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RESEARCH ARTICLE

Bone marrow stromal cells as a genetic platform for systemic delivery of therapeutic proteins *in vivo*: human factor *IX* model

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Abstract

Background Hemophilia B is an X-linked bleeding disorder that results from a deficiency in functional coagulation factor IX (hFIX). In patients lacking FIX, the intrinsic coagulation pathway is disrupted leading to a lifelong, debilitating and sometimes fatal disease.

Methods We have developed an *ex vivo* gene therapy system using genetically modified bone marrow stromal cells (BMSCs) as a platform for sustained delivery of therapeutic proteins into the general circulation. This model exploits the ability of BMSCs to form localized ectopic ossicles when transplanted *in vivo*. BMSCs were transduced with MFG-hFIX, a retroviral construct directing the expression of hFIX. The biological activity of hFIX expressed by these cells was assessed *in vitro* and *in vivo*.

Results Transduced cells produced biologically active hFIX *in vitro* with a specific activity of 90% and expressed hFIX at levels of ~497 ng/10^6 cells/24 h and 322 ng/10^6 cells/24 h for human and porcine cells, respectively. The secretion of hFIX was confirmed by Western blot analysis of the conditioned medium using a hFIX-specific antibody. Transduced BMSCs (8×10^6 cells per animal) were transplanted within scaffolds into subcutaneous sites in immunocompromised mice. At 1 week post-implantation, serum samples contained hFIX at levels greater than 25 ng/ml. Circulating levels of hFIX gradually decreased to 11.5 ng/ml at 1 month post-implantation and declined to a stable level at 6.1 ng/ml at 4 months.

Conclusions These findings demonstrate that genetically modified BMSCs can continuously secrete biologically active hFIX from self-contained ectopic ossicles *in vivo*, and thus represent a novel delivery system for releasing therapeutic proteins into the circulation. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords gene therapy; hemophilia; bone marrow stromal cells; mesenchymal stem cells; osteogenesis

Introduction

Ex vivo gene therapy strategies utilize autologous cells that are genetically modified prior to reimplantation [1]. In principal, this approach offers the potential to correct a wide spectrum of inherited and acquired human diseases. *Ex vivo* gene therapy methods have successfully corrected genetic defects in patients with severe combined immunodeficiency syndrome [2], and are also being developed to deliver therapeutic proteins

into the circulation [3–6]. Genetically modified mesenchymal cells such as fibroblasts and myoblasts have been used most frequently as vehicles to deliver recombinant proteins *in vivo* in part because they are readily accessible and can be easily expanded in culture [7–10]. However, several other cell types are being explored as alternative means to deliver therapeutic proteins *in vivo*. For example, genetically modified hematopoietic stem cells are effective in correcting genetic defects in animal models [11–13], hepatocytes are capable of long-term expression of transgenes [14–16], and neuronal cells can deliver neurotropic factors *in vivo* [17,18].

Hemophilia is a class of bleeding disorders that is actively being examined as a model for *in vivo* and *ex vivo* gene therapy [19]. Hemophilia B is an X-linked bleeding disorder that results from a deficiency in functional coagulation factor IX (hFIX). In patients lacking FIX, the intrinsic coagulation pathway is disrupted leading to a lifelong, debilitating and sometimes fatal disease. Treatment strategies include the infusion of plasma-derived clotting factor concentrates and the more currently available recombinant hFIX [20]. However, despite these therapies, substantial financial cost and significant morbidity result when excessive hemorrhage occurs in joints and muscle spaces.

Cloning of the cDNA encoding hFIX has facilitated molecular approaches to correcting the inherited defects associated with hemophilia B [21]. Because the liver is the normal site for FIX synthesis, it has been the target of several gene therapy approaches for hemophilia [22,23]. Therapeutic delivery of FIX in ectopic sites has also been studied by retroviral transduction of hematopoietic cells [24], myocytes [7] and bone marrow stromal cells that were reinfused into the peripheral circulation, iliac crest marrow or spleen [25–27]. More recently, a clinical trial of hemophilia B gene therapy using recombinant adeno-associated virus was carried out in humans [28]. In this study, a hFIX expression vector was delivered via injection into skeletal muscles and the results were modestly positive in two of the three patients enrolled. Although these approaches show some promise, each has its limitations.

We have developed an *ex vivo* strategy that may overcome some of the limitations of either parenteral or *in situ* delivery of vectors to correct inherited or acquired diseases that have deficiencies in plasma proteins. This *ex vivo* gene therapy system exploits the ability of bone marrow stromal cells (BMSCs) to form ectopic ossicles when transplanted *in vivo*. We hypothesized that these localized ossicles would serve as *in vivo* bioreactors and continuously secrete hFIX into the general circulation. BMSCs become the predominant adherent cell population when bone marrow is cultured *in vitro* [29]. When transplanted into ectopic sites within scaffolds that support cell adhesion and differentiation, BMSCs form self-contained, vascularized bone ossicles consisting of bone and marrow [30–33]. Here, we demonstrate that BMSCs genetically modified with a retrovirus engineered to express hFIX are capable of producing biologically active hFIX *in vitro* and can secrete detectable levels of hFIX into the peripheral circulation for up to 16 weeks. These findings demonstrate that genetically modified BMSCs can continuously secrete biologically active hFIX from self-contained ectopic ossicles *in vivo*, and thus represent a novel delivery system for releasing therapeutic proteins into the circulation.

Materials and methods

Vectors

Construction of the hFIX expression vector, MFG/hFIXc, is described elsewhere [34]. This vector consists of an MFG-type backbone and a hFIX minigene. The control virus construct, AdRSVntlacZ, is a recombinant adenovirus that contains the bacterial β-galactosidase gene and a nuclear localization signal sequence [35] that was purchased from the University of Michigan Vector Core Laboratory.

BMSC cultures

Bone marrow stromal cells were cultured from bone marrow aspirates of human and Yucatan minipigs and were cultured as described previously [32]. All procedures were performed under University of Michigan IRB approval. BMSCs were cultured in αMEM medium containing 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate and 10% fetal bovine serum (Life Technologies), 10^{-4} M L-ascorbic acid phosphate magnesium salt hydrate (AscP; Wako, Osaka, Japan) and 10^{-8} M dexamethasone (Dex; Sigma, St Louis, MO, USA). BMSC strains from passages 3–5 were used for this study.

Ex vivo viral transduction

Viral stocks were produced from the Phoenix packaging cell line as described [34]. BMSCs were cultured in 6-well plates for 24 h (approximately 30% confluent). The culture medium was removed and the cells were incubated with 2 ml of virus (6.1×10^6 CFU/ml) supplemented with Polybrene (hexadimethrine bromide, 8 µg/ml; Sigma). Following a 5-min incubation at 37 °C, the culture plates were centrifuged at 2500 rpm at 32 °C for 30 min and the retrovirus supernatant was replaced by culture medium for an overnight incubation. A second round of infection was performed as described and the cells were cultured until confluent.

Western blot analysis

Western blot analysis using human-specific antisera was used to confirm the secretion of hFIX. hFIX in aliquots (1 mL) of culture medium of BMSCs was precipitated with barium sulfate (100 mg) and subjected to Western

blot analysis as described previously [36]. Secreted hFIX was detected with a rabbit anti-hFIX polyclonal antibody and was visualized with goat anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (Bio-Rad Laboratories) and developed using chemiluminescent detection (Boehringer Mannheim).

Loading cells into the transplantation vehicles

Porcine BMSCs (2×10^6 cells) were loaded into 50–100 mm^2 cube blocks of Collagraft™ sponges (Zimmer, Warsaw, IN, USA) that consist of denatured bovine type I collagen and hydroxyapatite/tricalcium phosphate ceramic particles. After a brief centrifugation, the cell pellets were resuspended in 30–50 ml of medium. The Collagraft™ sponge was wetted with medium, compressed between sterile filter paper to eliminate remaining air bubbles, and immediately placed into the cell suspension where the cells were loaded by capillary action. The sponges containing the transduced cells were incubated at 37 °C for 30 min prior to transplantation.

Transplantation of BMSCs

Immunocompromised mice (N : NIH-*bg-nu-xid*BR, Charles River) served as subcutaneous transplant recipients to avoid graft rejection of porcine BMSC transplants. This strain of nude mice lacks T-cell function, is devoid of natural killer cells and has poor maturation of T-independent B-lymphocytes. Transplantation of transduced BMSCs was performed under anesthesia achieved by inhalation of methoxyflurane (Mallinckrodt Veterinary, Mundelein, IL, USA). Mid-longitudinal skin incisions of about 1 cm in length were made on the dorsal surface of each mouse and four subcutaneous pockets were formed by blunt dissection. A single transplant was placed into each pocket. The incisions were closed with surgical staples.

Evaluation of hFIX production into the circulation

Peripheral blood was collected from excised tails of mice with transduced BMSC transplants or non-transduced controls and circulating hFIX determined as previously described [37]. Following dissection of about 1 mm of the tail, approximately 100 µl of peripheral blood samples were collected, serum samples prepared and subjected to quantification of circulating hFIX by a hFIX-specific ELISA. The specific activity was also calculated using normal human plasma as a control.

Results

Transduced BMSCs secrete biologically active hFIX *in vitro*

BMSCs that were cultured from human or porcine bone marrow aspirates were infected with MFG/hFIXc, a retroviral construct directing the expression of hFIX. The transduced cells from both human and porcine origin were capable of producing biologically active hFIX *in vitro* (Table 1). The transduced BMSCs secreted biologically active hFIX with a specific activity of 90%, indicating that the overexpressed hFIX was appropriately post-translationally modified including γ-carboxylation [38,39]. BMSCs secreted hFIX at levels of 497 ± 17 and 365 ± 25 ng hFIX/10^6 cells/24 h for human and porcine cells, respectively, after 7 days in culture (Table 1). Western blot analysis confirmed that retrovirus-transduced BMSCs produced hFIX of the expected molecular size. Immunoblots confirmed the secretion of hFIX protein from the transduced cells (Figure 1, lane 1), whereas no immunoreactivity was observed in conditioned medium from non-transduced cells (Figure 1, lane 3). hFIX secreted from BMSCs migrated at approximately 68 kDa, a size that corresponds to the recombinant hFIX control (Figure 1, lane 2) and to hFIX produced from genetically modified myoblasts and endothelial cells [37,40].

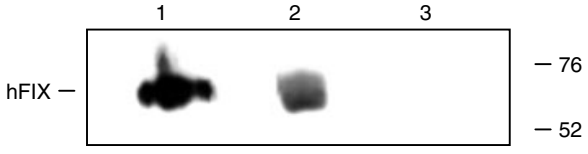

Figure 1. Western blot analysis of hFIX secreted from transduced porcine BMSCs *in vitro*. Protein samples from conditioned medium were resolved by SDS-PAGE. Lane1, MFG/hFIXc-transduced BMSCs; lane 2, recombinant hFIX control; lane 3, non-transduced cells. The numbers on the right indicate the molecular size markers in kDa

Table 1. Expression of hFIX in MFG-hFIX-transduced BMSCs *in vitro*

	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control (ng/10^6 cells/24 h)	0	0	0	0	0	0
Infected Human BMSCs (ng/10^6 cells/24 h)	nd	nd	nd	nd	nd	497.4 ± 16.8
Infected Porcine BMSCs (ng/10^6 cells/24 h)	358.4 ± 18.5	360.9 ± 28.8	366.0 ± 26.6	355.8 ± 26.6	362.7 ± 20.9	365 ± 24.7

nd = not determined.
Values are the average ± the standard deviation.
$n = 6$ for each experimental group.

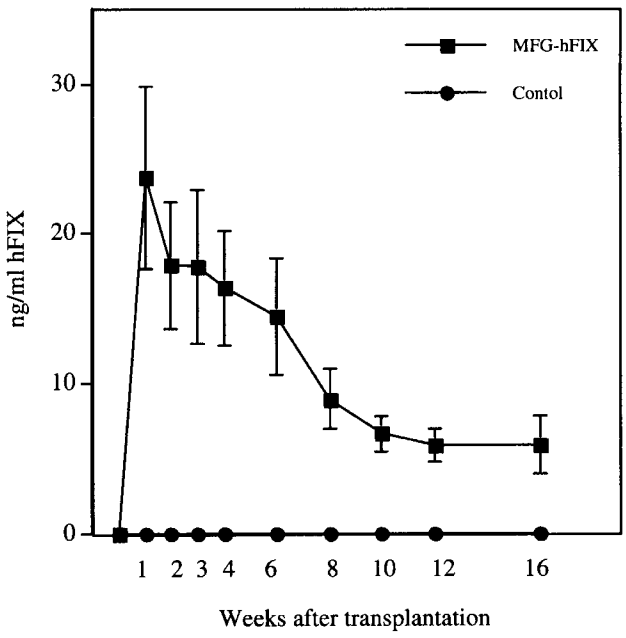

Figure 2. Production of hFIX in mice. Porcine BMSCs were transduced with MFG/hFIXc and were transplanted into immunocompromised mice. Peripheral blood was collected and the levels of hFIX secreted into the circulation from the implanted ossicles were determined for up to 16 weeks. Filled squares represent hFIX levels in MFG/hFIXc-transduced BMSCs. Filled circles represent hFIX levels in mice receiving transplants of non-transduced BMSCs ($n = 6$)

Factor IX expression *in vivo*

Self-contained bone ossicles are formed when BMSCs are transplanted into ectopic sites within scaffolds that support cell adhesion and differentiation [30,32,33,41]. When BMSCs harvested from transgenic mice carrying type I procollagen reporter genes are transplanted, continuous reporter gene activity can be detected from differentiated cells within the transplant suggesting that osteoblasts and osteoprogenitor cells derived from transplanted BMSCs exist in a dynamic state within the ectopic ossicles [32]. We took advantage of this dynamic state to investigate the ability of transplanted BMSCs to secrete biologically active proteins into the peripheral circulation from these localized sites. Transduced BMSCs (8×10^6 cells per animal) were loaded within scaffolds and transplanted into subcutaneous spaces in immunocompromised mice at four sites. Using this approach, BMSCs differentiated into self-contained, subcutaneous ossicles by 4 weeks. We therefore tested the hypothesis that transduced cells within the localized transplant would continuously secrete hFIX into the general circulation. Blood was collected from excised tails and serum samples were prepared and analyzed for hFIX activity (Figure 2). At 1 week post-implantation, serum samples contained hFIX at a concentration level of 25 ng/ml as analyzed by a human-specific ELISA. The hFIX levels in the circulation gradually decreased to 17.5 ng/ml at 4 weeks post-implantation and further declined to a stable level at 6.1 ng/ml at 10–16 weeks. No hFIX was detected in control animals. At 4 months post-transplantation, implants were harvested and subjected to histological analysis. All implants formed bone that was maintained at the approximate size of the original implant (Figure 3A).

Localization of hFIX-producing cells

The tissues within an ossicle formed by transplanted BMSCs exist as a chimera of donor and recipient cells. Several experimental methods, such as the use of species-specific antibodies, species-specific repetitive DNA sequences and reverse transplantation, have demonstrated that the bone-forming cells are of donor origin. By similar criteria, hematopoietic cells and macrophages originate from the recipient [32,33,42–44]. We therefore sought to identify the source of hFIX-producing cells within the implant by incubating tissue sections of hFIX-transduced BMSC transplants with human-specific, anti-FIX anti-bodies. Positive immunostaining was observed in osteoblasts and osteocytes in the newly formed ossicle (Figure 3C) supporting the hypothesis that osteoblasts derived from differentiating BMSC actively secrete hFIX *in vivo*. In contrast, no immunoreactivity above background was seen in the tissue sections incubated with non-immune serum (Figure 3B).

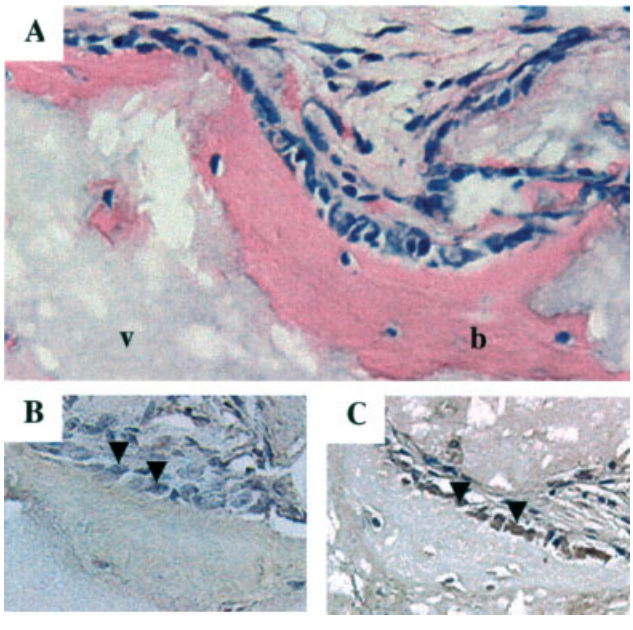

Figure 3. Localization of hFIX-secreting cells in transplants of transduced porcine BMSCs. BMSCs were infected with MFG/hFIXc *in vitro* and transplanted into immunocompromised mice. After 5 weeks *in vivo*, transplants were harvested and prepared for immunohistochemistry. (A) Hematoxylin- and eosin-stained section showing newly formed bone (b). (B) Tissue sections were incubated with non-immune serum and were counterstained with hematoxylin. (C) Tissue sections were incubated with a human-specific polyclonal antibody and were counterstained with hematoxylin. Note the brown color of osteoblasts (black arrowheads) demonstrating immunoreactivity to human-specific antibodies raised against hFIX. New bone is designated b and the hydroxyapatite scaffold is designated v (magnification 40×)

Discussion

The objective of this study was to test the feasibility of using genetically modified bone marrow stromal cells as a platform for sustained systemic delivery of therapeutic proteins into the circulation. Human factor IX served as the model system for this study. We found that human or porcine BMSCs could be effectively transduced with MFG/hFIXc retrovirus and were capable of secreting biologically active hFIX *in vitro* and *in vivo*. Consistent with previous reports that BMSCs form bone when transplanted within scaffolds that support cell adhesion and differentiation, the hFIX-transduced BMSCs formed new bone in the *in vivo* osteogenic assay. These self-contained, subcutaneous ossicles were also the source of biologically active hFIX that resulted in detectable levels of hFIX in the peripheral circulation for up to 16 weeks. These findings suggest that the use of BMSCs may serve as an alternative approach to delivering therapeutic proteins into the circulation from localized, ectopic sites.

Among the rationale for using bone marrow stromal cells as a strategy to deliver hFIX into the circulation is the observation that osteoblasts and hepatocytes share some post-translational modification processes including the vitamin-K-dependent γ-carboxylation of specific glutamic acid residues [45]. The vitamin-K-dependent proteins include several coagulation factors (II, VII, IX and X), proteins C and S [39], and the skeletal extracellular matrix proteins osteocalcin and matrix Gla protein [46]. γ-Glytamyl carboxylase catalyzes the post-translational modification of specific glutamic acid residues to γ-carboxyglutamic acid (Gla) and is required for biologic activity of the Gla proteins. *In vivo* manifestation of this requirement is observed in humans, where naturally occurring mutations in the factor IX propeptide region occur in warfarin-sensitive hemophilia B patients [47,48]. Therefore, our finding that the hFIX secreted by BMSCs exhibited a specific activity of 90% of the recombinant hFIX control demonstrates that the transduced cells are capable of functional post-translational modification. Because transplanted BMSCs can differentiate into functional osteoblasts under appropriate *in vivo* conditions, they are good candidates for synthesizing biologically active proteins from ectopic ossicles *in vivo*.

Hemophilia B has often been used as a model for developing gene delivery systems. The deficiency in plasma levels of factor IX in hemophilia B allows the development of strategies to ectopically express the protein in tissues such as muscle [49–52] and bone marrow [25–27,53], in addition to the natural site of expression in the liver [54,55]. A rather wide therapeutic window is also an advantage for using hemophilia B as a model for gene therapy. While circulating levels of hormones such as insulin must be tightly regulated to be safe and effective, the FIX plasma concentration may be as high as 150% of the normal median level ($\sim$5 μg/ml plasma), although achieving persistently high levels has been difficult. Conversely, an increase in the FIX plasma concentration as small as 1% of the normal level (approximately 50 ng/ml plasma) can convert a severe form of hemophilia to a more easily managed moderate form. Furthermore, mouse and canine models of hemophilia B that display a phenotype similar to hemophilia B in humans have been generated by inactivating the FIX gene by homologous recombination providing powerful model systems to study the effectiveness of gene therapies [56].

Experimental models using bone marrow stromal cells that were reinfused into the peripheral circulation, iliac crest marrow or spleen [25–27] have been developed as potential treatments for hemophilia. In each of these strategies, adequate expression of coagulation factor was dependent on engraftment of the transduced cells. The strategy described here does not depend on proper engraftment or homing to a particular tissue. Rather, the transduced cells differentiate into localized ectopic ossicles that are capable of expressing biologically active hFIX. The levels of hFIX produced in our *in vivo* studies represent near therapeutic levels for up to 2 months. Subsequently, circulating hFIX levels declined to a consistent level of 6 ng/ml for the duration of the study (Figure 2). The reasons for the lack of persistent high-level hFIX secretion are not yet understood. Potential factors include the self-inactivation of the viral promoter, the failure to transduce mesenchymal stem cells within the heterogeneous BMSC population, and the failure of fully differentiated osteoblasts within the ectopic ossicle to continuously secrete hFIX.

Our findings demonstrate that genetically modified BMSCs can continuously secrete hFIX from subcutaneous sites *in vivo* and thus represent a novel delivery system for releasing therapeutic proteins into the circulation. By titrating the number of transplanted cells, this *ex vivo* gene delivery system may provide better control of the number of cells expressing a transgene than does parenteral delivery of virus. This system may also be amenable to the use of inducible promoters to gain additional control of secretion. Other advantages of this new gene delivery approach in developing gene therapies for disorders such as hemophilia include: (1) the continuous delivery of transgene products by targeting mesenchymal stem cells for gene transfer, (2) avoidance of the difficulty in BMSCs homing to the bone marrow, and (3) the ability to retrieve the localized transplant in the event of encountering adverse effects due to them.

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