Myoblast-mediated gene transfer and its repeated applications were tested for achieving a long-term stable systemic production of human factor IX (hFIX) at a therapeutic level in SCID mice. Primary skeletal myoblasts were stably transfected with a hFIX expression plasmid vector, pdLMe4βA-hIXm1, which contains a hFIX minigene under the control of a β-actin promoter with muscle creatine kinase enhancers. Myotubes derived from the myoblasts produced 1,750 ng hFIX/10^6 cells/24 hours in culture. hFIX secretion by the myoblasts and thereof derived myotubes were equally efficient, and myotubes were shown to have a sufficient secretory capacity to handle a substantially elevated production of hFIX. After intramuscular injection of 5, 10, and 20 × 10^6 cells approximately 2 months later elevated the systemic hFIX levels to an average of 182 ± 21 ng/mL, a therapeutic level, which persisted for at least 8 months (end of the experiment). These results indicate that long-term, stable systemic production of hFIX at therapeutic levels can be achieved by repeated application of myoblast-mediated gene transfer.

Persisting Systemic Production of Human Factor IX in Mice by Skeletal Myoblast-Mediated Gene Transfer: Feasibility of Repeat Application to Obtain Therapeutic Levels

By Jian-Min Wang, Hong Zheng, Mila Blaivas, and Kotoku Kurachi

MATERIALS AND METHODS

Mouse. Pathogen-free, inbred, male SCID mice 5 to 6 weeks of age were obtained from Taconics (Germantown, NY) and housed in a sterile environment in the Unit of Laboratory Animal Medicine facility of this campus. All the animal studies were carried out in accordance with the institutional guidelines.

Vectors. Construction of the differentiated muscle cell-specific hFIX expression vector, pdLMe4/AhIXm1, was described elsewhere. It contains a hFIX minigene (hIXm1) with a shortened first intron sequence and a transcriptional control unit composed of a β-
Actin promoter (βA) and four copies of muscle creatine kinase gene enhancer (Me4) are present in pdLMe4/ΔAhixm1. Plasmid vectors pLIXSN (Fig 1) and pBAG contain hFIX cDNA and a bacterial β-galactosidase (β-gal) gene, respectively, in retroviral vector frames under the transcriptional control of the long-terminal repeat (LTR), and a neomycin resistance gene (Neo) under control of SV40 promoter.27,28 pSV2Neo is a Neo expression vector26 and pCH110 is a β-gal expression vector.27 Mouse primary skeletal myoblasts were isolated from muscles of SCID mice and grown in growth medium containing Dulbecco’s modified essential medium (DMEM), 20% fetal calf serum (FCS), 0.5% chicken embryo extract (GIBCO/BRL), and antibiotics (streptomycin and penicillin).21,23 hFIX assay medium was composed of the growth medium supplemented with NaCl-treated FCS (20%) and Vitamin K1 (10 μg/mL).21,23 Differentiation medium was the same as the hFIX assay medium except that FCS was replaced with NaCl-treated horse serum (2%). In all experiments, culture medium was changed every 24 hours for quantifying hFIX by enzyme-linked immunosorbent assay (ELISA) as previously described,27,28 and replaced with fresh medium. Differentiation of myoblasts to myotubes was induced by changing the medium to the differentiation medium for 3 days. All cells were kept at 37°C in a humidized incubator under 5% CO2.

Transient expression assay of hFIX expression vectors in muscle cells. Transient expression assays were carried out using LIPOFECTAMINE-mediated cell transfection as previously described.23 Myoblasts at a density of 2 × 10^5 per well in 6-well plates were transfected with 2 μg of a hFIX expression vector and 0.2 μg of pCH110 DNA. Myoblasts in one well from each group were collected at the end of day 2 and were used for determining cell number, β-gal activity, and total cellular protein content, which were used for normalizing the transfection efficiency as previously described.23 Cell number at the time of switching to the differentiation medium was approximately 2 × 10^6/well at 80% to 90% confluence.

Stable transfection of myoblasts and hFIX expression assay. Primary myoblasts at a density of approximately 1 × 10^6 per 10-cm dish were cotransfected with 10 μg pdLMe4/ΔAhixm1 and 0.8 μg pSV2Neo using LIPOFECTAMINE as described above, and subjected to G418 selection (1 mg/mL). Primary myoblasts transduced with LIXSN and BAG retroviruses were prepared as previously described.28 BAG was used as a background control, and LIXSN was used as a hFIX expression reference control because the cells carrying LIXSN were well characterized in our previous studies for their in vitro and in vivo hFIX expression and served for assessing any improvements in hFIX expression with the new vector. hFIX expression levels were determined by plating myoblasts stably transfected with pdLMe4/ΔAhixm1, or myoblasts transduced with LIXSN or BAG retrovirus at a density of about 4 × 10^5 cells per well in 6-well plates. hFIX protein produced into the medium was quantified by ELISA, and its activity was determined by one-stage clotting assay.25

Southern blot analyses. Genomic DNA prepared from cells was digested with KpnI or BamHI, and subjected to Southern blot analysis using 32P labeled hFIX cDNA as a hybridization probe.25 The transgene band intensity was quantified with PhosphorImager (Molecular Dynamics, Inc, model 400E). Transgene copy numbers in the genomic DNA were determined by using known amounts of vector DNA as the copy number standard.

Analyses of hFIX production and secretion efficiency. Stably transfected or retrovirus-transduced cells were plated at a density of about 6 × 10^4 cells per dish in 6-cm dishes. The media were collected daily, and cells were collected for preparing protein extracts and total RNA on days 2, 5, and 7. Cellular protein extracts were prepared as previously described23 except that the extraction solution contained Complete protease inhibitor (one Complete protease inhibitor tablet per 25 mL) (Boehringer Mannheim). Intracellular and secreted hFIX was detected by ELISA. Total cellular RNA was prepared using TRIzol total RNA isolation reagent (GIBCO-BRL) and subjected to Northern blot analysis using hFIX cDNA labeled with 32P as the hybridization probe. The filter was exposed to X-ray films, and radioactivity of RNA bands on the filter were quantified by a PhosphorImager. Intracellular and secreted hFIX was then stripped of the hFIX probe and rehybridized with 32P labeled 18S ribosomal RNA cDNA to confirm equal RNA loading to the lanes.

Intramuscular implantation of myoblasts. Myoblasts carrying hFIX or β-gal expression vectors were harvested and washed as described,27,28 and resuspended in serum-free DMEM supplemented with 1 μg/mL basic fibroblast growth factor (bFGF) (R&D systems) at 50 or 100 × 10^5 cells/mL. Cells were then injected into the limb muscles of SCID mice through a 30 G needle at five or ten different sites (10 μL per site).27 At various time points after cell injection, blood samples (0.1 to 0.3 mL) were collected by orbital sinus bleeding under brief anesthetization with inhalation of methoxyflurane (Pitman-Moore) and plasma samples were prepared for ELISA. At various time points, animals were killed by deep anesthesia with methoxyflurane. Muscles into which cells were implanted were removed for preparing muscle tissue sections and for isolating myoblasts carrying transgenes. The tissue sections were used for H & E staining and immunohistochiematic staining. Myoblasts isolated from the muscle tissue which received cell implantation were subjected to G418 selection and used for in vitro hFIX expression assays as previously described.27

Immunostaining analysis. Tissue sections were stained for hFIX using a murine anti-hFIX monoclonal antibody, AHIX-5041 (Haematologic Technologies, Inc), and Histostain-SP Kit (Zymed Laboratories, Inc) according to the manufacturer’s instructions. H & E and Masson trichrome stain were carried out using standard histologic methods.20

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Fig 1. hFIX expression vectors. pLIXSN contains a hFIX cDNA driven by long terminal repeat (LTR) promoter. pdLMe4/ΔAhixm1 contains a hFIX minigene (Δhixm1) under the transcriptional control of β-actin promoter (βA) and four copies of MCK enhancer (Me4). I to VIII indicates eight exons of hFIX gene. SVNeo indicates a neomycin resistant gene under the control of SV40 immediate early promoter. X in LTR indicates the deleted enhancer region. Thin lines with arrow show the predicted mRNA species; and zigzag line indicates the portion (hFIX intron) to be spliced.
Myotubes carrying pdLMe4 fold higher than those transfected with pLIXSN (Fig 2A). In vitro hFIX production levels of myoblasts transiently transfected with pdLMe4/AhIXm1 and LIXSN were determined. Following differentiation, intracellular and secreted hFIX levels of cells carrying pdLMe4/AhIXm1 increased by 2.8- and 3.3-fold, respectively, but only marginally increased for cells carrying LIXSN (Fig 4). In agreement with ELISA results, the hFIX mRNA level in myotubes with pdLMe4/AhIXm1 was 2.9-fold higher than that in the parent myoblasts (comparison of day 5 to day 2; mean of three experiments), while it was not significantly different for cells with LIXSN (1.1-fold increase in myotubes over that in myoblasts) (Fig 5). These results indicated that hFIX secretion from myoblasts is very efficient even at the level up to 2 µg/10^6 cells/24 hours.

Fig 2. hFIX production in culture by muscle cells carrying pdLMe4/AhIXm1 and LIXSN. (A) Transient expression of hFIX by muscle cells transfected with pdLMe4/AhIXm1 (■), LIXSN (○), and pSV2Neo (○). Medium was collected every 24 hours for ELISA and replaced with fresh medium. Arrow indicates the differentiation initiation time point. Vertical bars are standard deviations of triplicated assays. (B) Stable expression of hFIX. (■) indicates pdLMe4/AhIXm1; (○), LIXSN. Results shown are averages of three independent experiments with standard deviations shown in vertical bars. Signals in ELISA from cells transfected with BAG were subtracted as background from the results. The others are the same as described for transient assay (A).

Western blot analysis. hFIX in aliquots (1 mL) of day 5 culture medium of myotubes was precipitated with barium sulfate (100 mg) and subjected to Western blot analysis as previously described using a rabbit anti-hFIX polyclonal antibody and the chemiluminescence Western blot kit (Boehringer Mannheim).

**RESULTS**

Transient and stable expression of hFIX by muscle cells in vitro. In vitro hFIX production levels of myoblasts transiently transfected with pdLMe4/AhIXm1 were 6- to 10-fold higher than those transfected with pLIXSN (Fig 2A). Myotubes carrying pdLMe4/AhIXm1 secreted hFIX at a rate of 212 ± 21 ng/10^6 cells/24 hours on day 6, a 15-fold higher level than that of myotubes with pLIXSN (14 ± 5 ng/10^6 cells/24 h). This was consistent with the presence of the differentiated muscle cell-specific enhancer in pdLMe4/AhIXm1, but not in pLIXSN.

Myoblasts stably transfected with pdLMe4/AhIXm1 were prepared by cotransfection with pSV2Neo followed by G418 selection. Fifty-three myoblast colonies picked were individually expanded and their medium samples were subjected to ELISA screening for hFIX production. Nineteen of them were positive for hFIX expression, and six colonies expressed over 200 ng/10^6 myoblasts/24 hours. Myotubes derived from one of these myoblast colonies produced hFIX at 1,750 ± 61 ng/10^6 cells/24 hours on day 6, the highest level among the colonies tested. Myotubes derived from the myoblasts transduced with LIXSN produced 463 ± 114 ng hFIX/10^6 cells/24 hours (Fig 2B). Southern blot analysis of the genomic DNA indicated that the cells transfected with pdLMe4/βAhIXm1 or transduced with LIXSN had approximately four or two copies of the vector DNA per cell, respectively (data not shown). The recombinant hFIX protein produced from myoblasts and myotubes was biologically fully active with a specific activity ranging from 95% to 103% and a molecular size similar to natural hFIX (68 kD), agreeing well with our previous observations (Fig 3). A minor band with an apparent smaller size (about 52 kD) observed may be a degradation product of hFIX protein generated during the protein preparation procedure as we also observed for canine factor IX.

Characterization of production and secretion of hFIX by muscle cells. To test the capacities of primary myoblasts and myotubes in hFIX expression and secretion, hFIX mRNA as well as intracellular and secreted hFIX levels were determined. Following differentiation, intracellular and secreted hFIX levels of cells carrying pdLMe4/βAhIXm1 were 6- to 10-fold higher than that in the parent myoblasts (comparison of day 5 to day 2; mean of three experiments), while it was not significantly different for cells with LIXSN (1.1-fold increase in myotubes over that in myoblasts) (Fig 5). These results indicated that hFIX secretion from myoblasts is very efficient even at the level up to 2 µg/10^6 cells/24 hours.

Systemic delivery of hFIX in SCID mice after single and repeated cell implantations. hFIX production into the circulation of SCID mice after myoblast-mediated gene transfer is shown in Fig 6. Five groups of SCID mice (n = 4 per group) were implanted with various numbers of primary...
myoblasts stably carrying BAG, LIXSN, or pdLMe4βA-hIXm1. Animals in groups 1 and 2 were injected with 20 × 10^6 myoblasts transduced with BAG or LIXSN retroviral vectors. Animals in groups 3, 4, and 5 were injected with 5, 10, and 20 × 10^6 myoblasts with pdLMe4βAhIXm1, respectively. As assayed 26 days after the first cell injection, animals in groups 2, 3, and 4 produced hFIX at average levels of 23.1, 27.1, and 58.5 ng/mL serum, respectively. Animals in group 5 gave the highest hFIX production (94.1 ng/mL serum on average), 4- to 5-fold higher than those in group 2, which received myoblasts carrying LIXSN. These results were remarkably consistent with the in vitro observations (Fig 2B). hFIX expression levels in groups 3, 4, and 5 were approximately proportional to cell numbers injected (Fig 6). Stable hFIX production at levels of approximately 12, 18, and 60 ng/mL serum were observed for groups 2, 3, and 5, respectively, at least up to 10 months (end of observation).

To test the feasibility of repeat cell implantation, animals in group 4 were injected with additional doses of cells on day 43 (10 × 10^6, left leg muscle), day 52 (10 × 10^6, right leg muscle), and day 59 (20 × 10^6, both forearm muscles). Systemic hFIX levels in these animals were elevated to a range of 160 to 210 ng/mL serum, again approximately proportional to the implanted cell numbers, and stayed stable for an additional 8 months (end of the observation) (Fig 6). This range was approximately 2.5-fold higher than that of group 5, and approximately 15-fold higher than that of group 2 (cells with LIXSN).

Two hundred days after the first cell injection, primary myoblasts were obtained in culture from the muscle tissues of a representative animal in group 4. After G418 selection, cells showed a hFIX expression level equivalent to that of the original unimplanted, parent cells (Fig 7). Myoblasts isolated from another animal in group 1 3 months after implantation also gave similar results (data not shown). These results were consistent with our previous observations, demonstrating that a fraction of implanted cells can acquire the satellite cell status, and that little inactivation of LTR or Me4βA promoter took place in vivo. Although it is not likely, the possibility of partial inactivation of these promoters in vivo and regaining of their full activities in vitro cannot be denied. Immunohistochemical analysis of muscle tissue sections prepared from mice in groups 4 and 5 after 200 to 300 days of the original cell implantation also confirmed active hFIX production by the implanted cells (Fig 8, A and B).

Characterization of tumor. About 6 months post-cell implantation, one of the group 4 animals developed a tumor in the right leg muscle where cells were injected. The left leg muscle injected with the same batch and number of cells was normal and did not grow any tumor. This animal did not show any unusually elevated level of hFIX expression.
Fig 6. hFIX levels in the systemic circulation of SCID mice after intramuscular implantation of myoblasts carrying pdLMe4bAhIXm1 or LIXSN. The background ELISA signals obtained from group 1 (control group) mice, which were injected with $2 \times 10^7$ myoblasts transduced with BAG, were subtracted from the observed levels of other groups. Group 2 mice were injected with $2 \times 10^7$ myoblasts transduced with LIXSN. On day 0, group 3, group 4, and group 5 animals were injected with 5, 10, and $20 \times 10^6$ myoblasts stably transfected with pdLMe4bAhIXm1, respectively. Mice in group 4 were injected with additional cell doses (1, 1, and $2 \times 10^7$ cells, respectively) on days 43, 52, and 59 as shown with arrows. Results from individual animals in each group were shown.

Such an increase was invariably observed, when the implanted primary cells were transformed and proliferated in vivo. Cells isolated from the tumor biopsy expressed only a background level of hFIX (less than 10 ng/10^9/24 hours in culture), and they did not survive the G418 selection. Cells isolated from the other leg of the same animal, where the same batch and number of cells were also injected with no subsequent tumor development, survived the G418 selection well. The selected cells expressed hFIX at a comparable level with that of the unimplanted parent cells (Fig 7). Southern blot analysis detected substantially lowered level (23%) of the factor IX transgene in the genomic DNA prepared from the tumor tissue in comparison with that of the other leg where no tumor developed (Fig 9). The lowered transgene level is presumably due to the presence of a massive tumor tissue interspersed with a lesser amount of the normal muscle cells carrying the transgene in the muscle tissue removed for various analyses (Fig 8C). Masson trichrome stain (Fig 8D) and electron microscopic studies (data not shown) identified that this tumor is a fibrosarcoma. These results probably determined that the tumor is not derived from the implanted myoblasts.

DISCUSSION

In recent years, the promising potential of myoblast-mediated gene transfer for systemic production of therapeutic proteins has been reported by multiple groups including ours.\textsuperscript{3,5-8} In most of these studies, retroviral expression vectors combined with single dose cell implantation were used. In the present study, we have successfully demonstrated that the nonviral myoblast-mediated gene transfer and its repeat application can be used to achieve a long-term stable production of hFIX into the systemic circulation in mice at a therapeutic level. The systemic level achieved in this feasibility study is equivalent to 4% to 5% of the normal hFIX level, sufficient to change a clinically severe hemophilia B to a mild condition.

pdLMe4bAhIXm1, one of our refined hFIX expression vectors,\textsuperscript{23} was used as a plasmid vector in the present study. In transient assays, this vector, which contains a muscle specific transcription control unit, can express approximately 15-fold higher hFIX than LIXSN (our first generation retroviral vector) in myotubes (Fig 2A). Myotubes derived from $1 \times 10^6$ myoblasts stably transfected with this vector produced as high as 1,750 ng of hFIX per 24 hours, approximately 4-fold higher than those of the LIXSN transfected myotubes (Fig 2B). This is a relatively small increase in comparison to that observed in the transient expression assays (~15-fold) (Fig 2A). This difference cannot be simply explained by the transgene copy number, because cells trans-
Fig 8. Immunohistochemical analyses of sections of muscle tissue and a spontaneous tumor. (A) Tissue section of muscle with no cell injection. (B) Tissue section of muscle received myoblasts carrying pDLMe4/βAhIXm1. Tissue sections in panels A and B were stained with hFIX monoclonal antibody followed by counter-staining with hematoxylin (×630). Brownish-red color indicates the presence of hFIX. (C) Section of the tumor from a group 4 mouse stained with H&E (×500). The fibrosarcoma cells are small and spindle-shaped; one large muscle fiber is on the left. (D) Tumor section stained with Masson trichrome shows small spindled cells of fibrosarcoma and the collagen produced by the neoplasm in blue color; several muscle fibers are red or purple-red in color and large in size (×500).

Animals injected with various numbers of myoblasts carrying pDLMe4/βAhIXm1 or LIXSN persistently produced hFIX into the circulation for at least 10 months (end of the experiment) (Fig 6). In addition, hFIX levels in the systemic circulation were roughly proportional to the cell numbers injected. Importantly, this repeat cell implantation scheme works well, thus enabling us to achieve much higher, truly therapeutic levels of stable hFIX production into the circulation (Fig 6, group 4). The elevated hFIX levels were again approximately proportional to the total cell number injected. Rando and Blau31 also reported that, after intramuscular injection of myoblasts transduced with the β-gal expression retroviral vector, the expression levels of β-gal are proportional to the injected cell numbers in a given range. The cell numbers as well as cell concentration used by Rando and Blau,31 however, were much smaller than those used in the present study. The hFIX levels in the systemic circulation reported here were 10- to 15-fold higher than the stable levels we previously achieved in mice,7 and much higher than those (approximately 10 ng/mL of canine FIX in mice) reported by Dai et al.,8 who also used an approach of primary myoblast-mediated gene transfer with retroviral vectors. Kay et al9 reported a long-term stable canine FIX expression in dogs by in vivo delivery of FIX retrovirus through the portal vein, but only at low levels (<10 ng/mL plasma). Although we must be cautious in direct comparison of our results with those obtained by others because of the different experimental conditions used,9 the stable systemic hFIX level we have achieved and the possibility of its further elevation by repeated therapies are important.
Based on the two-compartment distribution kinetics, we have estimated that the plasma hFIX concentration level obtained for the group 4 animals is less than 5% of the theoretical steady-state level that could be expected with the total $5 \times 10^7$ cells injected. Here we assume that all cells can produce hFIX in vivo as efficiently as in vitro. Although it is a rough estimation, this suggests that most implanted cells are not efficiently contributing to hFIX production in vivo. This may be due to (1) the death of a large percentage of the implanted cells without successful fusion with existing myofiber cells or among themselves to form new myofiber cells, (2) the in vivo environment is substantially different from that in the culture and the transgene cannot be expressed as efficiently as in the cultured cells, (3) a combination of these possibilities, or (4) other yet unknown mechanisms. Generally low survival of nongenetically modified myoblasts implanted in humans (the above second possibility) was also reported by multiple groups suggesting that the myoblast implantation procedure and cell fusion processes are at least two critical steps that need further careful investigation for improving this gene transfer approach.

After 6 months of cell injection, one of the group 4 animals developed a tumor in the right hind leg muscle. The left hind leg muscle, which also received the same batch and number of genetically modified cells, was normal and did not grow any tumor. A series of biochemical and immunohistochemical analyses in addition to the lack of increased production in vivo of the recombinant FIX indicated that this tumor was fibrosarcoma, and not directly derived from the implanted cells. At this stage of the study, however, we should not disregard a possibility that the tumor might have been derived from the implanted cells due to an unknown mechanism. Other possible explanations for its development may include a random tumorigenesis incidence in the immune-deficient SCID mice, possible tumorigenic stimulation in the local tissue due to the damage by multiple needle stabbings or any other unforeseen mechanisms. The implanted cells were mixed with basic FGF just before their implantation to increase the hFIX expression in vivo. This effect is presumably due to a brief stimulation of proliferation or possibly augmentation of migration and/or invasion through basal lamina of the injected myoblast. Although no tumorigenic potential of bFGF has been reported, its angiogenic activity is well established, suggesting a possibility that once tumorigenesis is triggered due to unknown mechanism(s), it may support subsequent tumor growth. In our experimental conditions, however, bFGF should be cleared from the injection sites within minutes, making it unlikely for bFGF to be directly responsible for such an aberration(s). It has been reported that after subcutaneous implantation in athymic mice, C2 myoblasts (established cells), but not cloned primary myoblasts, develop tumors and also that intramuscularly injected C2 cells can proliferate in vivo. In addition, extensive human trials of myoblast implantation therapy for Duchenne muscular dystrophy (DMD) have strongly supported the general safety of myoblast implantation. Roman et al. reported that C57BL/J6 mice intramuscularly implanted with either C2C12 cells or primary myoblasts, which are transduced with recombinant retroviruses, invariably developed rhabdomyosarcomas after 60 days of cell implantation. Unfortunately, the investigators provided neither information regarding the potential contamination of replication-competent retrovirus in the viral stock used nor other explanations for the tumor development with the primary myoblasts.

The present study has demonstrated the feasibility to refine the myoblast-mediated gene transfer method in achieving both the long-term systemic production of therapeutic level recombinant FIX and improved safety. The results warrant further intensive studies on its safety aspects and improvement of the overall FIX systemic production, before its clinical application.

ACKNOWLEDGMENT

We thank Dr David L. Allen and Dr Atac Turkay for their critical reading of the manuscript and the morphology core facility of the Reproductive Science Program of the University of Michigan for preparing tissue sections.

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