Increased progression to kidney fibrosis after erythropoietin is used as a treatment for acute kidney injury

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Acute kidney injury; chronic kidney disease; ischemia-reperfusion; erythropoietin; EPO; fibrosis

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forming growth factor-β (TGF-β) and decreased fibrosis and epithelial-to-mesenchymal transition (EMT) (45, 60). Similar results have been published in the 5% nephrectomy CKD model (4, 61, 62) and in nephrotoxic animal models of CKD (33, 43). In the brain, EPO protected neurons after hypoxic injury but also stimulated growth of glial cells (69) and fibroblasts (53). An important unanswered question is that, in the acutely injured kidney, if rhEPO does decrease injury and promote repair in the tubular epithelium but also stimulates and activates interstitial profibrotic myofibroblasts, does the improved acute outcome translate to improved renal health in the long term? There have been no reports of the effects of early high-dose rhEPO treatment for IR-induced AKI on the long-term outcomes, whether they are good or bad. Thus we have examined the long-term effects of rhEPO therapy for AKI, using in vivo and in vitro models of IR-induced AKI.

MATERIALS AND METHODS

In Vivo Experiments

Experiments were approved by the University of Queensland Animal Experimentation Ethics Committee (RBWH/077/07/NHMRC). The IR rodent model was used previously by us for rhEPO studies (28, 65). Briefly, mature male Sprague-Dawley rats (200–220 g, n = 4) were anesthetized (Zoletil “100”/xylazine, 20 mg/ml; 50:50) and underwent either sham operation (controls) or bilateral renal artery occlusion for 40 min followed by reperfusion (IR). At various time points (4, 7, and 28 days) after IR, renal functional and structural changes and molecular correlates were studied. Rats were administered vehicle or rhEPO (Epoetin-α, Janssen-Cilag Pty, North Ryde, Australia) at 1,000 (25) or 5,000 IU/kg (11, 28, 46, 65) intraperitoneally at the time of reperfusion. For some of the 28-day IR animals, 1,000 IU/kg rhEPO was selected, in the context of a IU per kilogram dose similar to humans treated for AKI (17). At the end of the experiments, the animals were euthanized (pentobarbital sodium, 100 mg/kg), and blood samples were collected for determination of serum creatinine (SCr) as a marker of kidney function, lactate dehydrogenase (LDH) as a marker of kidney injury, and hematocrit (28, 38, 51, 65). Both kidneys were removed, bisected horizontally, half of each kidney was fixed in 10% formalin and prepared routinely for histology and immunohistochemistry (IHC), and half was frozen in liquid nitrogen and stored at −80°C for protein analysis.

In Vitro Experiments

Rat kidney epithelial cells (NRK52E) and fibroblasts (NRK49F; ATCC, Rockville, MD) were grown routinely in DMEM (Invitrogen, Melbourne, Australia) containing antibiotics (22). Cells were prepared in 12-well tissue culture plates on glass coverslips or in 10-cm Petri dishes for protein analyses (n = at least 3). For experiments, cells were grown for 24 h in serum-free medium (SF), SF plus hypoxia, or SF plus 1 mM H2O2 for oxidative stress. For hypoxia, 5% CO2 in nitrogen was bubbled through SF medium for 45 min (Po2 ∼50–100 mmHg) (22). This was then added to cells which were immediately placed into a humidified isolated incubator chamber filled with hypoxic gas. The sealed hypoxic chamber was then placed in a normal incubator for 24 h.

Western Blotting for Protein Analysis

Tissue or cell protein extracts were prepared, and protein concentrations were determined using our published methods (38, 51). Forty micrograms protein were electrophoresed on a 10–12% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membrane. For immunoblots, primary antibodies were α-smooth muscle actin (SMA; 1:1,000, Sigma-Aldrich, Sydney, Australia), GAPDH (1:1,000–1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA), vimentin (1:1,000, Santa Cruz Biotechnology), fibronectin (1:1,000, Transduction Laboratories, BD Biosciences, San Jose, CA), and phosphorylated or nonphosphorylated ERK1/2 (1:1,000, Santa Cruz Biotechnology). Positive cell extracts from antibody suppliers were used routinely as positive immunoblot controls. Appropriate secondary antibodies (1:2,000, Zymed Laboratories, San Francisco, CA) were applied, and protein bands were visualized using enhanced chemiluminescence. X-ray film was scanned (Hewlett Packard ScanJet, 300 dpi), and Scion Image software (vβ4.0.2) used to determine expression in arbitrary densitometry units (ADU). Protein bands were normalized against GAPDH expression patterns. In some cases, ADU were used to calculate a fold-change in control group expression. This was carried out by converting the normalized control value to a single unit and then calculating the fold value of the other groups against controls.

Histopathological Review

Grading and definitions for cellular degradation, necrosis, infiltration of neutrophils, and ED1 IHC for monocytes/macrophages are listed in Table 1. These were assessed at ×400 magnification (38) and carried out blinded to treatments. Paraffin sections were prepared using our published methods (22, 51). Sections were stained with hematoxylin and eosin (HE; normal morphological assessment) or Masson’s trichrome (collagen). Masson’s trichrome-stained sections were assessed for fibrotic index using digital pathology and morphometry (Aperio ScanScope XT, Aperio Technologies, Vista, CA). The percentages of positively stained (blue) areas were measured (Aperio), excluding vessel walls. Coverslip cultures were fixed in 4% buffered paraformaldehyde and stained with HE for microscopy. Cell death and mitosis were first assessed using HE staining and light microscopy and defined morphological characteristics (19, 26). Apoptosis was also quantified using the in situ enzymatic assay ApopTag (ApopTag Peroxidase In situ Apoptosis Detection Kit, Merck-Millipore, Bil-

Table 1. Parameters for semiquantitative assessment of histopathology

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell degradation (cellular swelling, vacuolization, mild nuclear condensation, casts in tubular lumens)</td>
<td>No change from normal</td>
<td>&lt; 5% of total field</td>
<td>5–30% of total field</td>
<td>&gt;30% of total field</td>
</tr>
<tr>
<td>Necrosis (pyknosis, karyorrhexis, karyolysis, extensive cell swelling, and membrane disruption)</td>
<td>Nil</td>
<td>Some single-cell necrosis</td>
<td>Dispersed focal necrotic tubules</td>
<td>Confluent necrosis in most tubules</td>
</tr>
<tr>
<td>Neutrophils (infiltration, using multilobed nuclear morphology)</td>
<td>Nil</td>
<td>1–3 cells/field</td>
<td>4–6 cells/field</td>
<td>Heavy infiltration</td>
</tr>
<tr>
<td>ED1 staining (monocytes/macrophages indicated by ED1-positive cells using IHC)</td>
<td>Nil</td>
<td>1–3 cells/field</td>
<td>4–6 cells/field</td>
<td>&gt;6 cells/field</td>
</tr>
</tbody>
</table>

ED1, monocytes/macrophages marker using immunohistochemistry (IHC); “field,” microscope field of view at magnification ×400 (∼40x objective, ∼10 eyepiece).
lerica, MA) (19). Mitosis was quantified using proliferating cell nuclear antigen (PCNA) and IHC. Labeled nuclei were counted per high-power microscope field (38, 60). Ten to twenty random microscope fields were scored in the outer stripe of the outer medulla (apoptotic or mitotic index = cells/mm² tissue).

**IHC and Immunofluorescence**

Published methods were used (38, 51). Primary antibodies were PCNA as a marker for cell cycling (1:50, Sigma-Aldrich), α-SMA as a marker for myofibroblasts (1:100, Sigma-Aldrich), vimentin for mesenchymal differentiation and ED1 as a marker for macrophages (both 1:100, Santa Cruz Biotechnology), profibrotic TGF-β and proinflammatory tumor necrosis factor-α (TNF-α; both 1:500, Santa Cruz Biotechnology), and 8-hydroxy deoxyguanosine (8-OHdG) as a marker for oxidative stress (1:100, Chemicon, Millipore Australia Pty, Kilsyth, Australia). Sections for each individual antibody were batch stained. Negative and positive controls were used routinely. Sections were lightly counterstained with hematoxylin or periodic acid-Schiff reagent (PAS), then dehydrated, cleared, and mounted in Depex. IHC expression was measured using Aperio ScanScope methods, as detailed for collagen histochemistry. Immunofluorescence was viewed after photographic records of changes using a FluoView FV1200 Confocal tag and DAPI nuclear staining, Confocal microscopy was used for photomicroscopy.

**Statistical Analysis**

Data are expressed as means ± SE and analyzed by one-way ANOVA and Dunnett’s multiple comparison test or Student’s t-test. A value of P < 0.05 was considered to be significant.

**RESULTS**

**In Vivo Experiments**

Are improved pathophysiological outcomes for rhEPO-treated AKI maintained long term? Rats having 40-in bilateral IR, with or without rhEPO, were examined at 4 and 7 days post-IR (5,000 IU/kg) and 28 days post-IR (5,000 or 1,000 IU/kg). Data for body weight, SCR, LDH, and hematocrit are detailed in Table 2. IR induced an increase in SCR at 4 days (**P < 0.001), which was decreased by rhEPO (**P < 0.01) compared with IR alone. LDH was significantly increased by IR at all time points (**P < 0.001; *P < 0.05). rhEPO reduced LDH significantly at 4 and 7 days post-IR (#P < 0.05) compared with the time-matched IR groups, but there were no differences with or without rhEPO between IR groups at 28 days, when LDH remained elevated. Hematocrit was moderately but significantly elevated over controls at 4 days in rhEPO-treated groups (P < 0.05) but normalized in the subsequent experimental times.

The results for semiquantitative assessment of histopathology are detailed in Table 3, with an explanation of the grading system found in Table 1. Cell degradation and necrosis were reduced at 4 days post-IR with rhEPO delivery, compared with IR alone (*P < 0.05), but there were no significant differences found at 7 and 28 days post-IR with or without rhEPO. There were no differences among experimental groups for neutrophil and monocyte/macrophage (ED1-positive) infiltration.

Counts for apoptosis and mitosis are presented graphically in Fig. 1. Compared with sham-treated control animals (negligible value represented by C in graph), IR induced significant tubular epithelial apoptosis at 4, 7, and 28 days (**P < 0.001). rhEPO reduced apoptosis at 4 days post-IR (**P < 0.01), but IR-induced apoptosis was not diminished by rhEPO therapy at 7 (5,000 IU/kg) and 28 (5,000 or 1,000 IU/kg) days post-IR. Tubular epithelial regeneration occurred at 4 days post-IR (P < 0.05), and this was promoted by rhEPO (**P < 0.05). Cell proliferation remained high at 7 days in the IR groups with and without rhEPO, but there was no significant increase with rhEPO. By 28 days post-IR, there was no difference in cell proliferation among sham-treated controls, IR, and IR treated with rhEPO. There was no significance in apoptotic or mitotic outcome at 28 days for 5,000 vs. 1,000 IU/kg. Figure 1 also demonstrates histology for apoptosis (A; HE), ApopTag enzymatic labeling for apoptosis (B and C), and PCNA IHC for cell proliferation, with PAS as a counterstain (D).

Is there any difference in fibrosis long term when AKI is treated with EPO? The translation of improved structure and function at 4 days post-IR with rhEPO, to reduced fibrosis at 7 and 28 days post-IR, was investigated. Figure 2 shows histo-

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**Table 2. Body weight, serum creatinine, lactate dehydrogenase, and hematocrit for in vivo experiments**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>SCR, mmol/L</th>
<th>LDH, U/L</th>
<th>Hct, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 4 days</td>
<td>185 ± 3</td>
<td>&lt;0.03</td>
<td>463 ± 110</td>
<td>37.3 ± 1.9</td>
</tr>
<tr>
<td>C EPO 4 days</td>
<td>180 ± 2</td>
<td>&lt;0.03</td>
<td>398 ± 148</td>
<td>42.8 ± 2.7†</td>
</tr>
<tr>
<td>IR 4 days</td>
<td>181 ± 2</td>
<td>0.15 ± 0.01†</td>
<td>2,472 ± 235†</td>
<td>36.3 ± 2.5</td>
</tr>
<tr>
<td>IR EPO 4 days</td>
<td>180 ± 4</td>
<td>0.07 ± 0.02‡</td>
<td>1,908 ± 263‡</td>
<td>44.1 ± 2.5‡</td>
</tr>
<tr>
<td>C 7 days</td>
<td>202 ± 2</td>
<td>&lt;0.03</td>
<td>653 ± 188</td>
<td>38.3 ± 1.7</td>
</tr>
<tr>
<td>C EPO 7 days</td>
<td>199 ± 3</td>
<td>&lt;0.03</td>
<td>604 ± 224</td>
<td>41.4 ± 1.2</td>
</tr>
<tr>
<td>IR 7 days</td>
<td>176 ± 5</td>
<td>0.08 ± 0.03</td>
<td>3,125 ± 490†</td>
<td>37.8 ± 1.6</td>
</tr>
<tr>
<td>IR EPO 7 days</td>
<td>182 ± 5</td>
<td>0.05 ± 0.02</td>
<td>1,191 ± 361‡</td>
<td>40.1 ± 1.3</td>
</tr>
<tr>
<td>C 28 days</td>
<td>229 ± 3</td>
<td>&lt;0.03</td>
<td>596 ± 201</td>
<td>38.8 ± 2.1</td>
</tr>
<tr>
<td>C EPO 28 days (5,000 IU)</td>
<td>236 ± 5</td>
<td>&lt;0.03</td>
<td>450 ± 117</td>
<td>37.4 ± 1.2</td>
</tr>
<tr>
<td>C EPO 28 days (1,000 IU)</td>
<td>238 ± 6</td>
<td>0.04</td>
<td>398 ± 129</td>
<td>39.3 ± 2.1</td>
</tr>
<tr>
<td>IR 28 days</td>
<td>229 ± 5</td>
<td>0.04</td>
<td>1,010 ± 164*</td>
<td>39.4 ± 1.7</td>
</tr>
<tr>
<td>IR EPO 28 days (5,000 IU)</td>
<td>233 ± 4</td>
<td>0.05 ± 0.01</td>
<td>1,237 ± 240*</td>
<td>38.4 ± 1.5</td>
</tr>
<tr>
<td>IR EPO 28 days (1,000 IU)</td>
<td>231 ± 5</td>
<td>0.05 ± 0.01</td>
<td>1,166 ± 231*</td>
<td>38.2 ± 2.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. C, controls; IR, ischemia-reperfusion; EPO, recombinant human erythropoietin; IR EPO, animals treated with both ischemia-reperfusion and erythropoietin; SCR, serum creatinine; LDH, lactate dehydrogenase; Hct, hematocrit; U/L, units per liter; IU, international units. *P < 0.05 compared with time-matched groups. †P < 0.01 compared with time-matched groups. ‡P < 0.05 reduction in LDH for IR EPO compared with time-matched IR only.
pathology (rows A–C; HE staining showing fibrosis), collagen deposition (rows D–F; Masson’s trichrome staining), and activation of myofibroblasts (rows G–I; /H9251-SMA staining). In each case, the first column in Fig, 2 shows examples of control animals; the second column shows IR at 28 days; and the third column shows IR plus rhEPO at 28 days post-IR. In each set, the greatest evidence of fibrosis was seen at 28 days post-IR (B vs. C; E vs. F; H vs. I). The graphs were formulated using morphometry of collagen-positive areas (Masson’s trichrome, blue) in D–F, and /H9251-SMA-positive areas (brown) in G–I. IR induced a significant increase in collagen and myofibroblasts at 7 days (**P < 0.05), with no difference seen between IR and IR treated with rhEPO. A similar increase was seen at 28 days, but in this case there was a significant increase in the IR-induced collagen deposition and myofibroblasts when 5,000 IU/kg rhEPO was used as a therapy for AKI (**P < 0.05). In the group receiving 1,000 IU/kg rhEPO at 28 days post-IR, there was no significant difference in collagen deposition and myofibroblast numbers over the IR groups without rhEPO. Of note, there were no /H9251-SMA-expressing tubular epithelial cells in any of the sections we examined, and so no support for EMT in vivo.

Table 3. Assessment of cell damage and inflammation

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell Degradation</th>
<th>Necrosis</th>
<th>Neutrophils</th>
<th>ED1 + Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>Grade 1</td>
<td>Grade 1</td>
<td>Grade 1</td>
<td>Grade 1</td>
</tr>
<tr>
<td>Controls+EPO</td>
<td>Grade 1</td>
<td>Grade 1</td>
<td>Grade 1</td>
<td>Grade 1</td>
</tr>
<tr>
<td>IR 4 days</td>
<td>Grade 3</td>
<td>Grade 4</td>
<td>Grade 1</td>
<td>Grade 3</td>
</tr>
<tr>
<td>IR EPO 4 days</td>
<td>Grade 3</td>
<td>Grade 3</td>
<td>1.33 ± 0.47</td>
<td>3.20 ± 0.67</td>
</tr>
<tr>
<td>IR 7 days</td>
<td>Grade 2</td>
<td>Grade 2</td>
<td>Grade 1</td>
<td>Grade 3</td>
</tr>
<tr>
<td>IR EPO 7 days</td>
<td>Grade 2</td>
<td>Grade 1</td>
<td>1.13 ± 0.33</td>
<td>3.15 ± 0.80</td>
</tr>
<tr>
<td>IR 28 days</td>
<td>Grade 1</td>
<td>Grade 1</td>
<td>Grade 1</td>
<td>Grade 3</td>
</tr>
<tr>
<td>IR EPO 28 days (5,000 IU)</td>
<td>Grade 1</td>
<td>Grade 1</td>
<td>Grade 1</td>
<td>Grade 3</td>
</tr>
<tr>
<td>IR EPO 28 days (1,000 IU)</td>
<td>Grade 1</td>
<td>Grade 1</td>
<td>Grade 1</td>
<td>Grade 3</td>
</tr>
</tbody>
</table>

Values are means ± SE. See Table 1 in MATERIALS AND METHODS for grading criteria. ED1-positive cells, monocytes and macrophages. *P < 0.05 compared with IR 4 days without human recombinant (rh) EPO.

Fig. 1. In vivo experiments: apoptosis and mitosis after recombinant human erythropoietin (rhEPO)-treated ischemia-reperfusion (IR). x- Axis: 4D and 7D, 4 and 7 days post-IR; 28D5 and 28D1, 28 days post-IR, treated with 5,000 or 1,000 IU/kg rhEPO, respectively. Apoptosis in control kidneys was almost negligible (marked as C for 4D and 28D5). Compared with sham-treated control animals, IR induced significant tubular epithelial apoptosis at 4, 7, and 28 days (**P < 0.001). rhEPO therapy (5,000 IU/kg) reduced apoptosis at 4 days post-IR (**P < 0.01). It had no effect on IR-induced apoptosis at any other time point. Tubular epithelial cell regeneration (mitosis) increased in IR animals at 4 and 7 days (**P < 0.05 compared with IR only). A dose of 5,000 IU/kg rhEPO increased cell proliferation significantly only at 4 days post-IR (**P < 0.05) compared with IR only. By 28 days post-IR, there was no difference in cell proliferation for any group. There was no significant difference (NS) in apoptosis or mitosis for 28 days IR treated with 5,000 vs. 1,000 IU/kg. Below the graphs are arrowed examples of histology for apoptosis (hematoxylin and eosin; A). ApopTag enzymatic labeling of apoptosis [at 4 days post-IR (B) and at 28 days post-IR (C)], and proliferating cell nuclear antigen (PCNA) immunohistochemistry with periodic acid-Schiff counterstain (D, 4 days post-IR with rhEPO). In D, the asterisk indicates a mitotic nucleus.
How does rhEPO stimulate fibrosis in IR-injured kidneys?

IHC and morphometry were carried out for profibrotic TGF-β, proinflammatory TNF-α, and 8-OHdG for oxidative stress (Fig. 3). The left column of each panel of IHC consists of control tissue stained with TGF-β, TNF-α, or 8-OHdG (A, D, and G, respectively). The middle and right columns consist of results of 28 days IR without, or with, rhEPO, respectively (B and C for TGF-β; E and F for TNF-α; H and I for 8-OHdG).

Morphometry revealed that IR caused an acute (4 days) and progressive (7 and 28 days) increase in TGF-β (*P < 0.01), which was significantly increased at 7 and 28 days post-IR when rhEPO was delivered (**P < 0.05 compared with IR only). TNF-α was increased by IR at 7 and 28 days (*P < 0.05), but rhEPO did not cause any further significant change. This result supports the lack of significance in temporal changes in ED1-positive cells (Table 3) between IR and IR...
with rhEPO. 8-OHdG was significantly increased at each time period with IR (*\(P < 0.05\), but only for IR with rhEPO at 28 days (**\(P < 0.05\) compared with IR at 28 days).

Expression of ECM protein fibronectin, phospho- and non-phospho-ERK1/2 and α-SMA are represented in Western blots in Fig. 4. The representative blots for fibronectin show increasing expression at 7 and 28 days for IR and IR treated with 5,000 IU/kg rhEPO (**\(P < 0.05\), but there was no significant increase in fibronectin in the IR plus rhEPO groups over IR alone. ERK1/2 and phospho-ERK were significantly increased in the IR groups with and without rhEPO compared with their respective controls (*\(P < 0.05\)). At 28 days IR with rhEPO,

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**Fig. 3.** In vivo experiments: mechanisms for rhEPO-induced progressive fibrosis. Morphometry and immunohistochemistry (IHC) are demonstrated for profibrotic transforming growth factor-β (TGF-β), proinflammatory TNF-α, and 8-hydroxy-deoxyguanosine (8-OHdG), a marker of oxidative stress. The left column of each panel of IHC consists of control tissue stained with TGF-β, TNF-α, or 8-OHdG (A, D, and G, respectively). The middle and right columns show results of 28 days IR without or with rhEPO, respectively (B and C for TGF-β; E and F for TNF-α; H and I for 8-OHdG). Morphometry is shown in the graphs, where each batch of columns represents controls, controls plus rhEPO, IR, and IR plus rhEPO, respectively. IR caused an acute (4 days) and progressive (7, 28 days) increase in TGF-β (**\(P < 0.01\) that was significantly increased at 7 and 28 days post-IR when rhEPO was delivered (**\(P < 0.05\) compared with IR only). TNF-α was significantly increased by IR at 7 and 28 days (*\(P < 0.05\), and rhEPO did not cause any further significant change. 8-OHdG was significantly increased at each time period with IR (*\(P < 0.05\), but only for IR plus rhEPO at 28 days (**\(P < 0.05\) compared with IR at 28 days).
phospho-ERK was significantly higher than all other rhEPO-treated groups (**P < 0.05). α-SMA expression was highest in the 28-day IR groups with and without rhEPO, with significance in the 1.5-fold increase in expression when IR plus rhEPO is compared with IR alone (P < 0.05).

In Vitro Experiments

Is cytoprotection of the renal tubular epithelium by EPO associated with stimulation of fibrosis? Renal tubular epithelial cells (NRK52E) and renal fibroblasts (NRK49F) treated with hypoxia or 1 mM hydrogen peroxide for oxidative stress were used as a model of IR injury in vitro to investigate rhEPO-induced fibrogenesis. Figure 5 demonstrates apoptosis, mitosis, and expression of α-SMA and vimentin. In epithelial cells (52E), hypoxia and oxidative stress induced an 8- to 10-fold increase in apoptosis compared with controls and this was significantly decreased by rhEPO (200 IU/ml) (*P < 0.05). Mitosis was almost negated by both treatments, and this was retrieved by rhEPO (**P < 0.05). rhEPO did not reduce the hypoxia-induced apoptosis in fibroblasts (49F), but it did significantly reduce oxidative stress-induced apoptosis (**P < 0.05). Mitosis was again almost negated by both treatments, and rhEPO retrieved fibroblast proliferation to normal levels (**P < 0.05) in both hypoxia and oxidative stress treatments. A representative Western blot shows the expression profile for α-SMA and vimentin in these experiments, and the fold-
changes on control expression of each protein are demonstrated in the graphs. In epithelial cells (52E), SMA in hypoxia-treated cells, and both SMA and vimentin in oxidative stress-treated cells had significant increases with rhEPO (*P < 0.05). Mitosis was almost negated by both treatments, and rhEPO retrieved fibroblast mitosis to normal levels (**P < 0.05 compared with HP- or OX-treated cells). Also shown is densitometry of Western blots for α-SMA and vimentin in these experiments, presented as ADU (bottom). In the 52E cells, both α-SMA and vimentin had significant increases in expression in the OX-treated cells with rhEPO. In the fibroblasts, rhEPO stimulated expression of α-SMA and vimentin when delivered with either HP or OX (*P < 0.05 compared with rhEPO vehicle).

DISCUSSION

In this study, a single high-dose treatment with rhEPO (1,000 or 5,000 IU/kg) or vehicle was administered to rats subjected to bilateral 40-min IR. Kidney structure and function were studied at 4, 7, and 28 days after IR. Injury, repair, regeneration, and fibrosis were compared, with and without rhEPO modulation. The results in our rat model indicate that rhEPO, on the one hand, protects against AKI, but on the other hand the supraphysiological dose (5,000 IU/kg) needed for renoprotection stimulates progressive fibrosis beyond that normally seen in repair with IR-induced AKI. Improved function and structure by rhEPO at earlier times after IR (1 and 2 days) have been demonstrated by us previously (28). During this research, increased myofibroblast numbers were seen in the cortex of rhEPO-treated animals with IR-induced injury (our unpublished data), and this made us suspicious of stimulation of a fibrotic pathway by rhEPO. CKD after IR-induced AKI has been demonstrated previously by Basile et al. (5, 6) at various times up to 40 wk post-IR, but our results showing exacerbation of the AKI-induced fibrosis by rhEPO are new. In parallel with the animal studies, renal tubular epithelial and fibroblast cells in culture were subjected to hypoxia or H2O2 as models for ischemia and oxidative stress, respectively, and the effects on cell death, regeneration, and fibrogenesis were ana-
lyzed with and without modulation by rhEPO. The hypoxia and oxidative stress–induced EMT was exacerbated by concurrent treatment with rhEPO, as was stimulation of the myofibroblast phenotype in treated fibroblast cells.

A dose of 1,000 IU/kg rhEPO was also used in the current research for some of the 28-day IR animals, in the context of milligrams per kilogram dose of rhEPO similar to humans treated for AKI (17). This dose had no significant profibrotic effects on AKI repair long term, although there was a concerning nonsignificant increase in fibrosis compared with IR only. However, human doses cannot be translated to rats on a milligrams per kilogram basis (55) because of differences in metabolic rate, and 5,000 IU/kg has been widely used as the typical renoprotective dose for rodent experiments (11, 28, 29, 46, 65). In addition, there was no renoprotection recorded at 2 × 500 IU/kg rhEPO for humans with AKI (17), and a higher dose may be needed for humans if rhEPO is to be used successfully in some cases of acute renal failure.

The potential of the kidney to repair itself after acute ischemic injury may be augmented with application of extrinsic factors that modulate hypoxia and/or stimulate reparative molecules (27, 38). The very stimulation of those factors, however, may predispose the kidney to advance to CKD after the acute injury has been repaired; that is, the kidney injury does not completely resolve but organizes with progressive tubulointerstitial fibrosis, and ultimate destruction of the functioning renal nephron. In humans, the timeline for progression from acute injury to CKD is very variable, but in most cases this is a slow and insidious process that may take years (64). The fibrogenesis described in the present work at 1 mo after AKI may seem mild, but there remain significant increases in parameters of fibrosis after IR, and significant stimulation of fibrosis with IR plus rhEPO. The myofibroblasts are a key cell population essential for repair but are also difficult to remove once activated (73). Their presence is closely associated with progressive renal fibrosis (32). In the kidney, the origin of these cells remains in dispute (27). However, regardless of whether their origin is the resident renal fibroblast, vascular pericytes, epithelial cells of the renal nephron via EMT, or other cell populations, their continued presence means continued production of ECM proteins, especially in the interstitial space of the damaged kidneys. In the current research, as well as significantly increased myofibroblast numbers, there was also increased deposition of collagen, and increased profibrotic TGF-β expression, after delivery of rhEPO for AKI. Although EMT of renal epithelial cells, as evidenced by increased a-SMA expression, was not seen in any renal sections from our IR in vivo study, it was identified by increased α-SMA and vimentin in hypoxia- or oxidative stress–injured kidney epithelial cells with rhEPO treatment in the in vitro experiments.

Sustained TGF-β activity in progressive development of CKD has been recorded previously: for example, in Yamamoto et al. (70) after glomerulonephritis; in Lane et al. (32) after analgesic nephropathy; in Wong et al. (67) in humans with type 2 diabetes mellitus; and in Yang et al. (72) after cisplatin nephrotoxicity. Basile et al. (6), using an IR model of AKI similar to our current investigation, demonstrated a fourfold increase in TGF-β1 expression 3 days post-IR which returned to basal levels at 4 and 8 wk post-IR. They then reported a secondary increase in TGF-β1 mRNA at 40 wk post-IR, where TGF-β1 mRNA was significantly elevated in the post-IR group compared with sham-operated rats. In the current investigation, the IR-induced fibrosis at 28 days post-IR was mirrored by increased TGF-β and its downstream product of fibronectin. However, the rhEPO-stimulated TGF-β increase was not represented by a significant increase in fibronectin with rhEPO. An improvement in the current investigation would have been to include evidence of other downstream indicators of TGF-β activity, like connective tissue growth factor and some of the Smad proteins (31, 50). However, as well as its known profibrotic role, TGF-β may be directly toxic to the tubular epithelium (47), causing nephron atrophy (72). With increased cell injury, verified by our rhEPO-stimulated increased levels of LDH in the in vivo study, comes increased oxidative stress, which we also found to be augmented in the IR animals treated with rhEPO at 28 days. Oxidative stress is also profibrotic (47). Thus, mechanistically, stimulation of TGF-β and oxidative stress by rhEPO may help explain the increased fibrogenesis at 28 days post-IR with this therapy. The sustained TNF-α expression at 7 and 28 days post-IR may stimulate a death receptor pathway that is linked to the sustained apoptosis and tubular atrophy at these times, but rhEPO did not cause any exacerbation of this outcome. The role of ERK in fibrosis is less clear. We have described increased phospho-ERK in fibrosis in the UUO and cell culture models of kidney fibrosis (47, 48), a result in concordance with the increased phospho-ERK in the animals with IR plus rhEPO at 28 days post-IR, seen in the current study. However, the overall decreased phospho-ERK with rhEPO administration compared with IR alone may indicate that another signaling pathway acts with phospho-ERK to determine the increased fibrosis when IR is treated with rhEPO, and this needs further study.

There is some published evidence for decreased fibrosis with EPO therapy, but this evidence comes mainly from rat models of primary CKD vs. CKD developing after repair of AKI. Using the 5⁄6 nephrectomy model, darbepoetin given once weekly after renal mass reduction reduced glomerular and tubular damage and renal scarring (4). In the rat UUO model, apoptosis, tubulointerstitial fibrosis, EMT, and TGF-β expression were reduced by rhEPO (45, 60). These CKD models differ greatly in mechanism, if not outcome, from our model of IR-induced AKI followed by CKD. The closest model to our IR-AKI model is one where there was a reduction in tubulointerstitial fibrosis after doxorubicin nephrotoxicity in rats (43). Darbepoetin was delivered once weekly 2 wk after the last dose of doxorubicin. Thus their dose regimen is distinctly different from ours. We delivered rhEPO as a single dose at the time of IR-induced AKI, akin to treating an intensive care patient in acute renal failure from IR. Differences in models may explain differences in outcome from rhEPO therapy. Nonetheless, there should be concern about any possible stimulation of fibrosis by the rhEPO therapy.

There are ample data, including from our own research, demonstrating that rhEPO is antiapoptotic and proregenerative in its renoprotective mechanism (3, 28, 56, 65). In the current investigation, the IR-induced increases in apoptosis, necrosis, and LDH were reduced by rhEPO at 4 days after IR, and regeneration was improved. However, at 7 and 28 days post-IR, epithelial cell apoptosis remained high, contributing to tubular atrophy as part of the process of CKD progression. Apoptosis did not decrease with rhEPO long term, but there
was also no rhEPO-stimulated increase in mitosis or tubular regeneration as tubular atrophy progressed.

EPO may be anti-inflammatory (24, 40, 74). This may occur indirectly because of rhEPO-induced modulation of cell death in the kidney after IR (1), but we did not find that rhEPO modulated acute or chronic inflammatory cell populations, or modulated the increased levels of TNF-α seen at 7 and 28 days post-IR. Lee et al. (33) reported a direct dampening effect by rhEPO on proinflammatory cytokine production, using a cyclosporine-induced nephrotoxic kidney injury model. In a rodent model of stroke, EPO reduced astrocyte activation, leukocyte recruitment, and infiltration of microglia in the infarct site. In contrast to all other results, Lifshitz et al. (37) reported a proinflammatory effect of EPO. They found that, in splenic macrophages, inflammatory peritoneal macrophages and bone marrow-derived macrophages in culture, EPO increased macrophage numbers and stimulated their function. Thus the differences in effects of EPO on inflammation may need to take into account tissue and model specificity.

Other concerns exist for administration of supraphysiologically-doses of rhEPO than the profibrotic ones we have presented here. One concern centers on stimulation of erythropoiesis with increased hematocrit, leading to thrombosis and vascular sludging (21). In our model, hematocrit levels increased significantly in the rhEPO-treated animals at 4 days posttreatment, but they decreased by 7 days, and normalized by 28 days post-rhEPO treatment. There was no evidence of effects of thrombosis in any animal. Another concern for use of rhEPO as a cytoprotective agent is the potential for EPO to stimulate the growth of various tumors through its antiapoptotic, pleiotropic actions (18, 35, 66). Clinical trials of rhEPO as a therapy for anemia in cancer patients have indicated that this therapy is associated with reduced tumor-free survival (36). In our experiments, although only taken to 28 days and not in kidneys with tumors, there was no indication of hyperproliferation or cellular transformation in the kidneys from animals treated with rhEPO, nor in any of the in vitro experiments.

In summary, there needs to be continuing debate about the short- and long-term benefits, or otherwise, of the use of high-dose rhEPO as a treatment for AKI. The present results verify the acute cytoprotective outcome of its use for this purpose but indicate that the long-term outcome may be worsened by delivery of a supraphysiological dose of rhEPO. The very reactive nature of injured renal tissue, and the ability of EPO to stimulate multiple lineages of cells including those of mesenchymal lineage, may mean that its use as a renoprotective works against renal health in the long term. In this study, progressive fibrosis seen after IR-induced AKI was not diminished by rhEPO therapy and was stimulated by the high (significantly) and moderate (nonsignificant but noticeable and concerning) rhEPO therapy. Thus, by acting in its known pleiotropic manner, rhEPO may ultimately reinforce the development of CKD after AKI, and accelerate and augment CKD progression.

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AUTHOR CONTRIBUTIONS


REFERENCES

17. Endre ZH, Walker RJ, Pickering JW, Shaw GM, Frampton CM, Henderson SJ, Hutchinson R, Mehrten JS, Robinson JM, Schollum...


