Erythropoietin Delivery by Genetically Engineered Bone Marrow Stromal Cells for Correction of Anemia in Mice with Chronic Renal Failure

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The goal of this research was to develop a strategy to couple stem cell and gene therapy for in vivo delivery of erythropoietin (Epo) for treatment of anemia of ESRD. It was shown previously that autologous bone marrow stromal cells (MSCs) can be genetically engineered to secrete pharmacologic amounts of Epo in normal mice. Therefore, whether anemia in mice with mild to moderate chronic renal failure (CRF) can be improved with Epo gene-modified MSCs (Epo+MSCs) within a subcutaneous implant was examined. A cohort of C57BL/6 mice were rendered anemic by right kidney electrocoagulation and left nephrectomy. In these CRF mice, the hematocrit (Hct) dropped from a prenephrectomy baseline of approximately 55% to 40% after induction of renal failure. MSCs from C57BL/6 donor mice were genetically engineered to secrete murine Epo at a rate of 3 to 4 units of Epo/10⁶ cells per 24 h, embedded in a collagen-based matrix, and implanted subcutaneously in anemic CRF mice. It was observed that Hct increased after administration of Epo+MSCs, according to cell dose. Implants of 3 million Epo+MSCs per mouse had no effect on Hct, whereas 10 million led to a supraphysiologic effect. The Hct of CRF mice that received 4.5 or 7.5 million Epo+MSCs rose to a peak 54 ± 4.0 or 63 ± 5.5%, respectively, at 3 wk after implantation and remained above 48 or 54% for >19 wk. Moreover, mice that had CRF and received Epo+MSCs showed significantly greater swimming exercise capacity. In conclusion, these results demonstrate that subcutaneous implantation of Epo-secreting genetically engineered MSCs can correct anemia that occurs in a murine model of CRF.


A nemia, a hallmark of ESRD, is due in part to the reduced production of erythropoietin (Epo) by the kidney. Recombinant human Epo (rhEpo) utilization has considerably improved the management of anemia of patients with ESRD and markedly reduced the required number of blood transfusions (1–3). With rhEpo treatment, patients also benefit from secondary advantages, such as improved cardiac function, enhanced exercise capacity, and better quality of life (3). However, rhEpo administration also includes undesirable aspects: the intermittent high-dose nature of rhEpo treatment unlike the normal continuous endogenous Epo production; the possibility of antibody formation against rhEpo and its cross-reacting with endogenous Epo, potentially leading to pure red cell aplasia; the possible noncompliance of patients to self-administer rhEpo injections or to follow recommended instructions; and the high cost and limited financial resources that confine rhEpo use to patients with severe anemia. In view of the aging population and consequently predicted rise in renal disease, rhEpo-related expenditures are anticipated to escalate, as will the need to develop cost-effective alternatives. One such alternative treatment that we propose herein is a strategy to couple cell and gene therapy, whereby patients with anemia would receive subcutaneous implantation of a neo-organoid that encloses autologous cells that have been genetically engineered to secrete Epo.

One autologous cell type, bone marrow stromal cells (MSCs), which are important in hematopoiesis and in the bone marrow microenvironment, also has valuable characteristics that render it a desired autologous cellular vehicle for the systemic delivery of therapeutic proteins (4–8). MSCs are plentiful in humans of all ages; can be obtained with minimal discomfort; can be expanded, genetically engineered, and clonally selected in vitro easily; and can be reimplanted in the donor without a conditioning regimen (9–12). We therefore hypothesized that MSCs that are harvested from a patient can be gene-modified in vitro to express Epo and subsequently implanted subcutaneously in the same patient as a neo-organoid that allows sustained, clinically relevant production of Epo to correct anemia, reducing morbidity and improving health status.

We previously published proof-of-concept studies with murine MSCs that were genetically engineered to secrete murine Epo (mEpo). In brief, we reported significant mEpo production from mEpo gene-modified MSCs (Epo+mMSCs) that were implanted in nonmyeloablated, immunocompetent mice via the
intraperitoneal or subcutaneous route of delivery (13). We showed that embedding these Epo−MSCs within a matrix before their subcutaneous administration led to a more significant and prolonged pharmaceutical effect, as ascertained by resulting hematocrit (Hct). We observed that this enhanced outcome that was achieved when Epo−MSCs were embedded within the mouse-compatible material Matrigel (13) also occurred when the human-compatible, FDA approved, bovine collagen-based matrix Contigen was used (14). Furthermore, we demonstrated a retrievability safety feature of our strategy, confirmed by our observation that upon retrieval of implants of Contigen-embedded Epo−MSCs, the effect on the Hct was reversed (14). The sum of these observations supported the notion that a cell and gene therapy approach with genetically engineered MSCs would be feasible and advantageous for the treatment of anemia in ESRD.

In this investigation, we tested our strategy in a mouse model of anemia secondary to experimental renal failure, a well-tolerated procedure, and consequently noted that subcutaneous implantation of Contigen-embedded Epo−MSCs in these anemic mice led to a cell dose−dependent increase in plasma Epo and in Hct levels, as well as significantly enhanced exercise capacity.

Materials and Methods

Generation of Retrovirus-Producing Cells and Gene-Modified Primary Murine MSCs

We generated a retroviral plasmid construct (pEpo; Figure 1) that contained the cDNA for mEpo and used it to prepare retrovirus-producing cells GP+E86-Epo, as reported previously (14). Control GP+E86-EmptyVector virus producers were similarly produced (14).

A female 15- to 20-g C57BL/6 mouse (Charles River, Laprairie Co., St. Constant, QC, Canada) was killed, and whole bone marrow was collected by flushing femurs and tibiae with complete media (DMEM supplemented with 10% FBS and 50 U/ml Pen/Strep); after 5 d at 37°C with 5% CO₂, the nonadherent hematopoietic cells were removed, and the adherent MSCs were maintained for approximately 15 passages. MSCs were genetically engineered by transduction with filtered retroviral supernatant from GP+E86-Epo, giving rise to the polyclonal population of mEpo gene-modified MSCs, as described previously (14). The monoclonal subpopulation of mEpo gene-modified MSC that was used in this study (Epo−MSCs) was generated after the polyclonal population was plated at limiting dilutions and one clone was isolated and expanded. A supernatant sample from Epo−MSCs was used in an ELISA that was specific for hEpo (Roche Diagnostics, Indianapolis, IN). Control EmptyVector, which lacks the mEpo cDNA, MSCs likewise were prepared and referred to as control MSCs (14). Animals were handled under the guidelines promulgated by the Canadian Council on Animal Care.

Differentiation of MSCs

To verify the in vitro differentiation capabilities, we exposed Epo−MSCs to specific media. For induction of osteogenic differentiation, Epo−MSCs were cultured for 4 wk in complete medium that was supplemented with β-glycerol phosphate (10 mM), ascorbic acid 2-phosphate (5 μg/ml), and dexamethasone (10−8 M; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) (15,16), and subsequently stained with Alizarin Red S (Sigma-Aldrich Canada; pH 4.1 using ammonium hydroxide) (16). For induction of adipogenic differentiation, Epo−MSCs were cultured for 7 d in complete medium that was supplemented with insulin (10 μg/ml), dexamethasone (1 μM), 3-isobutyl-methylxanthine (0.5 mM), and indomethacin (46 μM; Sigma-Aldrich Canada) (17), and then stained with Oil Red O (Sigma-Aldrich Canada) (15).

Generation of Murine Model of Anemia from Renal Failure

Renal failure was produced in female C57BL/6 mice (15 to 20 g) by electrocoagulation of the right kidney surface, leaving a small portion of the hilum intact, and 2 wk later surgical ablation of the contralateral left kidney, as previously published (18–21). Blood samples were collected from the saphenous vein, using heparinized micro-Hct capillary tubes (Fisher Scientific, Pittsburgh, PA), before and after induction of anemia, and used for determination of Hct by using standard micro-Hct method, of plasma urea nitrogen concentration by colorimetric method (Urea Nitrogen Reagent, Colorimetric Method; Teco Diagnostics, Anaheim, CA), and of plasma Epo concentration by ELISA (Roche Diagnostics). These test mice developed, within approximately 3 wk of left nephrectomy, mild to moderate anemia with Hct of approximately 40%. The Hct of normal C57BL/6 mice was 56 ± 0.4%. Moreover, this model of CRF with anemia has been described to induce in mice the development of a constellation of other abnormalities also noted in humans with CRF (18–20). In this well-characterized murine model, the anemia was reported to respond to rhEpo in a dose-dependent manner (18,19).

Implantation of Gene-Modified MSCs and Analysis on Recipient Mice

Once the Hct remained steady at its lowered levels at approximately 1 mo after left nephrectomy, i.e., induction of CRF, gene-modified MSCs were implanted subcutaneously within a matrix scaffolding, similar to our previous work (14). In this study, taking into account our published findings in normal mice, various doses (3 to 10 million cells per mouse) of Epo−MSCs were implanted in cohorts of anemic C57BL/6 mice (referred to as CRF mice; n = 6 to 8 per group) to allow...
for effect comparison. Normal C57BL/6 mice likewise received implants. More specific, Epo⁺MSCs were trypsinized; concentrated by centrifugation; and 3 × 10⁶, 4.5 × 10⁶, 7.5 × 10⁶, or 10⁷ cells were resuspended in 50 µL of RPMI medium (Wisent, Rocklin, CA) and then approximately 500 µL of Contigen (C.R. Bard Inc., Covington, GA). Contigen-embedded cells were injected subcutaneously in the right flank of CRF and of normal mice. In addition, control CRF mice were generated through implantation with Contigen-embedded control MSCs. Whole peripheral blood was collected from the saphenous vein before implantation and once per week or more after implantation and used for measurements of Hct, plasma urea nitrogen, and plasma mEpo levels. Furthermore, mice that received implants of either 4.5 × 10⁶ or 7.5 × 10⁶ cells were assessed for exercise capacity by recording of the length of time they were able to swim continuously when placed in a container with 32°C water.

Statistical Analyses
The t test was conducted to compare the various groups, and P < 0.05 was deemed significant.

Results
Characterization of Gene-Modified MSCs
Murine MSCs were genetically engineered by transduction with retroviral particles from virus-producing cells that were generated by transfection with retroviral plasmid pEpo (Figure 1). A subpopulation of resulting Epo⁺MSCs, which were shown to secrete in vitro 3 to 4 units of Epo/10⁶ cells per 24 h, was used in this study. Control MSCs, engineered with a control retroviral vector that lacks Epo, were shown by ELISA to secrete <0.02 units of Epo/10⁶ cells per 24 h. No difference was noted in the in vitro growth of Epo⁺MSCs and of control MSCs that were maintained and expanded in culture plates.

The cell surface marker phenotype of Epo⁺MSCs was analyzed previously by flow cytometry and shown to be CD31⁻, CD45⁻, Mac1⁻, CD34⁻, CD44⁺, CD73⁺, CD90⁺, and CD105⁺ (22). These phenotypic markers confirm that MSCs are not hematopoietic cells (by the absence of CD45) and do not express the endothelial marker CD31 (also known as plateletendothelial cell adhesion molecule-1). In fact, MSCs often are referred to as mesenchymal stem cells because of their ability to differentiate into mesenchymal cell types (23–25), and this functional cellular feature defines and distinguishes MSCs from endothelial cells and other end-differentiated somatic and progenitor cells that reside in marrow. For us to verify the mesenchymal differentiation capability of our cells, Epo⁺MSCs were exposed in vitro to specific agents that induce osteogenic or adipogenic differentiation. As shown in Figure 2, when our undifferentiated cells (Figure 2A) were exposed to β-glycerol phosphate, ascorbic acid 2-phosphate, and dexamethasone, they developed an osteogenic phenotype (Figure 2B), whereas when exposed to insulin, dexamethasone, 3-isobutyl-methylxanthine, and indomethacin, they acquired an adipogenic phenotype (Figure 2C).

Hct of Mice Implanted with Gene-Modified MSCs
Our experimental procedure to evaluate the effect of mEpo-secreting MSCs on the anemia of mice with experimental renal failure is illustrated in Figure 3 and is described in Materials and Methods. A cell-dose-finding series of experiments were performed to determine the optimal amount of Epo⁺MSCs in neo-organoids for physiologic correction of anemia in CRF mice. We tested implants that comprised 3 × 10⁶, 4.5 × 10⁶, 7.5 × 10⁶, and 10 × 10⁶ cells. As seen in Figure 4, a dose-response was observed between the number of Epo⁺MSCs that were implanted in CRF mice and the resulting effect on the Hct. A correlation was noted between the number of Epo⁺MSCs that were implanted in CRF mice and the percentage increase in Hct (R² = 0.911 at day 80 after electrocoagulation of right kidney). With the lowest dose of 3 × 10⁶ Epo⁺MSCs per mouse, no correction of anemia occurred, as no Hct change was noted in anemic mice, although an increase in Hct arose in normal mice, up to a maximum value of 71 ± 1.8% (mean ± SEM) at approximately 4 wk after implantation (Figure 4A). The dissimilarity in the response to the 3 × 10⁶

Figure 2. Differentiation ability of marrow stromal cells (MSCs). As described in Materials and Methods, Epo gene-modified MSCs (Epo⁺MSCs) undifferentiated (A), when 70 to 80 or 50 to 60% confluent, were cultured in conditions that were inductive of osteogenic or adipogenic differentiation, respectively. After osteogenic differentiation, calcium in the mineralized extracellular matrix was shown by Alizarin Red S staining (B). After adipogenic differentiation, lipid droplets were evident by light microscopy (C) as well as by their staining with Oil Red O (C, insert). Epo⁺MSCs, before and after differentiation, were photographed under bright-field light microscopy using an Axiosvert25 Zeiss microscope attached to a Contax167MT camera. Magnifications: ×100 in A, ×50 in B, ×400 in C.
Epo MSCs between normal and CRF mice indicated that although CRF mice had a deficiency in Epo, they, unlike normal mice, are resistant to its action. CRF mice that received $3 \times 10^6$ Epo MSCs or $3 \times 10^6$ control MSCs showed similar Hct over time. Epo resistance, as we observed here in CRF mice with this lowest tested amount of Epo MSCs, resembles observations made in humans with renal impairment and is attributed to the effect of the uremic environment on the bone marrow (26–28). Erythropoiesis may be influenced not only by Epo deficiency but also by other factors, such as intramedullary hemolysis and reduced red cell lifespan (29–31).

In contrast, with the highest dose of $10 \times 10^6$ Epo MSCs per mouse, Hct response was supraphysiologic both in normal and in CRF mice (Figure 4D). The Hct in CRF mice, as in normal mice, rose to levels $>80\%$, more precise, to $85 \pm 1.1\%$, by approximately 4 wk after Epo MSCs implantation, whereas the Hct was not significantly altered in CRF mice that received the control MSCs (Figure 4D).

The intermediate doses of $4.5 \times 10^6$ and $7.5 \times 10^6$ Epo MSCs led to more physiologic Hct levels in CRF mice, as shown in Figure 4, B and C, respectively. More specific, in CRF mice that received $4.5 \times 10^6$ Epo MSCs, the Hct that had fallen from basal $57 \pm 0.6$ to $40 \pm 1.1\%$ after induction of mild to moderate renal failure increased to $54 \pm 4.0\%$ at approximately 3 wk after Epo MSCs implantation and remained above $48\%$ for $>16$ wk more (Figure 4B). In CRF mice that received $7.5 \times 10^6$ Epo MSCs, the Hct that had declined from basal $55 \pm 0.7$ to $40 \pm 0.8\%$ after renal failure induction rose to $63 \pm 5.5\%$ at 3 wk after Epo MSCs implantation and persisted at $>54\%$ for more than another 16 wk (Figure 4C). In contrast, in CRF mice that each received $7.5 \times 10^6$ control MSCs, the Hct remained low, below normal, at values of 39 to 44% (Figure 4, B and C). A significant statistical difference was noted between the Hct data of CRF mice that received Epo MSCs at the cell doses of $4.5 \times$
10^6, 7.5 x 10^6, and 10 x 10^6 cells/mouse and that of CRF mice that received control MSCs (P < 0.005, T test; Figure 4, B through D). Moreover, in normal mice that received 4.5 x 10^6 or 7.5 x 10^6 Epo^+MSCs, the Hct rose to levels that were higher than the increased levels that were achieved in CRF mice, as revealed in Figure 4, B and C, pointing to the presence of a relative Epo resistance in CRF mice.

**Plasma Urea Nitrogen and Epo Concentrations in Relation to Hct and Exercise Capacity**

To ascertain whether our groups of experimentally induced CRF mice had comparable kidney dysfunction, we assessed blood plasma for urea nitrogen levels. As shown in Figure 5A, plasma urea nitrogen concentrations in CRF mice at 2 wk after implantation of Epo^+MSCs or of control MSCs were elevated similarly, approximately three-fold higher than normal, with no significant difference detected between CRF groups. More precise, CRF mice that received 4.5 x 10^6 Epo^-MSCs, 7.5 x 10^6 Epo^-MSCs, or 7.5 x 10^6 control MSCs had plasma urea nitrogen levels of 78 ± 5.8 (mean ± SEM), 76 ± 8.1, or 86 ± 4.7 mg/dl, respectively, in contrast to 25 ± 2.0 mg/dl in normal mice that did not receive implants (Figure 5A). In addition, the concentration of urea nitrogen in plasma samples of anemic mice that received 3 x 10^6 or 10 x 10^6 Epo^-MSCs was 69 ± 2.9 or 79 ± 5.3 mg/dl, respectively, at approximately 2 wk after implantation, similar to that of anemic mice that received 3 x 10^6 or 10 x 10^6 control MSCs (79 ± 8.0 or 72 ± 3.8 mg/dl, respectively; n = 6 to 9).

To detect any changes in plasma Epo levels in CRF mice that received Epo^-MSCs, we evaluated the plasma concentration of mEpo by ELISA for hEpo, as similarly used in other reported studies (13,32,33). The ELISA’s specificity for hEpo renders a poorer sensitivity for mEpo (34), indicating that the plasma mEpo concentrations that are detected are likely to be underestimations but nevertheless valuable for comparison purposes. As shown in Figure 5B, plasma Epo concentrations in CRF mice 2 wk after Epo^-MSCs implantation were significantly greater than in CRF mice that received control MSCs (P < 0.05, t test). In brief, levels were 35 ± 8.9 or 83 ± 32 mU/ml in CRF mice that received 4.5 x 10^6 or 7.5 x 10^6 Epo^-MSCs, respectively, in contrast to 17 ± 2.4 mU/ml in CRF mice that received control MSCs (Figure 5B).

We show that Hct response correlates with cell dose and plasma Epo levels in treated animals. In CRF mice that received 4.5 x 10^6 or 7.5 x 10^6 Epo^-MSCs, the Hct was 47 ± 2.7 or 57 ± 3.9%, respectively, 2 wk later, both significantly superior to the Hct of CRF mice that received control MSCs (P = 0.05, t test; Figure 5C). Moreover, the concentration of Epo in plasma of anemic mice that received 3 x 10^6 Epo^-MSCs embedded in Contigen was 6.8 ± 0.9 mU/ml at approximately 2 wk after implantation, similar to that of anemic mice that received 3 x 10^6 control MSCs (6.0 ± 1.7 mU/ml), whereas the plasma Epo concentration of anemic mice approximately 2 wk after implantation with 10 x 10^6 Epo^-MSCs was considerably increased at 160 ± 40 mU/ml, in contrast to that of anemic mice that received 10 x 10^6 control MSCs (11 ± 2.1 mU/ml).

It is recognized that anemia alters exercise ability in humans and that treatment with rhEpo of patients with renal failure provides improved energy and physical activity (35,36). Therefore, to demonstrate a physiologic correlate to increased Hct that was induced by mEpo-secreting MSCs, we subjected mice to a swimming endurance test. As displayed in Figure 5D, CRF mice that received Epo^-MSCs, as compared with those that received control MSCs, showed significantly greater swimming endurance at approximately 2 mo after implantation (P < 0.05, t test). CRF mice that each received 4.5 x 10^6 or 7.5 x 10^6 Epo^-MSCs swam for 155 ± 24 or 173 ± 19 s, respectively, whereas CRF mice that received control MSCs swam for 95.6 ± 13 s before stopping.

Recipient mice were weighed over time, and no significant difference was noted among the various groups of mice. In addition, no significant difference was observed in the survival of treated mice.
Discussion

Studies on the treatment of renal anemia, other than with rhEpo administration, have included gene therapy approaches, whereby the Epo gene is transferred directly to the host either through viral or nonviral means, as well as cell and gene therapy strategies, such as ours, whereby the Epo gene is transferred to cells in vitro and the cells subsequently are implanted in vivo to serve as Epo-releasing vehicles. Indeed, several studies, including our previous published work, have reported the use of autologous MSCs for the delivery of plasma-soluble therapeutic proteins in vivo in immunocompetent hosts and via various routes of administration (9,11,37–40). The main purpose of our study was to adapt this biopharmaceutical approach for the delivery of Epo in vivo for the treatment of anemia consequent to ESRD. The potential benefits of this biopharmaceutical strategy can be contextualized to past published experience in cell and gene therapy approaches to Epo-deficient anemia.

Gene therapy studies for anemia correction have included the utilization of plasmid DNA and of adenoviral and adeno-associated viral vectors that express Epo. With the use of plasmid DNA encoding for rat Epo, studies reported significantly increased Hct long term after plasmid electroporation into skeletal muscles in rats that were rendered anemic by 5/6 or subtotal nephrectomy. The Hct response was proportional to the amount of plasmid DNA delivered (41,42). It must be noted that the Epo gene in the Epo−MSCs retroviral construct is not under physiologic hypoxia-inducible factor-1 control, and, as such, constant, high-level production of Epo may lead to polycythemia in otherwise normal experimental mice (13,14), as well as in CRF mice (Figure 4D). One remedy to decrease the incidence of polycythemia is to control the therapeutic effect by the number of implanted Epo-secreting MSCs. As shown in Figure 4, we determined that the magnitude of the increase in blood Hct that was caused by Epo release in vivo depended on the amount of implanted Epo−MSCs. We controlled the rise in Hct and obtained a more physiologic effect in CRF mice (Figure 4, B and C) by adjusting the number of Epo−MSCs. Furthermore, the effect can be regulated by the number of neo-organoids implanted, and it also may be adjusted after implantation in vivo to serve as Epo-releasing vehicles. Indeed, several studies, including our previous published work, have reported the use of autologous MSCs for the delivery of plasma-soluble therapeutic proteins in vivo in immunocompetent hosts and via various routes of administration (9,11,37–40). The main purpose of our study was to adapt this biopharmaceutical approach for the delivery of Epo in vivo for the treatment of anemia consequent to ESRD. The potential benefits of this biopharmaceutical strategy can be contextualized to past published experience in cell and gene therapy approaches to Epo-deficient anemia.

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With the use of viral vectors, reports revealed increased Hct levels through intraperitoneal administration of a replication-defective adenoviral vector that expresses hEpo in mice with CRF from polycystic kidney disease (44), as well as through intravenous injection of a fully deleted helper-dependent adenoviral vector that expresses mEpo in subtotally nephrectomized rats (45). Amelioration of the anemia occurred through Epo but not of the uremia (45), as we also noted in our study. In a further investigation, the intramuscular injection of an adeno-associated virus vector construct with a hypoxia responsive promoter brought about physiologically regulated expression of mEpo and resulting long-term normalized Hct in anemic Epo-deficient Epo-TAGh transgenic mice (46). However, the use of adenoviral and adeno-associated viral vectors is associated with well-documented potential risks, including the threat of serious, possibly lethal, host immune response (47–50). Moreover, regardless of whether the gene delivery vehicle is viral or nonviral, efficiency of gene transfer in vivo is unpredictable. Unforeseen negative events in fact have arisen in gene therapy of anemia studies that used virus vectors directly in vivo. In one report, severe anemia occurred through an acquired autoimmune response to Epo after intramuscularly delivered adeno-associated virus vector that expressed Epo (51). We have not observed red cell aplasia or acquired anemia in any mouse—kidney failure or not—that received Epo-secreting autologous MSCs. A likely explanation is that MSCs are genetically engineered in vitro with nonimmunogenic replication-defective retroviral vectors. This gene expression system does not lead to expression of viral xenoproteins, and care was taken to avoid the co-expression of any reporter/selection xenoprotein (e.g., drug-resistance genes) that may lead to an immune response to gene-enhanced cells. Furthermore, because cells are gene-modified in vitro under highly controlled conditions, there is absolutely no risk for in vivo viral dissemination or transfer, and transgene expression level is measured before implantation.

Combined cell and gene therapy strategies for treatment of anemia have been reported using an array of cell types. Mesothelial cells have been investigated, specifically human and murine mesothelial cells that were transfected or transduced to produce Epo and subsequently implanted into 5/6 nephrectomized Balb/c or nude mice by intraperitoneal or intramuscular administration (52). Epo-secreting human mesothelial cells in nude mice, when affixed onto the peritoneum with a collagen matrix, led to detectable systemic levels of Epo (52). Epo gene-modified murine mesothelial cells corrected the anemia of uremic mice and led to a greater Hct rise in nonuremic mice, although transiently (52). The higher Hct enhancement in normal versus anemic mice, as we also noted, likewise seems to indicate an Epo resistance. In contrast to previous work in the field of gene-enhanced cell delivery, our platform technology led to a long-term pharmacological effect that persisted for >19 wk. Such a long-term beneficial outcome is desired when looking for a clinically applicable substitute to rhEpo injections for overcoming anemia. As we previously reported (14), we did not note any vasculogenic differentiation of MSCs when embedded in the human-compatible collagen-based matrix Conti-
gen, in contrast to our earlier observations with the use of Matrigel (13). However, this does not seem to interfere with the desirable biopharmaceutical characteristics of the Contigen organoid base in terms of its ability to provide a stable extracellular matrix environment for genetically engineered MSCs and to permit long-term, sustained delivery of the plasma-soluble gene product in vivo. The effect, however, does subside slowly over time and this may be associated with the physical decrease in size of the implant, likely as a result of gradual resorption of the Contigen material as we have noted previously (14). Therefore, our cell and gene therapy strategy as used here in this form does have its limitations as a long-term approach to anemia therapy. A loss of effect over time or, conversely a supraphysiologic response, may occur after a first treatment with our matrix-embedded Epo-secreting cells and consequently may necessitate further subcutaneous injections or a resection of the neo-organoid, respectively. Therefore, clinical relevance would be enhanced through the use of an improved matrix and/or via prolongation of in vivo cell survival.

Another cell type that is used in cell and gene therapy investigations is myoblasts. The C2 myoblast cell line that was transfected with hEpo and injected intramuscularly into nude mice with renal failure caused significant Hct augmentation (53). In another study, C2C12 myoblasts that were transfected with mEpo and implanted subcutaneously within semipermeable polyethersulfone hollow fibers restored hemopoiesis for up to 1 yr in anemic Epo-TAg<sup>b</sup> mice after their immunosuppression (54). With our subcutaneous implants consisting of collagen-embedded mEpo gene-modified primary murine MSCs, a long-lasting effect occurred in nonmyeloablated, immunocompetent mice. Therefore, our alternative to rhEpo utilization does not require any toxic conditioning or immunosuppression preparatory regimen of the host. We recently revealed that long-term use of MSCs for cell and gene therapy applications, at least in mice, is restricted to autologous settings, because a cellular and humoral immune response against allogeneic MSCs as well as against Epo arose in allogeneic hosts (22). Therefore, Epo gene-modified autologous MSCs, not allogeneic MSCs, would be used for Epo delivery for anemia treatment.

A clinical trial involving cell and gene therapy was performed in patients with anemia of CRF (55). Autologous dermal samples were genetically engineered with an adenoviral vector that expresses hEpo and injected into patients by subcutaneous implantation, subsequently revealing minimal morbidity, transient rise in Epo serum concentration, and increased reticulocyte numbers (55). The transient nature of the increased Epo levels, up to approximately 10 d, was attributed to the occurrence of a cellular immune response to the adenovector gene-modified dermal cores, likely against viral proteins that were expressed by the adenovirus (55). Besides adenoviruses, use of autologous terminally differentiated somatic cells, such as dermal fibroblasts, has its shortcomings. Skin fibroblasts may inactivate introduced vector sequences after transplantation (56,57) and may have an expansion ability restricted by the donor’s age (58), leading possibly to suboptimal amounts for clinical utilization. In contrast, MSCs possess a strong expansion ability in vitro (5), also allowing for high-efficiency retroviral engineering, and the selection, proliferation in culture, and utilization in vivo of selected populations of gene-modified cells.

CRF mice that received Epo<sup>+</sup>MSCs acquired improved swimming duration consequent to the persistent mEpo delivery (Figure 5). The beneficial effect that was observed on exercise capacity, as determined by swimming duration of CRF mice that received Epo<sup>+</sup>MSCs, points to advantages of such continuous Epo delivery that may be linked not only to the correction of anemia. Epo is a multifunctional trophic factor with different sites of endogenous expression, a tissue-specific regulation, and several mechanisms of action (59–62). Functional Epo receptors not only have been identified on erythroid progenitor cells but also have been detected on nonerythroid blood cells, such as megakaryocytes, lymphocytes, and myeloid cells, and on various nonhematopoietic cells, such as endothelial cells, myocardial cells, smooth muscle fibers, mesangial cells, cells of neural origin, and renal cells (62–64). The expression of Epo receptors on renal cells possibly may allow Epo to exert a reparative effect on damaged kidney (64). Therefore, although the effect on Hct delivered by Epo<sup>+</sup>MSCs may be similar to that of intermittent pulsed rhEpo, there may be additional clinical benefit on organ systems that express the Epo receptor, such as on myocardium. Epo’s many effects include cardioprotection and improved myocardial function (62,65–67). Therefore, the greater swimming time in CRF mice that received Epo-secreting MSCs may have arisen not only from the resulting increase in Hct but also from a direct biologic effect on myocardium that would be observed in a setting of continuous Epo delivery, such as provided by our neo-organoid system.

Conclusion

Gene-enhanced autologous MSCs can serve as an effective custom Epo production organoid to avoid pitfalls such as autoimmune complications that are associated with immunogenic vector systems and allogeneic cell source. The high ex vivo proliferative capacity of MSCs makes it a more desirable cellular source than senescence-susceptible autologous somatic cells. The subcutaneous retrievable implant strategy allows for cell dosing and avoids the complexities of replicating physiologically responsive hypoxia sensors. Last, continuous low-dose Epo delivery provided by the cell-based platform may lead to unheralded desirable effects on organ systems, such as myocardium, which directly use Epo as a means to preserve and enhance function in addition to the Epo-dependent erythroid effects.

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