Gene therapy for long-term expression of erythropoietin in rats

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ABSTRACT The injection of recombinant erythropoietin (Epo) is now widely used for long-term treatment of anemia associated with chronic renal failure, cancer, and human immunodeficiency virus infections. The ability to deliver this hormone by gene therapy rather than by repeated injections could provide substantial clinical and economic benefits. As a preliminary approach, we investigated in rats the expression and biological effects of transplanting autologous vascular smooth muscle cells transduced with a retroviral vector encoding rat Epo cDNA. Vector-derived Epo secretion caused increases in reticulocytes, with peak levels of 7.8–9.6% around day 10 after implantation. The initial elevation in reticulocytes was followed by clinically significant increases in hematocrit and hemoglobin for up to 11 weeks. Ten control and treated animals showed mean hematocrits of 44.9 ± 0.4% and 58.7 ± 3.1%, respectively (P < 0.01), and hemoglobin values of 15.6 ± 0.1 g/dl and 19.8 ± 0.9 g/dl, respectively (P < 0.01). There were no significant differences between control and treated animals in the number of white blood cells and platelets. Kidney and to a lesser extent liver are specific organs that synthesize Epo in response to tissue oxygenation. In the treated animals, endogenous Epo mRNA was largely downregulated in kidney and absent from liver. These results indicate that vascular smooth muscle cells can be genetically modified to provide treatment of anemias due to Epo deficiency and suggest that this cell type may be targeted in the treatment of other diseases requiring systemic therapeutic protein delivery.

Erythropoietin (Epo) is a 30-kDa glycoprotein hormone that serves as the primary regulator of red cell production in mammals (1, 2). The therapeutic potential for Epo in the treatment of anemia associated with renal failure was demonstrated initially by its administration to anemic uremic rats and sheep (3, 4). The availability of recombinant human Epo provided a major advance in the treatment of anemia in renal failure patients receiving dialysis (5). The attendant dangers of transfusion therapy were eliminated and the quality of life of these patients was significantly improved. This treatment, given two or three times weekly, raises hematocrit and hemoglobin levels and improves cardiovascular status (2, 6).

Adenoviral vectors have been used to achieve in vivo Epo gene transfer (7, 8). Studies of Epo gene transfer using transplantation of transduced cells have included myoblasts in mice (9, 10) and smooth muscle cells in rats (11). Vascular smooth muscle cells provide an attractive target tissue for gene therapy and have been studied by several investigators (12–17). These cells are easily obtained and cultured and can be efficiently infected with replication-defective retroviral vectors and returned to the donor by seeding in natural or synthetic blood vessels (11–14, 18). Since quiescent smooth muscle cells have a low turnover rate, their implants have the potential to survive and provide therapeutic gene expression for years (13, 19). Furthermore, the proximity of the transduced cells to the circulation may increase their therapeutic usefulness, especially for the systemic secretion of hormones. In previous work, we have shown that rat smooth muscle cells will express transduced marker genes for at least 1 year with no evidence of vector inactivation (13). This suggests the use of genetically modified vascular smooth muscle cells for systemic delivery of regulatory proteins. To pursue this potential clinical application of gene therapy, we investigated in rats the secretion of Epo by genetically modified vascular smooth muscle cells.

MATERIALS AND METHODS

Retroviral Vectors. The retroviral vector LrEPSN was made by inserting an EcorRI/BamHI fragment of the rat Epo cDNA into viral plasmid LXSN (20). A plasmid containing the rat Epo gene was kindly provided by Boissell and Bunn (21). The control retroviral vector LASN encoded human adenosine deaminase (ADA) (22).

Cell Culture, Transduction, and Transplantation. Ectropic PE501 and amphotropic PA317 retrovirus packaging cell lines (20, 23), NIH 3T3 thymidine kinase-negative cells (23), and primary cultures of rat smooth muscle cells were grown in Dulbecco-Vogt-modified Eagle’s medium with high glucose (4.5 g/liter) supplemented with 10% fetal bovine serum in humidified 5% CO2/95% air at 37°C.

Rat smooth muscle cell cultures were prepared by enzymatic digestion of the aorta from male Fisher 344 rats. These cells were characterized by positive staining for muscle cell-specific actins with HHF35 antibody (14) while staining negative for von Willebrand factor (14), an endothelial cell-specific marker. Early passage smooth muscle cells were exposed to 16-hr virus harvests from PA317-LrEPSN and PA317-LASN amphotropic virus-producing cell lines for a period of 24 hr in the presence of Polybrene (4 µg/ml). Vascular smooth muscle cells infected with LrEPSN and selected in G-418 antibiotic (1 mg/ml) secreted 6.7 milliunits per 24 hr per 106 cells of Epo. For these assays we used an ELISA procedure constructed to measure human Epo (R&D Systems), which probably underestimated the rat hormone. Biological activity of vector-encoded Epo was confirmed by proliferation of a murine erythroleukemia cell line (HCD-57) sensitive to recombinant human Epo (24). Transduced Epo-secreting smooth muscle cells showed the same growth characteristics as control cells both in vitro and in vivo, indicating the absence of any Epo-mediated autocrine effect (data not shown).

For cell seeding, rats were anesthetized, and the left carotid artery was temporarily isolated with ligatures and denuded of endothelium by passage of a balloon catheter introduced through an arteriotomy in the external branch (12, 13). Transduced vascular smooth muscle cells (106 cells in 50 µl of culture medium) were infused over 15 min into the isolated carotid segment by means of a cannula in the external carotid segment.

Abbreviations: Epo, erythropoietin; ADA, adenosine deaminase; WBC, white blood cell(s).

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after a brief irrigation with culture medium. The external carotid segment was ligated after removal of the catheter, blood flow was restored, and the wound was closed (12, 13). Anticoagulated blood samples (100 μl) were obtained from the tail vein, and reticulocyte count was determined by vital staining with brilliant cresyl blue and counting 1000 cells by standard techniques. Hematocrit, hemoglobin, platelet, and white blood cell (WBC) number were measured with a Coulter Counter.

**Epo mRNA Analysis.** Total RNA was isolated from rat liver and kidney after homogenization in the presence of RNezol. Using Moloney murine leukemia virus reverse transcriptase, 1 μg of total RNA was reverse-transcribed in the presence of random hexamer primers. PCR (35 cycles) was performed with rat Epo-specific primers (5'-AGG CGC GGA GAT GGG GGT GC-3' and 5'-CCC CGG AGG AAG TTG GAG TAG-3') to give a 540-bp amplified segment. An aliquot of the amplified reaction mixture was electrophoresed in a 2% agarose gel and, after Southern transfer, the membrane was hybridized with a 32P-labeled 500-bp Epo cDNA probe. As a control for RNA extraction, reverse transcription, and amplification, actin-specific primers (5'-GTG GGG CGC CCC AGG GAT CA-3' and 5'-CTC TTT AAT GTC AGC CAC GAT TTC-3') were used to amplify a 500-bp fragment from reverse-transcribed RNA.

**RESULTS**

In animals seeded with LrEPSN cells, the reticulocyte counts increased, with peak levels of 7.8–9.6% at about day 10 (Fig. 1A). In comparison, the reticulocyte counts of control animals, which were seeded with cells transduced with a vector encoding human ADA (LASN) or were subjected to balloon injury alone, did not show changes in reticulocyte counts (Fig. 1A). The mean reticulocyte count from 9 control rats was 2.9 ± 0.6%, in agreement with published values, which range from 1.5% to 3.5% (25). Hematocrit and hemoglobin levels in the treated rats gradually increased, reaching peak values at ~3 weeks that were sustained for up to 79 days (Fig. 1B and C). Comparison of blood cell measurements of 10 control and 10 treated rats from day 20 (when peak values were established) showed maintenance of highly significant elevations in the treated group (P < 0.001). The hematocrit had a mean increase of 13.8%, and hemoglobin had a mean increase of 4.2 g/dl (Table 1). WBC and platelet values were not different between the control and treated animals (P = 0.1). The control hematological levels were in agreement with normal rat blood values (25).

To determine whether vector-encoded Epo expression resulted in down regulation of endogenous Epo production (26), test rats were sacrificed when elevated hematocrit and hemoglobin levels were established. At a similar time point, control rats that received LASN-transduced cells also were sacrificed. RNA harvested from liver and kidney was subjected to reverse transcription PCR using rat Epo-specific probes (21). As shown in Fig. 2, endogenous Epo mRNA in the kidney of a rat seeded with LrEPSN-transduced cells was greatly reduced in comparison to a control kidney. Also, the hybridization signal was below the level found in control liver, the secondary tissue for Epo production. Epo mRNA was undetectable in treated rat liver. These results were confirmed in three other treated rats (data not shown). Southern band intensities from actin mRNA amplification of test and control tissues were similar, indicating equivalence in RNA isolation and amplification (data not shown). The large difference in hybridization signal between kidney and liver in control rat reflects the major and minor contribution, respectively, of these tissues to Epo biosynthesis.

To estimate the number of seeded transduced cells, we determined the secretion of Epo from carotid arteries removed from rats showing elevated blood counts. Two rats that received LrEPSN-transduced cells and two LASN cell seeded controls were sacrificed 2 months after transplantation. Their
carotid arteries were cultured and Epo secretion was measured by an ELISA constructed for human Epo measurement, which probably underestimates rat hormone. A mean Epo secretion of 8.3 milliliters per 24 hr was measured from the LrEPSN carotid arteries; Epo was not detectable from the controls, consistent with kidney and liver as the only source of Epo biosynthesis (26). Thus, based on the preimplantation level of 6.7 milliliters of Epo secreted from 10^5 cultured transduced cells, we estimated that the seeded carotid arteries contained \( \sim 1.2 \times 10^5 \) Epo-secreting cells, representing 10% of the cell number incubated in the seeding procedure. These data indicate a relatively efficient seeding procedure that results in a cell mass capable of providing sustained gene delivery at therapeutically significant levels.

**DISCUSSION**

Kidney and to a lesser extent liver are the specific organs that synthesize Epo in response to tissue oxygenation (1, 26, 27). In treated rats, the level of Epo expression we achieved resulted in significant down regulation of endogenous Epo biosynthesis in kidney and total suppression in liver, indicating that the red blood cell production we induced was mediated by vector-encoded Epo secretion.

In these experiments, the viral long terminal repeat promoter in LrEPSN virus expressed unregulated production of Epo. However, control elements from the human Epo gene have been defined (28-31) and if incorporated in a retroviral vector may allow delivery of a regulated supply of Epo. Hypoxia regulates Epo synthesis primarily through the rates of gene transcription in the kidney (1). The hypoxia-responsive cis elements of the Epo gene are localized to the 3′ region (29-35) and have been shown to interact with the Epo promoter (31). Of potential interest is the fact that the oxygen-sensing system initially identified in Epo-producing cells has been found in a wide variety of cell types (33-35). However, these up regulatory elements may not be useful with our vector construct, where Epo expression was driven by the strong viral long terminal repeat promoter. We propose, rather, that genetic elements to control potential overproduction of Epo and abnormally high hematocrits would be more appropriate (i.e., negative-regulatory elements), and these are currently not defined in a size suitable for insertion into a retroviral vector (36).

We observed no significant differences in WBC and platelet numbers between treated and control rats, indicating that constitutive vector-encoded Epo secretion enhanced red cell production without other hematological effects. A previous study reported decreased platelet counts in mice receiving large, chronic injections of Epo (37). However, the majority of human studies report no significant changes in leukocyte or megakaryocyte production from the long-term administration of recombinant Epo (5, 24, 38).

The constitutive level of Epo we achieved in this study would provide useful therapy for patients with renal failure. Although arterial seeding is not feasible in human subjects, we have recently shown in baboons that prosthetic vascular grafts can be used as a device to implant transduced cells (14). From the data produced in this rat model and our studies in dogs and baboons (14, 18), we estimate that 10^6 transduced vascular smooth muscle cells can provide a therapeutic dose of Epo to an 80-kg patient, and this cell number could be transplanted in a 10 mm \( \times \) 4 mm prosthetic graft. Such grafts are frequently used to provide dialysis access for patients with renal failure (2) and could be readily seeded with genetically modified autologous smooth muscle cells to secrete Epo. The estimated annual cost of recombinant Epo for the 85,000 patients in the United States with anemia of end-stage renal disease is in excess of $500 million (39). The ability to treat these patients, and others with Epo-responsive anemias, by gene therapy would provide major clinical and economic benefits.

These studies have demonstrated that gene therapy targeted at vascular smooth muscle cells may provide a useful approach to the treatment of anemias due to Epo deficiency and other diseases, such as the hemophilies, that are responsive to the administration of regulatory proteins.

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**Table 1. Control and treated rat blood values**

<table>
<thead>
<tr>
<th></th>
<th>Hb, g/dl</th>
<th>Hct, %</th>
<th>WBC per µl, ( \times 10^3 )</th>
<th>Plt per µl, ( \times 10^3 )</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.6 ± 0.1 *</td>
<td>44.9 ± 0.4 †</td>
<td>8.33 ± 0.76 †</td>
<td>708 ± 36 †</td>
</tr>
<tr>
<td>LrEPSN</td>
<td>19.8 ± 0.9 *</td>
<td>58.7 ± 3.1 †</td>
<td>7.83 ± 0.61 †</td>
<td>654 ± 10 †</td>
</tr>
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Hct, hematocrit; Plt, platelets; n = 10.

*, P < 0.001.
†, P > 0.1.

**Fig. 2.** Epo mRNA analysis. Total RNA was isolated from kidney and liver of rats receiving Epo-expressing LrEPSN-transduced cells and control rats receiving LASN-transduced cells expressing human ADA. Reverse transcription (RT) PCR was performed with rat Epo-specific primers to give a 540-bp amplified segment that was subjected to electrophoresis and hybridized with a 32P-labeled Epo probe.