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Delivery of erythropoietin by encapsulated myoblasts in a genetic model of severe anemia

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Background. Existing animal models of anemia inadequately reflect the hematocrit usually present in chronic renal failure (CRF) patients and do not permit long-term treatment studies. The transgenic mouse strain 134.3LC (Epo-TAg^{H}) displays a severe chronic anemia resembling that observed clinically during CRF, while displaying an active, normal life span. This phenotype makes it a particularly interesting mouse model for testing erythropoietin (Epo)-based gene transfer strategies.

Methods. Ex vivo gene therapy was employed to administer mouse Epo to homozygous anemic Epo-TAg^{H} mice. Encapsulated C_2C_{12} myoblasts genetically engineered to secrete 163 IU mouse Epo/10^6 cells/day were subcutaneously transplanted on the dorsal flank of the mice. Efficacy of delivered Epo was monitored by weekly measurements of animal hematocrit.

Results. Most treated homozygous Epo-TAg^{H} mice displayed only a transient rise in hematocrit before eventually decreasing to levels as low as 3%. Administering the immunosuppressor anti-CD4+ monoclonal antibody (mAb) to homozygous Epo-TAg^{H} mice, beginning at the time of implantation, permitted a rise in hematocrit that remained stable at elevated levels in cases of continued immunosuppression.

Conclusions. Mice having the T antigen insertion in both Epo alleles appeared to develop an immune response to the natural mouse Epo delivered by encapsulated cells. By preventing this reaction using immunosuppression, we demonstrate that encapsulated myoblasts can deliver therapeutic doses of mouse Epo systemically and restore hemopoiesis in a genetic model of severe anemia.

In situations of chronic renal failure, patients develop anemia due to inadequate erythropoietin (Epo) production by the kidney. Recombinant Epo, administered as a replacement therapy, restores hematocrit and blood hemoglobin concentration, eliminating the need for blood transfusions. Treatment entails regular injection of Epo, 2 to 4 times per week, given either intravenously or subcutaneously [1].

Gene transfer techniques simplify protein-based Epo therapies, as required molecules can be produced in situ, eliminating dependence on exogenous injections and facilitating the delivery of Epo to anemic patients. Methods include direct injection of viral or plasmid vectors expressing the *Epo* transgene [2–4] and the transplantation of cells or tissue genetically engineered to secrete Epo [5]. Using these gene therapy strategies, Epo may be produced in a continuous fashion and thereby replace the function of damaged endogenous Epo-secreting cells in the diseased kidney [2–5]. Anemic chronic renal failure (CRF) patients treated using gene therapy approaches will benefit from stable low levels of endogenously produced Epo, which may raise their hematocrit to normal levels as opposed to Epo injections that maintain patients at slightly depressed hematocrits.

Animal models have been developed using a partial nephrectomy procedure to reproduce conditions that lead to anemia in patients with CRF. These models suffer from shortcomings, including hematocrits that do not decrease to the levels observed in CRF and systemic toxicity due to renal failure, which leads to animal death [6].

The transgenic mouse strain (134.3 LC, *Epo-TAg^{H}*), which has a stable chronic anemia due to a relative deficiency of erythropoietin, may prove to be a valuable tool for refining gene transfer approaches for delivery of *Epo* [7]. Mice homozygous for the modified *Epo* allele are severely anemic, with hematocrits ranging from 13% to 20% [7, 8], but otherwise appear healthy and remain active. These mice display hematocrit levels comparable to those observed in patients with chronic renal failure, making them an interesting model for the study of *Epo*-based gene therapies.

An ex vivo gene therapy approach employing cell encapsulation for the transplantation of genetically modified cells was investigated for the delivery of mouse Epo

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to these anemic mice. With this method, cells are enclosed inside a semipermeable polymer membrane, which isolates encapsulated cells and prevents direct cell-to-cell contact with the host, thereby eliminating antigen recognition and immune rejection through direct presentation. Capsule geometry facilitates device implantation and retrieval. Genetically-modified allogeneic cell lines are attractive for this purpose as they can be screened for biosafety and banked in liquid nitrogen, permitting a standardized therapy that uses a universal donor cell.

The aim of the present study was to evaluate the Epo-TAg^{H} mouse strain as an anemic animal model resembling human CRF for testing encapsulated cell-based Epo delivery. For treatment, an allogeneic C_2C_{12} myoblast cell line engineered to secrete 163 IU Epo/10^6cells/day was encapsulated and implanted subcutaneously on the dorsal flank of Epo-TAg^{H} mice.

METHODS

Epo-TAg^{H} mouse line

The transgenic mouse strain employed for these studies was the product of the homologous recombination within the *Epo* gene, resulting in a modified *Epo* gene (*Epo-TAg^{H}*) containing a 2.7 kb fragment coding for the entire SV40 large T antigen inserted in the 5′ untranslated region upstream the ATG site [*www.mgu.har.mrc.ac.uk/fesa/fesa.html*; TgH(eposvT)1Pjr] [7]. Animal experimentation was conducted following the review and approval by the Swiss Cantonal Vetinarian Services. Wild-type, heterozygous and homozygous mice were differentiated from one another by polymerase chain reaction (PCR) of genomic DNA. The forward primer 5′-ATGG ATAAAGTTTTAAACAGAGAGG-3′, lying in the 3′ region of the inserted SV40 large T antigen, and the reverse primer 5′-GGAGGTCACAGGTCCTTAGCC TGTG-3′, present in the first intron of murine erythropoietin gene, were used to identify the inserted SV40 T antigen sequence.

RT-PCR of the murine Epo cDNA

Mice were placed in a hypoxic environment containing 7% oxygen for 24 hours as described previously [9]. Thereafter, total RNA was extracted from kidney tissue using the Trizol reagent (Life Technologies, Paisley, UK) and the murine Epo cDNA was amplified from cDNA prepared from isolated RNA by PCR using the mEpo forward primer: 5′-ATGGGGGTGCCCGAACGTCC CACCC-3′, containing regions in the first and second exons, and the mEpo reverse primer: 5′-TCACCTGTC CCCTCTCCTGCAGACC-3′.

Cell culture and cell encapsulation

Mouse C_2C_{12} myoblasts obtained from the American Type Culture Collection (CRL 1772; ATCC, Rockville, MD, USA) were transfected with the pPI-mEpo-ND plasmid [5] using calcium phosphate precipitation (mammalian transfection kit; Stratagene, Basel, Switzerland). C_2C_{12} mEpo myoblasts were resuspended at 1×10^5 cells/μL and then injected into semipermeable polyethersulfone (PES) hollow fibers (OD 720 μm; ID 524 μm; molecular weight cutoff 280 kD; Akzo Nobel Faser AG, Wupperthal, Germany) as previously described [9]. One-centimeter long capsules lacking matrix were loaded with C_2C_{12} mEpo myoblasts at a density ranging from 2×10^5 to 6×10^5 cells per capsule. Encapsulated cells were maintained in culture at least three days prior to implantation.

Measurement of Epo secretion

Mouse Epo (mEpo) secretion from encapsulated myoblasts was measured at pre-implant and post-explant by incubating capsules for one hour in 1 mL of medium. Epo levels in the conditioned media were measured using an enzyme-linked immunosorbent assay (ELISA; Quantikine IVD; R&D Systems, Minneapolis, MN, USA) with a detection limit of 2.5 mIU hEpo/mL. Cross-reaction of the kit allowed measurement of mEpo in culture supernatants [5]. The detection limit of mEpo released by encapsulated cells was approximately 250 mIU mEpo/day.

Capsule implantation

Animals were anesthetized by inhalation of isoflurane (Forene; Abbott Laboratories, Cham, Switzerland) and capsules were then implanted subcutaneously in the dorsal flank using a trocar (Abbocath-T 16 G; Abbott Laboratories) [9]. Upon recovery, the animals were returned to the animal care facility, where they had access to food and water ad libitum.

Western blot analysis

Cells and tissue were lysed in a RIPA buffer containing 1% Triton X-100, 0.15 mol/L NaCl, 10 mmol/L Tris (pH 7.4), 50 μg/mL phenylmethylsulfonyl fluoride (PMSF) and 2 μg/mL aprotinin at 4°C for 30 minutes. Immunoprecipitation was performed on 1 mg of total protein in 1 mL of radioimmunoprecipitation assay (RIPA) buffer containing 1 μg αTAG antibody (AB-1 monoclonal; Oncogene Research, Darmstadt, Germany) or 1 μg anti-human Epo (polyclonal; R&D Systems) overnight at 4°C. The AB-1 monoclonal antibody (mAb) is specific for the first exon of the SV40 T antigen. The anti-human Epo Ab also is employed in the ELISA kits that cross-react with mouse Epo. To this mixture 20 μL protein-A Sepharose (Pharmacia, Duebendorf, Switzerland) was added, followed by a one-hour incubation at 4°C and a wash (4×) in RIPA buffer. Protein precipitates were migrated on a 10% gel and transferred to a nitrocellulose filter. A rabbit polyclonal anti-SV40 T antigen antibody (R613) diluted 1/2000 (a kind gift of Dr. S. Efrat, Albert

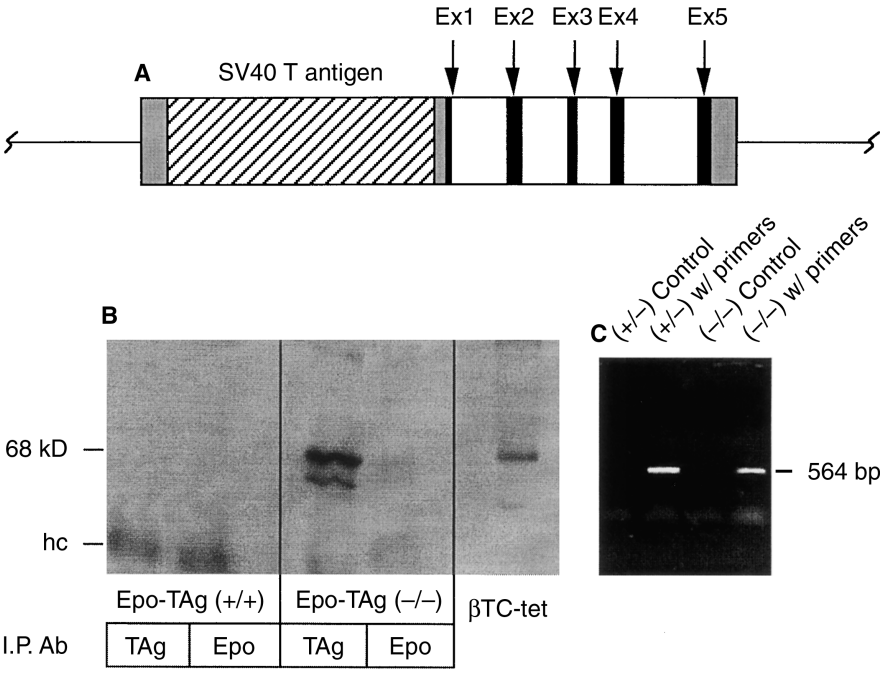

Fig. 1. (*A*) Transgenic construct employed for generating the 134.3 LC, Epo-TAg^H mouse line. Epo in the untranslated region is in the gray bars and in the translated region is solid black; the large T antigen is represented by the hatched area; Ex is exon. (*B*) Western blot of the immunoprecipitated SV40 large T antigen and Epo expressed in kidneys of anemic homozygous Epo-TAg^H mice (−/−) and wild-type (+/+) littermates. Kidney protein extracts were immunoprecipitated either with antibodies against the SV40 large T antigen or Epo. Cell lysates of bTC-tet cells expressing the SV40 large T antigen were employed as a positive control (hc is heavy chain) [10]. (*C*) rtPCR of mouse Epo expressed in the kidney tissue of homozygous (−/−) and heterozygous (+/−) Epo-TAg^H mice. The band migrating near 600 bp corresponds to the size of the mouse Epo cDNA. Reactions carried out in the absence of primers served as negative controls: (+/−) control and (−/−) control lanes.

Einstein College of Medicine, NY, USA) was employed to label the transferred SV40 large T antigen.

Immunosuppression with anti-CD4+ mAb

The anti-mouse CD4+ mAb, a depleting anti-mouse CD4+ mAb, was purified from conditioned media used to culture the rat GK1.5 hybridoma (ATCC). The mAb was resuspended in 0.1 mol/L glycine (pH 7.0) and sterile filtered for injection. Animals were administered 0.25 mg mAb per intraperitoneal injection either on days −3, 0, +3 or on days −3, 0, +3, +14, +28, +42 relative to capsule implantation.

Statistical analysis

Data are expressed as mean ± SEM. The data obtained for the different animal groups were analyzed for statistical significance using analysis of variance (ANOVA) followed by a Fischer's *post hoc* test. A $P < 0.05$ was considered to be statistically significant.

RESULTS

Characterization of the transgenic Epo-TAg^H anemic mouse line

The Epo-TAg^H mouse line was generated by the homologous recombination of the *Epo-TAg^H* transgene (Fig. 1) at the locus of the endogenous *Epo* gene [7]. Mice having one copy of this *Epo-TAg^H* allele were slightly anemic with a significantly ($P < 0.001$) lower hematocrit value (36.1 ± 0.4%, $N = 86$) than the wild-type background strain (41.2 ± 0.4%, $N = 56$). Those mice homozygous for the *Epo-TAg^H* insertion were severely anemic compared to heterozygotes ($P < 0.001$), having hematocrit values of 19.2 ± 0.2% ($N = 147$).

Western blot analysis of kidney extracts in homozygous Epo-TAg^H mice revealed the presence of the SV40 T antigen as a doublet, with the upper band corresponding to the expected size (80 kD) of the large T antigen (Fig. 1B). The lower band was believed to result from variations in protein phosphorylation. No signal was detected on Western blot analysis with anti-T antigen Ab when kidney extracts were first immunoprecipitated using an anti-Epo Ab. This suggests that the T antigen and Epo are not produced as a fusion protein. The presence of a full-length mEpo messenger RNA in homozygous Epo-TAg^H mice was verified using rtPCR (Fig. 1C), indicating that there is transcription beyond the SV40 T antigen polyadenylation site. Epo was not detected in the serum of homozygous Epo-TAg^H mice either by Western analysis or ELISA techniques, confirming that the endogenous Epo levels were extremely low.

Response of Epo-TAg^H mice to mEpo delivered by encapsulated cells

To correct the severe anemia in the homozygous Epo-TAg^H mice, animals were implanted subcutaneously with encapsulated C_2C_{12} myoblasts engineered to secrete mouse Epo. Wild-type littermates were implanted with identical devices for comparison purposes. Within one week, both animal groups responded to the implants by increasing their hematocrit levels (Fig. 2).

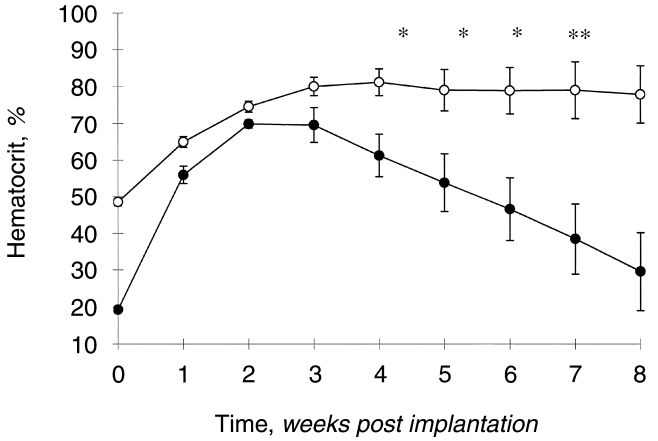

Fig. 2. Comparison of wild-type (+/+; ○) and anemic homozygous (−/−; ●) Epo-TAg^H mice to subcutaneously implanted capsules secreting mouse Epo (N = 7). Data are presented as mean ± SEM (*P < 0.05; **P < 0.01).

At four weeks the homozygous mice began to show a significant decline in their hematocrit as compared to wild-type mice. Differences in blood hematocrit increased with time, and by week eight, the wild-type group had stabilized at 78% with homozygous mice decreasing to an average of 30%. Capsules were explanted on week eight and analyzed for secretion of Epo. Devices retrieved from wild-type mice displayed on average a two-fold higher Epo secretion than those explanted from homozygous mice. The ratio of Epo released at explant versus implant was employed to measure relative cell survival within the devices (Table 1). Despite the divergence in the hematocrit of the two groups, no statistically significant difference was observed in the relative capsule secretion explant/implant ratio. In separate experiments lasting longer than eight weeks, implanted homozygous Epo-TAg^H mice became critically anemic compared to baseline levels (19.2%), with hematocrits dropping to as low as 3% (data not shown). This suggested that homozygous mice develop an immune response to the delivered natural mouse Epo that may cross-react with the endogenous Epo of the Epo-TAg^H strain. On the contrary, heterozygous mice never became anemic or showed any indication of an Epo-derived immune response following implantation of capsules releasing mouse Epo (data not shown). This was expected, as heterozygous mice still have one remaining copy of the unaltered *Epo* gene.

There were rare exceptions to this observation, probably due to heterogeneity in the immune response. Notably, two implanted homozygous Epo-TAg^H mice maintained elevated hematocrits for periods of 9 and 12 months (Fig. 3). Following removal of the capsules, animal hematocrit returned to pre-implant levels, demonstrating that the encapsulated myoblasts were responsible for the long-term correction of anemia. While clusters of viable cells were observed inside the explanted capsules, secreted Epo levels by the devices were below the 250 mIU/day detection level of the ELISA kit. This suggests that only very low levels of continuously released Epo were required to normalize anemic mice.

Immunosuppression of treated homozygous Epo-TAg^H mice

Further studies were pursued to verify whether the decreases in hematocrit observed in homozygous mice were related to an immune reaction to foreign Epo. The physiological response of homozygous Epo-TAg^H mice to mouse Epo delivered by encapsulated myoblasts was evaluated in a context where recipients were immunosuppressed. Mice were given a specific anti-CD4+ mAb for depletion of CD4+ T cells.

Homozygous Epo-TAg^H mice were treated with anti-CD4+ mAb either transiently, on days (−3, 0, +3) or in a repeated manner on days (−3, 0, +3, +14, +28, +42) relative to the moment of capsule implantation. Mice receiving transient depletion of CD4+ T cells became polycythemic, with hematocrits rising to 80% (Fig. 4A). During the period from weeks six to eight, two out of the five animals began to decrease their hematocrits (Fig. 4A), suggesting that transient immunosuppression was insufficient. At explant, all capsules secreted measurable levels of mEpo (Table 1). Homozygous Epo-TAg^H mice repeatedly administered anti-CD4+ mAb every 14 days following implantation increased their hematocrit levels and maintained an average near to 80% for the eight weeks investigated (Fig. 4B). Explanted capsules continued to produce mEpo at a slightly higher level, although not significantly, than those retrieved from mice transiently treated with anti-CD4+ mAb (Table 1). Histology of recovered capsules contained viable clusters of myoblasts dispersed within implants, with a characteristic necrosis at the center of the capsule (Fig. 5).

DISCUSSION

The aim of the present report was to explore the efficacy of Epo delivery by encapsulated engineered myoblasts in an animal model of severe anemia that most accurately reflects the blood hematocrit levels found in CRF patients. Previously, it was observed that Epo administered using cell encapsulation was able to induce polycythemia in healthy mice [5]. Bearing in mind the eventual clinical application of this technology, it is necessary to demonstrate that encapsulated Epo delivery can restore hemopoiesis in situations of chronic anemia.

To date, most Epo-based gene therapies have been conducted in healthy animals, which respond to delivered Epo by becoming polycythemic with hematocrits ranging from 70% to 90%. To reproduce the conditions leading to anemia in patients with chronic renal failure,

Table 1. Capsule Epo secretion at implant versus explant in Epo-TAg^{H} mice

Animal groups	Mouse Epo secretion *IU/day*		
	Implant	Explant	Expl/Impl %
Homozygous (−/−) (N = 7)	4.96 ± 0.42	1.11 ± 0.14	22.4
Wild-type (+/+) (N = 7)	6.13 ± 0.67	2.21 ± 0.57	36.1
Immunosuppressed homozygous (−/−) mice			
Anti-CD4+ mAb (days −3, 0, +3) (N = 5)	11.0 ± 1.7	1.4 ± 0.4	12.7
Anti-CD4+ mAb (days −3, 0, +3, +14, +28, +42) (N = 5)	9.4 ± 1.9	1.8 ± 0.5	19.1

Data are presented as average ± SEM.

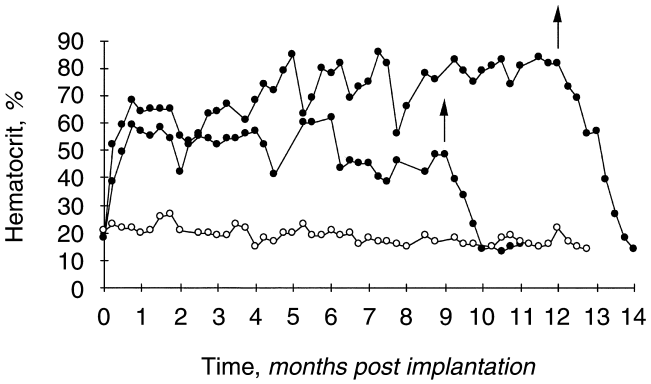

Fig. 3. Two of the homozygous mice maintaining elevated hematocrits were followed for a period of 9 and 12 months prior to explantation (arrows), at which point they returned to their normal baseline hematocrit levels. Symbols are: (●) mEPO; (○) control.

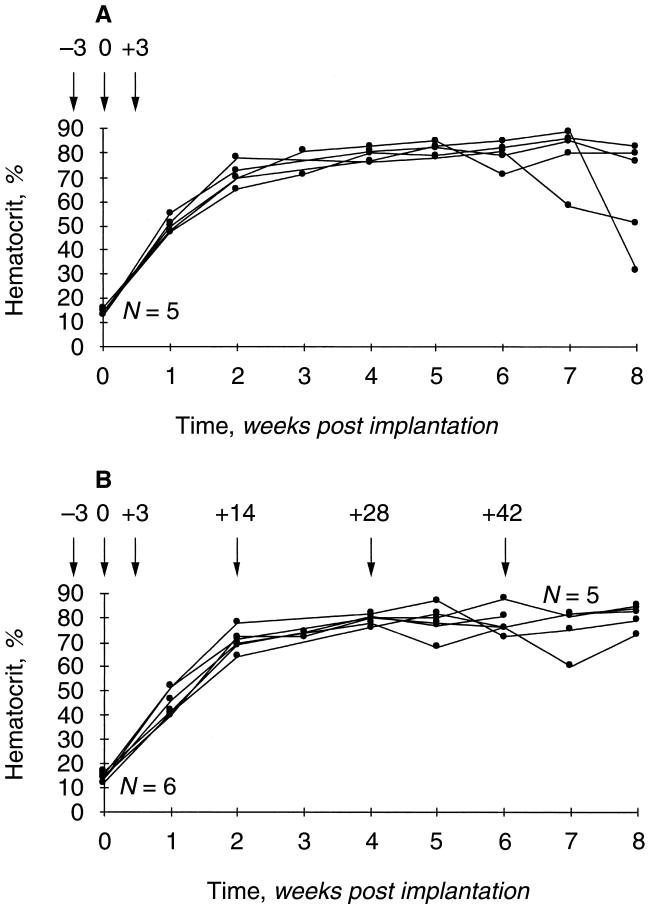

Fig. 4. Homozygous anemic Epo-TAg^{H} mice implanted with capsules secreting mouse Epo were treated with anti-CD4+ mAb. Efficacy of (*A*) transient (days −3, 0, +3) or (*B*) intermittent dosing (days −3, 0, +3, +14, +28, +42) was compared.

partially nephrectomized animals have been employed as a model for anemia. One study has been described in which an ex vivo gene therapy approach was effective in treating anemia in partially nephrectomized nude mice [6]. Unfortunately, the partial nephrectomy model is complicated as systemic toxicity due to renal insufficiency eventually leads to animal death, prohibiting long-term follow up subsequent to *Epo* gene delivery [6]. Hematocrits of nephrectomized animals lie near 34%, which is significantly higher than most cases of CRF observed clinically.

On the other hand, mice homozygous for the *Epo-TAg^{H}* insertion naturally have hematocrits near 20%, while leading a normal life span, making this strain clinically relevant for evaluating *Epo* gene transfer techniques. The response of the background, wild-type Epo-TAg^{H} mice to mouse Epo delivered by encapsulated myoblasts resembled that previously observed in C3H, DBA/2J, and C57/Bl6 mice strains [5], with animals increasing their hematocrit and maintaining elevated levels throughout the study period. Unexpectedly, treated homozygous Epo-TAg^{H} mice displayed only a transient rise in hematocrit before eventually becoming acutely anemic. This was not a result of insufficient Epo delivery, as explanted devices continued to release protein levels comparable to capsules retrieved from wild-type mice.

In certain circumstances, it has been observed that protein replacement therapy may lead to the development of host neutralizing antibodies against the therapeutic product. This includes the case where there are species incompatibilities between delivered and endogenous proteins [11]. In other situations, a host may be incapable of naturally producing a protein, such as factor IX, and hence develop antibodies against "foreign" recombinant factor IX given as a therapy [12].

Homozygous Epo-TAg^{H} mice produce undetectable

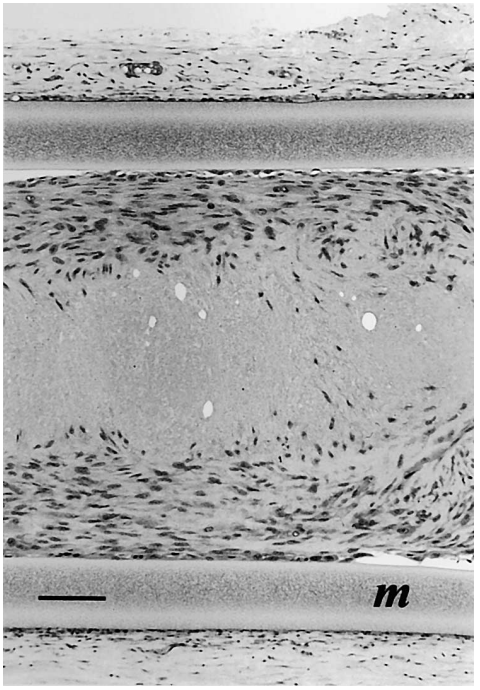

Fig. 5. Photomicrograph of encapsulated C_2C_{12} mEpo myoblasts following 8 weeks in vivo. Clusters of viable myoblasts were observed within the capsule's interior.

levels of mouse Epo due to the insertion of the large T antigen. Based on the effects of immunosuppression on animal reaction to Epo, it is believed that homozygous Epo-TAg^H mice develop an immune response to exogenously delivered mouse Epo. This is reinforced by the findings that a continued therapy using anti-CD4+ mAb was more effective than transient dosing regimens in maintaining elevated hematocrit levels. Treatment with anti-CD4+ mAb has been demonstrated to be one of the most efficacious means of preventing the development of neutralizing antibodies against a recombinant gene product in vivo [11].

It remains unknown why the majority of homozygous Epo-TAg^H mice display an apparent immune reaction against mouse Epo delivered by encapsulated cells. It has been previously demonstrated that Epo is highly species specific and that administration of human Epo to animals leads to the development of an immune response directed against both the human Epo and the endogenously produced mouse Epo [13]. In the present study, direct analysis of the immune response was not possible as neither endogenous Epo protein or anti-Epo antibodies could be detected in the treated homozygous mice due to the low levels present in the serum (data not shown). However, indirect evidence attained using immunosuppression provides strong support for the development of an Epo-directed immune response in treated homozygous mice.

While it is not our intent that systemic immunosuppression be used as a support therapy for Epo delivery by encapsulated cells, it was necessary to immunosuppress homozygous anemic Epo-TAg^H mice in order to follow the blood hematocrit response to administered Epo in the absence of an interfering antibody reaction. The doses of Epo required from encapsulated cells to correct anemic mice to normal hematocrit levels (45%) appears to be below 250 mIU/day, as these levels led to polycythemia (80%) in anti-CD4+ mAb treated mice. This suggests that perhaps as little as 10 to 50 mIU/day would be needed to treat these anemic mice.

In a few isolated cases, a long-term correction of anemia was observed in homozygous Epo-TAg^H mice in the absence of immunosuppression. As this was a rare phenomenon, it is likely due to the natural heterogeneity in a host's immune response to Epo. The capsule removed from the mouse with a hematocrit range from 40% to 60% secreted Epo levels below the 250 mIU/day detection limit, as might be predicted. Nevertheless, capsules containing Epo secreting cells restored hemopoiesis in anemic animals for periods as long as 12 months, validating this technology for use in prolonged therapies.

In the clinic, CRF patients are treated with bolus doses ranging from 1500 to 4000 IU Epo administered three times a week. Based on these values, we estimate that to restore hematocrit to acceptable levels, CRF patients will require capsules continuously delivering doses of at least 100 IU Epo per day. The positive effects of encapsulated Epo delivery in the anemic model have motivated us to scale up our system for human application. At present, we are in the pre-clinical phase of evaluating nontumorigenic human allogeneic cells that can secrete up to 1000 IU human Epo per day. We believe that these levels of production will allow us to show this first efficacy of an ex vivo Epo gene therapy in CRF patients.

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