The Fate of Myoblasts Following Transplantation into Mature Muscle

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Cell transplantation has potential benefits for tissue replacement in the enhancement of tissue regeneration and as cell-mediated gene therapy for systemic diseases. The transplantation of myoblasts into skeletal muscle also allows gene transfer into cells of the host since myoblasts fuse with host fibers thereby forming hybrid myofibers. The success of myoblast transplantation can be determined by a variety of measures, such as the percentage of myoblasts that fuse, the number of hybrid myofibers formed, or the level of transgene expression. Each measure is a reflection of the fate of the transplanted cells. In order to compare different measures of transplantation efficacy, we followed the fate of transplanted myoblasts expressing the marker enzyme β-galactosidase (β-gal) in two different assays. Two weeks after transplantation, the number of hybrid myofibers was determined histochemically, whereas transgene (β-gal) expression was measured biochemically. To control for variability of transplantation among different animals, we obtained both measurements from each muscle by using alternate cryosections in the two assays. Within each individual muscle, both hybrid fiber number and β-gal expression were maximal at the site of implantation and diminished in parallel with distance from the site. However, for determining the success of transplantation among groups of muscles, these two measures of efficacy yielded discordant results: the transplants with the highest number of hybrid fibers were not the transplants with the greatest β-gal activity. Such discrepancies are likely due to regional variations at the transplantation site that arise when cells are introduced into a solid tissue. These results demonstrate the importance of multiple measures of cell fate and transplantation efficacy for studies of cell transplantation and for the application of such studies to cell therapy and cell-mediated gene therapy.

INTRODUCTION

The application of myoblast transplantation for in vivo gene transfer in the treatment of genetic muscle diseases was recognized nearly 2 decades ago [1]. Myofibers, which are the mature skeletal muscle cells, are multinucleated syncytia formed by the fusion of hundreds to thousands of myoblasts during development. When cultured myoblasts are transplanted into mature muscle, the donor cells fuse with host myofibers thereby creating hybrid myofibers containing nuclei of both donor and host [2]. Myoblast transplantation has thus been used as a means of transferring genes, carried by donor cell nuclei, into muscle cells in vivo. Most animal studies in this area have focused on the introduction of normal genes into dystrophic muscle as a form of gene therapy [3–5]. Such studies attempt to demonstrate a correlation between transgene expression and modulation of the disease phenotype. Histological analysis of transplants is essential not only for determining disease progression, but also for assessing anatomical aspects critical to all cell transplantation studies such as the localization of transplanted cells in the host tissue, the further proliferation or differentiation of the transplanted cells, and the immunological response of the host [6–8]. Biochemical analysis of transplants provides quantitative measures of transgene expression not obtainable from histological analysis. Since in many studies there is tremendous variability from transplant to transplant (see, for example, [9, 10]), it would be ideal to be able to perform both types of analysis on each muscle rather than separately on two groups of muscles.

In this report we examine the fate of transplanted myoblasts and the efficacy of transplantation by both histological and biochemical analysis of transgene expression. By assaying β-gal expression in serial cryosections, anatomical information on transplanted cell fate and transplantation efficacy could be compared to biochemical measurements of transgene expression.

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There were excellent correlations within individual muscles. Among groups of muscles, these two measures indicated different relative levels of success of transplantation. This approach of being able to obtain two different measures of efficacy from the same tissue sample should be very useful in studies of cell transplantation, not only in muscle but in a broad range of tissues.

METHODS

Myoblast transplantation. We used primary mouse myoblasts transduced with the gene for β-gal by retroviral mediated gene transfer [6, 11], but the method described here is applicable for any myoblast population stably expressing the β-gal marker enzyme [10]. Myoblasts were grown in medium consisting of Ham’s F-10 nutrient mixture (GIBCO BRL, Gaithersburg, MD), 20% fetal calf serum (HyClone Laboratories, Inc., Logan, UT), 2.5 ng/ml basic fibroblast growth factor (Promega Corp., Madison, WI), penicillin (200 U/ml), and streptomycin (200 μg/ml).

The myoblast transplantsations were performed as described previously [6]. Briefly, β-gal-labeled myoblasts were suspended in transplant infection buffer (0.5% bovine serum albumin in F-10 nutrient mixture) at a concentration of 5 × 10⁶ cells/ml, and 5 μl of this suspension (10⁶ myoblasts) were injected into the tibialis anterior muscles of 4- to 6-week-old immunodeficient (nu/nu) mice. The mice were sacrificed 2 weeks later. The tibialis anterior muscles were removed, and those to be processed for cryosectioning were placed in cryomolds containing embedding medium (O.C.T. compound, Miles Laboratory, Elkhart, IN) and frozen in isopentane cooled in liquid N₂. Muscles to be homogenized were placed in plastic tubes and snap frozen in liquid N₂.

All animals were handled in accordance with guidelines of the Administrative Panel on Laboratory Animal Care of Stanford University.

Tissue sectioning. Frozen, embedded muscles were sectioned, yielding serial cross-sections from one end of the muscle to the other. The sections, which were 30 μm thick, were retained only at regular intervals (approximately every 300–500 μm) and the intervening sections were discarded. For histology, the cryosections were collected onto gelatin-coated glass slides; for biochemical assay, they were collected into 0.65-ml microcentrifuge tubes cooled to −20°C in the cryostat.

Histological analysis. The histological detection of β-gal-labeled cells in cryosections was done using X-gal (5-bromo-4-chloro-3-indoly)-β-D-galactopyranoside), a chromogenic substrate for β-gal [12]. This compound yields a blue reaction product in cells expressing high levels of β-gal. The sections were first fixed by dipping the slides in a cold (4°C) solution of 4% paraformaldehyde, 0.25% glutaraldehyde in 100 mM NaPO₄, pH 7.2, for 4 min. The slides were rinsed twice for 5 min in PBS. The sections were then stained with X-gal (Sigma Chemical Co., St. Louis, MO) at a concentration of 1 mg/ml in 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂ in PBS. The slides were coveredslipped using an aqueous mounting medium (Airvol; Air Products and Chemicals, Inc., Allentown, PA) and examined microscopically for the presence of β-gal-labeled (“blue”) myoblasts [6, 13]. The number of hybrid (i.e., β-gal labeled) fibers in a muscle was determined from the section with the maximum number of such fibers, and that was invariably at the site of implantation [6].

Biochemical analysis. Each whole muscle was homogenized in 1 ml of an extraction buffer consisting of 100 mM β-mercaptoethanol, 0.1% Triton X-100, 90 μg/ml phenylmethylsulfonyl fluoride, 0.2 μg/ml aprotinin, 100 μM leupeptin in 100 mM NaPO₄, pH 7.0, on ice. The homogenate was centrifuged at 4000g at 4°C for 10 min, and the supernatants were transferred to individual tubes and kept on ice.

FIG. 1. Hybrid myofibers formed by myoblast transplantation demonstrate an inhomogeneous distribution of donor cells. β-Gal-labeled myoblasts were transplanted into muscle, and the muscle was examined 2 weeks later for the presence of donor cells. A tissue cryosection was stained with X-gal, which produces a blue reaction product in cells expressing the marker enzyme. Most of the host fibers in this area have incorporated donor myoblasts and thus stain blue, although the intensity of staining varies among the hybrid fibers. Some of the fibers are darkly stained (curved arrow), which may indicate the incorporation of more donor cells than those fibers that stain lightly (straight arrow). Bar, 30 μm.

For cryosections, 100 μl of extraction buffer was added to the tube containing the sections. The tubes were vortexed and placed at 4°C overnight. Before the extracts were assayed, the tubes were centrifuged to pellet insoluble material.

The supernatants of whole muscle or cryosection extractions were analyzed for β-gal activity using a fluorescence-based assay [13, 14]. The substrate, MUG (4-methylumbelliferyl β-D-galactoside), yields a fluorescent product when cleaved by β-gal. For each sample, 10 μl of supernatant was added to 90 μl of assay buffer consisting of 10 mM KCl, 1 mM MgSO₄, in 100 mM NaPO₄, pH 8.0, in wells of a 96-well plate. Fifty microliters of 2 mM MUG (Sigma) was added to each well and the plate was placed at 37°C in the dark. After 30 min, 100 μl of a stop buffer (15 mM EDTA, 300 mM glycine, pH 11.2) was added to each well and the fluorescence was measured using a fluorescent plate reader (Fluoroskan; Flow Laboratories, Inc., McLean, VA) with an excitation filter of 355 nm and an emission filter of 460 nm. The β-gal activity is represented as fluorescence units (FU).

RESULTS

When β-gal-labeled myoblasts are transplanted into mature muscle of syngeneic or immunodeficient mice, the transplanted cells fuse with host myofibers yielding hybrid fibers which are stable for at least 6 months [6]. These hybrid fibers can be identified by staining tissue sections for the presence of β-gal [13], as shown in Fig. 1. Counting the number of hybrid myofibers provides a measure of the efficacy of the transplantation procedure if the goal is to introduce the transgene product (in this case, β-gal) into as many host fibers as possible [6]. Another measure of transplantation efficacy is the level of transgene expression, especially if one is trying to correlate histological outcome with transgene ex-
pression as would be the case for introducing normal gene products into diseased muscle. That kind of quantitation of gene expression cannot be obtained from histological analysis. The level of transgene expression in one hybrid fiber may be very different than the level in another hybrid fiber. It is likely that the more darkly stained fibers are expressing higher levels of β-gal than are the lightly stained fibers (Fig. 1), but the relative amounts are not discernible from histochemical analysis. Quantitative estimates of β-gal activity levels in the whole muscle can be derived from biochemical measurements of tissue extracts. However, tissue homogenization precludes being able to obtain important anatomical information such as the number of hybrid fibers formed, the regional distribution of implanted cells, the tissue integrity following cell implantation, and the immunological response of the host.

In order to be able to obtain both quantitative biochemical data as well as anatomical information from a single transplanted muscle, we tested whether biochemical analysis of transgene expression could be accurately performed on tissue cryosections. Six mice received β-gal-labeled myoblast transplants into both tibialis anterior muscles. Two weeks later, the mice were sacrificed and six of the muscles were processed for biochemistry by homogenization of the tissue. The remaining six muscles were processed for routine sectioning, and cryosections were collected for β-gal activity measurements. Cryosections were retained at regular intervals (see Methods), and the amount of tissue in the pooled sections represented only about 8% of the total muscle mass. The estimates of the total β-gal activity in the muscles extrapolated from whole muscle extracts or from cryosections were indistinguishable (Fig. 2).

The processing of muscle for cryosectioning did not appear to alter significantly the results of the MUG assay. Specifically, the embedding medium which was present in cryosections but not in muscle homogenates did not affect β-gal measurements. When increasing amounts of embedding medium were added to replicate tubes of β-gal-labeled myoblasts, the measured β-gal activity was unaffected even by a 10-fold excess of that present in the sections assayed in Fig. 2. Only when embedding medium was present at greater than 25-fold excess was there a reduction of the measured β-gal activity.

The reliability of measuring β-gal activity in cryosections was tested by assaying individual rather than pooled sections. If the MUG assay on a single 30-μm section is reliable, then the β-gal activity measured in adjacent sections should be nearly identical. For each muscle, two adjacent sections from the middle of the muscle, near the site of injection, were assayed. The β-gal activities of such serial sections differed by only 3.0 ± 2.4% (mean ± SD; n = 6) even though each section contained as little as 0.4% of the total muscle mass. Thus, β-gal measurements of cryosections reliably reflected the β-gal activity of the segment of muscle from which the sections were taken.

Even though transplanted myoblasts fuse with host muscle cells, it is possible to examine the fate of the cells by following the β-gal activity histochemically. It was apparent from histochemical staining as shown in Fig. 1 that myoblast transplantation did not lead to homogeneous distribution of the transplanted cells within the tissue. The implanted myoblasts appeared to partition unequally among the host fibers. The cells remained localized at the implantation site rather than spreading out evenly across the cross-sectional area of the muscle (6, 15). Furthermore, along the longitudinal axis of the fibers, there were clearly regional variations of the distribution of the β-gal label. The highest levels were found at the site of implantation and diminished with increasing distance from the injection site (see Fig. 3). This distribution was manifested both as a reduction in the number of β-gal-labeled fibers as well as a reduction in the intensity of the β-gal stain. This kind of spatial information is not obtainable from β-gal measurements of muscle extracts.

In order to compare two different measures of transplantation success, hybrid fiber formation and level of transgene expression, we tested whether they correlated regionally within an individual transplant. Serial cross sections were collected at 400-μm intervals from one end of each tibialis anterior muscle to the other. At each interval, one 30-μm section was collected for histochemistry to determine the number of hybrid fi-
FIG. 3. There is a direct correlation between the number of hybrid fibers and the segmental β-gal activity present in serial cryosections of an individual muscle. Hybrid myofibers were counted in cryosections at regular intervals (every 400 μm) along the length of individual muscles, and the β-gal activity was measured in adjacent sections (two 30-μm sections at each interval). The values were normalized to percentage maximal and aligned by their peaks since the transplantation procedure resulted in slight variations of the implantation sites. The range of maximal fiber number was 107–333; the range of maximal β-gal activity was 1485–3484 FU. Error bars represent mean ± SD (n = 6).

numbers, and two 30-μm sections were collected for measurement of β-gal activity in that segment. Figure 3 shows that the number of hybrid fibers closely paralleled the segmental distribution of β-gal activity along the length of the muscle. Thus, within an individual muscle, these two measures of transplantation efficacy showed similar regional variation.

We then tested whether ranking transplantation success among a group of muscles would be identical if success were gauged by hybrid fiber number and by transgene expression. In other words, we asked whether a higher number of hybrid fibers formed also meant a higher level of transgene expression. Although there was a close correlation between hybrid fiber number and β-gal activity within an individual muscle (Fig. 3), the correlation between muscles was poor (Fig. 4). The rank order of success as judged by hybrid fiber number did not correspond to the rank order of success as judged by β-gal activity. This probably reflects the fact that a single hybrid myofiber could represent the incorporation of one, tens, or hundreds of donor myoblasts, and thus the ratio of β-gal activity per hybrid fiber formed varied from muscle to muscle. The determination of hybrid fiber number and the quantitation of transgene expression provide complementary information and two different outcome measures of myoblast transplantation. This underscores the value of obtaining both sets of data from each transplant.

DISCUSSION

Among the goals of myoblast transplantation for the treatment of genetic muscle diseases are the delivery of a normal gene to as many muscle fibers as possible, the distribution of the gene product as widely as possible within those fibers, and the constitutive expression of that gene at sufficiently high levels. Most experimental work for intrinsic muscle diseases has been done using a mouse mutant, the mdx mouse, with a defect in the gene for dystrophin [4, 5]. Detection of the normal gene product has been used as a measure of the success of the transplantation. In studies to assess transplantation efficacy, not necessarily in diseased muscle, a variety of both endogenous and exogenous genetic markers that distinguish donor from host have been used (glucose-6-phosphate isomerase: [2, 16]; hu-

FIG. 4. The rank order of success of myoblast transplantation among a group of muscles differs for two different measures of transplantation efficacy (hybrid fiber formation and level of transgene expression). Both measures were obtained from cryosections at the myoblast implantation site. (A) A group of six transplants are presented in decreasing order of transplantation success, measured as the maximum number of hybrid fibers formed. (B) The six transplants presented in the same order as in A, but displaying maximal β-gal activity of the transplant. Clearly, the rank order of success as judged by the level of β-gal activity (rank order: transplant numbers 4, 3, 6, 1, 5, 2) has no correspondence with the rank order as judged by hybrid fiber number.
man leukocyte antigens: [17]; β-gal: [11, 13]). Although the detection of transgene products in tissue sections has been studied in detail, quantitation of transgene expression has received much less attention. Clearly, the level of expression of a gene product is critical to the correction of an intrinsic genetic defect in muscle [18]. Since many transplantation protocols result in tremendous variability of transplantation efficacy within a group of muscles [4, 9, 10], it is advantageous to be able to correlate transgene expression with histological outcome in the same muscle.

The results presented here demonstrate that measurements of transgene expression can be combined with and complement histological examination of a single transplant by using serial cryosections. Transgene expression was reproducibly measured in serial sections, and the process of tissue preparation for sectioning had no significant effect on the reliability of that measurement (Fig. 2). Adjacent sections could thus be used to determine both the magnitude and the localization of transgene expression. Furthermore, sections used for anatomical localization could also provide information on the effect of the transplantation on tissue architecture, on the immunological response of the host, which is a major issue in the success of myoblast transplantation [7, 19], and on the fate of the transplanted myoblasts. Transplanted myoblasts may have several different fates including remaining quiescent as mononucleated cells [20], differentiating and fusing with host fibers to form hybrid myofibers [2, 6], or proliferating to form tumors [21]. The biochemical studies likewise are not limited to measurement of the levels and regional variation of the protein product of the transgene. Cryosections could be used in biochemical and molecular analyses of other parameters of donor cells, such as the localization of the transgene itself or the localization and quantitation of mRNA levels.

The comparison of hybrid fiber number and transgene expression along the length of each individual muscle revealed that the two measures paralleled one another (Fig. 3). Both measures were maximal at the implantation site where the number of myoblasts is maximal after transplantation. That a direct relationship exists between the number of transplanted myoblasts and these measures of transplantation efficacy is supported by our previous results [6], which showed an approximate doubling, on average, of the number of hybrid fibers formed as the number of transplanted myoblasts was doubled. Likewise, we would expect a close correlation between transplanted cell number and maximal β-gal activity. The results shown in Fig. 2 demonstrate a small variation in total β-gal activity among a group of muscles receiving the same number of transplanted cells.

In the experiments described in this report, we present two complementary approaches to assessing the fate of transplanted myoblasts and thus the efficacy of the transplantation: histological and biochemical. The complementary nature of these two assessments is shown most clearly in Fig. 4, in which there is a discrepancy between the results obtained with the two assays. In these experiments, a constant number of myoblasts was injected into each muscle. Among the muscles analyzed, the numbers of hybrid fibers formed were within the range expected from our previous results. However, within that range, the muscle with the greatest number of hybrid fibers was not necessarily the one with the greatest β-gal activity (Fig. 4). This may present an apparent paradox with the results presented in Fig. 3, in which the two measures paralleled one another within an individual muscle. However, comparisons between muscles as shown in Fig. 4 must take into account variations from one implantation site to another. One injection, with cells distributed over a broad cross-sectional area of the muscle, could result in many hybrid fibers formed. Another injection, with the same number of myoblasts but distributed over a more restricted cross-sectional area, could yield fewer hybrid fibers (as transplanted cells would have access to fewer host fibers with which to fuse) while still having the same regional β-gal activity as the first implantation. Such a variability at the transplantation site could lead to poor correlations between individual muscles, as seen in Fig. 4, but would not be present within an individual muscle (Fig. 3) and would be averaged when comparing groups of muscles [6]. A technique to quantify the intensity of transgene expression in situ could provide further information on transplantation efficacy by quantifying transgene expression over the cross-sectional area of a muscle. Taken together, such diverse assays of spatial distribution and levels of transgene expression provide important information on the outcome of cell transplants into solid tissues.

In addition to assessing the regional effects of myoblast transfer described above, measures of the overall efficiency of the procedure are necessary. Efficiency could be assessed in different ways, but one important measure is the percentage of transplanted myoblasts that persist in the host tissue. In muscle, the syncytial nature of the tissue creates a problem for following donor cells labeled with a cytoplasmic marker. After fusing with host fibers, donor myoblasts lose their cellular identity as they become incorporated into the syncytium. Therefore, the nature of the marker of donor cells is critical to this assessment of transplantation efficiency. Detection of protein products specific to donor cells, such as those mentioned above, permits the identification of hybrid fibers but does not permit the actual counting of the number of donor cells. Similarly, semi-conserved cytoplasmic markers such as polystyrene or latex microspheres [15] are useful only for identifying hybrid fibers or unfused donor cells. Al-
though the cytoplasm of donor and host cells mix within the hybrid myofiber, the nuclei of donor and host remain distinct. The nuclear labels tritiated thymidine [5], bromodeoxyuridine [22], and fluorogold [23] have been used in myoblast transplantation studies to identify donor nuclei and distinguish them from host nuclei, but these markers are not conserved with cell division and reuptake of label into host nuclei from degenerating donor cells can be problematic [24]. Probes of conserved genetic markers, sequences on the Y chromosome, have been used to identify donor nuclei in myoblast transplantation studies [25]. The Y chromosome probes are both heritable and stable, as well as being endogenous markers, but detection and quantitation in histological sections is laborious and time consuming.

There are few estimates of the percentage of myoblasts that survive after transplantation using any of these donor cell markers. Using donor cells labeled with tritiated thymidine, Beauchamp et al. [26] arrived at an estimate of the survival of transplanted myoblasts of less than 5% 5 days after transplantation. Estimates of the survival of donor myoblasts in our studies, based on the total β-gal activity of the muscle and the β-gal activity in a known number of transplanted cells, would indicate a survival rate of approximately 70% 3 days after transplantation, and greater than 50% 6 days and 2 weeks after transplantation (unpublished observations). Discrepancies between our results and those of Beauchamp et al. [26] could be due to differences in transplantation techniques. We have found that the survival of transplanted cells is improved by reducing the amount of time cells are in suspension, by maintaining the cell suspension on ice, and by including protein (bovine serum albumin) in the transplantation buffer. However, discrepancies in cell survival estimates may also be due to the particular cell marker used. Incorporated tritiated thymidine does not provide a direct measure of transplanted cell number because the label is diluted with cell divisions and can be taken up by host cells after death of transplanted cells. Likewise, the relationship between β-gal activity and transplanted cell number may not be linear since changes in rates of transcription, rates of translation, and protein stability could alter this relationship. A study combining an enzyme marker (such as β-gal) with an intrinsic nuclear marker (such as a Y chromosome probe), both measurable in serial cryosections, would establish the quantitative relationship between donor cell nuclei and marker enzyme activity. Then, in future experiments, the number of donor nuclei could be determined from enzymatic activity alone with certainty, making such determinations far more rapid. For all studies aimed at improving and comparing the efficiency of myoblast transplantation using different protocols, an accurate assessment of cell survival or persistence is essential.

More generally, the approach described in this report, using serial cryosections for both biochemical and histocellular assays, may be broadly applicable to all cell transplantation studies in solid tissues. As with the prevention of myofiber degeneration in dystrophic muscle by myoblast transplantation, the desired outcome of many cell transplantation experiments is a local effect, such as the reestablishment of neurotransmitter release in neurodegenerative conditions [27] and the restoration of cartilage production in arthritic conditions [28]. These effects are clearly dependent on local incorporation of transplanted cells into the host tissue. An inhomogeneous distribution of the product of the transplanted cells, such as that shown in Figs. 1 and 3, obviously cannot be examined in tissue homogenates and yet will clearly lead to an outcome that varies regionally within the tissue. Within solid tissues, only by combining quantitative histological and biochemical analyses will measures of the efficacy of cell transplantation be reliably obtained and properly interpreted.

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