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Genetics

Gene therapy via primary myoblasts: Long-term expression of factor IX protein following transplantation *in vivo*

(retroviral vectors/tissue-specific enhancer/muscle)

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ABSTRACT We have explored the use of primary myoblasts as a somatic tissue for gene therapy of acquired and inherited diseases where systemic delivery of a gene product may have therapeutic effects. Mouse primary myoblasts were infected with replication-defective retroviruses expressing canine factor IX cDNA under the control of a mouse muscle creatine kinase enhancer and human cytomegalovirus promoter. The infected myoblasts were injected into the hindlegs of recipient mice and levels of secreted factor IX protein were monitored in the plasma. We report sustained expression of factor IX protein for over 6 months without any apparent adverse effect on the recipient mice.

A wide variety of somatic tissues are being explored for the introduction of foreign genes with a view toward gene therapy. A prime requirement for successful gene therapy is the sustained expression of the therapeutic gene without any adverse effect on the recipient (1–3). In the past, fibroblasts have been used for introducing genes whose products are secreted into the circulation (4–7). In most cases, however, the expression of the transgene, introduced via infection with a recombinant retrovirus either under the control of a viral long terminal repeat (LTR) or heterologous internal promoter, was shut off (8, 9). The use of a housekeeping gene promoter such as that of the dihydrofolate reductase gene led to sustained expression, albeit at very low levels, lending support to the notion that the choice of a suitable promoter–enhancer combination could be crucial for long-term expression (8). Recently several groups have suggested the use of myoblasts as a possible somatic tissue for gene therapy, partly because of their ready accessibility and ease of *in vitro* and *in vivo* manipulations and because skeletal muscles constitute nearly 40% of the body weight of an average individual (10–13). Data from a number of studies with the mouse C2C12 cell line suggest that (*i*) myoblasts can be efficiently infected *in vitro* with recombinant retroviruses, and the transduced gene is expressed; (*ii*) the product of the transgene can be processed and secreted; and (*iii*) upon formation of myotubes by fusion of the myoblasts, the expression of the transgene is not affected. Although long-term (85 days) expression of the transgene following transplantation of C2C12 cells in the muscle of the recipient mouse has been reported, the data do not apply to clinical gene therapy because C2C12 cells, which are tumorigenic, were used, rather than primary myoblasts (11). We have explored the use of primary myoblasts as a somatic tissue for gene therapy and report the sustained expression of biologically active factor IX protein for over 6 months without any apparent adverse effect on the recipient mice.

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MATERIALS AND METHODS

Animals and Cell Culture Conditions. Adult nude (*nu*/*nu*) athymic mice (6–8 weeks old) were obtained from The Jackson Laboratory. The retroviral packaging cell lines GPE86 and AM12 (14) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum. Mouse primary myoblasts were maintained in DMEM with 20% fetal calf serum and 1% chicken embryo extract (GIBCO; treated at 65°C for 10 min) on gelatin-coated dishes. Differentiation of myoblasts was induced by transfer of the cells to fusion medium, consisting of DMEM with 10% horse serum. Infected cells were selected in medium containing G418 at 400 μg/ml, for AM12 and GPE86 cells, or 200 μg/ml, for primary mouse myoblasts.

Vector Construction. LNCIXL (i.e., LNcdF9L) has been described previously (6). It contains canine factor IX cDNA linked to the 579-base-pair (bp) human cytomegalovirus (CMV) immediate-early gene enhancer/promoter (−524 to +55). LNMEaGIXL was constructed by replacing the CMV enhancer/promoter in LNCIXL with 207 bp of mouse muscle creatine kinase (MCK) enhancer (−1256 to −1050) (15), 650 bp of human α-globin promoter (16), and 55 bp of *Xenopus* β-globin 5′ untranslated region (17). LNMECIXL was constructed by inserting the 207-bp MCK enhancer into retroviral vector LNCIXL at the *Sal* I site, which is 250 bp upstream from the CMV enhancer/promoter (Fig. 1*A*).

Virus production. Ten micrograms of plasmid DNA was transfected into the ecotropic packaging cell line GPE86 by the calcium phosphate coprecipitation method. The medium was changed 24 hr later, and 48 hr after transfection the culture medium was harvested and used to infect the amphotropic packaging cell line AM12 in the presence of Polybrene (8 μg/ml). Single colonies of infected AM12 cells were isolated by selection in medium containing G418 and were expanded. Recombinant retroviruses were harvested from confluent culture dishes, filtered, and used to infect NIH 3T3 cells (mouse fibroblast line) to determine the viral titers. The presence of the helper virus was assayed by the marker rescue method (6). Assays for production of canine factor IX were carried out as described (6). Pooled normal canine plasma was used as a factor IX standard (10 μg/ml).

Isolation of Mouse Primary Myoblasts. Hindleg muscles were dissected from three to four neonatal (2-day-old) Swiss Webster mice (The Jackson Laboratory), minced, and dissociated for a total of 30–40 min by three successive treatments with trypsin (0.25%) (GIBCO) collagenase (100 μg/ml) (Worthington) in phosphate-buffered saline. The dissociated cells were seeded on the gelatin-coated dishes in DMEM supplemented with 20% fetal calf serum and 1% chicken embryo extract (treated at 65°C for 10 min). During the first

Abbreviations: CMV, cytomegalovirus; LTR, long terminal repeat; MCK, muscle creatine kinase.
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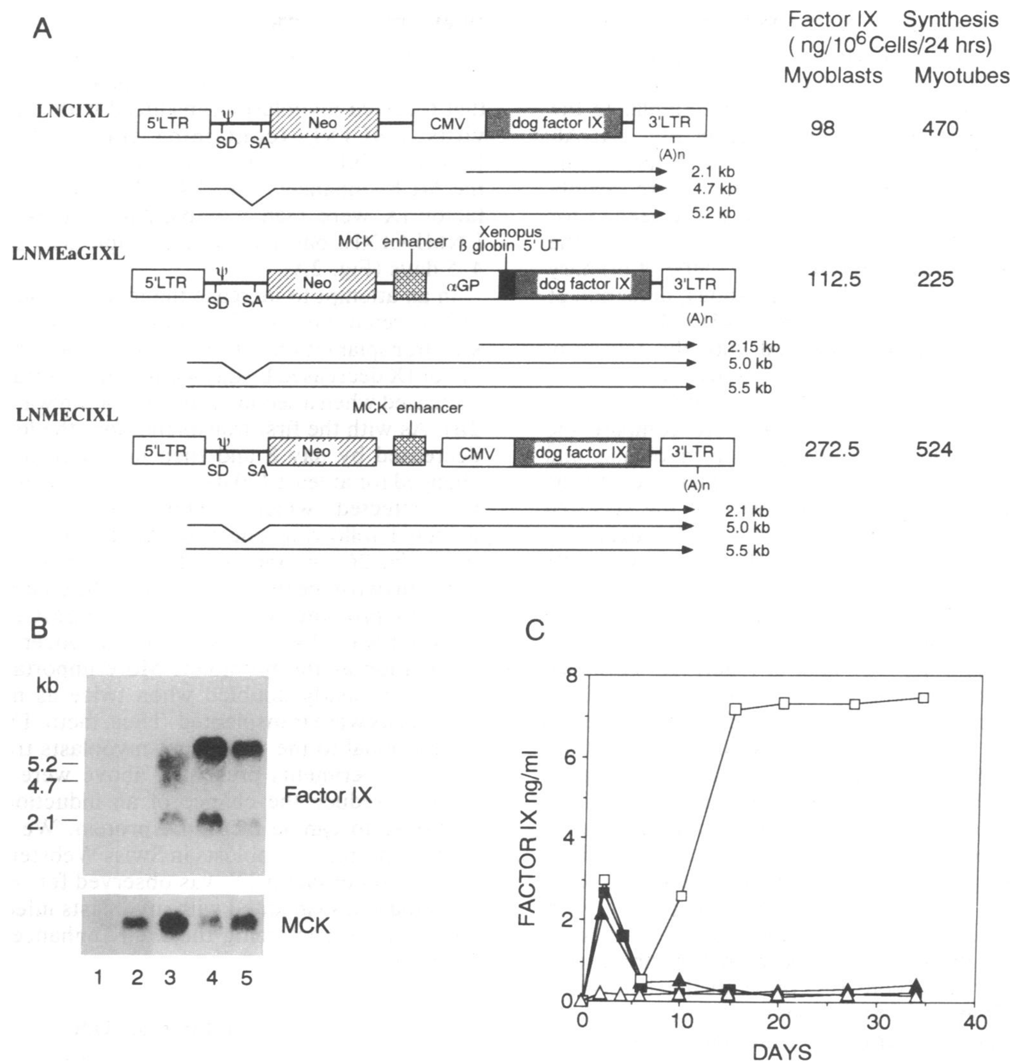

FIG. 1. Structure of retroviral vectors and production of recombinant canine factor IX by primary mouse myoblasts and in plasma from transplanted nude mice. (*A*) Structure of retroviral vectors containing different enhancer and promoter with canine factor IX cDNA. The expected sizes of the transcripts containing factor IX are indicated in kilobases (kb). The total amount of factor IX secreted into the medium by infected primary myoblasts and myotubes from Swiss Webster mice was determined by ELISA and is tabulated in the figure. Approximately 5×10^5 primary myoblasts were seeded on gelatin-coated 60-mm dishes in 5 ml of growth medium, and after the first 2 days, the cells were refed with fusion medium (DMEM plus 10% horse serum) to start fusion. Medium was removed every 2 days for factor IX assay by ELISA (6). Three to 4 days after addition of fusion medium, >90% of the cells transformed to multinucleated myotubes. SD, splice donor; SA, splice acceptor; Ψ, packaging signal; Neo, neomycin (G418)-resistance marker; (A)n, polyadenylylation site; αGP, α-globin promoter. (*B*) Northern blot analysis of RNA from uninfected mouse primary myoblasts (lane 1), differentiated myotubes (lane 2), or myotubes transduced with LNCIXL (lane 3), LNMEαGIXL (lane 4), and LNMECIXL (lane 5). Total cytoplasmic RNA was fractionated in a formaldehyde/agarose gel, transferred onto a nylon membrane, and hybridized to a random-primer-labeled, 1.5-kb canine factor IX cDNA probe (*Upper*) and a 0.6-kb *Pst* I fragment of rat MCK probe (18) (*Lower*). (*C*) Levels of canine factor IX in plasma from nude mice injected with ≈10^7 untransduced primary myoblasts (△) or primary myoblasts transduced with LNCIXL (■), LNMEαGIXL (▲), or LNMECIXL (□). Although data are shown for 34 days postimplantation, all four animals are alive and only the mouse transduced with LNMECIXL has continued to produce factor IX at nearly the same level for >180 days.

three passages, myoblasts were enriched by preplating the cells at 37°C for 20 min on a non-gelatin-coated dish to allow preferential adherence of fibroblasts. Most of the early-stage primary myoblasts were frozen at −80°C for further use. A small portion of these primary myoblasts were infected with an amphotropic virus from AM12 packaging cells and selected with G418 (200 μg/ml, active) (GIBCO). Approximately 100 G418-resistant myoblast colonies were pooled and expanded for all subsequent manipulations such as RNA isolation, factor IX assays, and injection into nude mice.

Injection of Infected Myoblasts into Nude Mice. Approximately 1–2 × 10^7 transduced mouse primary myoblasts were harvested, suspended at 5×10^7 cells per ml in phosphate-buffered saline, and injected into muscles of both hindlegs of anesthetized nude mice at 6–8 weeks of age (5–10 μl per site, 100 μl per leg). Multiple independent isolates of primary myoblasts were used for the experiments shown here, thereby eliminating the possibility that an immortalized cell expressed the recombinant retroviruses. Plasma was taken by bleeding the tail vein at the indicated times, and canine factor IX in mouse plasma was determined by ELISA (6).

RESULTS

Factor IX Expression in Transduced Primary Myoblasts. We have generated three recombinant retroviral vectors containing canine factor IX cDNA under the control of different enhancer–promoter combinations: the CMV promoter (LNCIXL), the MCK enhancer linked to the human α-globin promoter (LNMEαGIXL), or the MCK enhancer

linked to the CMV promoter (LNMECIXL) (Fig. 1*A*). High-titer helper-free recombinant retroviruses were generated in AM12 cells (14). The sizes of the mRNA transcripts in infected myoblasts (Fig. 1*B*, lanes 3–5) were similar to the expected sizes of the transcripts indicated in Fig. 1*A*. Although MCK-specific transcripts were detected only at low levels in primary myoblasts (lane 1), they were readily detected in myotubes (lane 2). Since the RNA needed for these studies was isolated from myoblasts 4 days after the addition of fusion medium, at a time when >90% of primary myoblasts had differentiated to form myotubes, detection of MCK-specific mRNA transcripts (lanes 3–5) indicated that primarily myoblasts had been infected with the retroviral vectors. The infected myoblasts, as well as myotubes formed *in vitro* following the addition of the fusion medium, secreted factor IX protein (Fig. 1*A*). It is difficult to compare the absolute amounts of factor IX produced in myoblasts and myotubes because the total number of cells increased during differentiation. We also found that >98% of the secreted factor IX could be absorbed by barium sulfate (6), suggesting that the myoblasts and myotubes synthesized biologically active factor IX.

Sustained *in Vivo* Expression of Factor IX. To study the expression of the transgene *in vivo*, we transplanted infected myoblasts into nude mice. The data in Figure 1*C* show that following transplantation the levels of canine factor IX in mouse plasma increased for 2–4 days and then declined to control levels (Fig. 1*C*), except in the case in which infections were carried out by recombinant retroviruses containing the MCK enhancer linked to the CMV promoter (LNMECIXL; Fig. 1*A*). This result is in contrast to *in vitro* differentiation of transduced myoblasts to myotubes, where the levels of factor IX protein generated by all three vectors increased (Fig. 1*A*). By day 7, the levels of secreted factor IX increased, reached a maximum by day 14, and remained unchanged for >34 days. Thus it appears that the presence of the MCK enhancer in the construct prevents the inactivation of transcription from the CMV promoter in myotubes.

We repeated these experiments using larger numbers of animals. Secretion of factor IX into circulation was observed for >90 days (Fig. 2*A*) in three animals transplanted with myoblasts infected with recombinant retrovirus containing the MCK enhancer linked to the CMV promoter (LNMECIXL; Fig. 1*A*). The expression declined for up to 4 days posttransplantation but increased thereafter until 9–10 days and remained essentially unchanged thereafter. With mice that received myoblasts transduced with recombinant retrovirus LNCIXL (containing the CMV promoter) or LNMEαGIXL (containing the α-globin promoter linked to the MCK enhancer; Fig. 1*A*), the initial levels of secreted factor IX were high, comparable to those observed with LNMECIXL, but decreased precipitously over the ensuing 4–5 days (Fig. 2*A*).

In an attempt to increase the levels of factor IX produced and secreted into the circulation, we attempted two successive transplantations after a hiatus of 4–5 days. Circulating factor IX decreased by day 4 after the first transplantation but increased when a second transplantation was performed (Fig. 2*B*). As with the first transplantation, the levels of factor IX decreased by day 8 and then increased and remained unchanged for at least 120 days. Up to 20 ng of factor IX per ml was detected, which is approximately twice the amount detected following one transplantation. In four additional mice (Fig. 2*C*), expression of factor IX was detected for >180 days. In two mice the orientation of the enhancer with respect to CMV promoter was reversed (LNEMCIXL). Higher expression was observed when the enhancer was in the same orientation as the promoter. More important, the levels of factor IX nearly doubled when twice as many transduced myoblasts were transplanted. Thus, factor IX expression was proportional to the number of myoblasts transplanted.

The experiments presented above were done with nude mice to reduce the chance of an induction of an immune response to canine factor IX protein. We have also transplanted primary myoblasts in Swiss Webster mice. Sustained expression of factor IX was observed for >180 days only in the mouse transplanted with myoblasts infected with recombinant virus containing the MCK enhancer (LNEMCIXL) (Fig. 3).

DISCUSSION

A principal requirement for successful gene therapy is long-term expression of the foreign gene. This has proven to be difficult, and to date *in vivo* long-term expression has been attained only with immortalized cell lines. Unfortunately,

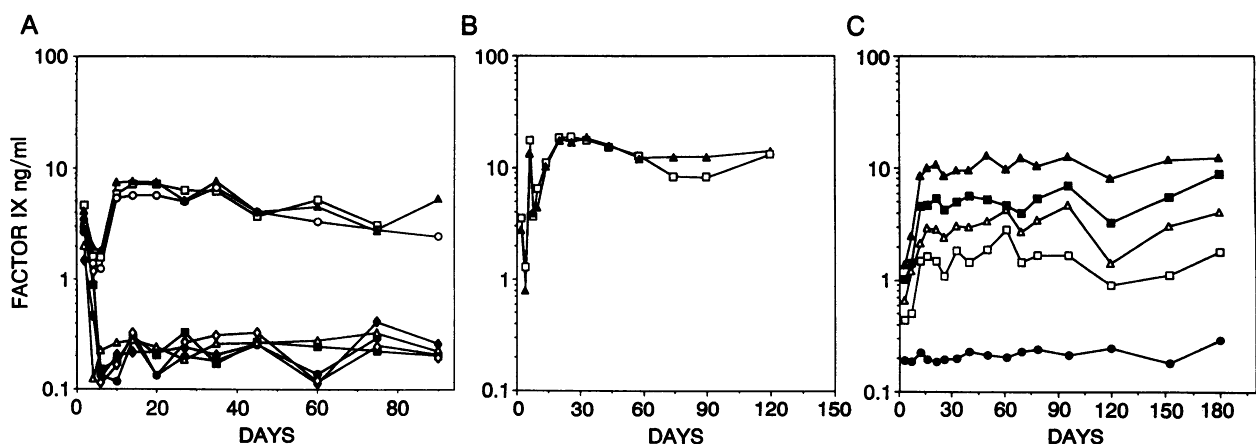

FIG. 2. Sustained *in vivo* expression of canine factor IX by retrovirus-transduced primary myoblasts implanted in muscles of nude mice. (*A*) About 8 × 10^{6} infected primary myoblasts were implanted into each of eight 7-week-old nude mice. Each symbol represents a different mouse implanted with primary myoblasts transduced with LNCIXL (●, ◇, ■), LNMEαGIXL (△, ◆), or LNMECIXL (○, □, ▲). One animal containing myoblasts transduced with LNMECIXL was sacrificed at day 76 to determine the expression of factor IX *in situ*. (*B*) About 10[7] LNMECIXL-transduced primary myoblasts were implanted into each of two 7-week-old nude mice (□, ▲) by injection at 20 sites in each hindleg, and the injections were repeated with the same amount of cells 5 days later. (*C*) About 6 × 10^{6} primary myoblasts transduced with LNMECIXL or LNEMCIXL (MCK enhancer in opposite orientation) were implanted into hindleg muscles of four 8-week-old nude mice by injection at 10 sites in each leg. Two of them received the second implantation with the same number of cells 4 days later. Each symbol represents a different mouse implanted with primary myoblasts transduced with LNMECIXL with single (■) or double (▲) implantation or LNEMCIXL with single (□) or double (△) implantation. ●, Control (no injections).

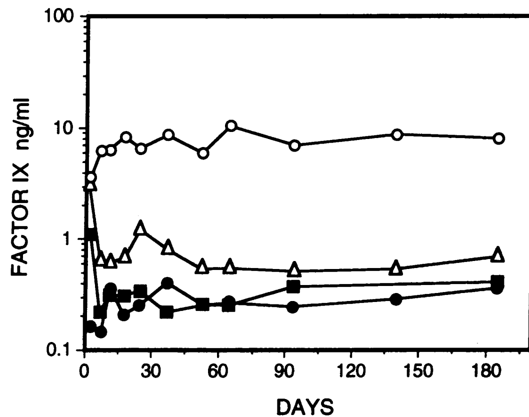

FIG. 3. Sustained *in vivo* expression of canine factor IX by retrovirus-transduced primary myoblasts implanted in muscles of Swiss Webster mice. About 2×10^7 infected primary myoblasts were implanted into each of three 8-week-old Swiss Webster mice. Each symbol represents a different mouse implanted with primary myoblasts transduced with LNCIXL (■), LNMEαGIXL (△), or LNEMCIXL (○), with an uninjected mouse as control (●). The levels of factor IX produced are lower because the myoblasts were infected with LNEMCIXL virus, which has the enhancer in opposite orientation and usually shows lower expression.

immortalized cell lines are tumorigenic and thus cannot be used for transplantation in humans. Our results indicate that primary myoblasts can be transduced by recombinant retroviruses and that long-term expression can be observed following transplantation into the skeletal muscles of recipient mice. While some degree of expression in myoblasts has been observed with a variety of recombinant retroviral vectors, we have found sustained expression following transplantation only from those vectors in which the MCK enhancer is linked to the CMV promoter. In our studies, the combination of MCK enhancer and CMV promoter proved to be most efficient in expressing stable levels of circulating factor IX. It is important to note that no neoplastic growth was observed in any animal where transduced myoblasts were implanted. The nearly constant amount of factor IX from day 10 to >180 days (Figs. 2*C* and 3) further confirms this notion; otherwise the levels of factor IX protein would have increased due to the increased mass of cells.

Sustained long-term expression *in vivo* following transplantation of myoblasts infected with only those recombinant retroviral vectors which contained MCK enhancers supports our expectation that myofibers had to form through myoblast fusion for MCK enhancers to be operational. Although other investigators have reported that transplanted myoblasts can fuse with endogenous myofibers (19–22), our data cannot exclude the possibility of fusion among transduced myoblasts, rather than fusion of transduced myoblasts with resident myotubes. It should also be emphasized that the data presented were obtained by transplantation of myoblasts obtained from 2-day-old mice into 7-week-old mice. We cannot predict whether transplantation of myoblasts prepared from older mice or injected into aged recipients will yield similar results. It should be noted that even though a large number of young myoblasts were injected at multiple sites, there were no long-term ill effects on the recipient animals.

Even though we detected stable long-term expression from vectors used in the experiments reported here, the steady-state levels of factor IX protein secreted into the plasma (10 ng/ml for 10^7 injected cells) are not sufficient to be of therapeutic value. We estimate that levels approaching 0.5–1 μg/ml of factor IX in plasma will be required for this approach to be effective in the gene therapy of hemophilia B (4, 6). That goal will require vectors that will express higher levels of factor IX protein, as well as a strategy for repeated injections. The data presented here, however, identify muscle as a somatic tissue in which long-term sustained secretion of a foreign protein can be achieved from a primary tissue rather than cell lines.

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