Temporal and Spatial Expression of Erythropoietin, Erythropoietin Receptor, and Common \( \beta \) Receptor in Wound Fluid and Granulation Tissue

Naveed Saqib, MD; Laura Duling, BS; Kelly Krier, MD; Thomas R. Howdieshell, MD

Abstract: Background. Erythropoietin (Epo) and its cognate receptor (EpoR) have been recently identified in nonhematopoietic cells. Epo structural variants, which possess tissue protective effects while exhibiting no effect on erythropoiesis, appear to require a second distinct receptor component, the common \( \beta \) receptor (\( \beta cR \)) of IL-3, IL-5, and GM-CSF for ligand signal transduction. The goal of this work was to determine the temporal and spatial presence of Epo, EpoR, and \( \beta cR \) in porcine wound fluid and granulation tissue. Methods. A ventral hernia, surgically created in the abdominal wall of female swine (n = 8), was repaired with silicone sheeting and skin closure. Over time, a fluid-filled wound compartment formed, bounded by subcutaneous and omental granulation tissue; its thickness was measured by ultrasonography. Serial wound fluid samples were obtained by percutaneous aspiration. On day 14, the animals were sacrificed. Protein isolated from skin, kidney, granulation tissue, and peritoneal and wound fluids was analyzed by Western blotting. Sections of formalin-fixed abdominal wall tissue were stained for immunoreactivity to Epo, EpoR, and \( \beta cR \). Results. A progressive increase in granulation tissue thickness was measured during the 14-day interval. Western blot analysis of serial wound fluid samples demonstrated an 8-fold increase in local wound fluid Epo concentration. Immunoblotting of day 0 skin and day 14 granulation tissue homogenates demonstrated presence of Epo, EpoR, and \( \beta cR \) in wound granulation tissue but not in control skin. Immunostaining demonstrated localization of Epo and its receptors in granulation endothelial cells, fibroblasts, macrophages, and smooth muscle cells. Conclusion. Temporal expression of soluble Epo was associated with a progressive increase in porcine granulation tissue formation. Receptor expression, spatially localized to cellular constituents of granulation tissue, increased in the wound environment compared to control tissue. Epo variants, which signal via a heteroreceptor complex including both EpoR and \( \beta cR \), may be an effective therapeutic approach to improve wound healing.

Erythropoietin (Epo) is a 30.4 kDa glycoprotein produced primarily in the adult kidney under the control of an oxygen-sensing mechanism. It regulates the daily production of \( 2 \times 10^{11} \) red blood cells in adult bone marrow to maintain the oxygen-carrying capacity of blood under phys-
iologic conditions. Epo has long been known to be the principle hematopoietic growth factor that regulates cellular proliferation and differentiation along the erythroid lineage.\(^1\) A recent study has shown that Epo is a pleiotropic cytokine that is proangiogenic and exerts broad tissue-protective effects in diverse nonhematopoietic organs.\(^2\)

The biological effects of Epo in hematopoietic cells are mediated through its binding to its specific cell surface receptor—the erythropoietin receptor (EpoR). EpoR is a member of the type I cytokine receptor family that includes cellular transmembrane receptors for factors such as granulocyte-colony stimulating factor, many of the interleukins, prolactin, as well as growth hormone.\(^2\) EpoR expression and signaling in hematopoietic tissues is essential for normal mammalian erythropoiesis during development. Targeted disruption of the genes encoding either Epo or EpoR in mice has revealed that knockout mouse embryos die in utero due to defects in angiogenesis and cardiac morphogenesis with increased apoptosis in endocardium and myocardium.\(^3,4\)

Functional EpoR expression has been documented in many nonhematopoietic cell types, including vascular endothelial cells, smooth muscle cells, skeletal myoblasts, cardiac myocytes, neurons, retinal photoreceptors, liver stromal cells, renal tubular cells, and macrophages.\(^5-7\) The tissue-protective effects of Epo in the brain and heart seem to require a second distinct receptor component, the common \(\beta\) subunit (\(\beta_cR\)) of the interleukin-3 (IL-3) receptor, also shared by interleukin-5 (IL-5) and granulocyte-macrophage colony stimulating factor (GM-CSF).\(^8\)

A series of recent preclinical studies have suggested that Epo may contribute to the regulation of physiological wound healing responses. In a rat model of wound healing, exogenous Epo administration into subcutaneous fibrin chambers was found to promote the formation of wound granulation tissue, an effect that was associated with stimulation of physiological angiogenesis and upregulation of iNOS expression.\(^9\) In other studies, systemic Epo administration was also found to promote wound healing associated with increased angiogenesis and wound collagen and VEGF content.\(^10,11\) In an experimental model of burn wounds, recombinant Epo promoted wound re-epithelialization and shortened the time to final wound closure.\(^12\)

**Methods**

A wound-healing model was devised to facilitate the serial analysis of wound fluid mediator composition and coincidental measurement of granulation tissue growth and wound physiology. This model was used to determine the temporal and spatial presence of Epo and its receptors, EpoR and \(\beta_cR\), in wound fluid and granulation tissue.

**Ethical considerations.** All animals were treated humanely in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals as part of a protocol approved by the institution’s animal review committee.

**Surgical procedures.** Following induction with Telazol, xylazine hydrochloride, and atropine, female Landrace swine (\(n = 8\), weighing 15 kg–20 kg) underwent anesthesia with isoflurane (1.5% to 3%) via inhalation with an endotracheal tube. After sterile preparation (povidone-iodine) and draping, an infracutaneous diagnostic peritoneal lavage was performed (Arrow DPL Kit, Arrow International, Reading, PA) with the percutaneous Seldinger technique to obtain pre-incision peritoneal fluid for molecular analysis. Post-surgical analgesia was provided with buprenorphine hydrochloride for 48 hours.

Next, a supraumbilical midline laparotomy was performed and biopsy samples of portions of the abdominal wall were used as controls. A full-thickness section of the abdominal wall (8 cm x 12 cm), excluding the skin, was surgically excised to create a hernia defect. A similar-sized piece of silicone sheeting (BioPlexus Corporation, Saticoy, CA) was sutured to the fascial edges with monofilament suture to effect abdominal wall closure. The midline skin incision was closed in two layers to cover the biomaterial and thus complete abdominal wound closure. Each animal was housed for 14 days after surgery and received water and food ad libitum.

On days 2, 4, 7, 9, 11, and 14 after surgery, following sedation, each animal was placed supine, and abdominal ultrasonography was performed with an Acuson Ultrasound Imager with 7.5-MHz linear array and 3.5-MHz sector probe (Acuson Corporation, Mountain View, CA). At each time point granulation tissue thickness was measured in millimeters with a cursor at multiple, consistent sites. A sample of wound fluid located in the compartment between the skin and silicone sheeting was percutaneously aspirated with a 23-gauge needle under sterile conditions. Wound and peritoneal fluid samples were centrifuged at 4°C at 5000 g for 10 minutes. The supernatants were stored at -80°C until assayed for total protein concentration and immunoblotting.

On postoperative day 14, the animals were sedated, an
infraumbilical diagnostic peritoneal lavage was performed to obtain pre-sacrifice peritoneal fluid for assay, and euthanasia was induced with pentobarbital. A renal biopsy was performed to obtain kidney tissue for control of molecular analysis and the abdominal wall was harvested en bloc. Histological and molecular analyses were performed on skin, kidney, peritoneal fluid, local wound fluid, and wound granulation tissue.

**Epo, EpoR, and βcR Western blot analysis.** Standard tissue lysates (skin, kidney, granulation tissue) were prepared for Western blotting from pulverized and homogenized frozen tissue. Lysate buffer (255 mM sucrose, 2 mM EDTA, and 10 mmol Tris-hydrogen chloride; pH 7.4) in the presence of protease inhibitors was used. Wound fluid, peritoneal fluid, and tissue homogenate supernatants were analyzed for protein concentration using the bicinchoninic acid method (Pierce, Rockford, IL). Proteins were separated on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to PVDF membrane with a wet-blotting apparatus (Trans-blot; Bio-Rad, Hercules, CA) for 60 minutes. The blots were allowed to air dry for 30 minutes and blocked with 5% nonfat dry milk diluted in TRIS-buffered saline (blocking buffer) for 1 hour at room temperature. The blots were then incubated with primary antibodies (anti-Epo, H-162, 1:200; anti-EpoR, H-194, 1:200; and anti-IL-3/IL-5/GM-CSFRβ, H-300, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in blocking buffer overnight at 4°C followed by two washes with blocking buffer at room temperature. The blots were then incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit antibody, sc-2214; EpoR, K-562 whole cell lysate, sc-2203; and cR, THP-1 cell lysate, sc-2238; Santa Cruz Biotechnology) were loaded into the initial lane of each Western blot. The specific bands were detected by using the enhanced chemiluminescence system (ECL; Pharmacia Corp., Piscataway, NJ).

The peritoneal and wound fluid samples were quantified by scanning densitometry using a bioimage analysis system (SigmaScan, SyStat Software Inc., San Jose, CA). An equal quantity of porcine protein (50 µg) was loaded into each lane. Densitized values of the immunoblot were normalized to the density of β-actin and expressed as a ratio to ensure comparable loading and integrity of proteins in each lane.

**Immunohistochemical tissue staining.** Serial sections (4 to 5 µm) of formalin-fixed, paraffin-embedded tissue were dewaxed in xylene, taken through a graded alcohol, and then hydrated in a phosphate-buffered saline (PBS) solution. Sections were treated with DAKO Target Retrieval Solution (S1700; DAKO Corporation, Carpinteria, CA) and steam heated for 30 minutes to improve antigen retrieval. The sections were incubated for 20 minutes with 0.75% hydrogen peroxide in methanol to block endogenous peroxidase activity, and were then washed and incubated with DAKO Protein Block serum-free for 30 minutes to reduce nonspecific binding, and also incubated with a primary antibody for 30 minutes at room temperature in a humidified chamber. Next, the sections were incubated with a secondary antibody with DAKO Envision Plus System and DAB kit according to the manufacturer's instructions. The sections were counterstained with Vector Hematoxylin QS (Vector Laboratories, Burlingame, CA) with quick immersion. The 3, 3′-diaminobenzidine (DAB) substrate-chromogen resulted in a brown-colored precipitate at the antigen site. For confirmation of the specificity of the immunohistochemical staining, secondary antibody only, normal rabbit IgG, and antigen excess stainings with blocking peptides or protein were performed. Primary antibodies used were: anti-Epo (H-162, 1:50) rabbit polyclonal (Santa Cruz Biotechnology); anti-EpoR (H-194, 1:50) rabbit polyclonal (Santa Cruz Biotechnology); and anti-IL-3/IL-5/GM-CSFRβ (H-300, 1:50) rabbit polyclonal (Santa Cruz Biotechnology).

**Statistical analysis.** Data are expressed as means ± standard error of the mean (SEM) in each group. Differences in Epo concentrations between peritoneal and serial wound fluids were determined with repeated-measures analysis of variance with Tukey significant difference test used for post hoc analysis. Differences among means were considered significant at $P < 0.05$.

**Results**

**Development of wound fluid compartment and ultrasonographic determination of granulation tissue thickness.** In previous studies, we observed that vigorous granulation tissue developed on both sides of the silicone implant when removed from animals on day 14. On the peritoneal side, the implant was completely enveloped and sealed off from the peritoneal cavity by a “reactive” omentum easily distinguishable from normal omentum by virtue of its opacity, increased thickness, and vasculature. On the subcutaneous side of the sili-
cone, a prominent layer of granulation tissue was present beneath the overlying layer of skin used to cover the implant at initial surgery. A fluid-filled wound compartment developed between the subcutaneous granulation tissue and the sheeting. Sonography, used to serially measure the thickness of the developing granulation tissue, demonstrated a progressive increase in granulation tissue thickness, reaching a maximum of 19 mm ± 2.3 mm at day 14 (Figure 1).

**Epo, EpoR, and βcR protein expression in control skin and granulation tissue.** Immunoblot analysis was utilized to determine the presence of each protein in control skin and day 14 granulation tissue. A single band corresponding to Epo was detected in tissue homogenates of porcine kidney and granulation tissue (Figure 2). The porcine tissue bands were detected at 30 kDa, the documented molecular weight of human Epo, and by an antibody which recognized a known Epo standard (Lane 1). Similar results for EpoR and βcR proteins are illustrated in Figures 3 and 4. Immunoblot analysis of day 0 porcine skin and subcutaneous tissue homogenates, which were used as wound controls, revealed no detectable comparable molecular weight bands.

**Peritoneal and wound fluid concentrations of Epo.** In addition to demonstrating a progressive increase in granulation tissue thickness, serial ultrasonography also demonstrated formation of an enlarging wound fluid compartment distinct from the peritoneal cavity. Therefore, Epo concentration in serial wound fluid samples was compared to its concentration in control peritoneal fluid. Densitometric analysis of serial wound fluids demonstrated a progressive 8-fold increase in soluble Epo concentration over 14 days (Figure 5).

**Immunolocalization of Epo, EpoR, and βcR.** Paraffin sections of control skin, kidney, and subcutaneous granulation tissue were examined microscopically after immunostaining with anti-Epo, anti-EpoR, and anti-βcR antibodies. Epo immunoreactivity was evident in porcine renal tubular cells as a positive porcine control. Examination of day 0 skin and subcutaneous tissue revealed weak immunoreactivity in dermal vasculature and fibroblasts (Figure 6). Day 14 subcutaneous granulation tissue was heavily stained with anti-Epo, especially where this tissue formed the boundary of the wound fluid compartment. The strongest staining was cell-associated, had a granular or punctate character, and was located within cell cytoplasm. Epo immunoreactivity was localized to granulation tissue fibroblasts, macrophages,
smooth muscle cells, and endothelium (Figure 7).

EpoR was immunolocalized to kidney and granulation tissue, but was not evident in control skin. Granulation tissue EpoR immunoreactivity was present in fibroblasts, macrophages, smooth muscle cells, and endothelium (Figure 8). Immunohistochemical analysis revealed βcR immunoreactivity in granulation tissue fibroblasts, macrophages, smooth muscle cells, and endothelium, localization identical to EpoR immunoreactivity (Figure 9).

Tissue sections incubated with rabbit IgG, secondary antibody only, and a premixed solution of each antibody and its associated protein or peptide in molar excess, were unstained (data not shown), confirming the specificity of the immunochemical staining.

Discussion

We report increased temporal expression of soluble Epo associated with a progressive increase in porcine granulation tissue formation. EpoR and βcR expression, spatially localized to cellular constituents of granulation tissue, increased in the wound environment compared to control tissue. Therefore, Epo’s wound effects may be mediated through a heteroreceptor complex comprising both EpoR and βcR.

Wound healing involves a complex interplay of physi-
ologic processes that include inflammation, proliferation and migration of different cell types, angiogenesis, matrix synthesis, and collagen deposition, resulting in re-epithelialization, neovascularization, and formation of granulation tissue.\textsuperscript{15} Impairment of blood supply may be a contributing factor in delayed healing or in chronic wounds such as diabetic foot ulcers, pressure ulcers, and other pathological conditions.\textsuperscript{16,17} Recent advances in the understanding of neovascularization have made granulation tissue a prime target for therapeutic manipulation in wound healing. Efforts have been made to induce or stimulate new blood vessel formation to enhance tissue repair or to reduce the unfavorable tissue effects caused by local ischemia.\textsuperscript{18}

Epo is a hematopoietic factor stimulating the production, differentiation, and maturation of erythroid precursor cells. These effects are mediated by specific binding with its cell surface receptor (EpoR), a type I cytokine receptor that is expressed in erythroid progenitor cells and numerous non-hematopoietic cells. Recombinant human Epo is therapeutically utilized for stimulation of the erythroid lineage to treat the anemia associated with chronic renal failure, HIV infection, cancer chemotherapy, and to reduce allogenic blood transfusion in burn and surgery patients.\textsuperscript{19}

However, in the last decade Epo has emerged as an important cytoprotective cytokine that possesses the ability to protect tissues including brain, heart, and kidney against ischemia and reperfusion injury.\textsuperscript{20–22} Epo receptors have been identified and appear to be widely distributed not only in erythroid precursor cells but also in numerous adult tissues, including most renal cell types, endothelial and smooth muscle cells, cardiomyocytes, and astrocytes, suggesting that Epo may exert autocrine and paracrine functions other than promoting erythropoiesis.\textsuperscript{5–7}

The GM-CSF, IL-3, and IL-5 receptors are all comprised of unique $\alpha$ chains that bind their specific ligands with low-affinity, and a shared $\beta$ chain that alone does not bind ligand, but is essential for high-affinity binding.\textsuperscript{23} Current research has demonstrated that the $\beta$ chain functionally and physically associates with EpoR. This suggests that these cytokine receptors exist as a large supercomplex and offers a molecular explanation for the synergistic effects of IL-3 and GM-CSF with Epo during erythropoiesis.\textsuperscript{24}

Recently, investigators have shown that the cytoprotective effects of Epo are mediated through its binding to heterodimers containing EpoR and $\beta$cR.\textsuperscript{25} Interestingly, carbamylated Epo binds to these heteroreceptors and exerts tissue protective effects, while it does not bind to the classical EpoR and does not stimulate erythropoiesis.\textsuperscript{26} This is the first evidence suggesting that erythropoietin receptors expressed in different tissues are not identical.

Recombinant human Epo is receiving increasing attention as a potential therapy for prevention of injury.
and restoration of function in nonhematopoietic tissues. However, the minimum effective dose required to mimic and augment these normal paracrine functions of Epo in some organs is higher than for treatment of anemia. Notably, in high-risk groups, a dose-dependant risk of adverse effects has been associated with recombinant human Epo administration including polycythemia-hyperviscosity syndrome, hypertension, and vascular thrombosis. 20

Conclusion
The development of compounds that lack the erythropoietic features of Epo but maintain its tissue protective effects, such as carbamylated Epo, may have therapeutic application in wound healing by binding to a heteroreceptor complex composed of EpoR and βcR present in granulation tissue.

Acknowledgement
This study was supported by an American Heart Association Southeast Affiliate Grant (T.R.H.).

References


