direct Akt activation is an important mechanism for tissue protection by EPO because we did not observe differences in VEGF expression in renal tissue of saline- and darbepoetin-treated animals. We also investigated the degree of apoptosis in renal tissue of RK rats from both groups. EPO in vitro inhibits apoptotic death–related pathways in some cell lines. We found a large increase of apoptotic activity in renal tissue of nephrectomized animals, and treatment with darbepoetin ameliorated this process. The effect of darbepoetin was particularly evident at day 14 after nephrectomy, ie, when massive endothelial activation and proliferation, followed by vascular obliteration, glomerulosclerosis, and ischemia-induced tubulointerstitial damage take place. In contrast, we found only low-grade apoptotic activity in animals of both groups surviving until week 6. We interpret these findings as evidence that darbepoetin treatment prevented the “first-strike” injury at the endothelial cell level and, in addition, reduced apoptotic cell death and preserved tissue integrity in the critical phase of tissue damage and destruction. These effects may be mediated, at least in part, by a persistent activation of the pro-survival Akt pathway.

In conclusion, chronic low-dose therapy with the long-acting rHuEPO analogue darbepoetin alpha conferred vascular and tissue protection, lowered blood pressure, preserved renal function, and improved survival in the RK rat model. The findings offer experimental and perhaps clinical perspectives. Other animal models of progressive ischemia-related organ failure must be tested, and the entire protective pathway must be elucidated. Conceivably, new clinically relevant therapies could be developed.

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References


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