Primary Mouse Myoblast Purification, Characterization, and Transplantation for Cell-mediated Gene Therapy

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Abstract. The transplantation of cultured myoblasts into mature skeletal muscle is the basis for a new therapeutic approach to muscle and non-muscle diseases: myoblast-mediated gene therapy. The success of myoblast transplantation for correction of intrinsic muscle defects depends on the fusion of implanted cells with host myofibers. Previous studies in mice have been problematic because they have involved transplantation of established myogenic cell lines or primary muscle cultures. Both of these cell populations have disadvantages: myogenic cell lines are tumorigenic, and primary cultures contain a substantial percentage of non-myogenic cells which will not fuse to host fibers. Furthermore, for both cell populations, immune suppression of the host has been necessary for long-term retention of transplanted cells. To overcome these difficulties, we developed novel culture conditions that permit the purification of mouse myoblasts from primary cultures. Both enriched and clonal populations of primary myoblasts were characterized in assays of cell proliferation and differentiation. Primary myoblasts were dependent on added bFGF for growth and retained the ability to differentiate even after 30 population doublings. The fate of the pure myoblast populations after transplantation was monitored by labeling the cells with the marker enzyme β-galactosidase (β-gal) using retroviral mediated gene transfer. Within five days of transplantation into muscle of mature mice, primary myoblasts had fused with host muscle cells to form hybrid myofibers. To examine the immunobiology of primary myoblasts, we compared transplanted cells in syngeneic and allogeneic hosts. Even without immune suppression, the hybrid fibers persisted with continued β-gal expression up to six months after myoblast transplantation in syngeneic hosts. In allogeneic hosts, the implanted cells were completely eliminated within three weeks. To assess tumorigenicity, primary myoblasts and myoblasts from the C2 myogenic cell line were transplanted into immunodeficient mice. Only C2 myoblasts formed tumors. The ease of isolation, growth, and transfection of primary mouse myoblasts under the conditions described here expand the opportunities to study muscle cell growth and differentiation using myoblasts from normal as well as mutant strains of mice. The properties of these cells after transplantation—the stability of resulting hybrid myofibers without immune suppression, the persistence of transgene expression, and the lack of tumorigenicity—suggest that studies of cell-mediated gene therapy using primary myoblasts can now be broadly applied to mouse models of human muscle and non-muscle diseases.

THE mature muscle cells of mammalian skeletal muscle, known as myofibers, are multinucleated syncytia that arise from the fusion of mononucleated precursors, or myoblasts. Myoblasts persist in mature muscle as satellite cells, continue to fuse to adjacent myofibers during postnatal growth, and provide a source of cells for new muscle formation during muscle regeneration after injury (Campion, 1984). The first indication that myoblasts could be used for therapeutic purposes was the finding that transplantation of minced muscle from one animal to another resulted in the formation of hybrid myofibers, which are essentially heterokaryons composed of nuclei from both animals (Partridge et al., 1978). This observation led to the idea that myoblasts, grown in vitro, could be used as "cell therapy" for hereditary muscle diseases (Law, 1982; Watt et al., 1982; Karpati et al., 1989; Partridge et al., 1989; Gussoni et al., 1992). By fusing with mature or regenerating fibers of the host, implanted myoblasts could form hybrid myofibers thus contributing to the syncytium a normal gene product that was missing from host muscle. This principle has been most successfully applied to muscular dystrophies in mice (Law et al., 1988*b*; Partridge et al., 1989). For example, the *mdx* mouse, which is the genetic homolog of the human disease Duchenne muscular dystrophy, has a defect in the structural gene, dystrophin (Sicinski et al., 1989). In the mouse, as in humans, the absence of dystrophin leads to focal muscle

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necrosis with cycles of muscle degeneration and regeneration (Tanabe et al., 1986). Transplantation of normal myoblasts into *mdx* muscle leads not only to the expression of dystrophin in hybrid fibers, but also to the protection of those fibers from the characteristic pathologic changes (Morgan et al., 1990).

In addition to the treatment of intrinsic muscle diseases, the principles of myoblast transplantation have been extended to the treatment of hormone deficiencies (Barr and Leiden, 1991; Dhawan et al., 1991), coagulation disorders such as hemophilia (Dai et al., 1992; Yao and Kurachi, 1992), and even neurodegenerative diseases (Jiao et al., 1993). For these conditions, myoblasts are engineered in vitro to produce an exogenous gene product. Upon transplantation back into muscle or to an ectopic site, the myoblasts act as cellular delivery vehicles by secreting a missing or deficient product that is generated as a result of the transgene expression.

The mouse is the most commonly used species for studies of cell-mediated gene therapy because of the many mouse models of human diseases. However, a major limitation of myoblast transplantation in the mouse has been the inability to obtain pure populations of primary myoblasts in sufficient quantity. Most studies have used primary cultures from dissociated muscle which are mixtures of myoblasts and non-myogenic cells, mostly fibroblasts (Watt et al., 1982, 1984; Law et al., 1988*b*; Partridge et al., 1989; Morgan et al., 1990; Dai et al., 1992). The non-myogenic cells in these cultures have a negligible propensity to fuse with muscle cells and thus are ineffective in correcting intrinsic muscle defects. Furthermore, under standard culture conditions, fibroblasts frequently overgrow the cultures and myoblasts tend to differentiate and fuse into postmitotic, multinucleated myotubes. As a result, the yield of myoblasts from primary cultures is limited and transplants of primary cultures are heterogeneous mixtures of cells (Morgan, 1988; Jones et al., 1990). To date, no culture conditions have been described that permit the preferential growth or extensive proliferation of primary mouse myoblasts in vitro.

As an alternative to heterogeneous mixtures of cells from primary cultures, many investigators have used pure myoblast populations from established cell lines for transplantation (Law et al., 1988*a*; Barr and Leiden, 1991; Dhawan et al., 1991; Yao and Kurachi, 1992). However, when transplanted into muscle in vivo, these cell lines have a propensity to form aberrant muscle fibers and even undifferentiated tumors (Wernig et al., 1991; Morgan et al., 1992). Moreover, substrate- and often anchorage-independent growth, unlimited proliferation potential, and low requirements for serum and growth factors are all typical of cell lines and atypical of primary cells. A notable exception is the myogenic MM14 line which requires basic bFGF for growth (Clegg et al., 1987). These observations suggest that studies of cell growth and differentiation properties in vitro may foretell the behavior of cells in a more complex environment such as differentiated tissue in vivo (Rastinejad et al., 1993).

The transplantation of myoblasts raises questions regarding muscle immunobiology. There are strains of mice with genetic muscle disorders for which no histocompatible myogenic cell lines exist (e.g., the *mdx* and the *dy* mice) yet may be amenable to treatment by cell-mediated gene therapy. Thus the use of cell lines would require immune suppression of the host, which itself may affect the behavior of transplanted myoblasts (Watt, 1982; Hardiman et al., 1993). In theory, if primary myoblasts were derived from syngeneic strains, then myoblast transplantation could be performed without immune modulation of the host. However, numerous observations have indicated immune suppression enhances the retention of transplanted primary cultures (mixtures of myogenic and non-myogenic cells) even in syngeneic strains (Watt, 1990; Partridge, 1991; Labrecque et al., 1992). The mechanism by which syngeneic cells induce a host immune response remains obscure, but several investigators have postulated that the inclusion in the transplants of antigen presenting cells such as fibroblasts may contribute to their rejection (Watt, 1990; Watt et al., 1991). Taken together, these findings suggest that pure populations of myoblasts from primary cultures could overcome the limited host range of currently available myogenic cell lines since they can be derived from any strain, and could avoid the problems of immunogenicity of mixed primary cultures.

Successful treatment of intrinsic muscle diseases by myoblast transplantation requires that host myofibers incorporate donor myoblasts. Hybrid myofiber formation may also be critical to efficient systemic delivery of recombinant proteins by transplanted, genetically engineered myoblasts. The assessment of the efficacy of transplantation depends on the ability to detect such hybrid fibers and distinguish them from host fibers that have not incorporated donor cells. For transplantation of normal myoblasts into *mdx* mice, hybrid myofibers were identified in situ by the expression of dystrophin, the genetic marker expressed by donor cells but not by host fibers (Karpati et al., 1989; Partridge et al., 1989). An analogous marker of donor cells is necessary to assess the consequences of myoblast transplantation for hereditary muscle diseases of unknown genetic basis and for acquired muscle diseases. Retroviral vectors provide such a method of marking donor cells (Sanes et al., 1986; Price et al., 1987). Because these vectors are expressed only in the transduced cells and their progeny, the expression of a retrovirally encoded protein in a host myofiber would indicate that donor cells had been incorporated. This marking method allows hybrid fibers to be easily distinguished from fibers only of host origin (Barr and Leiden, 1991; Dhawan et al., 1991). However, a concern has been that primary cells may extinguish expression of retroviral vectors after transplantation into animals (Palmer et al., 1991; Scharfmann et al., 1991).

To overcome the problems associated with transplantation of mixed primary cultures and myogenic cell lines, we have established conditions for the purification and propagation of pure populations of primary myoblasts. The cells were first characterized in vitro with regard to properties of growth and differentiation. Then their differentiation in vivo and immunobiology were studied in transplantation assays of hybrid fiber formation and tumorigenicity in different strains of mice. For monitoring cell fate after transplantation, cells were labeled with the marker enzyme β-galactosidase (β-gal)[1] by retroviral mediated gene transfer. After enrichment, more than 95% of the cells expressed β-gal. After injection into the muscle of syngeneic mice, primary myoblasts

1. *Abbreviations used in this paper*: β-gal, β-galactosidase; H&E, hematoxylin and eosin; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

formed hybrid myofibers with host muscle. The hybrid fibers were stable for up to six months even when there was no immune suppression of the host, and there was persistent expression of the retrovirally encoded transgene. In allogeneic strains, the transplanted cells were rapidly rejected. The ability to derive, label, and transplant primary myoblasts from a variety of mouse strains should now permit analysis of parameters of myoblast transplantation critical to the treatment of intrinsic muscle diseases, the enhancement of muscle regeneration, and the systemic delivery of recombinant proteins by genetically engineered myoblasts. Furthermore, the study of primary myoblasts as muscle stem cells can be extended to myoblasts derived from different developmental stages, from normal and mutant strains of mice, and from strains of mice used as models for human diseases, thus expanding the range of myoblast phenotypes that can be analyzed in vitro.

Materials and Methods

Animals

Mice were obtained from Simonsen Laboratories, Inc. (Gilroy, CA) (strains C57BL/6N, C3H/HeN, and BALB/cAnN), from the Jackson Laboratory (Bar Harbor, ME) (strains C57BL/10, B6C3Fe), or from the Department of Comparative Medicine, Stanford Medical Center (BALB/c/nu/nu). All animals were handled in accordance with guidelines of the Administrative Panel on Laboratory Animal Care of Stanford University.

Preparation of Primary Cultures

The forelimbs and hindlimbs were removed from neonatal mice (2–5 d old) and the bones were dissected away. The remaining muscle mass was weighed. A few drops of PBS were added and the muscle was minced into a coarse slurry using razor blades. Cells were enzymatically dissociated by the addition of 2 ml per g of tissue of a solution of dispase (grade II, 2.4 U/ml, Boehringer Mannheim Corp., Indianapolis, IN) and collagenase (class II, 1%; Boehringer Mannheim Corp.), supplemented with $CaCl_2$ to a final concentration of 2.5 mM. The slurry, maintained at 37°C for 30–45 min, was triturated every 15 min with a 5-ml plastic pipette, and then passed through 80 μm nylon mesh (Nitex; Tetko, Inc., Monterey Park, CA). The filtrate was spun at 350 *g* to sediment the dissociated cells, the pellet was resuspended in growth medium, and the suspension was plated on collagen-coated dishes. During the first several passages of the primary cultures, myoblasts were enriched by preplating (Richler and Yaffe, 1970).

Culture Conditions

Growth medium for primary myoblasts consisted of Ham's F-10 nutrient mixture (GIBCO BRL, Gaithersburg, MD) supplemented with 20% FBS (HyClone Laboratories, Inc., Logan, UT) and 2.5 ng/ml bFGF (Promega Corp., Madison, WI). Differentiation medium consisted of DME (GIBCO BRL) supplemented with 2% horse serum (HyClone). All media contained penicillin G (200 U/ml) and streptomycin (200 μg/ml). Tissue culture plastic dishes were coated with 0.01% type I collagen (Sigma Chemical Co., St. Louis, MO). Cells were grown in a humidified incubator at 37°C in 5% CO_2.

In the process of establishing these growth conditions for primary myoblasts, other culture conditions were tested. In early experiments, DME, Waymouth's, or M199 media (GIBCO BRL) were used instead of F-10 in the growth medium. Other growth factors tested included acidic FGF (Promega), epidermal growth factor (Promega), platelet derived growth factors AA and BB (UBI, Lake Placid, NY), insulin-like growth factors I and II (UBI), and leukemia inhibitory factor (gift of AMRAD Laboratories, Victoria, Australia). Finally, plastic culture dishes were coated with substrates other than type I collagen and included laminin (0.02%, UBI), fibronectin (0.03%, Collaborative Biomedical Products, Bedford, MA), and type IV collagen (0.02%, Collaborative Research Products).

Primary fibroblast cultures were obtained by growing primary cultures in DME with 10% FBS. Under such conditions, there was preferential growth of fibroblasts which exceeded 99% of the total population after two weeks. C2 myoblasts were from the C2C12 subclone derived in this laboratory (Blau et al., 1983) and were grown in a medium of DME supplemented with 10% calf serum (HyClone) and 5% FBS.

Cloning of Primary Myoblasts

Primary cultures enriched for myogenic cells (see Results) were plated at clonal density and individual clones were isolated using cloning rings when the colony size reached ~50–200 cells. During expansion of the colonies, the clones were tested for myogenicity by immunostaining for desmin (see below) and by assessing myotube formation in differentiation medium.

Assessment of Growth and Fusion Indices of Primary Myoblasts

To assess growth properties, 5×10^4 cells were plated in 35-mm dishes either with different concentrations of bFGF or on different substrates. At different times after plating, the number of cells in each dish was determined by hemacytometer counts after the cells were suspended by trypsinization. All conditions were tested in triplicate cultures. The concentration of bFGF that produced half-maximal stimulation of growth was calculated as the ED_{50}. The population doubling times of the cells on different substrates were determined during periods when cells were in log-phase growth.

To assess differentiation potential, 5×10^4 cells were plated in 35-mm laminin-coated dishes in growth medium. After 24 h, the growth medium was replaced by differentiation medium. The cells were maintained in differentiation medium with daily medium changes, and all measurements were obtained from triplicate cultures. The fusion index, measured daily, was determined as the ratio of the number of nuclei in myotubes (cells with ≥3 nuclei) to the total number of nuclei. Ten randomly chosen fields from each dish were counted at a magnification of 250×.

Retroviral Infection of Myoblasts and Selection of Infected Cells

Retroviral producer lines were grown in DME supplemented with 10% calf serum. The producer cells (ψCRIP or ψCRE) were grown to confluence and the media containing the replication-defective retroviruses BAG (Price et al., 1987) or α-SGC (Dhawan et al., 1991) were collected. Both viruses contain the lacZ gene encoding the bacterial β-gal enzyme. The media were filtered through 0.45 μm filters, supplemented with 10% FBS and 10 μg/ml polybrene, and transferred to dishes of primary myoblasts in log-phase growth. The period of exposure to retrovirus ranged from 4 to 12 h, and from 2 to 4 infections were performed at 24-h intervals. Each transduced culture was tested for helper virus production. Cultures of primary fibroblasts and C2 myoblasts were similarly infected.

β-gal-labeled cells were further enriched by one of two techniques. For cultures infected with the BAG virus, transduced cells were selected in growth medium supplemented with 50 μg/ml G418 (GIBCO BRL) since the virus also expresses the neomycin phosphotransferase gene. For cultures infected with the α-SGC virus (which does not express a drug resistance gene), infected cells were selected by fluorescence-activated cell sorting. Cells expressing β-gal produce a fluorescent product from the substrate fluorescein-di-β-D-galactopyranoside and can be purified on that basis (Nolan et al., 1988). Infection and selection efficiencies were assessed by staining for β-gal activity (see below).

Immunofluorescence and Histochemistry on Cultured Cells

Desmin staining. Cells were fixed in 2% formaldehyde in PBS for 5 min at room temperature followed by 100% methanol at −20°C for 5 min. The cells were then rinsed in PBS. All further incubations were carried out at room temperature and all rinses and dilutions were with a blocking solution consisting of 2% horse serum and 0.5% Triton-X 100 in PBS. An initial blocking step was performed with this solution for 30 min. An antibody to desmin (rabbit polyclonal, Sigma) was applied for 30 min at a dilution of 1:400. After rinsing, a fluorescein-coupled secondary antibody (1/250, Cappel Research Products, Durham, NC) was applied for 30 min. The cells were rinsed and stained with the bisbenzimide dye, HOECHST 33342 (Molecular Probes, Inc., Eugene, OR) at 0.2 μM in PBS for 15 min to stain individual nuclei. The percentage of myogenic cells was determined microscopically as the ratio of desmin expressing cells to the total number of cells in 10 randomly chosen fields at a magnification of 250×.

β-gal staining. Cells were fixed with 4% paraformaldehyde, 0.25%

gluteraldehyde, 100 mM NaH_2PO_4, pH 7.4, for 5 min at 4°C. After rinsing with PBS, the cells were incubated with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in a solution of 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ in PBS for 8–12 h at 37°C (Sanes et al., 1986). Cells expressing β-gal produce a blue reaction product from the substrate X-gal. The percentage of β-gal positive cells was determined microscopically as the ratio of the number of "blue" cells to the total number of cells in 10 randomly chosen fields at a magnification of 250×.

Cell Transplantations

Cultured cells were prepared for transplantation by trypsinization, resuspension in growth medium, washing two times with serum-free medium, and resuspension in a solution of F-10 with 0.5% BSA. The final cell density for implantation was $2 \times 10^6 - 2 \times 10^7$ cells/ml, and the suspension was kept on ice. Cell viability in suspension, determined by Trypan blue exclusion, was greater than 90% after 4 h under these conditions. If the solution did not contain BSA, or if the cells were maintained at room temperature, cell viability was reduced. Immediately before implantation, the cell suspension was drawn up into a 25-μl Hamilton syringe and a 27 gauge needle was attached. Mice, ages 2.5–4 weeks old, were anesthetized with pentobarbital (60 mg/kg, intraperitoneally) and an incision was made in the skin overlying the anterior muscles of the leg, exposing the tibialis anterior muscle. All further procedures were done under a dissecting microscope which insured that the injections would be targeted to the center of the muscle and not to the periphery or between muscles. The needle was inserted into the belly of the tibialis anterior muscle along the rostro-caudal axis, and 5 μl of the cell suspension was slowly injected while the muscle was examined for any leakage. When there were multiple injections into a single muscle, the needle was withdrawn slowly and reinserted in the same orientation, parallel to the first injection. After the final injection, the skin was sutured closed. This procedure of injecting myoblasts into an exposed muscle gave much more reproducible results than did injecting the cells through the skin into the muscle.

Tumor Assays

The cells were prepared for injection in the same manner as for transplantation into muscle. For tumor assays, the cells were implanted subcutaneously in the flanks of athymic (*nu/nu*) mice, lightly anesthetized by inhalation of methoxyflurane. Into each site, 10^6 cells were implanted in a volume of ~50 μl. The animals were examined for the appearance of tumors weekly up to 10 weeks. Animals were sacrificed if tumors arose bilaterally.

Histological Procedures on Frozen Sections

The animals were sacrificed at different times after the transplantation. The tibialis anterior muscles were dissected free, embedded in mounting medium (O.C.T. compound, Miles Laboratory, Elkhart, IN), and frozen in isopentane cooled in liquid nitrogen. Cryostat sections were collected on gelatin-coated glass slides at regular intervals (every 300–500 μm) along the entire length of the tibialis anterior muscle. At each interval, 30-μm and 10-μm sections were collected. The 10-μm sections were stained with hematoxylin and eosin (H&E) and mounted in Pro-Texx mounting medium (American Scientific Products, McGraw Park, IL). The 30-μm sections were fixed and stained for β-gal activity with X-gal as described above for staining of cells in culture. The sections were rinsed in PBS and mounted in Airvol (Air Products and Chemicals, Inc., Allentown, PA). The 30-μm sections were analyzed and photographed using Nomarski optics, and the number of β-gal positive fibers in a muscle was counted in the cross-section with the greatest number of labeled fibers. All analysis and photography was performed on a Zeiss Axiophot microscope.

Results

Enrichment and Cloning of Myoblasts from Primary Cultures

We sought to obtain pure populations of myoblasts from primary cultures of mouse skeletal muscle which were mixtures of myoblasts and fibroblasts. The two cell populations were distinguished by staining for the intermediate filament protein desmin, which is expressed only in myogenic cells (Kaufman and Foster, 1988). Although the percentage of myoblasts could be maintained at greater than 70% by preplating the cells at each passage (Richler and Yaffe, 1970), fibroblasts doubled more rapidly and after several days became the predominant cell type when the growth medium consisted of DME supplemented with 20% FCS. Without preplating, growth of primary cultures resulted in fibroblast-enriched populations in which less than 1% of the cells expressed desmin. To overcome this problem of fibroblast overgrowth, we tested different culture conditions that might preferentially enhance the growth of myoblasts.

First, different nutrient mixtures were tested for their effects on myoblast and fibroblast growth. When DME was replaced with Waymouth's or M199 media, fibroblasts continued to overgrow the cultures. However, when DME was replaced with F-10, myoblasts appeared to have a selective growth advantage and became the predominant cell type in the culture, accounting for more than 95% of the cell population within two weeks (Fig. 1). This myogenic enrichment increased further with time in culture. One reason for the improved growth of myoblasts in F-10 compared with the other nutrient mixtures was the low rate of spontaneous myoblast

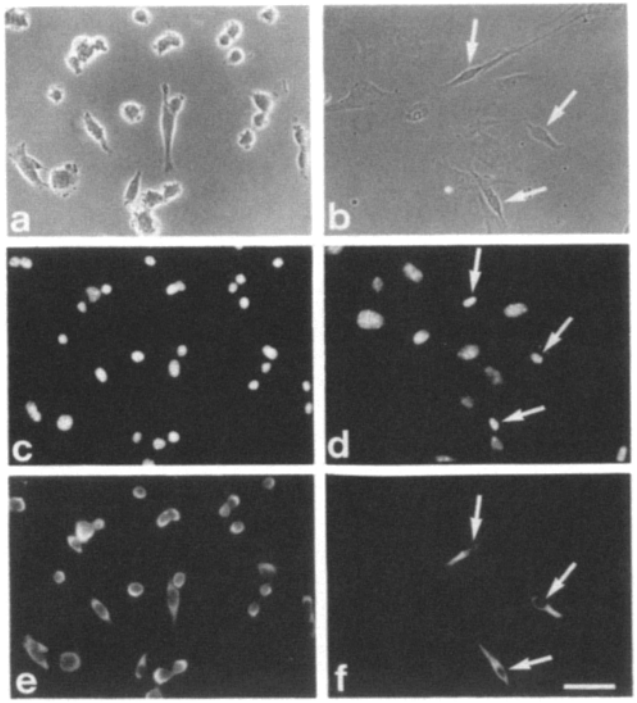

Figure 1. Myogenic enrichment by selective growth conditions. Primary cultures from muscle of neonatal C57 mice were grown in medium with either F-10 or DME as the nutrient mixture. After two weeks of growth and several passages, one dish of each was fixed and stained with an antibody to desmin to identify myogenic cells and with HOECHST nuclear stain to identify all cells. A single field is shown for cells in F-10 (*a*, *c*, and *e*) or DME (*b*, *d*, and *f*), and each is photographed to show morphology (*a* and *b*: *phase contrast*), total cell number (*c* and *d*: *HOECHST nuclear stain*) and myogenic cells (*e* and *f*: *desmin staining*). Cultures grown in F-10 were highly enriched for myogenic cells, here 100%, whereas a minority of cells grown in DME were myogenic. The three myogenic cells in the DME panels are indicated by arrows. Bar, 50 μm.

differentiation in F-10. For the cultures shown in Fig. 1, less than 3% of the myogenic cells in F-10 expressed myosin heavy chain, a marker of the differentiated state (data not shown). In DME, more than 30% of the myogenic cells expressed myosin heavy chain and multinucleated myotubes were present in the culture. This high rate of differentiation in DME removed a large percentage of the cells from the proliferating population and allowed fibroblasts to become the dominant cell type. When grown in F-10, myoblast enriched cultures had a doubling time of about 30% less (i.e., a higher growth rate) than that of fibroblast enriched cultures. This difference in growth rate accounted for the selective growth advantage and progressive enrichment of myoblasts in media with F-10.

Second, we found that bFGF stimulated the growth of primary myoblasts to a greater extent than did other growth factors. bFGF produced its maximal mitogenic effect at ~2.5 ng/ml which resulted in a stimulation of the growth rate by more than 25%. The ED_{50} of bFGF, a measure of potency determined as the concentration producing half-maximal growth stimulation, was ~0.5 ng/ml (Table I). Other growth factors, including acidic FGF, platelet-derived growth factors AA and BB, insulin-like growth factors I and II, epidermal growth factor, and leukemia inhibitory factor, were less efficacious and less potent than bFGF in stimulating the growth of primary mouse myoblasts (our unpublished observations).

Finally, we compared the effect of different substrates on the growth rate of primary myoblasts. Primary myoblasts grew poorly on tissue culture plastic because the cells detached easily from the dish. When dishes were coated with either type I collagen or laminin, cells remained adherent and the rate of growth of the myoblast population increased as evidenced by a reduction in the population doubling time (Table I). Type IV collagen and fibronectin were less effective as substrates in promoting primary myoblast growth.

The culture conditions described above, optimized for the enrichment and growth of bulk cultures of primary myoblasts, also allowed the cloning of myoblasts from primary cultures. When myoblast-enriched cultures were plated at low density to achieve clonal growth, the colonies were nearly all myogenic as determined by desmin staining. Myogenic clones from several different strains of mice, including C57, C3H, Balb/c, and B6C3Fe have been obtained. In each case, the properties of clonal myoblast cultures were compared to myoblast-enriched cultures. The cell populations were indistinguishable morphologically. Added bFGF stimulated equally the growth of enriched and clonal cultures of primary myoblasts with an ED_{50} of ~0.5 ng/ml (Table I). When grown on type I collagen or laminin, both cell populations had a doubling time of ~24 h; both grew more slowly on tissue culture plastic. When switched to medium with low serum to induce differentiation, both clonal and enriched myoblasts formed twitching myotubes (see Fig. 2 *b*), although the clones generally formed myotubes more slowly than enriched cultures. In sum, except for a difference in the rate of differentiation, the properties of clonal and enriched cultures of mouse myoblasts were similar.

Table I. Comparison of the Phenotypes of Myoblasts Enriched or Cloned from Primary Cultures

	Enriched myoblasts	Cloned myoblasts
bFGF response: ED_{50}	0.55 ± 0.10 ng/ml	0.48 ± 0.17 ng/ml
Population doubling time		
On collagen (type I)	23 ± 2 h	22 ± 3 h
On laminin	23 ± 1 h	24 ± 2 h
On plastic	44 ± 4 h	40 ± 4 h
Fusion index		
Day 3	66% ± 5%	33% ± 3%
Day 5	72% ± 5%	50% ± 9%

All values are the averages of 3–4 separate experiments and the errors represent ± SD.

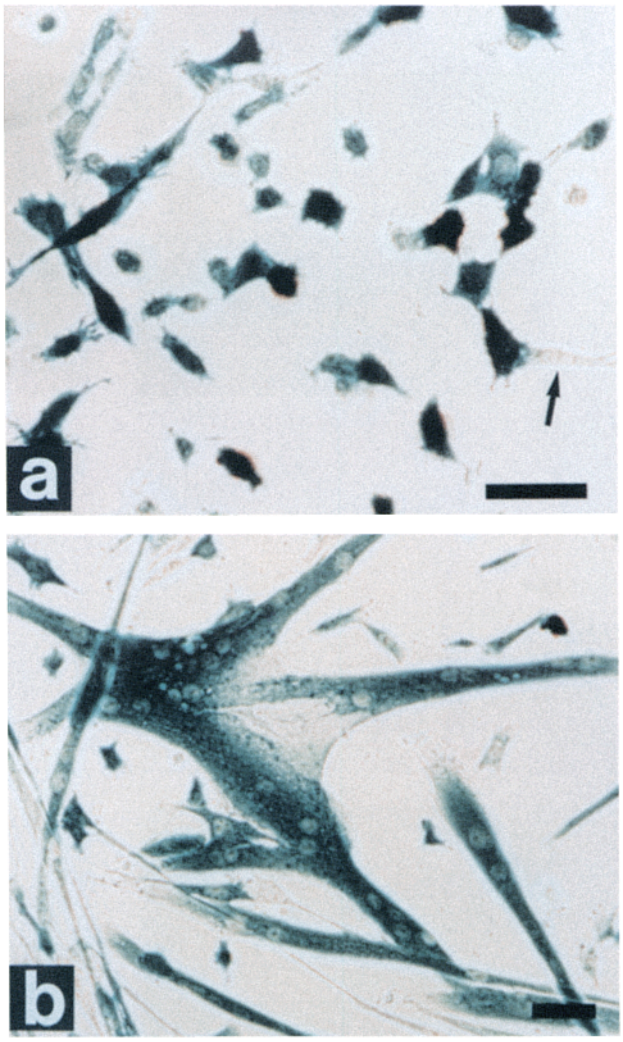

Figure 2. Retroviral labeling of primary myoblasts. (*a*) Cloned myoblasts were infected with the BAG virus, selected by addition of G418 to the growth medium, and then stained with X-gal to determine the percentage of cell expressing β-gal (cells with a blue reaction product). More than 95% of the cells were β-gal positive. A cell not expressing β-gal is indicated by the arrow. (*b*) Retrovirally labeled myoblasts were induced to differentiate in vitro by growth in low serum medium. Even after cloning, expansion in culture, retroviral infection, and selection, the cells readily differentiated to form linear and branching multinucleated myotubes. β-gal staining shows persistent expression of the transgene in differentiated cells. Bars, 80 μm.

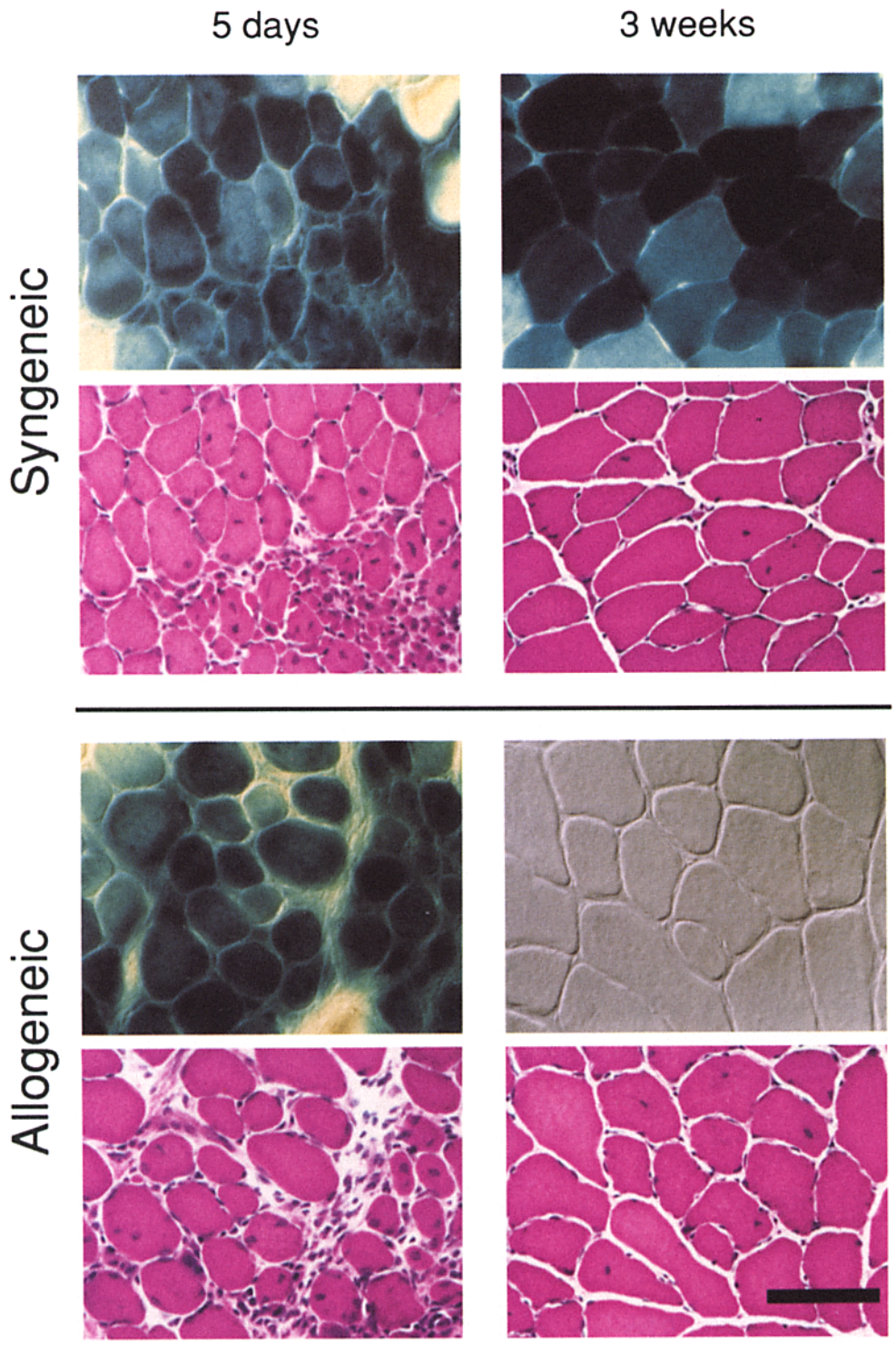

Figure 3. Histology of primary myoblast transplantation into syngeneic and allogeneic hosts. β-gal-labeled primary myoblasts were transplanted into the muscles of C57 mice and the muscles were examined at different times thereafter. Muscles were frozen and cryostat sections were collected for histological analysis. Photomicrographs are from muscles taken 5 d (*left*) and 3 wk (*right*) after transplantation. For each transplant, a pair of adjacent sections is shown: the first (*top*) is stained for β-gal activity to follow the fate of implanted cells; the second (*bottom*) is stained with H&E for routine histology. The results of transplantation of syngeneic (*C57*) and allogeneic (*C3H*) myoblasts are shown in the top four panels and bottom four panels, respectively. Bar, 60 μm.

Both clonal and enriched cultures of primary myoblasts displayed remarkable proliferation potential. Pure myoblast cultures from several mouse strains have been expanded beyond 40 population doublings, and some clonal cultures have been expanded beyond 50 population doublings (>10^{15} cells per clone). There were no detectable changes of growth rate or cell morphology even after extensive proliferation in vitro. Because of the extraordinary growth potential of primary myoblasts under these culture conditions, the transplantation studies described below were made possible and were not limited by the number of available cells.

Retroviral Infection of Primary Myoblasts

Primary myoblasts were infected with replication-defective retroviruses encoding β-gal (see Methods) in order to follow their fate of transplantation. The efficiency of retroviral infection was assessed by staining the cells for β-gal activity

with X-gal; transduced cells were identified by the blue reaction product formed. In most cases, between 30 and 50% of primary myoblasts expressed β-gal after retroviral infection. Both drug selection and fluorescence-activated cell sorting (see Methods) were used to purify retroviral-labeled myoblasts from the population. With either method, over 95% of the selected population expressed β-gal (Fig. 2 *a*). This high enrichment was maintained during extended growth in vitro. In addition, when the cells were induced to differentiate in low serum medium, β-gal continued to be expressed in the resulting multinucleated myotubes (Fig. 2 *b*).

Hybrid Myofibers Formed by Primary Myoblast Transplantation

The success of myoblast transplantation for correction of intrinsic muscle defects depends on the ability of implanted myoblasts to fuse directly to host muscle cells, thus forming hybrid myofibers. To determine whether primary myoblasts would form such hybrids, retrovirally labeled myoblasts were implanted into the tibialis anterior muscles of syngeneic host mice and the muscles were examined five days later (Fig. 3). Hybrid fibers were identified as fibers of normal diameter (>30 μm) that were β-gal positive, and were evident surrounding the implantation site as well as along the needle track. These large fibers, which had diameters comparable to neighboring unlabeled host fibers, most likely arose from the fusion of implanted cells with host fibers. By contrast, at the center of the implantation site where there was injury to host muscle, there were discrete foci of β-gal labeled myotubes and small regenerating fibers (<15 μm in diameter) among β-gal labeled mononucleated cells. The myotubes and small fibers may have arisen from the fusion of implanted cells with each other or with host muscle that was regenerating. Because it is not possible to distinguish hybrid regenerating fibers from fibers only of donor myoblast origin, these small fibers were not included in the analyses of hybrid myofiber formation.

When muscles were examined three weeks after transplantation, hybrid myofibers were evident amidst the normal muscle architecture (Fig. 3). At this time, mononucleated cells expressing β-gal were rarely seen. There still remained small diameter, β-gal positive fibers (see Fig. 7 for an example) at the center of the implantation site, and these fibers never achieved normal caliber but remained as a central cluster of aberrant (small) fibers even months later. The numbers of hybrid myofibers determined five, ten, and twenty-one days after transplantation were essentially constant (Fig. 4). Thus the hybrid fibers counted five days after transplantation probably represent a population that is constant and distinct. The formation of hybrid fibers appears to be the predominant fate of implanted primary myoblasts.

As a control that the labeling of host fibers with β-gal arose from the fusion of implanted myoblasts and not from the direct transfer of genetic material (Kaleko et al., 1990), we performed syngeneic transplants of fibroblast enriched cultures that were β-gal labeled by retroviral transduction. Muscles were examined two days to six weeks after transplantation of $10^5 - 4 \times 10^5$ fibroblasts. Within several days of transplantation, the muscle had a mass of β-gal labeled mononucleated cells at the site of implantation (data not shown). In most of the muscles examined, no β-gal labeled myofibers were detected, and the maximum number of β-gal labeled fibers in any given muscle was four. Previous work has shown that fibroblasts may fuse spontaneously with myogenic cells at a low frequency (Chaudhari et al., 1989) which would account for the occasional β-gal labeled fibers seen here. Alternatively, the few β-gal labeled fibers arose from the fusion of rare myogenic cells present in the cultures (<1% of cells in the fibroblast enriched cultures expressed desmin). In either case, the paucity of labeled fibers after fibroblast transplantation indicate that the results with transplanted myoblasts, namely a large number of labeled fibers, are unlikely to be due to a rearrangement and uptake of the lacZ gene within the tissue. Rather, the detection of β-gal within myofibers after myoblast transplantation clearly reflects the propensity of the implanted cells to undergo cell fusion.

Long-Term Stability of Hybrid Myofibers without Immune Suppression

Previous studies of transplantation of mixed primary cultures from muscle indicated that long-term survival of transplanted cells required immunosuppression, even in syngeneic hosts (Watt, 1990; Labrecque et al., 1992). It was suggested that the immune response generated against donor tissue was due to the presence of donor immune cells or antigen presenting cells, such as fibroblasts and endothelial cells, in the transplanted cell population (Watt, 1990; Watt et al., 1991). We therefore performed experiments to determine whether the use of pure populations of primary myoblasts could overcome this problem. Moreover, the lack of evidence of immune rejection three weeks after transplantation in our experiments suggested that it might be possible to achieve long-term stability of hybrid myofibers without immunosuppression. To test this possibility, we determined

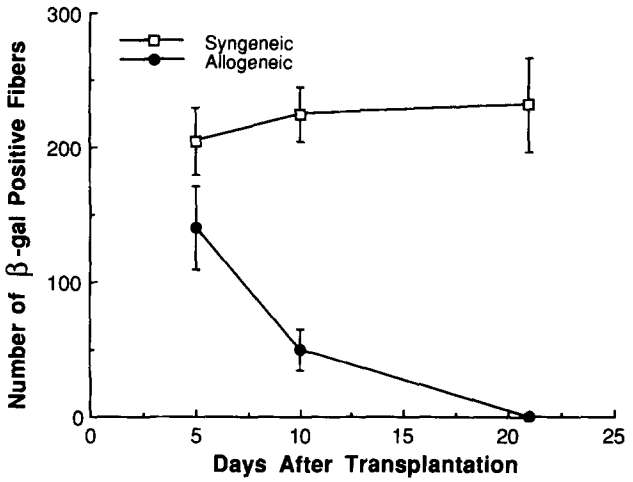

Figure 4. Hybrid fibers formed by syngeneic and allogeneic myoblast transplantation. From experiments such as those shown in Fig. 3, the numbers of hybrid fibers (normal diameter and β-gal positive) were counted for each transplant. For syngeneic myoblast transplantation, hybrid myofibers were formed within 5 d of transplantation and the number of these fibers changed little during the next two weeks. For allogeneic myoblast transplantation, despite early hybrid myofiber formation, there was complete rejection of transplanted cells by three weeks. Error bars, ± SD; $n = 6$ for each point.

the number of hybrid myofibers in muscles up to six months after transplantation of β-gal labeled primary myoblasts. There was no diminution of the intensity of β-gal staining between one and six months after transplantation (Fig. 5 *a*) indicating that expression of the retroviral vector was not shut off, and that retrovirally encoded β-gal could be used as a permanent marker of hybrid fibers for long-term experiments. The number of hybrid fibers remained within a remarkably constant range up to six months after transplantation (Fig. 5 *b*). These results suggest that hybrid myofibers formed by incorporation of syngeneic primary myoblasts may be stable indefinitely without immune suppression.

We hypothesized that hybrid fibers formed by the transplantation of allogeneic myoblasts might also be stable without immunosuppression. There is substantial evidence that mature myofibers express neither class I nor class II major histocompatibility molecules (Ponder et al., 1983; Karpati et al., 1988). From the data with syngeneic transplants, we knew that implanted myoblasts became incorporated into host fibers within five days of transplantation. We therefore postulated that allogeneic cells would also rapidly become incorporated into mature fibers and would thus be protected from immune rejection because of the lack of expression of major histocompatibility molecules on the hybrid fibers. To test this possibility, we transplanted β-gal labeled myoblasts into the muscles of allogeneic host mice. The muscles were examined for hybrid fiber formation five, ten, and twenty-one days after transplanta-

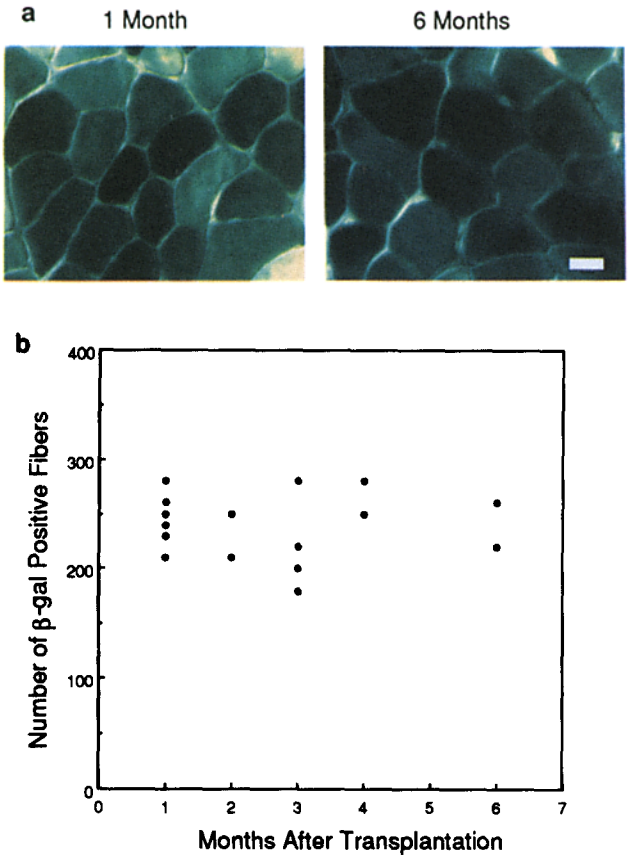

Figure 5. Long-term stability of hybrid myofibers from syngeneic myoblast transplants without host immune suppression. β-gal-labeled primary myoblasts were transplanted into syngeneic hosts and the muscles were analyzed at different times up to 6 months after transplantation. (*a*) There is no change in the appearance of the hybrid fibers or the intensity of β-gal staining between one and six months after transplantation. Bar, 30 μm. (*b*) The number of hybrid fibers remained within a narrow range without any diminution up to six months after transplantation. Each point represents an individual muscle.

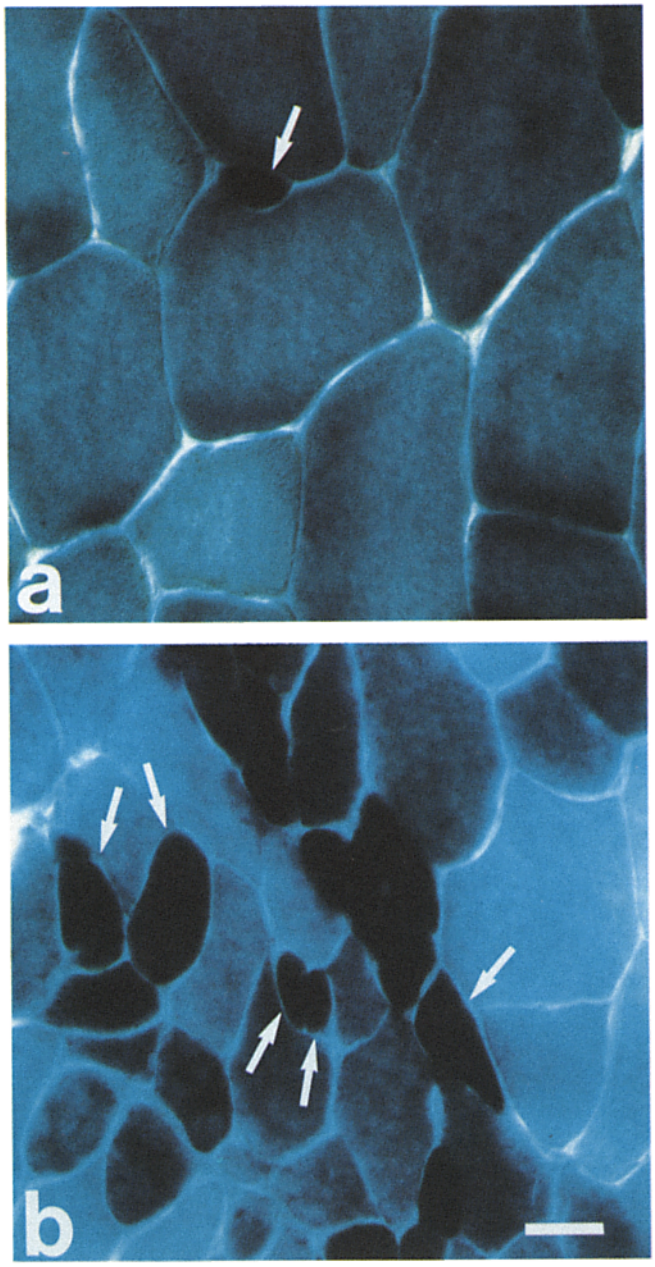

Figure 7. Comparison of primary and C2 myoblast transplants. Cloned primary myoblasts and C2 myoblasts, both labeled with the β-gal marker, were transplanted into muscles of athymic mice, and the muscles were analyzed for β-gal positive fibers at different times thereafter. Both panels are from muscles examined six weeks after transplantation. (*a*) Primary myoblast transplant: nearly all of the β-gal positive cells are normal caliber fibers. A single small diameter fiber in this field is indicated by the arrow. (*b*) C2 myoblast transplant: A majority of β-gal-labeled cells were fibers of small diameter and intensely stained for β-gal activity (*arrows*). These small fibers most likely arose from C2 myoblasts fusing with each other. Bar, 15 μm.

tion, hybrid myofiber formation was evident (Fig. 3), and the number of such fibers was only ~20% less than after syngeneic transplants (Fig. 4). Five days later (day 10), however, there was massive infiltration of the transplant by inflammatory cells with local tissue destruction and a reduction in the number of hybrid fibers (Fig. 4). By three weeks after transplantation, all evidence of implanted myoblasts had vanished and the immune response had completely resolved (Figs. 3 and 4). Clearly, for allogeneic transplants, even myoblasts that had fused to mature fibers were not protected from immune rejection.

Enhancement of Hybrid Myofiber Formation

Because the beneficial effects of myoblast transplantation for intrinsic muscle diseases depends on the fusion of the implanted cells with the diseased fibers, it is desirable that implanted myoblasts fuse with as many hosts as possible. We explored two ways to increase the number of hybrid fibers formed. The first method was to determine whether incorporation into fibers could be increased by increasing the number of myoblasts implanted. When individual muscles were given one, two, or three injections, the number of hybrid myofibers formed correlated with the number of injections and thus the number of implanted myoblasts (Fig. 6 *a*). The number of 5 μl injections was essentially limited to three because the tibialis anterior muscle, swollen with the 15 μl of cell suspension, could not retain much more volume. As an alternative, the density of the cells in suspension for a single injection was varied. Again, the number of hybrid fibers increased with the number of cells injected for cell densities between 5×10^4 and 2×10^5 myoblasts per 5 μl (Fig. 6 *b*). Higher cell densities did not yield proportionate increases of hybrid fibers. These data show that the number of hybrid fibers formed can be increased by increasing the number of myoblasts implanted within a given range.

As a second method to increase the number of hybrid myofibers formed, we attempted to induce implanted myoblasts to migrate to distant regions of the muscle. Recent data have suggested that implanted myoblasts have the capacity to migrate from the site of implantation to areas of muscle injury, even into adjacent muscles (Morgan et al., 1993). To test for such migration and perhaps to induce the formation of hybrid myofibers throughout the muscle, we injured the tibialis anterior muscle either lateral to the myoblast implantation site or caudal to it, i.e., along the axis of the host fibers. Injuries were made either by forceps crush or by local application of solid CO_2. In different groups of mice, muscles were injured 3 d before, simultaneously with, or 3 d after myoblast implantation. The muscles were examined 2 wk later when most muscle regeneration was complete. In no case were β-gal labeled fibers or mononucleated cells seen in the regenerated muscle, and the numbers of β-gal labeled fibers at the implantation site were never greater than in uninjured muscle. Under these experimental conditions, we were unable to detect any injury-induced migration of implanted myoblasts or consequent enhancement of hybrid myofiber formation.

In Vivo Proliferation and Tumor Formation by C2 Myoblasts But Not by Primary Myoblasts

Our data indicated that the predominant fate of primary myoblasts was to form hybrids with host myofibers. The fusion of implanted cells exclusively with each other was uncommon except at the very center of the implantation site, and the persistence of implanted cells as mononucleated cells was rare. In contrast, transplantation of myoblasts from established cell lines has resulted both in new muscle formation and undifferentiated tumors (Wernig et al., 1991; Morgan et al., 1992), although no marker of implanted cells was used in these studies. To compare directly the fates of primary myoblasts and myoblasts from the C2 myogenic cell line, each population was labeled with β-gal and transplanted

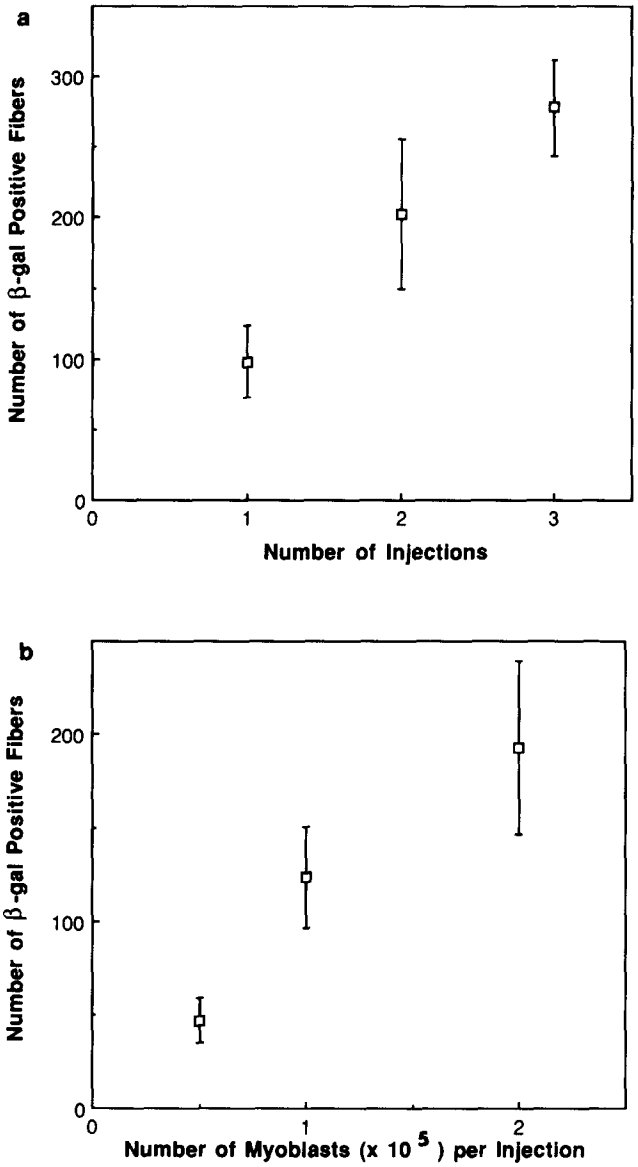

Figure 6. Relationship between the number of myoblasts transplanted and the number of hybrid fibers formed. Syngeneic β-gal-labeled myoblasts were transplanted into either C57 or C3H hosts, and the number of β-gal positive fibers were counted 2–3 wk after transplantation. The number of hybrid myofibers formed could be increased either by (*a*) increasing the number of injections (with a constant number of cells per injection); or (*b*) increasing the number of cells implanted via a single injection. Error bars represent $\pm$ SD; n = 4–6 for each point.

into the muscles of athymic mice. Muscles were examined from two weeks to three months later.

As in syngeneic hosts, primary myoblasts formed hybrid myofibers with host muscle (Fig. 7 *a*), and the number of hybrid fibers remained stable for months. Persisting β-gal positive myoblasts were rare, and small diameter fibers expressing β-gal (Fig. 7 *a*) constituted a small percentage of all β-gal labeled fibers. Therefore, just as in syngeneic hosts, fusion to host fibers again appeared to be the predominant fate of transplanted primary myoblasts.

The fate of implanted C2 myoblasts was more variable than that observed for primary myoblasts. Even though fusion of C2 myoblasts to host fibers was evident from the presence of β-gal labeled fibers of normal caliber, a majority of the β-gal labeled fibers were of small diameter (Fig. 7 *b*). These small fibers were more intensely stained for β-gal, their numbers increased with time, and they were densely packed at the center of the implantation site, features that all suggested that they arose from the fusion of C2 myoblasts with each other. As a result, the muscle architecture became increasingly distorted (Fig. 7 *b*). Such new muscle formation, arising from the fusion of implanted cells with each other, would be a fruitless outcome in an attempt to form hybrid myofibers for the correction of intrinsic muscle deficits.

A propensity to form tumors is undesirable for any cells transplanted for cell-mediated gene therapy. There was no evidence of uncontrolled proliferation of primary myoblasts after transplantation into muscle. To test tumorigenic potential more stringently, we implanted primary and C2 myoblasts into subcutaneous sites in athymic mice and assessed the mice for tumor formation for 10 wk. Implantation of C2 myoblasts produced tumors in three of four sites (Table II). These tumors were first visible at 5, 7, and 8 wk. In contrast, primary myoblasts produced no detectable tumors at any of twelve implantation sites. This low tumorigenic potential is further evidence that primary myoblasts are preferential to myoblasts from established cell lines for studies of cell-mediated gene therapy.

Discussion

The formation of hybrid myofibers by the fusion of implanted myoblasts with host muscle is the basis of myoblast transplantation for the correction of intrinsic muscle diseases (Partridge, 1991). The fusion of donor myoblasts to host myofibers allows donor cells to contribute their normal gene products to the syncytial myofiber, thus replacing any missing or defective gene product in the host. For efficient systemic delivery of recombinant proteins by genetically engineered myoblasts, it may also be necessary for donor myoblasts to fuse to host fibers. The results presented here demonstrate that pure populations of primary myoblasts are an excellent source of such donor cells: (*a*) primary myoblasts have a remarkable proliferative capacity in vitro; (*b*) their predominant fate after transplantation is fusion to host fibers; (*c*) the hybrid fibers formed are stable in syngeneic hosts without immune suppression; and (*d*) primary myoblasts appear to have no propensity to continue proliferating in an uncontrolled manner in vivo or to form tumors. The conditions described here allow purification and expansion of primary myoblast populations in a manner previously not possible. As a result, these cells can now be used to overcome the limitations of mixed primary cultures and myoblasts from established cell lines used in previous studies of myoblast transplantation (Watt et al., 1982; Law et al., 1988*a*, *b*; Partridge et al., 1989; Morgan et al., 1990). Thus myoblasts can be taken full cycle from the animal to the culture dish and back to the animal.

Table II. Tumorigenicity of Different Myoblast Populations

Cell type	Tumor formation (# sites with tumors/total # sites)
Enriched primary myoblasts	0/6
Cloned primary myoblasts	0/6
C2 myoblasts	3/4

The benefits of being able to obtain pure populations of primary mouse myoblasts extend beyond studies of myoblast transplantation. Primary myoblasts can be viewed as stem cells or self-renewing tissue precursors. As such, they offer the opportunity to study the stages of muscle differentiation in vitro. The ability to purify myoblasts from various strains of mice, from different developmental stages, and from mice with various hereditary muscle diseases expands the phenotypic range of muscle cells amenable to analysis in vitro. These include myoblasts from mutant mouse strains that serve as models for human myopathies (Partridge, 1991). To such, mutant strains can be added transgenic and "knock-out" strains of mice that further expand the genotypic range of myoblasts whose study may shed light on important aspects of cell growth, migration, and differentiation (Braun et al., 1992; Rudnicki et al., 1992). Finally, the proliferative capacity of primary myoblasts allows genetic engineering of these cells in vitro potentially providing an unlimited number of variants, including myogenic mutants derived from gene targeting in culture.

Although culture conditions have been established for the clonal growth of chick, rat, and human myoblasts (Konigsberg, 1961; Richler and Yaffe, 1970; Blau and Webster, 1981), no previous reports have described conditions for the successful cloning of mouse myoblasts. Investigators have therefore turned to techniques for enriching myoblasts from mouse primary cultures (Richler and Yaffe, 1970; Morgan, 1988; Jones et al., 1990). The major drawback of all of these techniques has been that the enrichment has been transient since fibroblasts eventually overtake the cultures. Furthermore, all of the techniques lead to a substantial reduction in the total yield of myogenic cells. By contrast, the culture techniques described here allow the preferential growth of myoblasts over fibroblasts, and the yield of myogenic cells from the initial primary culture is very high. Moreover, the percentage of myogenic cells increases with time in culture. Thus, from a mixed culture of myoblasts and fibroblasts, a nearly pure culture of myoblasts (>99%) is achieved within three weeks. Though not rapid, the enrichment is permanent and proceeds as the cultures are expanded. Equally importantly, the same culture conditions allow the growth of primary mouse myoblasts at clonal density. Thus, the culture conditions described here are suitable for studies of either bulk or clonal populations of mouse myoblasts.

Cloned and enriched myoblast populations had similar growth properties in vitro including bFGF dependence, substrate dependence, and growth potential. As an extension of

the in vitro characterization, the in vivo studies of hybrid fiber formation and tumorigenicity also revealed little difference between these populations. By contrast, C2 myoblasts had much less stringent requirements for growth in vitro and were more disruptive of normal muscle architecture after transplantation. If the implantation of cultured myoblasts is to be used as a model system for the study of normal muscle differentiation and repair (Morgan et al., 1992), then in vitro characterization may indicate which myoblast populations are best suited for that purpose. Despite their remarkable growth potential, primary myoblasts do not have the propensity to form aberrant muscle fibers or undifferentiated tumors typical of cells from established myogenic cell lines (Wernig et al., 1991; Morgan et al., 1992).

Introduction of exogenous genes into cells using retroviral vectors has allowed the constitutive expression of the genes in transduced cells and their progeny (Sanes et al., 1986; Price et al., 1987). The observation of inactivation of retroviral genes in primary cells after transplantation has been viewed as a potential obstacle for the use of primary cells for cell-mediated gene therapy (Palmer et al., 1991), but other reports have been more encouraging (Scharfmann et al., 1991). Dai et al. (1992) used retroviral mediated gene transfer to engineer mixed primary muscle cultures to produce factor IX. After transplantation of the cells in vivo, the transgene was expressed for up to six months. However, the percentage of myogenic cells in the injected population was unknown and there was no evidence that the implanted myoblasts had fused to host fibers since the fate of the implanted cells was not examined. We found that retroviral transduction of primary myoblasts with the lacZ gene led to persistent expression of β-gal in vitro and in vivo. The intensity of β-gal staining in mature myofibers was as robust six months after transplantation as after two weeks. Thus, constitutive transgene expression after retroviral transduction appears to be as feasible with primary myoblasts as with established cell lines, which is promising for the use of primary myoblasts for cell-mediated gene therapy.

Retroviral labeling of primary myoblasts with β-gal permitted the elucidation of their fate after transplantation. The possible fates of transplanted myoblasts include fusion to mature fibers of the host, fusion to regenerating muscle cells of the host, fusion to other implanted myoblasts, persistence as mononucleated cells (either quiescent, proliferating, or differentiated), or death. We do not know the extent of cell death among the implanted population, but the predominant fate of surviving cells is fusion to mature, host fibers. The persistence of primary myoblasts as mononucleated cells lying outside muscle fibers was rarely observed. Cells from mixed primary cultures are unlikely to have such a uniform fate after transplantation. Non-myogenic cells such as fibroblasts present in the population persist in the muscle interstitium and could produce adverse effects on muscle tissue such as increased interstitial connective tissue. Thus, the use of pure populations of myogenic cells is clearly preferable.

Of particular importance is the demonstration of long-term survival of the implanted primary myoblasts without immunosuppression. Several previous reports have indicated poor survival of myoblasts or minced muscle transplants even when donor and host were matched at major histocompatibility loci (Watt, 1990; Partridge, 1991; Labrecque et al., 1992). This poor survival has been interpreted as immune rejection related to antigen presenting cells such as fibroblasts or endothelial cells in mixed cultures, or to the inclusion of donor inflammatory cells in minced muscle (Watt, 1990; Watt et al., 1991). Thus myoblast transplantation studies are routinely performed with immunosuppression even in syngeneic hosts, in immunodeficient mice, or in mice made tolerant neonatally to donor cells (Morgan et al., 1990; Chen et al., 1992; Dai et al., 1992). The use of pure populations of myoblasts avoids the potential problem of contaminating cells. In our studies, even after weeks in culture and introduction and expression of a foreign gene, the primary myoblasts were incorporated into mature muscle and stably expressed a transgene for up to six months after transplantation without any immune modulation of the host. The use of immunocompetent hosts is not only simpler, but also preferable since altering the immunologic state of the host could potentially affect the fusion of transplanted myoblasts (Watt, 1982; Hardiman et al., 1993). Furthermore, immunosuppressive agents may be directly toxic to host muscle (Le Quintrec and Le Quintrec, 1991) and may themselves have effects on muscle disease processes. It is thus best to avoid the complications of immunosuppressive therapy in studies of muscle diseases using myoblast transplantation.

Although myoblasts transplanted into syngeneic hosts were retained without immune suppression, myoblasts transplanted into allogeneic hosts were subject to immune rejection. Watt et al. (1991) assessed the long-term survival of mixed cells from primary cultures injected into non-histocompatible hosts. The cells were injected into a minced autograft of host muscle and were rapidly rejected in more than 90% of the experiments. However, in a few cases, there was evidence of survival of donor myogenic cells seven weeks after transplantation. It may be possible for some allogeneic myoblasts to become incorporated into host fibers and persist in an immunologically protected environment since mature fibers express neither class I nor class II major histocompatibility molecules (Ponder et al., 1983; Karpati et al., 1988). In our experiments, transplantation of allogeneic myoblasts resulted in complete elimination of implanted cells within three weeks despite the fact that extensive fusion to mature fibers was seen five days after transplantation (Figs. 3 and 4). It may be that the specific strain combinations of host and donor may account for the differences in results (Butcher and Howard, 1982). We are currently investigating whether transient immune suppression, until the time when all persisting cells have become incorporated into mature fibers, could prevent rejection and allow primary myoblasts to persist indefinitely in allogeneic hosts without sustained immune suppression.

Several investigators have reported that endogenous muscle precursor cells are able to migrate toward areas of muscle damage to participate in the regenerative process (Schultz et al., 1985; Phillips et al., 1990). We found no evidence of the movement of implanted myoblasts to nearby areas of muscle regeneration. Recently, Morgan et al. (1993) reported evidence of migration of implanted myoblasts into adjacent muscles. The results are not directly comparable to ours because of the added variables in their studies: the host animals were immunodeficient, their limbs were exposed to high levels of X-irradiation before implantation to kill endogenous muscle precursor cells, and the muscle was dystrophic. When no irradiation was given, no migration was observed.

Previous work by these authors also suggested that migration of endogenous muscle precursor cells occurred only with widespread degeneration of host muscle and that evidence of such migration was more likely to be observed in immunodeficient hosts (Watt et al., 1987). Together with our results, these observations suggest that the mobility of implanted myoblasts may indeed be quite limited except under specific experimental conditions.

These studies of the characterization in vitro and in vivo of primary myoblasts are encouraging for their use in transplantation studies in mice. Investigations into muscle immunobiology beyond those presented here should unravel some of the conflicts of retention and rejection of transplanted myoblasts under different conditions. The ease of isolation, expansion, and retroviral infection of pure populations of mouse myoblasts should lead to further improvements in the efficiency as well as a better understanding of the immunology and tissue biology of myoblast transplantation. Such advances are critical to the improvement of current methods used for human trials of myoblast-mediated gene therapy, such as those for Duchenne muscular dystrophy (Gussoni et al., 1992; Karpati et al., 1993; Tremblay et al., 1993).

The α-SGC virus was generously provided by Drs. P. D. Robbins, B. Gould, and R. C. Mulligan. We are grateful to our colleagues, Dr. G. K. Pavlath, Dr. J. Dhawan, and Dr. M. B. McCormick, for critical discussions of the manuscript.

This work was supported by grants from the National Institutes of Health (HD18179) and the Muscular Dystrophy Association to H. M. Blau. T. A. Rando is a Howard Hughes Medical Institute Physician Research Fellow.

Received for publication 21 January 1994 and in revised form 22 March 1994.

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