Systemic Delivery of Recombinant Proteins by Genetically Modified Myoblasts

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The ability to stably deliver recombinant proteins to the systemic circulation would facilitate the treatment of a variety of acquired and inherited diseases. To explore the feasibility of the use of genetically engineered myoblasts as a recombinant protein delivery system, stable transfectants of the murine C2C12 myoblast cell line were produced that synthesize and secrete high levels of human growth hormone (hGH) in vitro. Mice injected with hGH-transfected myoblasts had significant levels of hGH in both muscle and serum that were stable for at least 3 weeks after injection. Histological examination of muscles injected with β-galactosidase–expressing C2C12 myoblasts demonstrated that many of the injected cells had fused to form multinucleated myotubes. Thus, genetically engineered myoblasts can be used for the stable delivery of recombinant proteins into the circulation.

A VARIETY OF ACQUIRED AND INherited diseases are now treated by repeated intravenous or subcutaneous infusions of recombinant or purified proteins. These include diabetes mellitus, treated with subcutaneous or intravenous injections of insulin, hemophilia A, treated with intravenous infusions of factor VIII, and pituitary dwarfism, treated with subcutaneous injections of growth hormone (*1*). The development of cellular transplantation systems that can stably produce and deliver such recombinant proteins into the systemic circulation would represent an important advance in our ability to treat such diseases. The ideal recombinant protein delivery system would use a cell that can be easily isolated from the recipient, grown, and transduced with recombinant genes in vitro, and conveniently reimplanted into the host organism. This cell would produce large amounts of secreted recombinant protein, and after secretion, this protein would gain access to the circulation. Finally, such implanted, genetically engineered cells should survive for long periods of time and continue to secrete the transduced protein product without themselves interfering with the function of the tissue into which they were implanted. Different cellular systems have been used to produce recombinant proteins in vivo. These include keratinocytes (*2*), skin fibroblasts (*3*), hepatocytes (*4*), lymphocytes (*5*), and bone marrow (*6*). Although several of these systems have produced detectable amounts of circulating proteins, it has proven difficult to produce stable, physiological levels of circulating recombinant proteins in normal animals.

Genetically engineered myoblasts represent a potentially useful system for the in vivo delivery of recombinant proteins into the circulation. Myoblasts can be readily isolated from muscle biopsies and expanded in vitro to large cell numbers (*7*). Cultured myoblasts can be transfected or infected in vitro and will synthesize large amounts of recombinant proteins (*8*). Previous studies have demonstrated that cultured myoblasts can be injected intramuscularly and will survive and fuse into adjacent normal muscle fibers at the site of injection (*9*). Finally, skeletal muscle is a highly vascular tissue. Thus, proteins secreted from myoblasts might be expected to readily enter the circulation.

To explore the feasibility of using myoblasts for the systemic delivery of recombinant proteins in vivo, we cotransfected (stably) murine C2C12 myoblasts (*10*) with two plasmids: the pRSVGH plasmid, containing the hGH gene under the control of the Rous sarcoma virus long terminal repeat (RSV LTR), and the pRSVneo plasmid, which encodes resistance to the antibiotic G418. C2C12 is a continuous cell line that has been shown to differentiate into nondividing, multinucleated myotubes in vitro (*10*). These myotubes express the full complement of myofibrillar proteins and display contractile activity. After exposure to G418 to select for stable transfectants, 2 out of 24 clones were shown to produce and secrete relatively high levels of hGH in vitro, and one of these, G19, was expanded for further studies.

In an initial series of experiments, the in vitro production and secretion of hGH by G19 cells was quantitated by use of a sensitive radioimmunoassay (RIA). This assay was linear over a range of hGH concentrations between 0.05 and 50 ng/ml (Fig. 1A). Levels of hGH in the culture medium of G19 cells increased in a linear fashion between 2 and 24 hours, with a mean rate of production of 12 ng/hour per 10^6 cells (Fig. 1B). After secretion, there was almost no degradation of hGH as evidenced by the finding that the levels of hGH from culture supernatants of G19 cells did not decrease significantly after incubation for 24 hours on monolayers of nontransfected C2C12 cells (Fig. 1C). After differentiation into myotubes in vitro, the hGH-transfected G19 cells continued to secrete hGH at a rate of 6 ng/hour per 10^6 cells (*11*). Thus, nondividing myotubes retain the ability to produce secreted proteins. Finally, the RIA used in these experiments was specific for hGH and did not cross-react with murine GH (Fig. 2B).

To determine whether G19 cells could produce circulating levels of hGH in vivo,

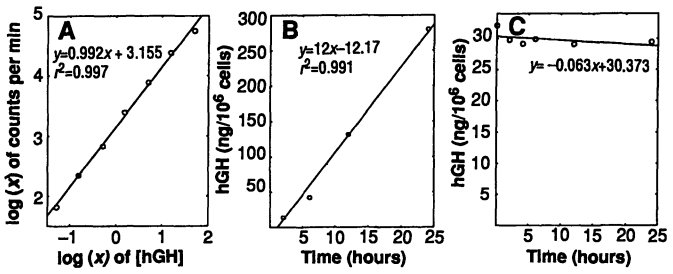

Fig. 1. Production of hGH in vitro by the G19 clone of pRSVGH-transfected C2C12 myoblasts. C2C12 myoblasts were cotransfected with the pRSVGH and pRSVneo plasmids by use of calcium phosphate as described previously (*17*). After selection in 1 mg/ml of G418 (Gibco, Grand Island, New York), clones were assayed for hGH production and secretion by a commercially available RIA (Nichols Institute Diagnostics, San Juan Capistrano, California). One clone, G19, was expanded for further studies. (**A**) Validation of the RIA for hGH. Dilutions of recombinant hGH containing between 0.05 and 50 ng/ml were assayed in duplicate by RIA as per the manufacturer's instructions. (**B**) Kinetics of hGH secretion by G19 cells in vitro. The 10^6 G19 cells were seeded onto replicate 100-mm dishes containing 10 ml of Dulbecco's minimum essential medium (DMEM) + 20% fetal bovine serum and 1% chick embryo extract (Gibco). The 100-μl aliquots of culture medium were assayed for hGH by RIA at the time points shown. All assays were performed in duplicate using culture medium from two different plates, and mean values were normalized for cell number to eliminate differences caused by cell division. (**C**) Kinetics of hGH decay during incubation on C2C12 monolayers. Cultures of 10^6 normal C2C12 cells were incubated in hGH-containing culture medium previously conditioned by exposure to G19 cells for 24 hours. Duplicate 100-μl aliquots of culture medium from two different plates were assayed for hGH at the time points shown. All statistical calculations were performed using Statview II software (Abacus Concepts, Berkeley, California) on an Apple Macintosh IIcx computer.

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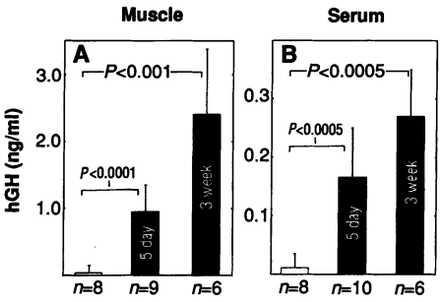

Fig. 2. Levels of hGH in muscle and serum of C2C12 myoblast–injected mice. Four-week-old C3H mice were injected intramuscularly with 6×10^6 G19 cells (solid bars) or control F17 cells (open bars) in a total volume of 240 μl of DMEM medium (Gibco) at six separate sites with a 30-gauge needle. Mice were euthanized 5 days or 3 weeks after injection and muscle samples from the sites of injection and serum samples were assayed for hGH by RIA. (**A**) Levels of hGH in muscle homogenates (*18*) from control ($n = 8$) and G19-injected ($n = 9$ at 5 days and $n = 6$ at 3 weeks) mice. (**B**) Levels of hGH in serum from control ($n = 8$) and G19-injected ($n = 10$ at 5 days and $n = 6$ at 3 weeks) mice. All samples were assayed in duplicate. The data are presented as mean ± SD.

we injected a total of 6×10^6 G19 cells intramuscularly into six separate sites in the lower limbs of normal 4-week-old syngeneic C3H mice. Control mice received identical injections of F17 cells, a G418-resistant clone of C2C12 that does not produce hGH. To prevent potential cellular or antibody-mediated immune responses by the C3H mice to the hGH produced by the G19 cells, we gave all mice daily injections intramuscularly of cyclosporin A (5 mg per kilogram of body weight). Mice were killed 5 days or 3 weeks after injection, and muscle from the site of injection as well as serum were assayed for hGH (Fig. 2). Muscle lysates from mice injected with the G19 hGH-producing cells contained 1.01 ± 0.34 ng/ml (mean ± SD) of hGH at 5 days and 2.43 ± 0.97 ng/ml at 3 weeks as compared with control-injected mouse muscle which contained 0.01 ± 0.01 ng/ml ($P < 0.0001$). The serum from G19-injected animals contained 0.16 ± 0.08 ng/ml and 0.28 ± 0.08 ng/ml of hGH at 5 days and 3 weeks after injection, respectively. These values were significantly different from those of control mice (0.01 ± 0.02 ng/ml) ($P < 0.0005$) at both time points. Thus hGH expression appeared to be stable for at least 3 weeks in these animals.

It was of interest to compare the levels of hGH in the myoblast-injected mice to physiological levels of hGH in human serum. GH is secreted in a pulsatile fashion in humans with normal physiological levels ranging between 0.1 and 25 ng/ml (*12*). Absolute levels of hGH also vary depending on the type of sample tested and the particular assay system used. Serum samples from normal human volunteers ($n = 7$) contained 0.27 ± 0.1 ng/ml of hGH as compared with 0.28 ng/ml, the mean serum level of hGH in the G19-injected mice. Thus, serum from the G19-injected animals contained physiological levels of hGH 3 weeks after a single injection of 6×10^6 hGH-transfected myoblasts. Finally, animals were also injected with 6×10^6 G19 cells without concomitant immunosuppression with cyclosporin A. Serum from these animals ($n = 4$) contained 1.0 ± 0.25 ng/ml of hGH 3 weeks after myoblast injection. Thus, immunosuppression does not appear to be necessary for the short-term production of recombinant proteins after myoblast injection.

An important question regarding the long-term feasibility of myoblast injections concerns the fate of the myoblasts after intramuscular injection. To address this question, we injected C3H mice with C2C12 myoblasts that had been previously infected in vitro with the β-galactosidase expressing β-*gal*-at-*gag* (BAG) retrovirus (*13*) and shown to express high levels of intracellular β-gal (Fig. 3A). The β-galactosidase-expressing–blue C2C12 cells were observed as clusters within areas of normal muscle (Fig. 3, C through E). Whereas the BAG-infected C2C12 myoblasts displayed a mononuclear fibroblast-like appearance when grown in high levels of serum in vitro (Fig. 3A), after injection many of these cells fused into multinucleated myotubes (Fig. 3, C through E) similar to those observed after the differentiation of C2C12 cells by growth in low serum in vitro (Fig. 3B) (*10*). Tumors were not detected in any case in the muscle or other organs of the C2C12 myoblast–injected animals at either 5 days or 3 weeks after injection. Moreover, lysates from the noninjected upper limbs, hearts, livers, kidneys, and lungs of the G19-injected animals were devoid of hGH activity, demonstrating that the injected cells remained localized to the site of injection (*11*). However, because C2C12 is a continuous cell line, an accurate assessment of the malignant potential of the injected G19 cells will require long-term follow-up of these animals. Finally, the β-galactosidase expression seen in vivo was not because of the infection of endogenous muscle with helper virus from the BAG-infected C2C12 cells because no helper virus could be detected by cocultivation assays with these cells (*14*) and because retroviruses are unable to infect nondividing cells such as myotubes (*15*). Therefore these results suggest that, as is true of primary myoblasts, the genetically modified C2C12 cells can become incorpo-

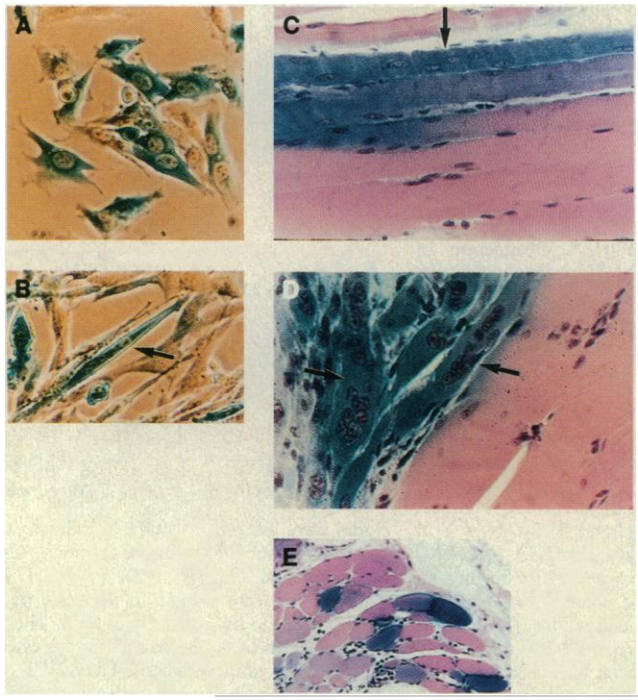

Fig. 3. Photomicrographs of BAG-infected C2C12 cells and muscle from C3H mice injected with BAG-infected C2C12 cells. (**A**) C2C12 cells that had been infected with the BAG retrovirus and selected by growth in G418 (1 mg/ml) were grown at low density in DMEM + 20% fetal bovine serum and 1% chick embryo extract (Gibco) before staining with 1 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) [as described (*19*)]. (**B**) C2C12 cells infected with the BAG retrovirus were grown to confluence and allowed to differentiate for 48 hours by growth in DMEM + 2% horse serum (Gibco) before staining with X-gal [as described (*19*)]. (**C** through **E**) Four-week-old C3H mice were injected intramuscularly with 6×10^6 BAG-infected C2C12 cells in 240 μl of DMEM medium at six separate sites. Mice were euthanized 5 to 7 days after injection, and muscle samples were fixed with 1.25% glutaraldehyde for 10 min at room temperature and stained with X-gal (*19*). The 3-mm sections were embedded with glycomethacrylate, and 7-μm sections were cut and counterstained with hematoxylin and eosin (*19*). Photomicroscopy was performed using Kodak Ektachrome 200 film and a Leitz Laborlux D microscope. Arrows denote multinucleated myotubes (×200).

rated into the injected muscle by differentiating into multinucleated myotubes in vivo.

The studies described in this report suggest that genetically engineered myoblasts may represent a useful delivery system for recombinant proteins in vivo. These cells can produce large amounts of secreted recombinant proteins. They can be stably introduced into muscle by simple intramuscular injection, allowing their secreted protein products to gain access to the circulation. The finding that the technique can be used to produce detectable levels of hGH is especially encouraging given the short half-life of hGH (less than 20 min) (*16*) as compared with those of other serum proteins. When taken together with previous reports demonstrating that primary human myoblasts can be readily isolated, expanded in vitro, and reinjected into muscle (*7, 9*), these data suggest that such a system may be adaptable to human therapy.

A number of questions must be answered before myoblast therapy can be considered for humans. First, more needs to be learned about the longevity of gene expression in this system. In addition, it remains to be determined if this system can be used to produce physiological levels of circulating proteins in large animals. Studies with primary myoblasts in larger mammals should answer many of these questions and allow an accurate assessment of the usefulness of this approach for the treatment of human disease.

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Systemic Delivery of Human Growth Hormone by Injection of Genetically Engineered Myoblasts

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A recombinant gene encoding human growth hormone (hGH) was stably introduced into cultured myoblasts with a retroviral vector. After injection of genetically engineered myoblasts into mouse muscle, hGH could be detected in serum for 3 months. The fate of injected myoblasts was assessed by coinfecting the cells with two retroviral vectors, one encoding hGH and the other encoding β-galactosidase from *Escherichia coli*. These results provide evidence that myoblasts, which can fuse into preexisting multinucleated myofibers that are vascularized and innervated, may be advantageous as vehicles for systemic delivery of recombinant proteins.

MAJOR ADVANCES HAVE BEEN made in the design of vectors that allow cells to express and secrete recombinant proteins at high concentrations in vitro. Yet, a general problem encountered with a number of cell types is that expression is not sustained after implantation of genetically engineered cells in vivo (*1–3*). Properties unique to myoblasts appear to enhance the long-term survival and function of these cells after implantation. Myoblasts have the unusual capability of crossing basal lamina (*4*). As a result, they contribute progeny to multiple, multinucleated myofibers in the course of normal postnatal mouse development (*5*). In addition, myoblasts injected into genetically deficient *mdx* mice fuse into the muscle fibers of the host and provide a missing endogenous muscle gene product, the intracellular protein dystrophin (*6–8*). Thus, injected myoblasts, unlike other cell types, become incorporated into a preexisting structure, which is sustained by neuronal activity and in contact with the circulation.

To examine whether myoblasts could deliver nonmuscle gene products to the circulation, we selected hGH for study. Sensitive assays distinguish mouse from human hormones and hGH has a short half-life of 4 min in serum (*9*), providing a stringent test for continuous production, secretion, and access to the circulation over time. To introduce the hGH gene into myoblasts, we used the MFG retroviral vector (*10*). The efficiency of stably introducing DNA into human myoblasts afforded by this approach is at least three orders of magnitude greater than by stable transfection of plasmids (*11*). A second retroviral vector, α-SGC, was used to introduce the *E. coli lacZ* gene which encodes β-galactosidase (β-gal) into the same myoblasts, providing an independent marker of implanted cell number and location. Recombinant retroviruses were generated in the ψ-CRIP line that produces replication-incompetent virus with an amphotropic host range, allowing infection of mouse and human cells. Cells of the C2C12 mouse myogenic line were infected with a

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