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Ligand-regulatable erythropoietin production by plasmid injection and in vivo electroporation

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Ligand-regulatable erythropoietin production by plasmid injection and in vivo electroporation.

Background. The development of an in vivo gene transfer approach to deliver physiologic levels of recombinant proteins to the systemic circulation would represent a significant advance in the treatment of protein deficiency disorders. However, the ability to regulate transgene expression is of paramount importance for safe and effective gene transfer therapy.

Methods. We developed two plasmids, one encoder of chimeric GeneSwitch protein, and the other an inducible transgene for human erythropoietin (Epo). The level of secretion of Epo into the serum was modulated by intraperitoneal administration of mifepristone (MFP). Rats were divided into four groups: one group administered Epo plasmid with MFP for 50 days, a second group administered Epo plasmid with MFP for 15 days and then again from day 30 to day 50, a third group administered Epo plasmid without MFP, and a fourth group administered control plasmid. A pair of electrodes was inserted into the muscle of the right thigh, 100 mg of each plasmid was injected, and in vivo electroporation (8 pulses at 100 V for 50 msec) was performed.

Results. The presence of vector-derived Epo mRNA was detected by RT-PCR only in the Epo plasmid and MFP(+) groups. The hematocrit levels increased continuously, from the pre-injection level of 41.2% to 55.0% on day 30 and 53.8% on day 50 in the Epo plasmid and MFP(+) groups. In the MFP re-challenged group, the hematocrit levels rose up to day 15, fell after 20 to 30 days, and then rose again after MFP re-administration. The serum Epo levels increased only in the Epo plasmid and MFP(+) groups. There were no significant changes in hematocrit levels and Epo levels in the Epo plasmid and MFP(−) group.

Conclusion. Epo gene transfer with the GeneSwitch system by in vivo electroporation is a useful procedure for efficient drug-regulated delivery of Epo.

Key words: gene therapy, plasmid, mifepristone, renal failure, electroporation, protein deficiency disorders, glycoprotein, GeneSwitch system.

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Erythropoietin (Epo) is a 30-kD glycoprotein synthesized in adult renal cells and functions as the major regulator of mammalian erythropoiesis [1]. Serum human erythropoietin (hEpo) levels are controlled transcriptionally in response to tissue hypoxia or anemia [2]. The secreted protein increases erythrocyte production by stimulating the proliferation and preventing the apoptosis of erythroid precursors. Most patients with end-stage renal disease (ESRD) who are treated with hemodialysis suffer from severe anemia resulting from inappropriately low levels of serum hEpo [3]. These patients are currently treated with repeated intravenous or subcutaneous infusion of recombinant hEpo [3]. The Epo-responsive anemia represents an excellent model for studies designed to develop an in vivo gene transfer approach for the treatment of serum protein deficiencies.

The permanent and regulated systemic delivery of therapeutic proteins from engineered tissue appears to be one of the most attractive goals of gene therapy. Different strategies can be considered for installing a source of recombinant protein in a mammalian organism. Intramuscular adenovirus injection has been reported to effectively induce Epo in rats. [4–7]. The autologous cells previously engineered in vitro by retrovirus-mediated gene transfer can be engrafted into syngeneic recipients. This technique has been used to achieve prolonged Epo delivery in mice [8–10]. Several novel methods recently have been used to regulate the expression of therapeutic hormones such as growth hormone and Epo over long periods. In subjects treated by an intramuscular injection of adeno-associated vector, doxycycline was found to adequately control Epo production [11]. Stable long-term delivery of Epo and growth hormone was developed under pharmacological control of rapamycin using two adeno-associated virus vectors [12, 13]. Several reports demonstrated that adenovirus vector induced an efficient expression of Epo in immunodeficient mice [14]. However, the immunoresponse to adenovirus prevents long and stable gene expression in immunocompetent mice [13].

Recently, an improved helper-dependent adenoviral vector allowed persistent gene expression after intramuscular delivery [15]. Naked DNA of Epo injection and muscle electroporation have been reported to cause efficient Epo production and elevation of hematocrit [16]. However, regulation of Epo production is not well controlled by adenovirus-mediated gene transfer or plasmid injection with in vivo electroporation. The ability to regulate transgene expression will become paramount for the safety and efficacy of gene transfer therapy. The GeneSwitch system for ligand-dependent transgene regulation is based on the unusual properties of a C-terminal truncation of the ligand-binding domain (LBD) of the human progesterone receptor [17]. A ligand-dependent, site-specific transcription factor is generated by linking the modified LBD to a heterologous DNA-binding domain (GAL4) and a human transcription activation domain [nuclear factor-κB (NF-κB) p65]. The chimeric protein is activated by antiprogesterone to form a homodimer, which binds to the promoter of a target gene with GAL4 consensus sequence and activates its transcription. In this report we describe the construction of an efficient and ligand-regulatable Epo production system using this GeneSwitch system with naked plasmid and in vivo electroporation.

METHODS

GeneSwitch system plasmid construction

We constructed a mifepristone (MFP)-inducible hEpo expression system, the GeneSwitch system, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). An inducible expression plasmid, pGene/V5, was under the control of a hybrid promoter containing GAL4 upstream activating sequences (UAS) and the adenovirus E1b TATA box. We inserted a full-length BamH1-Not1 fragment of the coding region of hEpo into this inducible expression plasmid (pGene/V5-hEpo). A regulatory plasmid, pSwitch, which encodes a fusion protein consisting of the yeast GAL4 DNA binding domain (DBD), a truncated human progesterone receptor ligand binding domain (hPR-LBD), and the human p65 activation domain (AD) from NF-κB, was used for in vivo electroporation (Fig. 1).

The GeneSwitch system contains a hybrid regulatory protein containing a DBD from the yeast GAL4 protein, a truncated LBD from the human progesterone receptor, and AD from the human NF-κB protein. This hybrid regulatory protein binds to the synthetic steroid, MFP, and functions as a ligand-dependent transcription factor to induce the expression of hEpo. As shown in Figure 1, in the presence of MFP, the GeneSwitch protein, GAL4-DBD-hPR-LBD, binds to MFP and acts as a transcription factor for the pGene/V5-hEpo. In the absence of MFP, GAL4-DBD-hPR-LBD does not bind to the GAL4 UAS site of pGene/V5-hEpo plasmids, and does not stimulate the transcription of hEpo.

In vitro transfection and hEpo assay in LLC-PK1 cells

LLC-PK1 cells originally purchased from the American Type Culture Collection (Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 50 IU/mL penicillin, 50 μg/mL streptomycin, and 10% heat-inactivated fetal calf serum (FCS; Gibco). Cells were cultured at 37°C in 5% CO_2. LLC-PK1 cells were transfected with plasmid DNAs [15 μg of pGene/V5-hEpo or pGene/V5 (control) plus 15 μg of pSwitch] by the electroporation method as previously described [18]. After 24 hours to 14 days, cell culture supernatants were assayed for secreted hEpo using radioimmunoassay (RIA; Amersham Pharmacia Biotech, Buckinghamshire, UK).

Intramuscular plasmid injection and electroporation

Male Sprague-Dawley rats weighing 100 to 150 g were anesthetized with pentobarbiturate sodium. The medial side of the right thigh was used for in vivo electroporation. A 1-cm incision was made in the skin and the muscle was exposed. Plasmid DNAs (100 μg of pGene/V5-hEpo and 100 μg of pSwitch) were injected intramuscularly with a 27-gauge needle into the central portion of the two needle electrodes, and electric pulses were immediately delivered to the muscle. Because electroporation with eight electric pulses of 100 V at 50 msec apart has been reported to induce efficient gene transfer [16], this protocol was used here. The electric pulse was delivered by an electric pulse generator (Electro Square Porator T820; BTX, San Diego, CA, USA) and monitored by a graphic analyzer (MVC540r; BTX). The electrodes consisted of a pair of stainless steel needles measuring 8 mm in length and 0.4 mm in diameter, with a fixed distance of 4 mm between them. Blood samples (200 μL) were collected at the indicated days.

Western blot analysis

Cells and muscle tissues were lysed in sodium dodecyl sulfate (SDS) sample buffer [50 mmol/L Hepes, pH 7.5, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L egtazic acid (EGTA), 10% glycerol, 1% Triton X-100, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.1 mmol/L sodium orthovanadate] at 4°C. After incubation for five minutes, lysates were centrifuged at 4°C for 15 minutes at 10,000 × *g*. The soluble lysates were mixed at a 1:4 ratio with 5× Laemmli buffer, heated for five minutes at 95°C, loaded into lanes (30 μg per lane) and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 5 and 20% acrylamide for stacking and resolving gels, respectively. Protein was transferred to nitrocellulose (pore size 0.45 μm; Schleicher and Schuell, Keene, NH, USA) and probed with polyclonal antibodies against hEpo

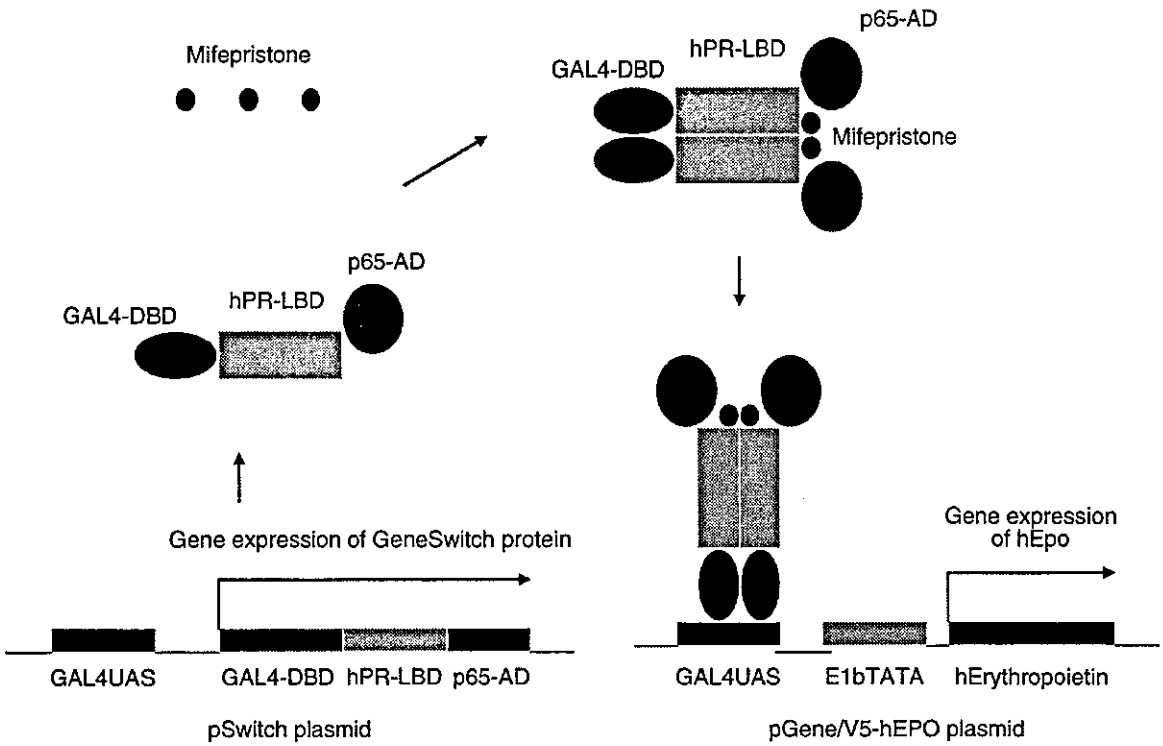

Fig. 1. Schema of the GeneSwitch system. An inducible expression plasmid, pGene/V5, under the control of a promoter containing GAL4 upstream activating sequences (UAS) and the adenovirus E1b TATA box. The full length of the coding region of human erythropoietin (hEpo) was inserted (pGene/V5-hEpo). A regulatory plasmid, pSwitch, which encodes a fusion protein consisting of the yeast GAL4 DNA binding domain (DBD), a truncated human progesterone receptor ligand binding domain (hPR-LBD), and the human p65 activation domain (AD) from nuclear factor-κB (NF-κB) was used for in vivo electroporation. The hybrid regulatory protein containing a DBD from the yeast GAL4 protein, a truncated ligand-binding domain (LBD) from the human progesterone receptor, and AD from the human NF-κB protein is expressed. This hybrid regulatory protein binds to the synthetic steroid, mifepristone, and functions as a ligand-dependent transcription factor to induce the expression of hEpo.

(Santa Cruz Biotechnology, Santa Cruz, CA, USA). The anti-human Epo antibody (Santa Cruz Biotechnology), diluted 1/1000, were detected using horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG and visualized by the Amersham enhanced chemiluminescence (ECL) system (Amersham Corp., Arlington Heights, IL, USA) after extensive washing of the membranes.

Detection of anti-hEpo antibody in rat serum

Anti-human erythropoietin antibody was detected from rat serum by absorbance experiments as described previously [19]. Briefly, LLC-PK1 cell lysate containing hEpo was incubated with hEpo-transferred rat serum or control rat serum, and the samples were then examined by Western blot analysis by the method described earlier. The anti-hEpo antibody was considered to be present when the signal for hEpo was significantly lower in the cell lysate incubated with hEpo-transferred rat serum than in that incubated with control rat serum.

PCR and RT-PCR analysis

DNA and RNA were isolated from LLC-PK1 cells or rats hind-limb muscles using Triozol™ (Gibco). Polymerase chain reaction (PCR) was performed using the GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer Cetus, Norwalk, CT, USA), with pGene/V5-hEpo, pSwitch, and hEpo cDNA specific primers prepared on a DNA synthesizer (Applied Biosystems Inc., Tokyo, Japan). The sequence of pGene/V5-hEpo primer 1 (antisense) was 5′-TCGA CCCGCGTAAGAGCTCGG-3′, and that of primer 2 (sense) 5′-GCTAGCAGTAATACTAACGG-3′. The predominant cDNA amplification product was predicted to be 426 bp in length (the distance between primers plus the primer length). The sequence of pSwitch primer 1 (antisense) was 5′-GAATGATCTCCCGATCCGTC-3′, primer 2 (sense) was 5′-GCTAGCAGTAATACTAA CGG-3′. The predominant cDNA amplification product was predicted to be 688 bp in length.

For the detection of hEpo RNA, to digest pGene/V5-hEpo DNA, the RNA solution was incubated with 1 U/mL RNase-free DNase (Pharmacia Fine Chemicals, Uppsala, Sweden) at 37°C for 30 minutes. The samples were heated to 95°C for five minutes to inactivate the DNase. Reverse transcription (RT) components produced using avian myeloblastoma virus reverse transcriptase were added to the reaction tubes as described previously [20]. The

reaction tubes were incubated at 42°C for 60 minutes. The sequence of hEpo primer 1 (antisense) was 5′-AGC CAGGCAGGACATTCGTA-3′, and that of primer 2 (sense) was 5′-AACTCTTCCGAGTCTACTCC-3′. The predominant cDNA amplification product was predicted to be 511 bp in length (Gene Bank #NM017001). RT and PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a positive control. The primers were defined by the following cDNA base sequences [21]: primer 1 (antisense) was 5′-AGATCCACAACGGAT ACATT-3′; primer 2 (sense) was 5′-TCCCTCAAGAT TGTCAGCAA-3′. The cDNA amplification product was predicted to be 309 bp in length. When GAPDH was used as an internal control primer, after reverse transcription, 20 μL samples were divided into 15 μL for each plasmid or hEpo mRNA and a 5 μL sample for GAPDH. After adjusting the volumes of the sample to 20 μL with sterile water, parallel PCR reactions were run with each set of primers. To examine the effectiveness of DNAse treatment in degrading residual plasmid DNA, PCR amplification of plasmid DNA was performed using primers to a non-transcribed region of the plasmid. The primers had the following cDNA base sequences: primer 1 (antisense), 5′-GGTGCATTGGAACGCGCATT-3′; primer 2 (sense), 5′-TGGCTGGCAACTAGAAGGCA-3′. The predominant cDNA amplification product was predicted to be 598 bp in length.

To carry out the PCR, 80 μL of a PCR reaction buffer was added to each tube directly. Fifty picomoles of each of primers 1 and 2 were used per reaction. Deoxynucleotides were added to a final concentration of 0.20 mmol/L each. The reaction buffer (10×) was diluted (1/10) to a final composition of: 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$ and 0.001% (wt/vol) gelatin, 2.5 U of Taq DNA polymerase. The tubes were placed in the Programmed Tempcontrol System programmed as follows: (*1*) incubation at 94°C for three minutes (initial melt); (*2*) 30 cycles of the following sequential steps: 94°C for one minute (melt), 60°C for one minute (anneal), and 72°C for three minutes (extend); and (*3*) incubation at 72°C for seven minutes (final extension). The treated samples were kept at 4°C until the time of analysis.

PCR product analysis

The PCR products were size-fractionated by 2% agarose gel electrophoresis. After electrophoresis and ethidium bromide staining, DNA bands were visualized with an ultraviolet transilluminator (Funakoshi, Tokyo, Japan).

To confirm that the PCR products were really pGene/V5-hEpo, pSwitch, and hEpo cDNA, the PCR products were sequenced. PCR products were subcloned into pGEM-3Zf(−) vector (Promega, Biotec, Madison, WI, USA) as described by Finney [22]. After cutting the pGEM-3Zf(−) vector at the Sam I site, thymine was attached at the 3′ end using Taq DNA polymerase (Perkin-Elmer Cetus), because Taq DNA polymerase has terminal transferase activity. The PCR product has adenine in its 3′end. We ligated PCR products and pGEM-3Zf(−), and then sequenced using the dideoxynucleotide chain-termination reaction of Sanger, Nicklen and Coulson [23].

Isolation of muscle tissue and histological examination

Rats were anesthetized with pentobarbital at indicated times after in vivo electroporation. The hind-limb muscles were quickly excised, fixed in formalin overnight, and then dehydrated and paraffin embedded. Thin sections were examined with periodic acid-Schiff (PAS) staining as described previously [24]. Immunohistochemical staining was performed by a streptavidin and biotin technique using hEpo-specific antibody (Santa Cruz), as described previously [19].

Statistics

The results were given as means ± SEM. The differences were tested using two way-analysis of variance (ANOVA) followed by the Scheffe test for multiple comparisons. Two groups were compared by the unpaired *t* test. $P < 0.05$ was considered significant.

RESULTS

Expression and regulation of hEpo in LLC-PK1 cells

We first examined the regulation of hEpo gene expression by MFP in LLC-PK1 cells (Fig. 2). LLC-PK1 cells were transfected with plasmid DNAs (15 μg of pGene/V5-hEpo and 15 μg of pSwitch) by electroporation. When MFP 10 nmol/L was included in the medium, Epo secretion into the medium was shown to have increased from 0 to 35.5 (U/10^6 cells) at three days. The cell counts were more prominently increased in MFP-treated group (1.5×10^7 cells/dish) than in the MFP(−) group (4.5×10^6 cells/dish). Next, the cells were divided into two experimental groups ($N = 5$, each group), one cultured with MFP and the other cultured without MFP. The Epo secretion of the MFP group continued to increase, reaching 45.4 U/10^6 cells at 9 days (Fig. 2A). On the other hand, the Epo secretion of the MFP-free group showed a decrease to 3.0 U/10^6 cells at nine days. After obtaining this result, we added MFP to the MFP-free group, and showed an Epo value of 28.6 U/10^6 cells at 12 days (Fig. 2A).

We further performed an immunoblot analysis of human Epo using anti-hEpo antibody (Fig. 2B). No positive signals were detected in either the control plasmid (control plasmid, pGene/V5 and pSwitch with MFP) or the hEpo-plasmid (pGene/V5-Epo) plus pSwitch without MFP. However, a clear hEpo signal was detected in the cell lysate of the pGene/V5-Epo plus pSwitch with MFP. These results demonstrate that MFP regulates the hEpo expression by the GeneSwitch system, at least in vitro.

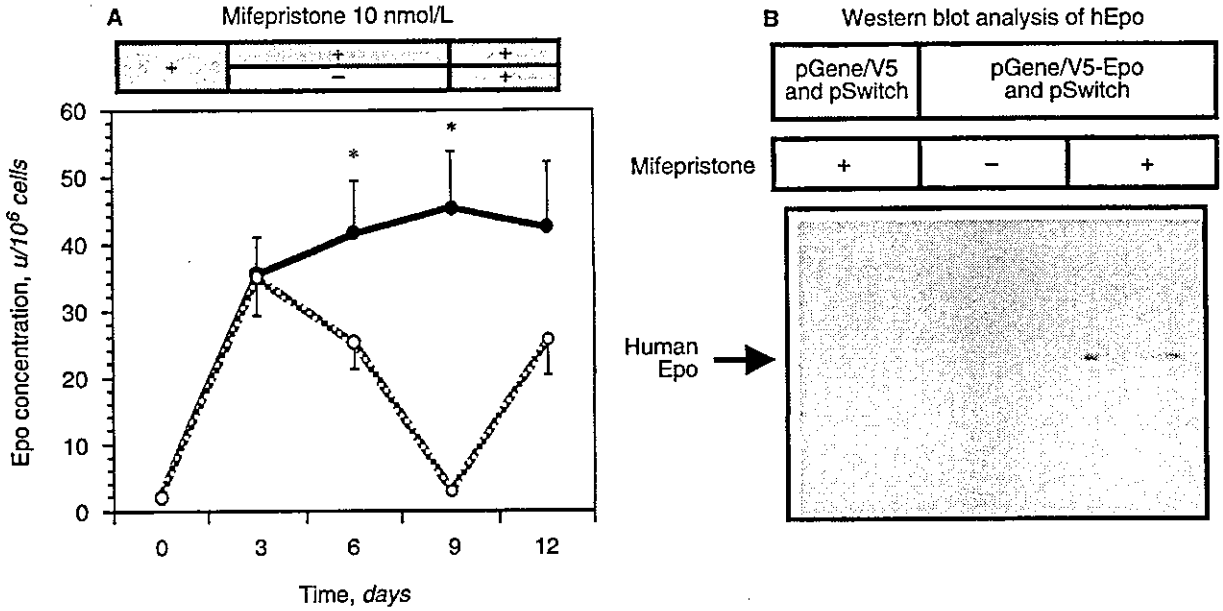

Fig. 2. Expression and regulation of hEpo in LLC-PK1 cells. (*A*) LLC-PK1 cells were transfected with plasmid DNAs (15 μg of pGene/V5-hEpo and 15 μg of pSwitch) by electroporation. When mifepristone (MFP; 10 nmol/L) was included in the medium, Epo secretion into the medium was increased at 3 days. We then divided the cells into two experimental groups: one group cultured with MFP (●) and one without MFP (○) (N = 4, each group). At day 9, MFP was added to the both cultures groups. Data are shown as means ± SEM. *$P < 0.05$ comparisons between the MFP(+) and MFP(−) groups. (*B*) Immunoblot analysis of hEpo from cell lysate using anti-hEpo antibody. No positive signals were detected in either the control plasmid group (control plasmid, pGene/V5 plus pSwitch with MFP, left lane) or pGene/V5-Epo plus pSwitch without MFP (middle lane). A clear hEpo signal was detected in the cell lysate of the pGene/V5-Epo plus pSwitch with MFP (right lane).

RT-PCR detection of hEpo mRNA and PCR detection of pGene/V5-hEpo and pSwitch plasmids in rat thigh muscle in vivo

Rats (N = 3 at each of seven time points, for a total of 21 rats) were injected intramuscularly with the following plasmid DNAs: 100 μg of pGene/V5-hEpo plus 100 μg of pSwitch. Electroporation was performed immediately after plasmid injection. MFP was injected every three days up to 50 days. The muscles injected with pGene/V5-hEpo plus pSwitch plasmids were resected at indicated times after injection. The positive PCR signals of pGene/V5-hEpo plus pSwitch plasmids were detected up to 50 days after the plasmid injection into the thigh muscle. RT-PCR was used to determine the presence of hEpo mRNA. The positive signal of hEpo mRNA also was detected up to 50 days after the plasmid injection in the presence of MFP administration (Fig. 3A). The control lane of Figure 3A is PCR amplification of DNA and RNA from an uninjected muscle specimen. To examine the effectiveness of DNAse treatment in degrading residual plasmid DNA, PCR amplification of plasmid DNA was performed using primers to a non-transcribed region of the plasmid. As shown in Figure 3B, hEpo plasmid DNA was not detected with DNAse treatment. We examined the protein expression and presence of mRNA of hEpo in the thigh muscle 10 days after injection in three different experimental conditions; that is, in muscles injected with pGene/V5 (control plasmid) plus pSwitch plasmids with MFP; in muscles injected with pGene/V5-hEpo plus pSwitch plasmids without MFP; and in muscles injected with pGene/V5-hEpo plus pSwitch plasmids with MFP. Positive hEpo protein and mRNA were detected only in the third group (Fig. 3C). To compare the efficiency of Epo mRNA and protein expression with or without electroporation, we examined the protein expression and presence of hEpo mRNA in the thigh muscle 10 days after injection in two different experimental conditions: in muscles injected with pGene/V5-hEpo plus pSwitch plasmids with electroporation and MFP; and in muscles injected with pGene/V5-hEpo plus pSwitch plasmids with MFP but without electroporation. The control group received neither the plasmid injection nor electroporation. Strong positive hEpo protein and mRNA were detected in the group treated with electroporation and MFP (Fig. 3D). In the group treated with MFP but without electroporation, a weak positive band was detected by RT-PCR, however, none was detected by immunoblot analysis (Fig. 3D).

Changes of hematocrit and serum human erythropoietin levels in rats

Rats (N = 24) were injected intramuscularly with the 100 μg of pGene/V5-hEpo and 100 μg of pSwitch, and then were divided into three groups: one group adminis-

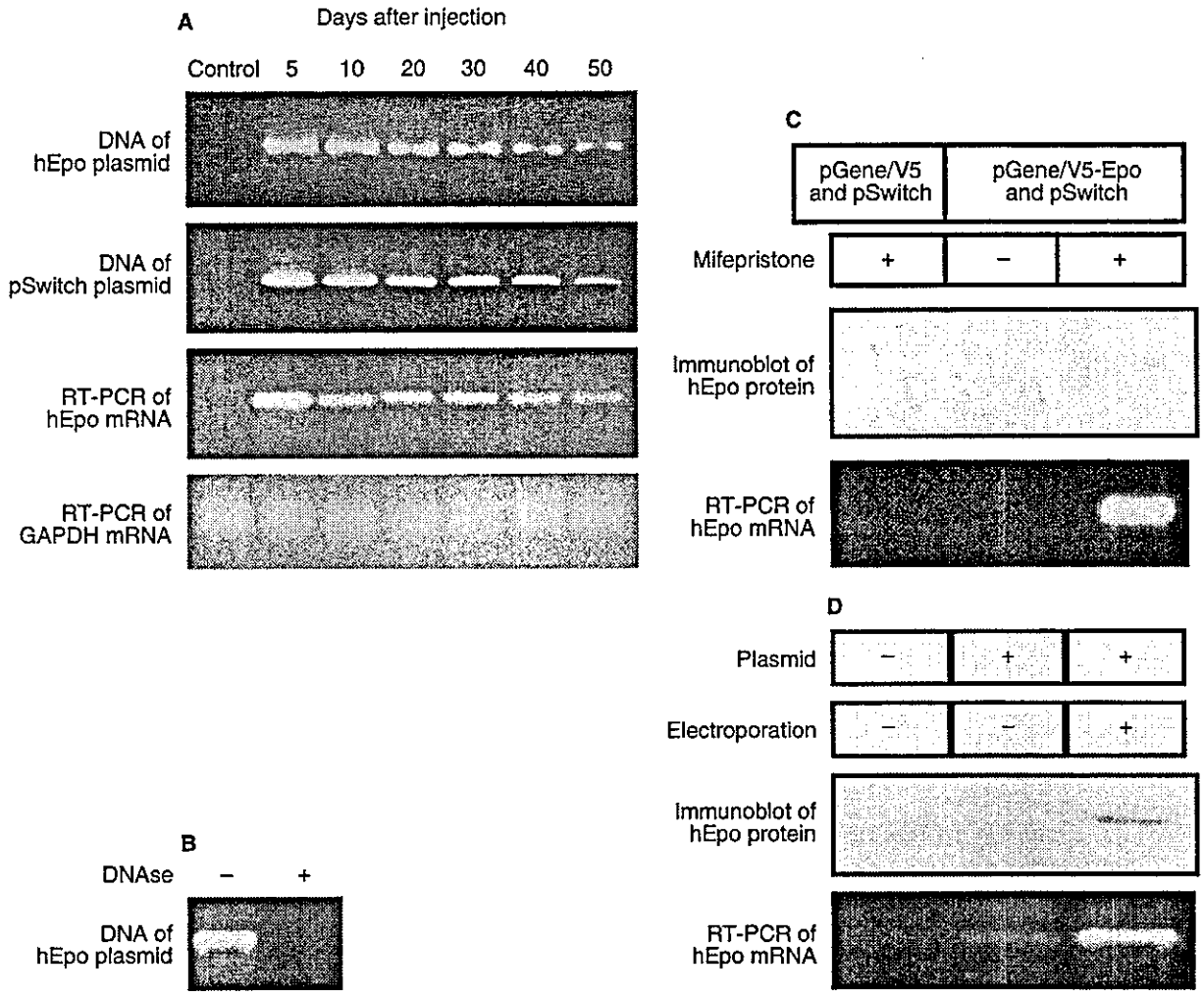

Fig. 3. Reverse transcription-polymerase chain reaction (RT-PCR) detection and immunoblot analysis of hEpo expression and PCR detection of pGene/V5-hEpo and pSwitch plasmids in rat thigh muscle in vivo. (*A*) Rats were injected intramuscularly with the following plasmid DNAs: 100 μg of pGene/V5-hEpo and 100 μg of pSwitch. MFP was injected every 3 days up to 30 days. DNA and total RNA were extracted from injected muscle at the indicated times. PCR analyses were performed for pGene/V5-hEpo and pSwitch plasmids, while RT-PCR analyses were performed for Epo mRNA and GAPDH mRNA (positive control). The control lane is PCR amplification of DNA and RNA from an uninjected muscle specimen. (*B*) The efficacy of DNAse treatment in degrading residual plasmid DNA was examined. We performed PCR amplification of plasmid DNA using primers to non-transcribed regions of the plasmid. Human Epo plasmid DNA was not detected with DNAse treatment. (*C*) Immunoblot and RT-PCR analyses for hEpo expression were performed from injected muscle at 10 days after injection. We examined three different experimental conditions ($N = 3$, in each experiments): in muscle injected with pGene/V5 (control plasmid) plus pSwitch plasmids and MFP; in muscle injected with pGene/V5-hEpo plus pSwitch plasmids without MFP; and in muscle injected with pGene/V5-hEpo plus pSwitch plasmids with MFP. (*D*) Effects of electroporation on Epo mRNA and protein expression in rat muscle. The protein expression and presence of hEpo mRNA were examined in the thigh muscle at 10 days after injection under two different experimental conditions: in muscle injected with pGene/V5-hEpo plus pSwitch plasmids with electroporation and MFP; and in muscles injected with pGene/V5-hEpo plus pSwitch plasmids with MFP but without electroporation. The control received neither plasmid injection nor electroporation. ($N = 3$, in each group)

tered MFP for 50 days; a second group administered MFP for the first 15 days and re-challenged from days 30 to day 50 days; and a third group not administered MFP. A fourth group of rats ($N = 6$) was injected intramuscularly with 100 μg of pGene/V5 (control plasmid) and 100 μg of pSwitch. Electroporation was performed immediately after plasmid injection. In the MFP-treated rats ($N = 6$ of the pGene/V5 group, $N = 12$ of the pGene/V5-hEpo group), 70 mg/kg MFP was injected intraperitoneally every three days. In the first group [pGene/V5-hEpo and MFP(+) for 50 days] the hematocrit levels increased continuously, rising from 41.2% before the injection to 52.7% on day 15, to 54.9% on day 25, to 55.0% on day 30, to 54.0% on day 40, and to 53.8% on day 50 (Fig. 4). In the second group [pGene/V5-hEpo and MFP(+) for 15 days, and re-challenged

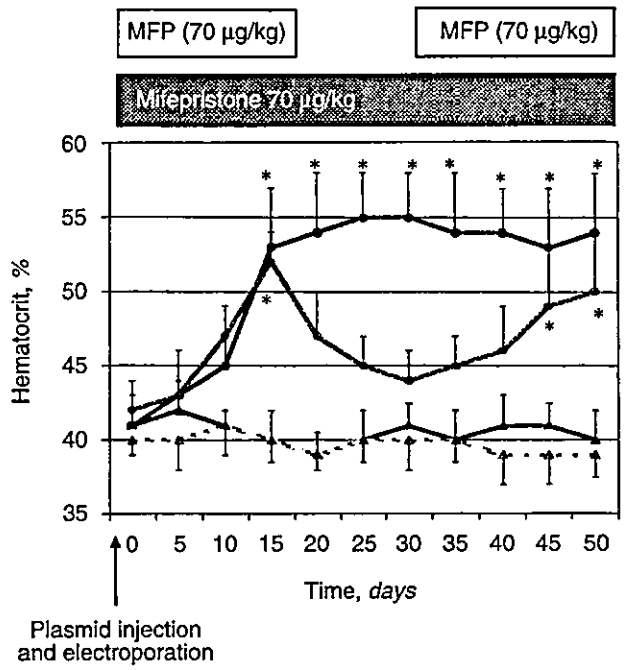

Fig. 4. Increase in hematocrit in rats injected with pGene/V5-hEpo plus pSwitch plasmid with MFP treatment. Rats (N = 18) were injected intramuscularly with 100 μg of pGene/V5-hEpo and 100 μg of pSwitch, and then divided into three groups: one group administered MFP for 50 days (●); a second group administered MFP for the first 15 days and then re-administered MFP from day 30 to day 50 (○); and a third group not administered MFP (▲). A fourth group, (△) of rats (N = 6) was injected intramuscularly with 100 μg of pGene/V5 plus 100 μg of pSwitch and treated with MFP. In the MFP-treated rats (N = 6 of the pGene/V5 group, N = 12 of the pGene/V5-hEpo group), 70 mg/kg MFP was injected intraperitoneally every 3 days. Data are shown as means ± SEM. $*P < 0.05$ comparisons with the control rats (fourth group injected with pGene/V5 plus pSwitch).

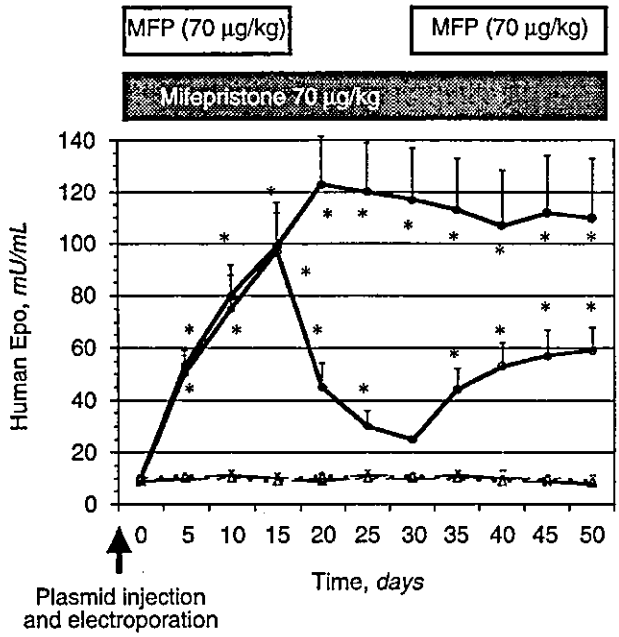

Fig. 5. Increase in the serum human Epo level in rats injected with pGene/V5-hEpo and pSwitch plasmid with MFP treatment. Rats (N = 18) were injected intramuscularly with 100 μg of pGene/V5-hEpo and 100 μg of pSwitch, and then divided into three groups: one group administered MFP for 50 days (●); a second group administered MFP for the first 15 days and then re-administered from day 30 to day 50 (○); and a third group not administered MFP (▲). Another group (fourth group, △) of rats (N = 6) was injected intramuscularly with 100 μg of pGene/V5 plus 100 μg of pSwitch and treated with MFP. In the MFP-treated rats (N = 6 of the pGene/V5 group, N = 12 of the pGene/V5-hEpo group), 70 mg/kg of MFP was injected intraperitoneally every 3 days. Data are shown as means ± SEM. $*P < 0.05$ comparisons with the control rats (fourth group injected with pGene/V5 plus pSwitch).

from 30 to 50 days] the hematocrit levels fluctuated, rising steadily from 42.7% before the injection to 52.5% on day 15, then falling to 47.1% on day 20 and 43.9% on day 30, then rising to 46.5% on day 40 and to 50.0% on day 50 (Fig. 4). In the third [pGene/V5-hEpo and MFP(−)] and fourth [pGene/V5 and MFP(+)] groups, the hematocrit levels did not change significantly (Fig. 4). In further tests to determine how hematocrit levels responded to the injection of MFP alone (MFP injection every 3 days for 50 days with no plasmid injection), no significant changes in the hematocrit levels were found (data not shown).

The serum hEpo levels increased from the preinjection level of 11.0 to 82.3 and 79.8 mU/mL on day 10 in the first group [pGene/V5-hEpo and MFP(+) for 50 days] and second group [pGene/V5-hEpo & MFP(+) for 15 days, and re-challenged from 35 to 50 days], respectively (Fig. 5). In the first group, the serum hEpo level continued to gradually increase from day 10, up to a peak of 123.6 mU/mL on day 20, and then it fell back slightly to 117.1 mU/mL on day 30, and 110.2 mU/mL on day 50. In the second group (in which MFP was stopped at 15 days, and re-challenged from 30 to 50 days), the serum hEpo levels fluctuated after day 15, falling to 23.5 mU/mL on day 30, rising again to 54.3 mU/mL on day 40, and 59.2 mU/mL on day 50. There was no significant change in the hEpo level in the pGene/V5-hEpo and MFP(−) group or pGene/V5 and MFP(+) group. Using absorbance experiments, we detected anti-hEpo antibody from the serum of four rats in the pGene/V5-hEpo and MFP(+) group at 50 days (data not shown). We also examined how the hEpo levels responded to the injection of MFP alone (MFP injection every 3 days for 50 days with no plasmid injection). In these MFP-treated rats, the hEpo levels did not significantly change (data not shown).

Expression of hEpo in rat thigh muscle in vivo

An immunohistological examination also was performed using anti-human Epo antibody in the thigh muscle of the pGene/V5-hEpo and MFP(+) group. Human Epo expression was detected in the thigh muscle of the

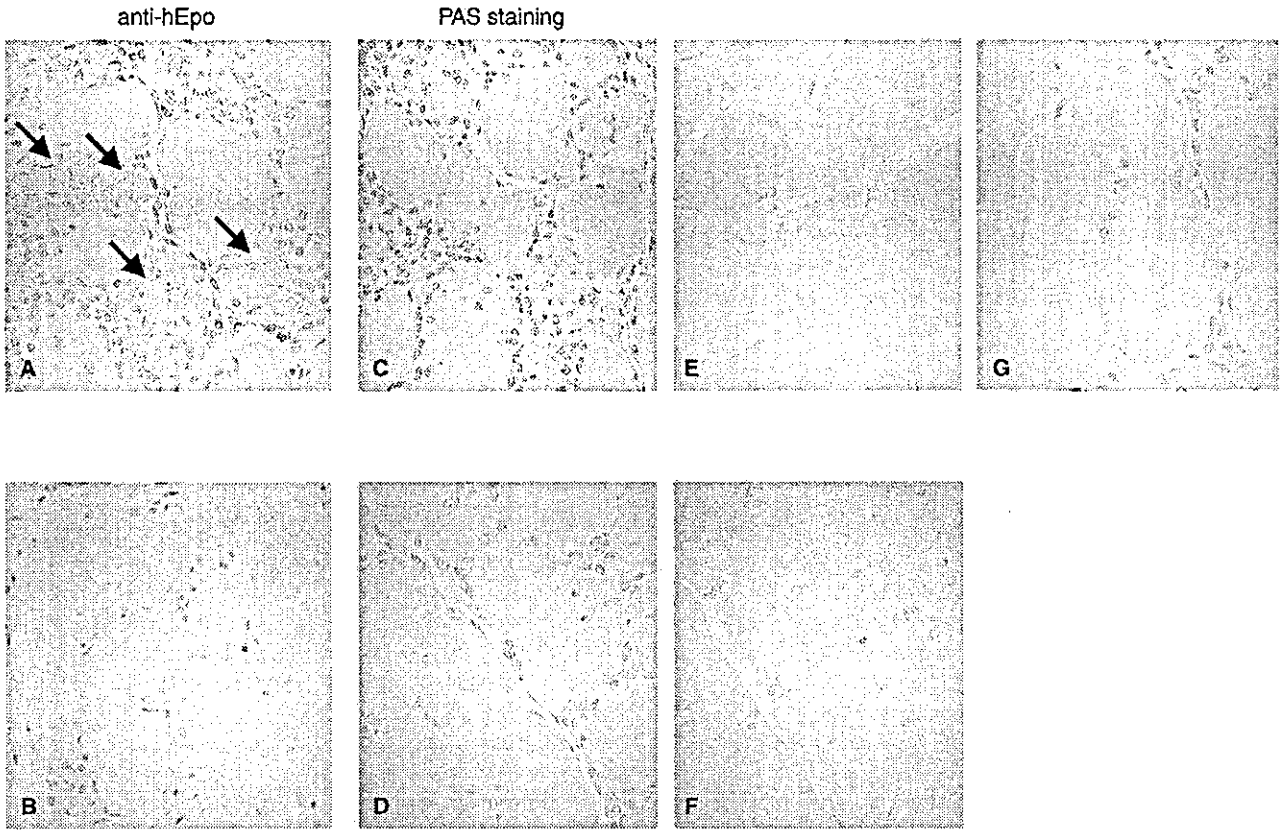

Fig. 6. In vivo human Epo expression in rat thigh muscle. (*A*) Immunohistological examination using anti-human Epo antibody in the thigh muscle injected with pGene/V5-hEpo, pSwitch and MFP(+) group rats at day 14 (×400). Brown staining (arrowheads) indicates hEpo expression in muscle fiber. (*B*) Immunohistological examination using anti-human Epo antibody in the thigh muscle of the control (no treatment) rats. (*C*) PAS staining of the thigh muscle of the pGene/V5-hEpo, pSwitch & MFP(+) group rats at day 14 (×400). Cell infiltration was observed around the area where transgene was expressed. (*D*) PAS staining of the thigh muscle of the pGene/V5-hEpo, electroporation(−) and MFP(+) treated rats. Slight neutrophil infiltration is observed in the muscle. (*E*) PAS staining of the thigh muscle of the pGene/V5-hEpo, electroporation(+) and MFP(−) treated rats. (*F*) PAS staining of the thigh muscle of the pGene/V5-hEpo, electroporation(−) and MFP(−) treated rats. (*G*) PAS staining of the thigh muscle of the plasmid(−) and electroporation(+) treated rats. No remarkable neutrophil infiltration is observed in panels E-G.

pGene/V5-hEpo and MFP(+) treated rats on day 14 (Fig. 6A). In contrast, no such signal was detected in the control (no treatment, no plasmid, no electroporation) rats (Fig. 6B), pGene/V5-hEpo and MFP(−) treated rats, or pGene/V5 and MFP(+) treated rats on day 14 (data not shown). In the rat skeletal muscle of the pGene/V5-hEpo, electroporation(+) and MFP(+) treated rats, neutrophil and fibroblast infiltration was observed around the area of transgene expression at day 14, as shown in Figure 6C. There was only slight neutrophil infiltration in the pGene/V5-hEpo, electroporation(−) and MFP(+) treated rats (Fig. 6D), the group that expressed low amounts of hEpo (Figs. 3C and 7). There was no remarkable neutrophil infiltration in the pGene/V5-hEpo, electroporation (+) and MFP(−) treated rats (Fig. 6E), the pGene/V5-hEpo, electroporation (−) and MFP(−) treated rats (Fig. 6F), or plasmid(−) and electroporation (+) treated rats (Fig. 6G). These data indicated that the neutrophil infiltration was due to transgene expression rather than electroporation.

In vivo electroporation dramatically increased the hematocrit level and hEpo level

Next, the hematocrit levels were compared in rats injected with different DNA doses (10, 50, and 100 μg) of pGene/V5-hEpo plus pSwitch and MFP (+) with or without in vivo electroporation on day 25. The hematocrit level increased in proportion to the dose of DNA, as shown in Figure 7. The increases in hematocrit levels were significantly higher in the rats treated by in vivo electroporation than in the rats that received no electroporation (at doses of 50 μg and 100 μg). The increases in hEpo levels were significantly higher in the rats treated by in vivo electroporation (120.1 mU/mL) than in the rats that received no electroporation (18.1 mU/mL) at doses of 100 μg of plasmid on day 25.

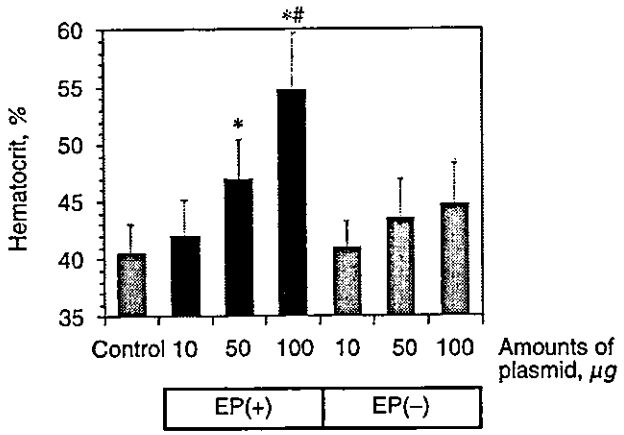

Fig. 7. Comparison of hematocrit levels in rats injected different DNA doses with or without in vivo electroporation (EP). The hematocrit levels in rat injected with different DNA doses (10, 50, and 100 μg) of pGene/V5-hEpo plus pSwitch with MFP(+) with or without in vivo electroporation at day 25 are shown. The data are mean ± SEM in 5 animals. *Significantly different from saline control ($P < 0.05$); #significantly different between EP(+) and EP(−) of same DNA doses ($P < 0.05$).

DISCUSSION

This study demonstrates that ligand-dependent regulation of hEpo expression can be achieved after the delivery of a two-plasmid GeneSwitch system to the hindlimb muscles of rats. Our results also demonstrate that in vivo electroporation dramatically increases the efficiency of the gene expression.

Treatment of patients with end-stage renal failure by recombinant hEpo has proven effective in correcting anemia, but it incurs substantial costs. An alternative method to deliver Epo continuously is by gene therapy, either by introducing the Epo gene into autologous cells ex vivo [9, 10, 25] and transferring the modified cells back into the individual, or by directly transferring the Epo gene in vivo using an adenovirus vector, adeno-associated vector, or naked plasmid [6, 12, 26–31].

However, ex vivo gene transfer is both labor intensive and expensive because it requires the isolation, growth, and transduction of primary human myoblasts from each patient. Several novel methods have proven successful in attaining long-term regulated expression of therapeutic hormones such as growth hormone and Epo. In subjects treated by intramuscular injection of adeno-associated vector, tetracycline was found to adequately control Epo production [11]. Bohl et al reported that a single intramuscular injection of an adeno-associated virus vector containing the components of the tetracycline-inducible regulatable system and a mouse Epo cDNA resulted in elevation of hematocrit and serum Epo concentration for up to 30-weeks in immunocompetent mice [11]. Other groups recently demonstrated a rapamycin-regulatable gene expression system [12, 13]. Ye et al conducted experiments using a mixture of two adeno-associated virus vectors, one expressing the transcription factor chimeras and one containing Epo under the control of a promoter responsive to the transcription factor. After injecting this system into the skeletal muscle of immunocompetent mice and rhesus monkeys, serum Epo was successfully elevated under the control of rapamycin for up to 150 days [12]. While these adeno-associated virus systems have proven very useful for gene delivery, the methods require relatively large amount of adeno-associated virus, as well as intensive labor to purify the virus. Rivera et al reported a similar rapamycin-regulatable growth hormone gene therapy [13]. In their report using immunodeficient mice, long-term regulated expression of growth hormone was achieved within the use of separate adenovirus and adeno-associated virus vectors, one encoding an inducible human growth hormone target gene, and the other encoding a bipartite rapamycin-regulated transcription factor. In immunocompetent mice, the expression of the adenovirus-directed growth hormone was quickly extinguished. Several reports demonstrated that adenovirus vector elicited an efficient expression of Epo in immunodeficient mice [14]. Thus, the utilization of adenovirus for therapeutic purposes is limited by the transient nature of the transgene expression and systemic toxicity, both of which are due to the inflammatory and immune responses triggered by the residual expression of viral proteins [15, 31]. The development of adenovirus vectors with all viral coding sequences deleted offers the prospect of a safer and more efficient way to deliver genes [13, 32]. This improved helper-dependent adenoviral vector allowed persistent gene expression after intramuscular delivery and overcame preexisting immunity to adenovirus [15].

Ex vivo gene transfer also has been investigated for Epo gene transfer. Bohl, Naffakh and Heard reported that retrovirus-engineered myoblast expressing rtTA (coding sequences for the tetracycline reverse transactivator), the chimeric transactivator conferring doxycyclin-inducible gene expression, can be stably engrafted in mice, thus allowing long-term control of Epo secretion in vivo [33]. It is less expensive and easy to perform. Currently available vectors for the in vivo transfer of the Epo gene into muscle include plasmid DNA, replication-defective adenovirus vectors, and adeno-associated virus vectors. A large quantity of highly purified plasmid DNA is easily and inexpensively obtained, and gene transfer can be repeated without apparent immune response to the DNA vector. However, plasmid vectors are inefficiently taken up by muscle cells and manifest relatively low-level expression compared with viral vectors [34, 35]. Indeed, there are only a few reports on the use of plasmid vectors for the in vivo transfer of the Epo gene [36].

Gene transfer by in vivo electroporation has been shown to be effective for introducing DNA into mouse skin [37], chicken embryos [38], rat liver [39], mouse

melanoma [40], rat brain [41], and rat corneal endothelium [42]. Moreover, it was demonstrated that gene transfer into mouse muscles by this approach is much more effective than that by simple intramuscular DNA injection [43]. Epo gene transfer to rat muscle using Epo plasmid with in vivo electroporation recently was reported by Maruyama et al [16]. The transfection efficiency was very high in their report. They used pCAGGS-Epo plasmid, a rat Epo cDNA with CAG promoter and a 3'-flanking sequence of the rabbit β-globin gene in the pCAGGS expression vector, but with no regulatory elements. The hematocrit was increased to as high as 67% in their report, but it was difficult to regulate the transferred Epo gene expression in their system. Previous reports using adenovirus also demonstrated a 60 to 70% elevation of hematocrit, but with a similar failure to establish controllable regulation of Epo expression [6, 7].

We demonstrated the ability of the autoinducible GeneSwitch system to regulate the expression of Epo gene in rats in a biologically relevant manner. Following the delivery of two plasmids to skeletal muscle with electroporation, the mRNA expression of hEpo in muscle tissue was induced by administration of MFP up to at least 50 days. There was no detectable mRNA expression of hEpo in the muscle or serum hEpo in the absence of MFP. Thus, a tight, drug-dependent transgene regulation was achieved in both in vitro and in vivo experiments. A recent report by Abruzzese et al demonstrated that the autoinducible GeneSwitch system regulates the expression of the Epo gene in mice [44]. However, they used a relatively small amount of Epo plasmid and observed relatively little elevation of Epo [44]. Our novel Epo gene delivery system may be a suitable subject for clinical trials on Epo gene therapy. However, before reaching the stage of clinical application, techniques should be found to reduce the frequency of MFP administration. We further performed immunohistological examinations of gene-transferred muscle and confirmed the induction of hEpo in the muscle in the presence of MFP. In rat skeletal muscle, cell infiltration was observed around the area where transgene was expressed, as shown in Figure 6. There are at least two possible explanations for this cell infiltration: tissue injury produced the electroporation, or an immunoreactive response induced by the anti-hEpo antibody detected in the late phase of the in vivo experiments. The second explanation may be more plausible, as the electroporation caused only a slight cell infiltration into the muscle (Fig. 6).

This is the first study on drug-regulated hEpo gene transfer combined with electroporation. Our novel methods have many advantages; not least of which are the safety of the plasmids compared to adenovirus, and the regulation of gene expression by MFP. Our results demonstrate that Epo gene transfer using the GeneSwith system and in vivo electroporation is a useful procedure for efficient drug-regulated delivery of Epo.

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Report

Association between Single-Nucleotide Polymorphisms in Selectin Genes and Immunoglobulin A Nephropathy

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Although intensive efforts have been undertaken to elucidate the genetic background of immunoglobulin A nephropathy (IgAN), genetic factors associated with the pathogenesis of this disease are still not well understood. We designed a case-control association study that was based on linkage disequilibrium among single-nucleotide polymorphisms (SNPs) in the selectin gene cluster on chromosome 1q24-25, and we found two SNPs in the E-selectin gene (SELE8 and SELE13) and six SNPs in the L-selectin gene (SELL1, SELL4, SELL5, SELL6, SELL10, and SELL11) that were significantly associated with IgAN in Japanese patients. All eight SNPs were in almost complete linkage disequilibrium. SELE8 and SELL10 caused amino acid substitutions from His to Tyr and from Pro to Ser ($\chi^2 = 9.02$, $P = .0026$, odds ratio = 2.73 [95% confidence interval {CI} 1.38–5.38] for His-to-Tyr substitutions; $\chi^2 = 17.4$, $P = .000031$, odds ratio = 3.61 [95% CI 1.91–6.83] for Pro-to-Ser substitutions), and SELL1 could affect promoter activity of the L-selectin gene ($\chi^2 = 19.5$, $P = .000010$, odds ratio = 3.77 [95% CI 2.02–7.05]). The TGT haplotype at these three loci was associated significantly with IgAN ($\chi^2 = 18.67$, $P = .000016$, odds ratio = 1.88 [95% CI 1.41–2.51]). Our results suggest that these eight SNPs in selectin genes may be useful for screening populations susceptible to the IgAN phenotype that involves interstitial infiltration.

Immunoglobulin A nephropathy (IgAN [MIM 161950]) is the most common form of glomerulonephritis, a principal cause of end-stage renal disease worldwide (Maisonneuve et al. 2000). Surveys of patients with primary glomerulonephritis that were conducted in 1985 and 1993 by the Research Group on Progressive Renal Diseases in Japan revealed a high prevalence of IgAN and a relatively poor prognosis for patients (Koyama et al. 1997). The rate of survival for the 502 patients with IgAN who were monitored, with renal-related death taken as the end point, was 61% at 20 years from the time renal abnormalities were first detected. The strong evidence from human studies of a role for genetic factors in the development and progression of IgAN has been provided by descriptive reports of familial aggregations of IgAN and by analyses of affected sib pairs and parent-child pairs from multiple ethnic groups (Hsu et al. 2000).

Accumulation of leukocytes within the glomerulus and interstitium of the kidney is considered to be a key pathogenetic mechanism in various types of glomerulonephritis (Adler and Brady 1999). The three major groups of adhesion molecules involved in these interactions are the selectins, the integrins, and certain proteins belonging to the immunoglobulin supergene family. To date, three selectins have been characterized: E-selectin is expressed predominantly in cytokine-activated

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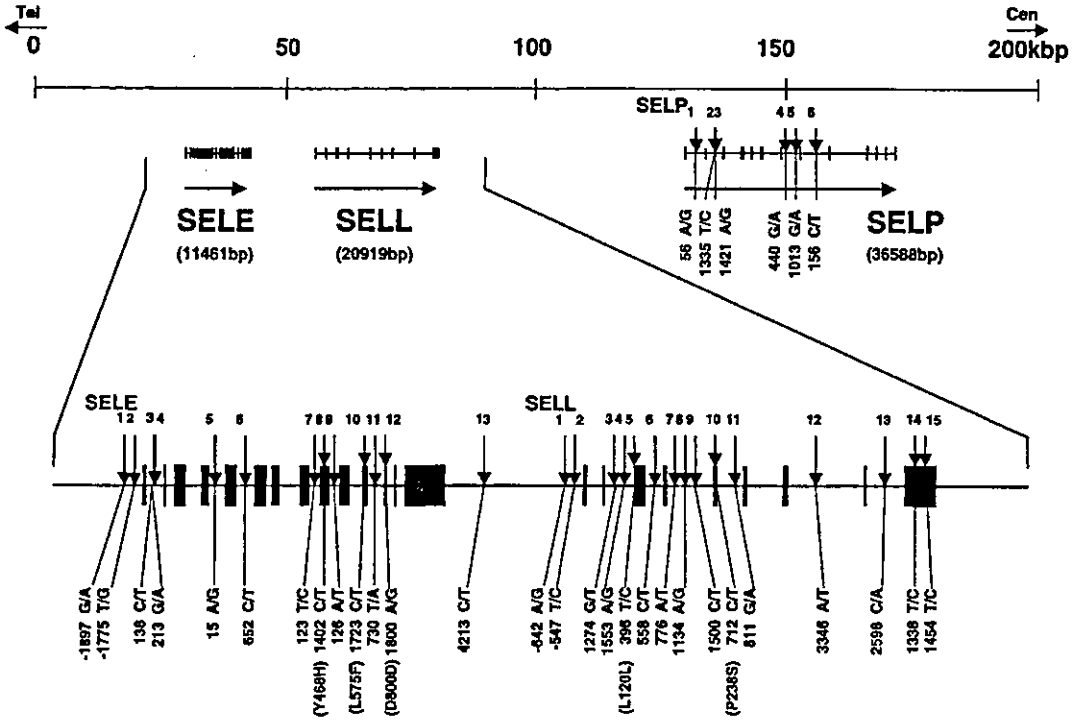

Figure 1 High-density map and association with IgAN of SNPs in the selectin gene cluster at chromosome 1q24-25. SNPs (*downward arrows*) are numbered for E-selectin (SELE1–13), L-selectin (SELL1–15), and P-selectin (SELP1–6), in the telomeric to centromeric direction.

endothelium, L-selectin in circulating leukocytes, and P-selectin in activated endothelial cells and platelets. Chaudhury et al. (1996) reported a marked increase in E-selectin and P-selectin expression on the extraglomerular vascular endothelium, with prominent interstitial infiltrates, in some biopsy samples from patients with IgAN; and a large number of L-selectin–positive cells were present within glomerular and interstitial infiltrates in affected kidneys. Lai et al. (1994) demonstrated an apparent increase in serum levels of E-selectin that was associated with increased histopathologic grade, when patients with IgAN were stratified according to the severity of their glomerular and interstitial lesions. Moreover, increased expression of E-selectin has been observed on renal interstitium that contains lymphocytes and macrophages, along with an increase in circulating soluble L-selectin (Kennel-De March et al. 1999).

Single-nucleotide polymorphisms (SNPs) in the human genome have great potential for application to association studies of complex diseases (Kruglyak 1999). Because SNPs are the most frequent type of genetic variation, we have been screening SNPs on a genomewide scale to clarify various complex diseases, including IgAN. For the work reported here, we designed a case-control association study, using SNPs found in the selectin genes (GenBank), and estimated haplotypes that might serve to identify regions containing loci responsible for IgAN phenotypes (Takeoka et al. 2001).

Peripheral blood samples were obtained from 346 patients (196 female and 150 male, mean age 44 years) who were diagnosed with IgAN on the basis of clinical manifestations as well as renal-biopsy findings at several surgical centers in Japan (Department of Medicine, Kidney Center, Tokyo Women's Medical University; Department of Urology, Iwate Medical University; Department of Urology, Sanai Hospital; Department of Urology, Iwate Prefectural Ofunato Hospital; and Department of Medicine, Niigata University School of Medicine). The mean value of serum creatinine at the time of renal biopsy was 1.07 mg/dl, ranging from 0.3 to 2.5 mg/dl. We analyzed blood from 408 control subjects randomly selected from population-based volunteers. DNA was prepared from each sample, according to standard protocols.

SNPs in the three selectin genes were screened according to methods described elsewhere (Ohnishi et al. 2000; Yamada et al. 2000). Information about each SNP we discovered can be obtained at the Japanese SNPs (JSNP) Web site.

We amplified multiple genomic fragments, using 20 ng of genomic DNA for each PCR, as described elsewhere (Ohnishi et al. 2001). Sequences of the primers

Table 1
Statistically Significant Differences in Eight SNPs among Patients with IgAN versus Control Subjects

	No. [%] of Subjects with							
Zygosity	SELE8	SELE13	SELL1	SELL4	SELL5	SELL6	SELL10	SELL11
IgAN:								
Major homozygotes	187 [57.89%]	189 [57.27%]	185 [54.41%]	157 [45.38%]	166 [48.26%]	133 [46.18%]	156 [53.24%]	161 [57.50%]
Heterozygotes	109 [33.75%]	115 [34.85%]	114 [33.53%]	138 [39.88%]	131 [38.08%]	106 [36.81%]	101 [34.47%]	95 [33.93%]
Minor homozygotes	27 [8.36%]	26 [7.88%]	41 [12.06%]	51 [14.74%]	47 [13.66%]	49 [17.01%]	36 [12.29%]	24 [8.57%]
Total	323 [100.00%]	330 [100.00%]	340 [100.00%]	346 [100.00%]	344 [100.00%]	288 [100.00%]	293 [100.00%]	280 [100.00%]
Major allele	483 [74.77%]	493 [74.70%]	484 [71.18%]	452 [65.32%]	463 [67.30%]	372 [64.58%]	413 [70.48%]	417 [74.46%]
Minor allele	163 [25.23%]	167 [25.30%]	196 [28.82%]	240 [34.68%]	225 [32.70%]	204 [35.42%]	173 [29.52%]	143 [25.54%]
Total	646 [100.00%]	660 [100.00%]	680 [100.00%]	692 [100.00%]	688 [100.00%]	576 [100.00%]	586 [100.00%]	560 [100.00%]
Control								
Major homozygotes	269 [66.92%]	274 [67.16%]	246 [61.65%]	218 [53.96%]	227 [56.05%]	218 [58.76%]	235 [62.67%]	262 [67.88%]
Heterozygotes	120 [29.85%]	121 [29.66%]	139 [33.53%]	159 [39.36%]	154 [38.02%]	132 [35.58%]	126 [33.60%]	112 [29.02%]
Minor homozygotes	13 [3.23%]	13 [3.19%]	14 [3.51%]	27 [6.68%]	24 [5.93%]	21 [5.66%]	14 [3.73%]	12 [3.11%]
Total	402 [100.00%]	408 [100.00%]	399 [100.00%]	404 [100.00%]	405 [100.00%]	371 [100.00%]	375 [100.00%]	386 [100.00%]
Major allele	658 [81.84%]	669 [81.99%]	631 [79.07%]	595 [73.64%]	608 [75.06%]	568 [76.55%]	596 [79.47%]	636 [82.38%]
Minor allele	146 [18.16%]	147 [18.01%]	167 [20.93%]	213 [26.36%]	202 [24.94%]	174 [23.45%]	154 [20.53%]	136 [17.62%]
Total	804 [100.00%]	816 [100.00%]	798 [100.00%]	808 [100.00%]	810 [100.00%]	742 [100.00%]	750 [100.00%]	772 [100.00%]

Table 2
Statistically Significant Differences in Eight SNPs among Patients with IgAN versus Control Subjects

	Statistical Significance in Subjects with							
Stratified Comparison	SELE8	SELE13	SELL1	SELL4	SELL5	SELL6	SELL10	SELL11
χ^2:								
Allele frequency	10.686095	11.572843	12.354986	12.242864	11.007155	22.701719	14.379202	12.292278
Genotype frequency (2 × 3)	11.704698	11.980555	19.773932	14.392873	13.899272	24.560263	18.609493	12.969646
Major homozygotes/others	6.244945	7.624198	3.961227	5.494191	4.530285	10.306812	6.018957	7.538820
Minor homozygotes/others	9.025162	8.026010	19.481165	12.982878	12.975893	22.013996	17.377607	9.470623
P:								
Allele frequency	.001079	.000669	.000440	.000467	.000908	.000002	.000149	.000455
Genotype frequency (2 × 3)	.002873	.002503	.000051	.000749	.000959	.000005	.000091	.001526
Major homozygotes/others	.012455	.005759	.046560	.019080	.033300	.001325	.014153	.006038
Minor homozygotes/others	.002663	.0046111	.000010	.000314	.000316	.000003	.000031	.002088

Note — The χ^2 test was used to evaluate possible associations between different genotypes, allele frequencies, or patients.

Table 3

LD Coefficients between Eight SNPs

	SELE8	SELE13	SELL1	SELL4	SELL5	SELL6	SELL10
SELL11	.95284	.95969	.85654	.73947	.78009	.80668	.97308
SELL10	.85928	.88022	.93324	.82487	.87217	.89132	
SELL6	.77048	.78813	.87736	.89847	.94944		
SELL5	.76206	.78182	.88943	.95107			
SELL4	.72427	.74805	.85863				
SELL1	.84004	.86222					
SELE13	.95929						

NOTE.—LD coefficients were calculated and expressed as the $D'=D/D_{max}$, as described elsewhere (Devlin and Risch 1995)

used in the present study are available at JSNP. Each PCR reaction was performed in a 20-μl solution containing 50 pmol of each primer, 10 units of Ex-*Taq* DNA polymerase (TaKaRa Shuzo), and 0.55 μg of *Taq*Start (Clontech Laboratories) in the GeneAmp PCR system 9700 (Applied Biosystems). Initial denaturation was at 94°C for 2 min, followed by 37 cycles of amplification at 94°C for 15 s and annealing at 60°C for 45 s, with final extension for 2 min at 72°C.

We genotyped a total of 34 SNPs present in three selectin genes—13 for E-selectin (SELE 1–13), 15 for L-selectin (SELL 1–15), and 6 for P-selectin (SELP 1–6) (fig. 1)—by means of the Invader assay that combines a structure-specific cleavage enzyme with a universal fluorescent resonance energy transfer system (Mein et al. 2000).

Comparison of allelic and genotypic frequencies in patients who have IgAN versus frequencies in control subjects disclosed significant associations between IgAN and eight SNPs present in two of the selectins (table 1 and table 2). Among the disease-associated SNPs, two were in the E-selectin gene (SELE8 in exon 9 and SELE13 in the 3′ flanking region). The other six were in the L-selectin gene (SELL1 in the 5′ flanking [promoter] region, SELL4 in intron 2, SELL5 in exon 3, SELL6 in intron 3, SELL10 in exon 5, and SELL11 in intron 5). These eight SNPs all lie within a 24-kb span of genomic DNA in the region harboring the three-gene cluster of selectins on chromosome 1q24-25 (fig. 1).

Homozygosity for minor alleles was significantly more common among patients with IgAN than among control subjects. The most significant association of a single SNP with IgAN involved SELL6 (χ^2=22.0, P = .000003, odds ratio 3.42 [95% CI 2.00–5.85]). Among the eight SNPs for which we found positive associations, two alter amino acid sequences: SELE8 substitutes tyrosine for histidine at codon 468 in the short consensus-repeat domain of E-selectin (χ^2 = 9.02, P = .0026, odds ratio = 2.73 [95% CI 1.38–5.38]), and SELL10 substitutes serine for proline at codon 238 in a short consensus-repeat domain of L-selectin (χ^2 = 17.4, P = .000031, odds ratio = 3.61 [95% CI 1.91–6.83]). A third SNP in the promoter region, SELL1, could affect promoter activity of the L-selectin gene (χ^2 = 19.5, P = .000010, odds ratio = 3.77 [95% CI 2.02–7.05]).

Analysis of linkage disequilibrium (LD) coefficients among these eight SNPs indicated quasi-complete LD (table 3). When we estimated haplotypes for three SNPs (SELE8, SELL1, and SELL10) that may affect the quality and quantity of the gene products, the TGT haplotype revealed a stronger association with the IgAN phenotype (χ^2 = 18.67, P = .000016, odds ratio of 1.88 [95% CI 1.41–2.51]) than did the genotype at either locus singly (table 4).

To investigate a potential role of E-selectin and/or L-selectin in the pathogenesis of IgAN, we performed immunohistological staining of these molecules in biopsy specimens (fig. 2). Strong staining of both E-selectin and L-selectin was observed in interstitial infiltrates of renal tissue from a patient with IgAN, whereas staining of both selectins was very weak in the renal tissues without infiltration.

In the present study, we have demonstrated significant association of eight SNPs in selectin genes with IgAN, as well as accumulation of two selectin proteins in renal tissues. Our findings suggest that one or more of the

Table 4

Estimated Haplotype Frequencies of SNPs SELE8, SELL1, and SELL10

Haplotype	IgAN	Control	Total
CAC	374.55 [68.10%]	568.9 [81.97%]	943.45 [75.84%]
CAT	6.05 [1.10%]	2.01 [.29%]	8.06 [.65%]
CGC	9.9 [1.80%]	2.02 [.29%]	11.92 [.96%]
CGT	20.9 [3.80%]	17.07 [2.46%]	37.97 [3.05%]
TAC	3.3 [.60%]	3.09 [.45%]	6.39 [.51%]
TAT	3.3 [.60%]	.03 [.00%]	3.33 [.27%]
TGC	0 [.00%]	.98 [.14%]	.98 [.08%]
TGT*	132 [24.00%]	99.9 [14.39%]	231.9 [18.64%]
Total	550 [100.00%]	694 [100.00%]	1,244 [100.00%]

NOTE.—Haplotype frequencies for pairs of alleles were estimated by the Estimating Haplotype-Frequencies software program, as described elsewhere (Terwilliger and Ott 1994).

* χ^2 (TGT/others) = 18.67, P = .000016.

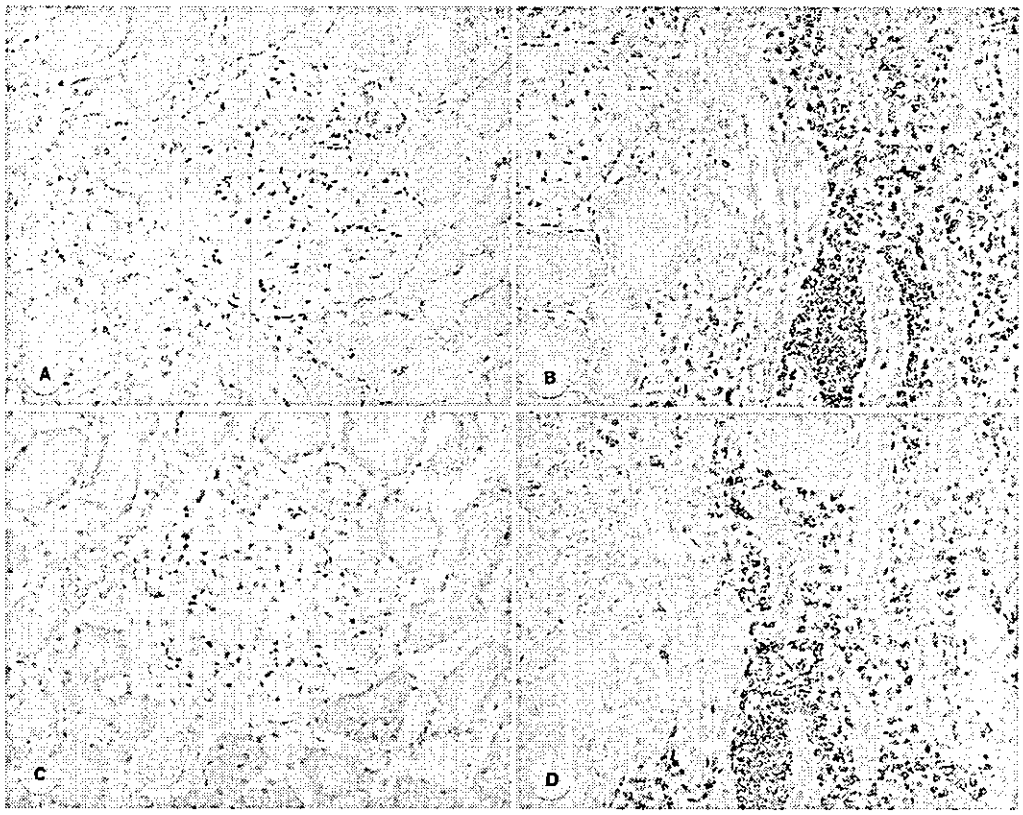

Figure 2 Immunohistochemical localization of E-selectin and L-selectin in the renal tissues of patients with IgAN. Control tissues were normal portions obtained from patients who underwent nephrectomy because of renal tumor. The monoclonal antibodies used in this study were anti-human E-selectin (clone 1.2B6) and anti-human L-selectin (clone FMC46) (DAKO Japan). *A*, Control E-selectin. *B*, IgAN E-selectin. *C*, Control L-selectin. *D*, IgAN L-selectin. Original magnification ×200.

three specific SNPs could affect the quality or quantity of the gene products and could play a significant role in the etiology of IgAN.

Although a genomewide analysis that used familial cases has been reported (Gharavi et al. 2000), most patients with IgAN have no familial history of the disease. IgAN is a complex disease whose etiology involves immunological, environmental, and genetic factors (Hsu et al. 2000). Since it was hard for us to locate patients with familial histories of IgAN, we chose to perform a case-control association study, using SNPs at the three-gene selectin complex on chromosome 1q24-25.

One study suggested that LD in the human genome was likely to extend only a few kilobases (Dunning et al. 2000), but other investigators have suspected that it could extend much farther, to distances >100 kb in some cases (Abecasis et al. 2001). In fact, we now know that distances encompassing LD can vary according to the chromosomal regions and/or the populations examined (Reich et al. 2001). We found that LD at the selectin locus extended over ~24 kb. This result implies that ~125,000 SNPs will be required for whole-genome scanning in the Japanese population.

Selectins are cell-cell adhesion molecules involved in the leukocyte–endothelial cell interaction that is required for extravasation at sites of tissue injury. They have a single C-type lectin domain at their extracellular amino-termini, followed by an epidermal growth factor (EGF)-like domain, short consensus repeat domains, a transmembrane domain, and a short cytoplasmic tail. In the E-selectin gene, substitutions from Ser to Arg at codon 149 in the EGF-like domain and from Leu to Phe at codon 575 in the transmembrane domain have been associated with severe atherosclerosis in German patients (Wenzel et al. 1996). However, in our Japanese population sample, we found no substitutions at codon 149 and no significant association between IgAN and the SNP (SELE10) at codon 575 of E-selectin. Instead, in patients with IgAN we found a significant increase in tyrosine alleles at codon 468 in E-selectin,

and in serine alleles at codon 238 in L-selectin, both of them in short consensus-repeat domains. Certain alleles of these two SNPs are likely to increase susceptibility to the IgAN phenotype with interstitial infiltration. The accumulation of these two isoforms of selectin that we observed in IgAN kidneys may provide a useful clue for development of novel treatments for this form of glomerulonephritis.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for selectin genes [accession numbers AL021940.1, NT000026.1, and AL022146.1])
JSNP, http://snp.ims.u-tokyo.ac.jp/ (for SNPs and primers)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for IgAN [MIM 161950])

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RESEARCH ARTICLE

Introduction of DNA enzyme for Egr-1 into tubulointerstitial fibroblasts by electroporation reduced interstitial α-smooth muscle actin expression and fibrosis in unilateral ureteral obstruction (UUO) rats

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The phenotypic alteration of interstitial fibroblasts into 'myofibroblasts', acquiring characteristics of both fibroblasts and smooth muscle cells is a key event in the formation of tubulointerstitial fibrosis. The up-regulation of the early growth response gene 1 (Egr-1) preceded the increased interstitial expression of α-smooth muscle actin (αSMA), a marker of phenotypic changes, in obstructed kidney, a model of interstitial fibrosis. To target Egr-1 expression in the interstitium of obstructed kidneys, we introduced a DNA enzyme for Egr-1 (ED5) or scrambled DNA (SCR) into interstitial fibroblasts by electroporation-mediated gene transfer. Northern blot analysis confirmed an increase in the cortical mRNA expression of Egr-1 in the obstructed kidneys from untreated or SCR-treated rats, while ED5 transfection blocked Egr-1 expression with a concomitant reduction in TGF-β, αSMA and type I collagen mRNA expression. Consequently, ED5 inhibited interstitial fibrosis. In conclusion, electroporation-mediated retrograde gene transfer can be an ideal vehicle into interstitial fibroblasts, and molecular intervention of Egr-1 in the interstitium may become a new therapeutic strategy for interstitial fibrosis.
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Keywords: *electroporation; interstitial fibrosis; erg-1; αSMA; TGF-β*

Introduction

Tubulointerstitial inflammations and fibrosis are common features in a variety of progressive renal diseases. It may determine the degree of impairment of renal function and predict long-term prognosis more accurately than glomerular injury.[1,2] Interstitial fibrosis is characterized by accumulation of matrix protein in the renal tubulointerstitial compartment.[3] During the development of interstitial fibrosis, a high proportion of 'myofibroblasts' was found to express α-smooth muscle actin (αSMA).[3–6] αSMA, one of six actin isoforms identified in mammalian cells, has received considerable attention as a specific marker for cells of smooth muscle origin.[7–10] αSMA is normally expressed by smooth muscle cells, myoepithelial cells and vascular pericytes.[7] The expression of this marker by mesenchymal cells with features of fibroblasts has been used to define the myofibroblast, a cell type intermediate in phenotype between fibroblasts and smooth muscle cells, thereby acquiring a contractile feature similar to smooth muscle cells. These cells have been identified in healing wounds and scarring processes in several organs including the kidney. Increased expression of αSMA in the interstitium has been noted in several progressive models of renal injury.[3–6] In addition, it was proposed that up-regulated interstitial expression of αSMA might serve as a predictor of progressive renal dysfunction.[11] Following the αSMA expression in the tubulointerstitium, several events occur in parallel, including an influx of monocytes/macrophages and accumulation of various extracellular matrix proteins.

Rupprecht *et al*[12] demonstrated a very close correlation between the proliferative response of mesangial cells and the induction of early growth response gene 1 (Egr-1) *in vitro* and *in vivo*. Egr-1 is a zinc finger transcription factor encoding 80–82 kDa that bind to target DNA that interacts with a consensus GC rich region, GCG(T/G)GGGCG, to influence the transcription of a diverse set of genes.[13–15] Egr-1 has been implicated in the induced expression of several growth factors such as transforming growth factor-β (TGF-β),[16] basic fibroblast growth factor (FGF),[17] platelet derived growth factor-A (PDGF-A)[18,19] and PDGF-B,[20] and adhesion molecule, such as intercellular adhesion molecule 1 (ICAM-1)[21] and CD44,[22] which was suggested to play an important role in renal interstitial inflammation and fibrosis. The induction of PDGF[4,23] and TGF-β[24,25] could contribute to myofibroblast transformation, proliferation and extracellular matrix accumulation that are the hallmarks of progressive interstitial fibrosis. Therefore, targeting Egr-

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1 expression in the interstitium could be an ideal strategy for interstitial fibrosis.

Targeting interstitial cells has proven to be challenging, and inhibition studies in animal models of progressive interstitial fibrosis largely remain to be performed. We pioneered a new strategy of gene transfer to the renal interstitial fibroblasts using the HVJ liposome method.[26] We then demonstrated that the introduction of antisense oligodeoxynucleotides (ODN) for TGF-β into renal interstitial fibroblasts could inhibit interstitial TGF-β expression and thereby prevent interstitial fibrosis.[27] More recently, we developed a new gene transfer system by electroporation *in vivo*: DNA injection via the renal artery followed by application of electric fields.[28] Electroporation is free from oncogenicity, immunogenicity and cytotoxicity of viral vectors. On histological examination, we observed few harmful effects on transfected kidney except small burns on the surface in contact with the electrode. There was no histologic damage in glomeruli and tubular epithelial cells by electoric pulses. In addition, the electroporation-mediated gene transfer technique resulted in significantly higher transfection efficiency than HVJ liposome method.[28]

In the present study, we examined the possibility of gene manipulation targeting interstitial Egr-1 expression by electroporation-mediated retrograde gene transfer into interstitial cells using DNA enzyme. DNA enzyme,[29] a new generation of catalytic nucleic acid composed of DNA, can potentially cleave RNA at any purine–pyrimidine junction and offer greater substrate specificity than hammerhead ribozymes. Here, we first demonstrated that the Egr-1 expression was increased in the obstructed kidneys. The introduction of a DNA enzyme for Egr-1 into interstitial fibroblasts by electroporation reduced Egr-1, TGF-β, αSMA, and type I collagen expressions and consequently suppressed the fibrosis in obstructed kidney.

Results

Effects of the DNA enzyme on Egr-1 expression in vitro

It was reported that serum stimulation elevates Egr-1 expression on mesangial cells.[12] To target Egr-1 expression in interstitial fibroblasts, we first examined the Egr-1 mRNA expressions on normal rat kidney (NRK) cells, a fibroblast cell line derived from rat kidney. Northern blot analysis demonstrated that Egr-1 mRNA levels were elevated in a fetal calf serum (FCS) concentration-dependent manner (Figure 1a) and that peak expression was observed at 60 min after serum stimulation (Figure 1b).

To examine the effects of the DNA enzyme on Egr-1 expression, NRK cells were incubated with ED5 or SCR before the serum stimulation. ED5 blocked the FCS induced Egr-1 mRNA expression, while SCR had no effect (Figure 1c). Western blot analysis demonstrated that ED5 also inhibited the serum stimulated Egr-1 protein expression (Figure 1d).

Electroporation-mediated retrograde gene transfer into interstitial cells

We examined the possibility of transferring foreign genes into renal interstitial fibroblasts by electroporation-mediated gene transfer. Fluorescein isothiocyanate

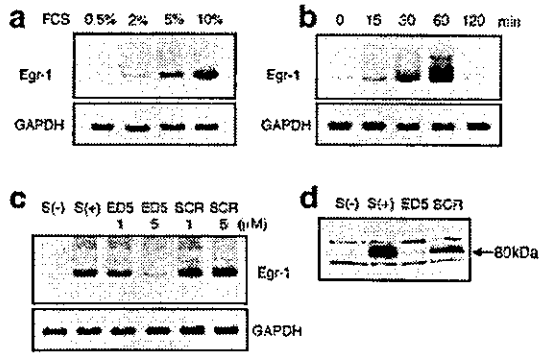

Figure 1 The effect on Egr-1 expression was examined in cultured NRK cells. Northern blot analysis demonstrated that treatment with FCS-induced Egr-1 expression in a concentration-dependent manner (a) with a peak at 60 min (b). The DNA enzyme, ED5 blocked the FCS-induced Egr-1 mRNA expression, while the scrambled ODN, SCR, showed no effect (c). Western blot analysis showed that the Egr-1 protein expression was also blocked by ED5 treatment (d).

(FITC)-labeled ODN were retrogradely infused into the left kidney via the ureter and the left kidney was electroporated (six times at 75 V) using the tweezer-type electrode. FITC-labeled ODN were diffusely observed in the nuclei of interstitial cells 10 min after transfection (Figure 2a). To examine the cellular localization of the transfected ODN, rabbit polyclonal anti-laminin antibodies and Texas red-conjugated anti-rabbit IgG were used to stain the basement membrane (Figure 2b). FITC (green)-positive cells were observed outside the basement membrane (red). To identify the transfected cells, the antibodies for RECA-1 (Figure 2c, d), and monoclonal antibodies ED1 (Figure 2e), and ER-TR7 (Figure 2f) were used. Transfected interstitial cells were not endothelial cells stained with anti-RECA-1 antibody (Figure 2c), nor macrophages stained with ED1 (Figure 2e), but were shown to be fibroblast-like cells stained with ER-TR7 (Figure 2f). Under the triple exposure of the green fluorescence of ODN, red fluorescence of endothelial cells and blue fluorescence of the nuclei, the color of ODN-transfected nuclei changed to sky blue, while the nuclear edge of endothelial cells appeared violet (Figure 2d).

Effect of ED5 on Egr-1 and αSMA expressions in obstructed kidneys

In obstructed kidneys, cortical Egr-1 expression preceded the increased expression of αSMA (Figure 3). The early expression of Egr-1 could be seen 12 h after ureteral ligation. It declined thereafter but remained strong 7 days after obstruction. αSMA expression, following the Egr-1 expression, started at day 2 and peaked from days 4 to 7.

To examine the effect of the DNA enzyme on obstructed kidneys, ED5 or SCR (200 μg) were retrogradely transferred via the ureter by electroporation and then ureteral obstruction was induced by ligation. Northern blot analysis demonstrated that cortical Egr-1 mRNA levels were markedly suppressed in ED5-treated obstructed kidneys on day 7 compared with those in untreated or SCR-treated obstructed kidneys (Figure 4).

The effective suppression of Egr-1 by ED5 limited the increased mRNA expression of TGF-β1, αSMA, and, type I collagen on day 7 (Figure 4).

Immunohistochemical examination showed that