

Sustained and therapeutic levels of human factor IX in hemophilia B mice implanted with microcapsules: key role of encapsulated cells

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Abstract

Background A gene therapy delivery system based on microcapsules enclosing recombinant cells engineered to secrete a therapeutic protein was explored in this study. In order to prevent immune rejection of the delivered cells, they were enclosed in non-antigenic biocompatible alginate microcapsules prior to being implanted intraperitoneally into mice. We have shown that encapsulated C2C12 myoblasts can temporarily deliver therapeutic levels of factor IX (FIX) in mice, but the C2C12 myoblasts elicited an immune response to FIX. In this study we report the use of mouse fetal G8 myoblasts secreting hFIX in hemophilia mice.

Methods Mouse G8 myoblasts were transduced with MFG-FIX vector. A pool of recombinant G8 myoblasts secreting ~1500 ng hFIX/10⁶ cells/24 h *in vitro* were enclosed in biocompatible alginate microcapsules and implanted intraperitoneally into immunocompetent C57BL/6 and hemophilic mice.

Results Circulating levels of hFIX in treated mice reached ~400 ng/ml for at least 120 days (end of experiment). Interestingly, mice treated with encapsulated G8 myoblasts did not develop anti-hFIX antibodies. Activated partial thromboplastin time (APTT) of plasmas obtained from treated hemophilic mice was reduced from 107 to 82 sec on day 60 post-treatment, and whole blood clotting time (WBCT) was also corrected from 7–9 min before treatment to 3–5 min following microcapsule implantation. Further, mice were protected against bleeding following major trauma. Thus, the FIX delivery *in vivo* was biologically active.

Conclusions Our findings suggest that the type of cells encapsulated play a key role in the generation of immune responses against the transgene. Further, a judicious selection of encapsulated cells is critical for achieving sustained gene expression. Our findings support the feasibility of encapsulated G8 myoblasts as a gene therapy approach for hemophilia B. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords microcapsules; alginate; myoblasts; hemophilia B; factor IX; gene therapy

Introduction

Hemophilia B is an X-linked bleeding disorder caused by a deficiency of functional coagulation factor IX (FIX), a disease that affects 1 in 25 000 born males [1]. According to the level of functional circulating FIX, the disease

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state is classified as severe (<1% of normal level), moderate (1–5%), or mild (5–30%). Almost half of hemophiliacs are severe cases with frequent bleeding events that can be life-threatening and often cause chronic joint damage. Current treatment for hemophiliacs is the life-long regular infusion of plasma-derived or recombinant human FIX (hFIX) [2–7]. However, plasma-derived FIX has the potential to transmit blood-borne pathogens, like HIV and hepatitis virus. Although heat-inactivated plasma has become very safe, the presence of novel pathogens cannot be ruled out. Recombinant proteins are considered safer, but come at a high cost (>120 000 per patient/year). Therefore, an alternative treatment would be highly desirable.

Several aspects make hemophilia a suitable disease model for the development of gene therapy strategies. First, the expression of FIX is not tightly regulated and the delivery of therapeutic agent is required only in the circulation. Second, even the supply of low levels of FIX antigen can improve the quality of life and clinical management of hemophilic B patients [8,9]. Finally, there are excellent murine and canine animal models of hemophilia B available for pre-clinical studies that accurately mimic the human condition.

Six gene therapy clinical trials, including both *in vivo* and *ex vivo* approaches, have been conducted during the last few years [10–14]. The direct injection of viral vectors for gene therapy has uncovered new safety concerns, such as potential germline transmissions, tumourigenesis and undesirable inflammatory reactions [15–18]. An autologous *ex vivo* approach offers an alternative to the use of viral vectors [19], although it must be tailored to each patient, thus increasing the cost of the procedure. Our laboratory has previously described the encapsulation of allogeneic recombinant cells secreting a therapeutic product followed by their implantation in mice [20,21]. The ultimate goal of using non-antigenic and biocompatible alginate-polylysine microcapsules is to prevent immune rejection of the transplanted cells. We have previously described the use of implantable alginate microcapsules enclosing recombinant fibroblasts or myoblasts to deliver hFIX in mice [20–24].

Factor IX is produced by the liver, where it undergoes substantial post-translational modifications before it fully becomes biologically active. Myoblasts also perform the necessary post-translational modifications, including γ -carboxylation, required to produce biologically active hFIX [25,26]. We have previously shown that murine C2C12 myoblasts engineered to secrete hFIX encapsulated in alginate-polylysine-alginate, and subsequently implanted into mice, delivered therapeutic levels of hFIX [21,22]. However, antibodies to hFIX appeared 14 days after microcapsule implantation in immunocompetent mice. Anti-hFIX antibody production coincided with decreased hFIX in mice plasma. Our results also showed that encapsulated C2C12 myoblasts, which are transformed cells, caused tumour development in nude mice [23]. This tumour development highlights a limitation of the use of a transformed cell line for gene therapy,

and also exemplifies the need to develop suitable new cell alternatives for non-autologous *ex vivo* gene therapy.

Transplanted fetal cells often do not trigger immune responses in a host, and thus might be a suitable cell line to be used in transplantation and *ex vivo* gene therapy strategies [27,28]. In this study, we describe the implantation of G8 murine myoblasts of fetal origin. We show a safe and sustained high-level secretion of hFIX in normal C57BL/6 and hemophilic mice following the implantation of recombinant mouse G8 myoblasts secreting hFIX enclosed in alginate microcapsules. These findings are encouraging for the potential of allogeneic cell-based therapy treatment.

Materials and methods

Cell culture

G8 is a myoblast cell line derived from the fetal skeletal muscle tissue of Swiss Webster mice (obtained from ATCC, CRL-1456). G8 cells were grown on 0.01% collagen-coated plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 10% horse serum, penicillin (100 unit/ml) and streptomycin (100 μ g/ml). This cell line can differentiate into myotubes under certain conditions [29]. C2C12 murine myoblasts secreting hFIX (C2C12-hFIX, clone #18) have been described previously [22].

Engineering of G8 myoblasts

Retroviral plasmid MFG-IXI is derived from the MFG retrovirus. This construct contains the hFIX cDNA with a mini-intron I, and has been described previously [22].

The retroviral packaging cell line, ecotropic pack 293 cells (obtained from ATCC, CRL-1573), was transfected with MFG-IXI plasmid using Infectine (Qiagen) according to the manufacturer's instructions. The tissue culture supernatant containing viral particles was collected at 48 and 96 h post-transfection. Following centrifugation of the supernatant at 4°C and 3000 g for 10 min, and filtration through a 0.45 μ m filter, the retrovirus concentrate was aliquoted and stored at –70°C until use.

Approximately 1×10^5 G8 myoblasts were seeded in a 3 cm plate and 1.5 ml of retrovirus stock medium was added together with Polybrene (8 μ g/ml), additional 10% FBS, and 10% horse serum. After 2 h incubation at 37°C, in 5% CO₂ atmosphere, the supernatant was removed. The cells were grown in fresh tissue culture medium for an additional 8 h, before additional rounds of transduction were performed. The transduction of G8 myoblasts was repeated again following the same procedure for 4–5 rounds of infection. After each round of transduction, hFIX secretion was quantified by enzyme-linked immunosorbent assay (ELISA) as previously described [30].

Cell encapsulation

Cells were mixed with 2% alginate (Improved Kelmar, ISP, San Diego, CA, USA) at a concentration of 5×10^6 cells/ml and extruded to form microcapsules of 300–500 μm diameter. Microencapsulation was performed with an encapsulator (Nisco Engineering Inc., Zurich, Switzerland) according to a protocol previously described [20,21]. After the procedure, the resulting encapsulated myoblasts were incubated under regular tissue culture conditions overnight before being implanted into mice.

We keep at McMaster University a colony of hemophilia B C57BL/6 mice (founding pair kindly provided by the Salk Institute, La Jolla, CA, USA [31]). Non-hemophilic C57BL/6 mice were purchased from Charles River (Montreal, Canada). For *in vivo* implantation of microcapsules, mice were anaesthetised with isoflurane (Bimeda-MTC, Animal Health Inc., Richmond Hill, Canada) in a small animal anaesthetic machine (Med-Vet Anaesthetic System Inc., Toronto, Canada). Intraperitoneal injection of the microcapsules was accomplished with a G18 catheter. Each mouse received a total of 20×10^6 cells. All animal procedures were conducted in accordance with the Animal Ethics Guidelines of McMaster University.

Factor IX determination

Blood samples were taken periodically from mouse retro-orbital plexus using either heparinised capillary tubes, or through regular capillaries into sodium citrate for the activated partial thromboplastin time (APTT) assay. Plasma was obtained by centrifugation and stored at -80°C until assayed. Circulating hFIX antigen and anti-hFIX antibodies were detected by ELISA. Human FIX antigen was detected using the reagents and protocol from Affinity Biologicals Inc. (Hamilton, Canada). For the detection of hFIX antibodies, a previously described protocol was used [21].

Functional assays

Activated partial thromboplastin time (APTT) was used to determine hFIX activity in mouse plasma or in cell culture medium using a Fibrinometer (Start4, Diagnostica Stago). A volume of 6 μl of testing mouse plasma diluted in 54 μl of veronal buffer was incubated at 37°C for 2 h. A single stainless steel stirring bar was added to the disposable reaction tube (Diagnostica Stago). A total of 50 μl of the above mix was put into the tube with 50 μl of hFIX-deficient plasma and 50 μl of APTT reagent, and incubated for 3 min at 37°C . The APTT reaction was initiated by the addition of 50 μl of 25 mM CaCl_2 .

The whole blood clotting time (WBCT) test was performed as follows: a volume of 35 μl of blood freshly taken from the retro-orbital plexus through ordinary micro-hematocrit capillary tubes (Fisherbrand)

was incubated at room temperature in a 6×50 mm glass tube (Kimble), and monitored for clotting every 30 s using a smooth glass bar. The time that lapsed before the blood clotted in the tube was recorded.

For determining tail-cut bleeding time, tails of anaesthetised mice were transected in a standard manner, cutting approximately a 1 cm tail segment. The tail tip was put in contact with a Whatman filter paper every 30 s to determine when bleeding had stopped, for up to 15 min. Mice that did not stop bleeding after 15 min were cauterised. In this test, hemophilic mice bleed for 15 min, whereas non-hemophilic mice typically stop bleeding within 3 min.

Results

Secretion of hFIX by G8 myoblasts

In order to genetically engineer cells to secrete high levels of hFIX, G8 murine myoblasts were transduced with ecotropic retrovirus MFG-hFIX, for a total of five rounds of transductions. The secretion of hFIX in the pool of transduced cells gradually increased with the number of transduction rounds (Figure 1). The first round of transduction resulted in the pool of recombinant myoblasts secreting *in vitro* 570 ng/ 10^6 cells/24 h of hFIX. The percentage of recombinant cells increased with each successive transduction, enhancing hFIX secretion to 1540 ng/ 10^6 cells/24 h after the fourth round. The fifth round of transduction did not yield any marked increase in hFIX secretion, indicating that most of the myoblasts were already transduced after the fourth round. A pool of recombinant myoblasts (G8-hFIX) subjected to four rounds of transductions was used throughout this study.

Table 1 shows that naked G8-hFIX cells secreted ~ 1500 ng FIX/ 10^6 cells/24 h *in vitro*. Expression of hFIX

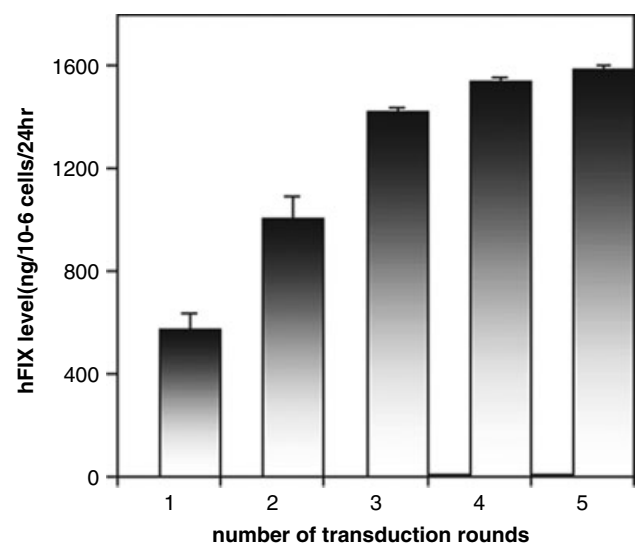


Figure 1. Effect of multiple rounds of retrovirus transductions on hFIX secretion by G8 cells. Human FIX secretion increased with the number of transduction rounds, reaching a plateau after four to five rounds

Table 1. Human FIX secretion under the different cell states *in vitro*. The hFIX present in myoblast-conditioned culture media was measured by ELISA. The results show that hFIX secretion by G8 cells is maintained under various cell conditions

Cell state	hFIX secretion <i>in vitro</i> (ng/10 ⁶ /24 h)
G8-hFIX cells	1500–1600
Differentiated G8-hFIX cells	1200–1400
Encapsulated G8-hFIX cells	1200–1500/ml capsule

was very stable, and did not substantially differ when differentiated G8-hFIX cells were assayed. Similarly, encapsulated G8-hFIX myoblasts continued to secrete hFIX vigorously, albeit at a somewhat reduced rate. Human FIX was undetectable in conditioned media from untransduced G8 cells (data not shown).

Delivery of hFIX in C57BL/6 mice

A group of adult (6–8 weeks old) immunocompetent C57BL/6 mice ($n = 12$) was implanted intraperitoneally with 4 ml of microcapsules containing 5×10^6 G8-hFIX cells/ml capsules per animal. A control group of C57BL/6 mice ($n = 4$) was implanted intraperitoneally with an equal amount of free G8-hFIX cells, without the protection of the alginate microcapsules. Another control group of C57BL/6 mice ($n = 4$) received empty capsule implantation. Finally, a control group of C57BL/6 mice ($n = 5$) received encapsulated C2C12-hFIX myoblasts.

At regular time intervals the mice were bled and the levels of circulating hFIX determined by ELISA. Human FIX was detected in the plasma of all the mice implanted with G8-hFIX throughout the entire experimental period, being on average 371 ng/ml on day 3, which is equivalent to 7% of the 5 μ g/ml of physiological FIX levels in healthy humans (Figure 2A). Furthermore, hFIX levels

were maintained at 186 ng/ml on day 120, still within the therapeutic range. The peak of hFIX in some mice was over 600 ng/ml. In contrast, hFIX was temporarily detected in plasma of mice that received encapsulated C2C12-hFIX cells on day 3, with a concentration of 147 ng/ml, that quickly disappeared by day 14. Similarly, hFIX was temporarily detected in plasma of control mice receiving free G8-hFIX cells on day 3, with a concentration of <10 ng/ml that quickly disappeared by day 7. Control mice receiving empty capsules never had detectable hFIX at any stage of the study. No adverse effects were noted in any of the treated mice by the staff of McMaster's Central Animal Facilities.

We have previously shown that mice implanted with encapsulated C2C12-hFIX myoblasts generated antibodies against the transgene [21,22]. Therefore, the presence of anti-hFIX antibodies was evaluated in this study. In agreement with our previous data, there was strong anti-hFIX IgG detected in mice that received encapsulated C2C12-hFIX cells (Figure 2B). Interestingly, no anti-hFIX antibodies were detected in any of the mice treated with encapsulated G8-hFIX (Figure 2B).

Stable expression of biologically active hFIX in hemophilic mice

Encouraged by the therapeutic antigen levels of hFIX detected in C57BL/6 mice, we estimated the biological activity of the secreted hFIX by assessing the potential of encapsulated G8-hFIX to reverse the hemophilia phenotype. Thus, a group of hemophilia B FIX-deficient immunocompetent C57BL/6 mice [31] was implanted intraperitoneally with 20×10^6 encapsulated G8-hFIX per animal ($n = 8$). The levels of circulating hFIX in treated mice were therapeutic, with an average of 323 ng/ml on day 3, and were sustained until at least day 60 (Figure 3A). In contrast, no hFIX was detected in control

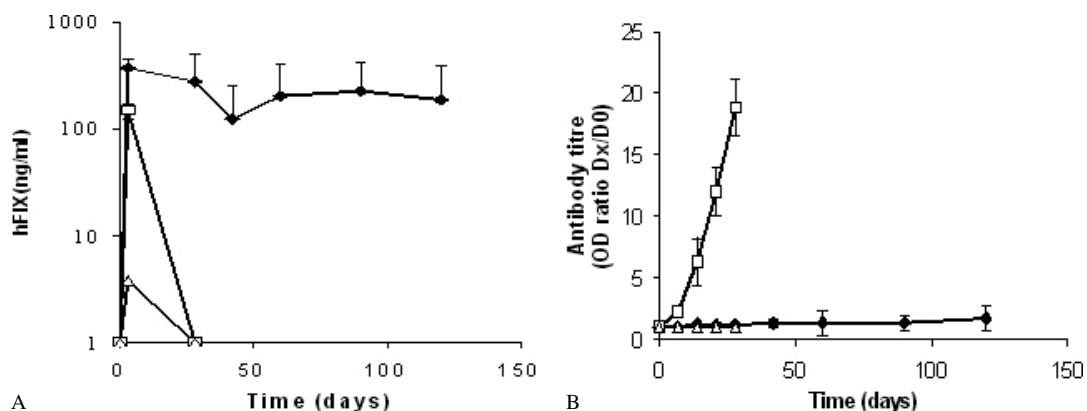


Figure 2. Human FIX antigen and anti-hFIX antibodies in the plasmas of immunocompetent C57BL/6 mice. (A) Circulating hFIX antigen was sustained in mice receiving encapsulated G8-hFIX (◆). In contrast, hFIX delivery was transient in mice receiving encapsulated C2C12-hFIX (□), while mice injected with free (unencapsulated) G8-hFIX cells (△) had a transient and modest levels of hFIX on day 3. Control mice receiving empty capsules had no detectable hFIX in their plasma (data not shown). Error bars represent standard deviations. (B) Anti-hFIX antibody in plasmas of C57BL/6 mice, expressed as OD ratio in relation to OD on day 0 (Dx/D0). Error bars represent standard deviations. Mice implanted with encapsulated C2C12-hFIX (□) had developed a vigorous immune response by day 14, while mice implanted with free C2C12-hFIX (△) and encapsulated G8-hFIX myoblasts (◆) had low to undetectable anti-hFIX antibodies. Control mice receiving empty capsules had no detectable antibodies (data not shown)

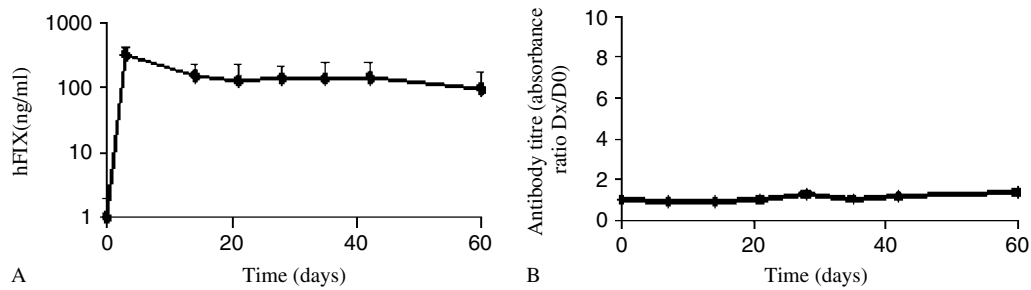


Figure 3. Circulating hFIX antigen and anti-hFIX antibody levels in hemophilic C57BL/6 mice implanted with encapsulated G8-hFIX myoblasts ($n = 8$). (A) Detection of hFIX in plasma. Human FIX antigen was detected (up to 400 ng/ml) in all treated mice by day 3 after capsule implantation and was maintained above 100 ng/ml throughout the experiment. In contrast, control mice receiving free (unencapsulated) G8-hFIX cells had no detectable antibodies (data not shown). Error bars represent standard deviation. (B) Anti-hFIX antibody in hemophilic mice, expressed as OD ratio in relation to OD on day 0 (D_x/D_0). Error bars represent standard deviation. Treated hemophilic mice had no detectable anti-hFIX antibodies throughout the whole experimental period

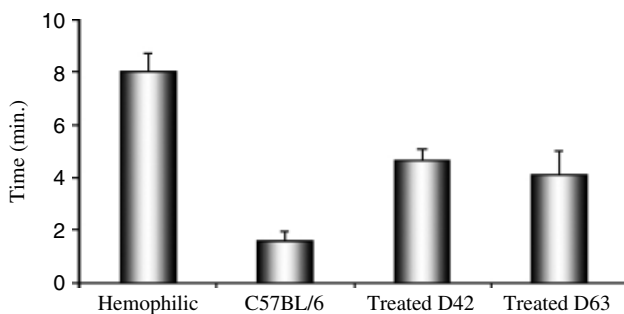


Figure 4. Measurement of whole blood clotting time (WBCT) in mice implanted with encapsulated G8-hFIX cells ($n = 8$) at 42 days (Treated D42) and 63 days (Treated D63) post-implantation. As control, hemophilic and non-hemophilic C57BL/6 mice are displayed. Bars represent average WBCT of the mice, while error bars represent standard deviations. Statistical difference between treated and untreated hemophilic mice is extremely significant by t-test ($p < 0.0001$)

mice that received free unencapsulated recombinant cells (data not shown). Of note, and in agreement with our previous findings in non-hemophilic C57BL/6 mice, there were no detectable anti-hFIX antibodies in any of the treated hemophilic mice during the whole experimental period (Figure 3B). No adverse effects were detected in any of the treated mice.

Given the FIX deficiency of these mice, the biological activity of the delivered hFIX was evaluated. Therefore, whole blood clotting time (WBCT) was measured. The average value of WBCT for the untreated hemophilic mice was 7–9 min, while WBCT for the normal mice was 1–3 min (Figure 4). The WBCT for the hemophilic mice implanted with microcapsules enclosing G8-hFIX cells shortened extremely significantly from 7–9 min before implantation to 4–5 min on day 42 ($p < 0.0001$), and to 3–5 min on day 63 ($p < 0.0001$) (Figure 4), indicating that the circulating hFIX produced by the G8-hFIX cells was biologically active.

In order to further determine the biological activity of hFIX, APTT was measured. APTT of the plasma in the treated mice decreased from 107 s prior to the implantation to an average of 84 s on day 42 and 82 s on day 60, a reduction that is statistically significant

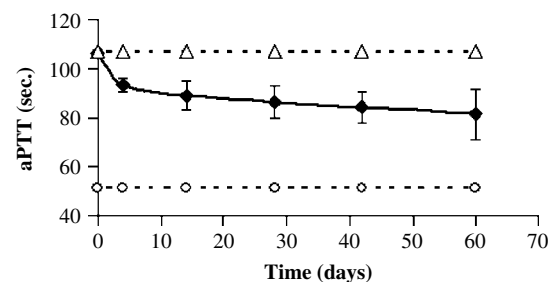


Figure 5. Activated partial thromboplastin time (APTT) of plasmas of hemophilic mice implanted with encapsulated G8 mouse myoblasts ($n = 8$). The APTT of the plasmas of untreated hemophilic mice was approx. 107 s, while the APTT of control mice was 51.5 s. The APTT of treated mice by day 60 was significantly reduced ($p < 0.01$). Values represent average, while error bars represent standard deviation

($p < 0.01$). The average APTT for normal mice was 51.5 s (Figure 5). These findings further confirmed the partial correction of the disease suggested by the circulating antigen and WBCT.

The ultimate goal for any treatment for hemophilia is to offer protection against bleeding following trauma. Thus, a standardised tail-cut test was performed on days 49 and 56 to determine the protection of treated hemophilic mice against severe trauma. Untreated hemophilic mice subjected to this test bled to death, and must be cauterised at 15 min post-injury. Non-hemophilic mice bleed on average for 2.8 min before bleeding stops. Hemophilic mice implanted with G8-hFIX showed evidence of being protected, and spontaneously stopped bleeding on average after 5 min on day 49 ($p < 0.0001$) and 3.7 min on day 56 ($p < 0.0001$) (Figure 6), further indicating the therapeutic effect of the treatment.

Discussion

In previous reports we have described the use of encapsulated transformed C2C12 myoblasts to deliver hFIX in mice [21,22]. Although successful, hFIX delivery in treated mice was transient. Furthermore, the disappearance of circulating transgene coincided with increasing

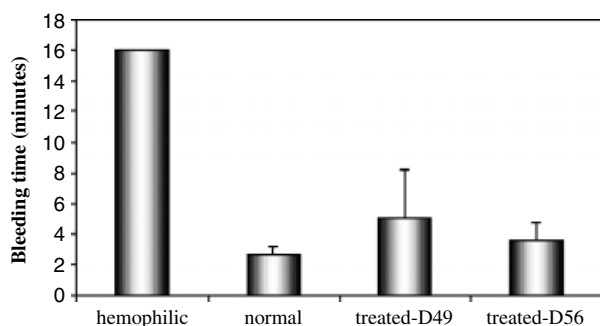


Figure 6. Protection against trauma. Following tail transection, the bleeding times of mice implanted with encapsulated G8-hFIX cells were measured for half the mice ($n = 4$) on day 49 post-treatment (Treated D49), and the other treated mice ($n = 4$) on day 56 post-implantation (Treated D56). As control, bleeding times of untreated hemophilic ($n = 8$) and non-hemophilic ($n = 8$) C57BL/6 mice are displayed for comparison. Bars represent average tail bleeding time of mice, while error bars represent standard deviation. Statistical difference between treated and untreated hemophilic mice is extremely significant by t-test ($p < 0.0001$)

titre of anti-hFIX antibodies. Thus, a less immunogenic cell line would be necessary to assure sustained delivery of hFIX *in vivo*. Importantly, transformed C2C12 myoblasts also gave rise to tumours in a model of immunodeficient hemophilic mice [23]. Thus, a safer cell line must be chosen for this *ex vivo* gene therapy strategy before a human trial can be considered.

This study describes the use of allogeneic G8 murine myoblasts as a suitable alternative. G8 cells were originally obtained from fetal skeletal muscle tissue and are not tumourigenic in immunocompromised mice (ATCC). Interestingly, therapeutic and sustained levels of hFIX were detected in treated C57BL/6 mice and FIX-deficient hemophilic mice. Of note, no tumours developed in any of the mice implanted with naked or encapsulated G8-hFIX cells, suggesting the safety of G8 myoblasts and the potential for this gene therapy strategy for hemophilia B.

Other gene therapy approaches using encapsulated cells to deliver therapeutic transgenes have been described. A variety of transformed recombinant cells have been used in gene therapy, including myoblasts, fibroblasts, kidney and gut epithelial cells, mesenchymal and haematopoietic stem cells, endothelial cells and adipocytes [32–39]. A common finding of these studies has been the detection of antibodies directed against the transgene, including immune responses against FIX, not considered to be a strong immunogen [20,40,41]. Antigen presentation and persistence are known key factors for eliciting an immune response [42]. This is a matter of great concern, particularly for the treatment of chronic diseases such as hemophilia that requires long-term delivery of FIX. Any undesirable immune responses could rapidly clear the available hFIX from the circulation and render the treatment ineffective.

Interestingly, and in contrast to our previous results using transformed C2C12 myoblasts enclosed in alginate

microcapsules [21,22], G8 myoblasts elicited undetectable anti-hFIX antibodies in either C57BL/6 or hemophilic mice in the present study (Figures 2B and 3B). The ability of C2C12 to act as antigen-presenting cells *in vivo* has also been reported in other studies in which C2C12 were effective in antigen presentation and in stimulating secretion of cytokines such as IL-2 [43]. In contrast, a myoblast rhabdomyosarcoma TE671 cell line induced immune tolerance due to the expression of B7-H1, suggesting this cell line does not produce co-stimulatory signals to induce lymphocyte proliferation [44,45]. These findings illustrate the broad spectrum of the influence that different types of myoblasts can play in defining the final outcome of a specific immune response against a transgene.

This study demonstrates that encapsulated G8 only elicit a background humoral immune response to hFIX in mice. The mechanisms by which encapsulated G8 myoblasts behave differently than C2C12 in mice require further study. Recently, we have characterised the immune responses elicited in mice by encapsulated C2C12 secreting hFIX [46]. Immune responses to hFIX were both humoral and cellular. Experiments with CD4^{-/-}, CD8^{-/-}, MHC I^{-/-}, as well as MHC II^{-/-} knockout mice indicated that the immune responses to hFIX were CD4⁺ helper dependent, and mediated mainly via MHC II [46]. The finding that this antigen presentation is not apparent for G8 myoblasts highlights how the choice of encapsulated cells can influence the outcome of this gene therapy strategy, and indicates the potential of G8 myoblasts for the sustained delivery of hFIX.

The concentration of circulating hFIX averaged 370 ng/ml on day 3 (Figure 2A). If a similar level were achieved in hemophilic patients, severe and moderate hemophiliacs would be converted to the mild form of the disease. The levels of circulating hFIX correlated with the partial reduction in APTT observed in the treated mice (Figure 6). More importantly for the potential clinical application of this technology, the levels were also concordant with the protection of the treated hemophilic mice against major trauma, only seen after a clinically relevant level of biologically active hFIX has been achieved. However, higher levels of hFIX leading to not only therapeutic but curative effect in hemophilic mice would be desirable. An increase in the level of hFIX expression by the encapsulated cells and/or in the number of encapsulated cells that are implanted would theoretically lead to an increase in circulating hFIX. However, recent clinical trials for hemophilia A based on the implantation of recombinant fibroblasts did not yield high levels of hFVIII in treated patients [19]. Therefore, whether encapsulated myoblasts can achieve a therapeutic level of transgene delivery in larger animals still remains to be determined.

Although not optimal, hemophiliacs currently enjoy a safe treatment. Thus, any alternative treatment for hemophilia must be at least as safe as the protein replacement products. In this regard, implantable microcapsules offer an alternative to gene therapy strategies based on the direct injection of viral vectors.

The unique nature of this strategy offers the possibility of reversing the treatment by peritoneal lavage, should this become necessary [47–49]. Furthermore, this immunoisolation approach excludes the potential risk of genotoxicity, since the host cells are not genetically modified. This is always a source of concern in gene therapy protocols based on the use of viral vectors. The use of allogeneic, well-characterised recombinant cells represents a potentially more cost-effective strategy than the implantation of *ex vivo* gene therapy approaches based on autologous cell transplantation.

In this study we show evidence of the immunoisolation properties of alginate microcapsules enclosing allogeneic cells such as murine G8 myoblasts. Myoblasts are attractive cells for encapsulation. Proliferative cells such as fibroblasts eventually fill the entire capsular space, thus reducing the diffusion rate of nutrients and leading to cell death. Myoblasts have the potential to differentiate into non-proliferative myotubes, hence retaining high cell viability for at least 7 months [21]. As a result, encapsulated myoblasts are suitable as a stable and sustained source for the delivery of therapeutics. However, the long-term goal of this research is the treatment of human patients. Murine myoblasts would obviously not be considered for implantation into hemophiliacs. Thus, there is a need to develop human cells expressing high levels of biologically active hFIX with similar properties to those of the G8 myoblasts described in this study.

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