Gene therapy for renal anemia in mice with polycystic kidney using an adenovirus vector encoding the human erythropoietin gene

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Gene therapy for renal anemia in mice with polycystic kidney using an adenovirus vector encoding the human erythropoietin gene.

**Background.** Recombinant human erythropoietin (rHuEPO) is primarily used for patients with anemia associated with end-stage renal disease. We evaluated the efficacy of EPO gene therapy using adenovector for chronic renal failure mice expressing severe renal anemia.

**Methods.** Recombinant HuEPO gene transfer to mesothelial cells was performed in vitro and in vivo. Recombinant replication-deficient adenoviruses containing rHuEPO cDNA (AdCMVEPO), E. coli lacZ gene (AdCMVlacZ), or a nonexogenous gene (AdNull as control vector) driven by the cytomegalovirus promoter/enhancer were constructed. The oligosaccharides associated with the rHuEPO from AdCMVEPO-treated mesothelial cells were analyzed. For in vivo study, the DBA/2FG-pcy mouse, a model for human autosomal recessive combined immunodeficient mice [11-13].

**Results.** The sialylated oligosaccharides associated with the rHuEPO produced in AdCMVEPO-treated mesothelial cells occupied 78 ± 0.7% of the total oligosaccharide pool. A single intraperitoneal administration of AdCMVEPO induced rHuEPO synthesis in the peritoneal cells and a marked increase in erythrocyte production. The maximal increase in hematocrit (43 ± 4%) was observed on day 28, and it remained elevated for 40 days.

**Conclusion.** These results indicate that intraperitoneal administration of AdCMVEPO improves renal anemia in mice with chronic renal failure and that the mesothelial cell is an appropriate target cell for gene transfer.

**Key words:** recombinant human erythropoietin, mesothelial cell, DBA/2FG-pcy mouse, gene therapy, end-stage renal disease, polycystic kidney disease.

Erythropoietin (EPO) synthesized in renal peritubular cells in response to hypoxia regulates erythropoiesis in the bone marrow [1-3]. The activity of EPO has been reported to depend on the number of sialic acid residues [4, 5]. Intravenous or subcutaneous injection of recombinant human EPO (rHuEPO) once to three times weekly results in an improved quality of life for patients with end-stage renal disease [6-8]. As an alternative persistent EPO therapy, implantation of genetically modified cells secreting rHuEPO has been applied in nephrectomized nude mice and other experimental animals [9, 10]. Replication-deficient adenovirus vectors have also been used for in vivo transfer of rHuEPO genes into the peritoneal cells of normal rats or into the muscle cells of severe combined immunodeficient mice [11-13].

For gene therapy studies, appropriate experimental animal models play a key role in the evaluation of physiological effects of transgene expression [14]. In this study, we used DBA/2FG-pcy mice with chronic renal failure and severe anemia for the assessment of EPO gene therapy. The DBA/2FG-pcy mice have been established as a good model for polycystic kidney disease. These animals develop progressive anemia caused by chronic renal failure [15, 16]. We have previously reported that stimulation of erythropoiesis by intraperitoneal administration of a replication-deficient adenovirus bearing the rHuEPO gene is stronger than that by subcutaneous administration in experimental animals with intact renal function. In this study, we assessed the bioactivity and excretion of transgene-encoded protein in mesothelial cells and their appropriateness as target for in vivo gene transfer in uremic animals. Our results demonstrated that mesothelial cell-mediated EPO gene therapy may provide a new development in the treatment of end-stage renal disease.
METHODS

AdCMV-EPO construction

A recombinant replication-deficient adenovirus bearing the rHuEPO cDNA (AdCMVEPO; kindly provided by Genetics Institute Co., Cambridge, MA, USA), the E. coli lacZ gene (AdCMVlacZ), and nonexogenous gene (AdNull as control vector) driven by the cytomegalovirus promoter/enhancer was constructed as described previously [12]. Briefly, these three vectors were propagated in the human embryonic kidney cell line 293 (ATCC CRL-1573; ATCC, Rockville, MD, USA), purified by cesium chloride density gradient centrifugation, dialyzed, and stored in multiple aliquots at −80°C. Viral titers were determined by plaque assay on 293 cells.

In vitro adenovirus-mediated gene transfer

A rat mesothelial cell line, 4/4RM-4 (ATCC CRL-216) and a Chinese hamster ovary (CHO) cell line (ATCC CRL-1793) were prepared to evaluate the suitability of AdCMVEPO for synthesis of rHuEPO in vitro. AdCMVEPO, AdNull (5 × 10^8 pfu) or phosphate-buffered saline (PBS) was added to mesothelial cells (1 × 10^6 cells) in serum-free Ham’s F12K medium (Sigma, St. Louis, MO, USA) or to CHO cells (1 × 10^6 cells) in serum-free Ham’s F12K medium or to CHO cells (1 × 10^6 cells) in serum-free α-modified Eagle’s medium (MEM; GIBCO-BRL, Gaithersburg, MD, USA). The secretion of rHuEPO in AdCMVEPO-infected mesothelial cells and in CHO cells was determined by radioimmunoassay using rabbit antibody against epoetin α (Recombigen EPO kit; Japan DPC, Tokyo, Japan) [17].

To evaluate the glycosylation of EPO, AdCMVEPO-infected mesothelial cells were treated with or without tunicamycin (2.5 μg/ml; Sigma), an inhibitor of glycosylation. Forty-eight hours after inoculation, the supernatant was collected. The molecular weight of rHuEPO secreted from AdCMVEPO-infected mesothelial cells was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. Three units of recombinant rHuEPO produced by modified CHO cells (EPOCH®; Chugai Pharmaceutical Co., Tokyo, Japan) as a positive control and the those secreted from AdCMVEPO-infected mesothelial cells were electrophoresed in 12.5% sodium dodecyl sulfate polyacrylamide gel. Proteins fractionated by electrophoresis were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Tokyo, Japan). Immunoblot analysis was performed using 1 μg/ml mouse anti-rHuEPO antibody (Genzyme, Cambridge, MA, USA). Antibodies bound to the proteins on the membrane were detected with horseradish peroxidase-conjugated goat antimouse IgG (1:1000; Zymed Inc., San Francisco, CA, USA) and were visualized by an ECL Western blot analysis kit (Amersham, Buckinghamshire, UK).

Analysis of oligosaccharides associated with human erythropoietin produced by AdCMVEPO-treated 4/4RM-4 cells

A 400 ml supernatant of the cultured medium of AdCMVEPO-treated 4/4RM-4 cells was collected and concentrated with YM10 (Amicon, Beverly, MA, USA). An approximately 20 μg glycoprotein sample was subjected to rigorous preparation. After exhaustive microflow dialysis against 0.1% trifluoroacetic acid, the lyophilized sample was eluted on GlycoPrep™ 1000 (Oxford GlycoSystems, London, UK), and the released oligosaccharides were recovered. The sample was fluorescently labeled by reductive amination with 2-aminobenzamide, applied to a Whatman 3 MM disc (Whatman International, Maidstone, UK), and subjected to chromatographic purification. The 2-aminobenzamide–labeled oligosaccharide pool was subjected to high-performance liquid chromatography (HPLC) on a GlycoSepC column (Oxford GlycoSystems) using acetonitrile and ammonium acetate as the eluent. To determine the characteristics of the acidic substituents of the oligosaccharides, an aliquot of the total pool of fluorescently labeled oligosaccharides was incubated with neuraminidase and then subjected to GlycoSepC chromatography.

In vivo adenovirus-mediated gene transfer

To evaluate the adenovirus-mediated in vivo gene transfer, AdCMVlacZ, AdNull (10^8 pfu/animal), or saline was administered to the peritoneal cavity of 20- to 22-week-old male or female DBA/2FG-pcy mice (N = 5 in each group) with uremia (BUN more than 60 mg/dl) and severe anemia (Hct less than 25%). These mice were provided by the Laboratory Animal Center (Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Japan). Twenty-four hours after the administration, the mice were anesthetized by diethyl ether inhalation (Wako Chemicals, Tokyo, Japan) and killed. The peritoneal tissues were fixed in 5% formaldehyde and 0.5% glutaraldehyde/PBS at 4°C for 30 minutes. The tissues were stained with 5 mM K3Fe(CN)6, 2 mM K4Fe(CN)6, and 0.5% glutaraldehyde/PBS at 4°C for 30 minutes. The tissues were stained with 5 mM K3Fe(CN)6, 2 mM MgCl2, and 200 mg/ml X-Gal reagent (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Boehringer Mannheim, Mannheim, Germany) for one hour at 37°C as reported previously [18]. The tissues were identified as positive for β-galactosidase activity by the blue stain of the X-Gal reaction [18].
lected, and total RNA was extracted by the phenol/chloroform purification method as described previously [19]. Recombinant human EPO mRNA in the peritoneum was analyzed by Northern blotting using 10 μg of total RNA hybridized with a random-primed 32P-labeled rHuEPO cDNA. For control, the same membrane was subsequently hybridized with a mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe labeled in the same fashion.

To assess the effects of intraperitoneal administration of AdCMVEPO on red blood cell production, DBA/2FG-pcy mice were killed on days 0, 21, 28, and 40. Fifty microliters of blood were collected on each day, and the hematocrit was measured.

Serum rHuEPO levels were measured by radioimmunoassay using the same antibody as in the in vitro study. Blood cells were prepared to count erythroid precursors in bone marrow from the femur and to count reticulocytes in peripheral blood. For each mouse, bone marrow samples stained with Wright-Giemsa and peripheral blood samples stained with 0.5% methylene blue were prepared in duplicate. Bone marrow and peripheral blood smears were examined by light microscopy to determine the proportion of erythroid precursors among the nucleated bone marrow cells (a minimum of 500 cells counted per animal) and the proportion of reticulocytes among red blood cells (a minimum of 500 cells per animal). Each sample was prepared in duplicate.

Statistical analysis

Results are expressed as mean ± sd. Statistical analysis was performed using analysis of variance, with P < 0.05 accepted as statistically significant. For hematocrit analysis, three groups were cross compared using Bonferroni’s method.

RESULTS

In vitro adenovirus-mediated gene transfer

Recombinant human EPO (54,300 ± 8100 mU/ml) was synthesized in 4/4RM-4 mesothelial cells (1 × 10^6) 48 hours after the inoculation with AdCMVEPO (5 × 10^7 pfu), which is significantly higher than 5440 ± 420 mU/ml rHuEPO synthesized in CHO cells (P < 0.001). In contrast, the 4/4RM-4 cells treated with PBS or with AdNull produced little rHuEPO (<10.0 mU/ml).

Western blot analyses of rHuEPO in the supernatant of 4/4RM-4 cells infected with AdCMVEPO showed the presence of 30.3 kDa rHuEPO, which was the same molecular weight as recombinant HuEPO produced in the modified CHO cells (EPOCH®). In the presence of 2.5 mg/ml tunicamycin, AdCMVEPO-infected 4/4RM-4 cells synthesized rHuEPO with two different molecular weights: approximately 21 and 22 kDa. These smaller bands show EPO with insufficient glycosylation. The 4/4RM-4 cells treated with PBS or with AdNull as the control vector did not show any bands specific for rHuEPO (Fig. 1).

The relative molar content of neutral and acidic oligosaccharides in the total pool was determined by integration of chromatographic peaks (data not shown). Anion exchange chromatography showed that oligosaccharides associated with rHuEPO produced by AdCMVEPO-treated 4/4RM-4 cells consisted of 22 ± 1.0% of neutral components and 78 ± 0.7% of acidic components. After incubation with neuraminidase, no acidic oligosaccharides were detected by chromatography (data not shown).

In vivo adenovirus-mediated gene transfer

AdCMVlacZ, AdNull as the control vector, or PBS as control was administered to the peritoneal cavity of DBA/2FG-pcy mice (Fig. 2A, a-c). Twenty-four hours after the administration of AdCMVlacZ, blue coloration by X-gal staining was observed only in the visceral and parietal mesothelial cells, as reported previously [20].
Fig. 2. In vivo gene transfer to mouse peritoneal mesothelium. (A) In vivo transfer of the E. coli lacZ gene to DAB/2FG-pcy mouse peritoneum. Two days after AdCMVEPO was administered intraperitoneally, the peritoneum was examined to assess the presence of the lacZ product (β-galactosidase) by using X-Gal stain (Blue staining). (a) Peritoneal section covering the surface of the abdominal muscle from a mouse infected with AdCMVLacZ demonstrates β-galactosidase expression in mesothelial cells. (b) Peritoneal section from a mouse infected with the control vector AdNull. (c) Peritoneal section from a mouse treated with phosphate-buffered saline (×400). (B) Hematoxylin-eosin staining of peritoneal section from a mouse infected with the control vector AdNull (×400). No toxic changes due to administration of adenovirus and no inflammatory changes such as cell infiltration or sclerotic changes were observed in the peritoneum.

No other cells accessible to the virus in the peritoneum showed lacZ gene expression. The administration of AdNull or PBS resulted in an absence of blue coloration by X-gal staining. No inflammatory changes, including cell infiltration and fibrotic changes, and no toxic changes caused by adenoviral administration were observed in the histology of the peritoneum obtained from AdNull- or AdCMVEPO-inoculated mice (Fig. 2B).

Northern blot analysis using rHuEPO cDNA showed rHuEPO cDNA transcripts in the peritoneum of mice treated with AdCMVEPO (Fig. 3, lane 3). In contrast, no rHuEPO cDNA transcripts were detected in the peritoneum of uninfected mice or AdNull-infected mice (Fig. 3, lanes 1 and 2). Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was similar in each sample (Fig. 3, lanes 4–6).

Figure 4 shows serum rHuEPO levels measured on days 0, 10, 21, 28, and 40 after intraperitoneal administration of 10^8 pfu AdCMVEPO, AdNull, or PBS. The intraperitoneal administration of AdCMVEPO resulted in mean serum rHuEPO levels of 2559 mU/ml for 40 days, with a maximum level of 14,730 ± 930 mU/ml on day 10 after administration. Serum rHuEPO levels remained high with a mean level of 154 mU/ml on day 40. However, in control mice treated with PBS or AdNull, serum rHuEPO levels were less than 10.0 mU/ml.

Erythroid precursors within the bone marrow of the untreated mice were 13 ± 1% of all nucleated hematopoietic cells, whereas they were reached 24 ± 1% on day 4 after treatment with AdCMVEPO (P < 0.001) and decreased thereafter. Reticulocytes in untreated mice were 1.5 ± 0.2% of the total circulating red blood cells. However, reticulocytes in mice treated with AdCMVEPO reached 20 ± 3.4% on day 21 (P < 0.001) and decreased to 5% by day 40. In control mice treated with PBS or AdNull, no significant changes were noticed in the number of reticulocytes (Fig. 5). The hematocrit was also increased significantly in mice treated with
AdCMVEPO on days 28 and 40 compared with that in control mice treated with PBS or AdNull. Before the administration of AdCMVEPO, the mean hematocrit level was 24 ± 3% at 20 weeks. Following a single administration of AdCMVEPO, the average hematocrit reached 43 ± 4% on day 28 and remained elevated on day 40 (42 ± 5%). Thus, the hematocrit levels increased twofold by the intraperitoneal administration of the adenovirus vector containing rHuEPO cDNA (Fig. 6).

DISCUSSION

The bioactivity and the regulation of the transgene-encoded protein were not examined in our previous study [12, 20]. Glycosylation depends on host cells and the peptide structure of glycoprotein [21]. This study showed that mesothelial cells modified by AdCMVEPO synthesize bioactive rHuEPO with nearly the same glycosylation pattern as EPOCH [448]. In our in vitro study, the mesothelial cells transfected with AdCMVEPO secreted 10-fold more glycosylated rHuEPO than the modified CHO cells transfected with AdCMVEPO.

Tunicamycin, an inhibitor of N-linked glycosylation, was previously reported to abolish the secretion of EPO in the human hepatoblastoma cell line [22]. In this study, AdCMVEPO-infected 4/4RM-4 cells with tunicamycin synthesized rHuEPO with two different smaller molecular weights. This suggests that AdCMVEPO-infected mesothelial cells synthesize glycosylated rHuEPO. The terminal sialic acid residues in the carbohydrate chains are essential for the bioactivity of rHuEPO in vivo [4, 5]. In this study, all acidic components were sialylated, and the sialylated saccharides made up 78% of the total oligosaccharides of rHuEPO produced by AdCMVEPO-treated mesothelial cells.

The advantage of using DBA/2FG-pcy mice in EPO gene therapy for renal anemia is that they provide a good model of polycystic kidney disease with eventual severe anemia [16, 23, 24]. The anemia in DBA/2FG-
Fig. 6. The increase in hematocrit from the baseline for each mouse administered AdCMVEPO, AdNull, or phosphate-buffered saline (PBS) intraperitoneally. Data are shown after the intraperitoneal administration of AdCMVEPO (●), AdNull (○), or PBS (△), and are means ± se. *P < 0.05; **P < 0.01.

Difficulties, immunosuppressive agents were used or E1/E4-deleted adenovirus vectors and E3-expressed adenovirus vectors were constructed [27, 28]. The construction of a new generation of adenovirus vector with lower immunogenicity is now in progress in our laboratory. There were some nonresponding animals in our study. Some possible explanations include a different host immunologic response to the foreign transgene, instability of the efficiency of adenovirus-infection to peritoneum in vivo.

There are two major advantages in using adenovirus-mediated in vivo gene transfer. The transfer of exogenous genes is simple: a single injection of replication-deficient adenovirus vector. No effort is required to control overexpression of the transgene in terms of transient expression. An ex vivo gene therapy approach is rather laborious because it requires the isolation, growth, and transfection of primary human myoblasts from each patient to be treated.

Our results demonstrate that a single intraperitoneal administration of rHuEPO cDNA with an adenovirus vector can produce a physiologically effective expression of recombinant human erythropoietin that promotes erythropoiesis and can ameliorate progressive renal anemia successfully in DBA/2FG-pcy mice with chronic renal failure. This study also suggests that the mesothelial cell may be an appropriate target cell for gene transfer.

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