SUCCESSFUL MYOBLAST TRANSPLANTATION IN RAT TONGUE RECONSTRUCTION

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Abstract: Background. Controversy exists regarding the success of myoblast transplantation. The purpose of this study was to determine the survival of transplanted myoblasts in a rat tongue reconstruction model by using fluorescently labeled myoblasts and surgical stains to mark the location of the pocket into which transplanted cells were delivered. We evaluated tongue histology after myoblast transplantation under the hypothesis that myoblast transplantation will promote muscle regeneration and result in minimal scar tissue formation.

Methods. Sterile solutions of 1:10 India ink, 1% methylene blue, and 1% crystal violet were applied to the inner lining of a left-sided mucosa-sparing hemiglossectomy pocket. After air-drying, the hemiglossectomy defect was filled with collagen gel and closed. The tongues were evaluated histologically at 6 weeks. Next, myoblasts were cultured and labeled with three commercially available fluorescent dyes, 5-chloromethyl-fluorescein diacetate (CMFDA), chloromethylbenzamido (OM-Dil), and fluorescently labeled microspheres (FLMs), to determine which would optimally label myoblasts in a tongue reconstruction model. Next, Lewis rats underwent left hemiglossectomy, and the created pockets were coated with 1:10 India ink. Control animals received collagen gel alone, whereas experimental animals received labeled myoblast/collagen constructs into the tongue defect. Tongues were harvested at intervals to determine the presence of labeled fluorescent cells, the relative numbers of viable myoblasts, and the degree of scarring.

Results. India ink coating of the hemiglossectomy pocket caused minimal inflammation and lasted longer than the other tested dyes. CMFDA and FLMs both successfully label myoblasts for transplantation. In vivo, donor cells were observed in all specimens at week 0 with increasing numbers of cells and muscle formation, determined by desmin immunofluorescence, after 6 weeks. There was less scar tissue contracture in the experimental group and a significant increase in the amount of desmin-stained muscle in the surgical defect.

Conclusions. India ink coating is an appropriate vehicle for intra-operative marking of a hemiglossectomy cavity. The introduction of myoblast/collagen constructs into the rat hemiglossectomy defect increases the amount of regenerated muscle, results in less scar contracture, and may increase meaningful tongue function. © 2006 Wiley Periodicals, Inc. Head Neck 28: 517–524, 2006

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Myoblast transplantation refers to the in vivo implantation of normal or genetically modified myogenic cells after culture and expansion in vitro. Investigations of myoblast transplantation in animal models to date have given various results in terms of the survival of implanted myoblasts in host muscle. The results obtained in human clinical trials have been disappointing.1–5 In the mouse, rejection is very rapid and efficient after major histocompatibility complex (MHC) — incompatible cell transplantation, because it involves both cellular and humoral immunity.6–13 Most success has been achieved using immuno-deficient mice14–16 and with immunosuppression.17–20 These studies suggest that differences of only a few minor antigens, even those linked to the Y chromosome, are enough to trigger the rejection process and may explain why myoblast transplantation evaluated with probes against the Y antigen have had relatively little success.21

The tongue is a multifunctional organ that plays a critical role in articulation, airway protection, and deglutition. To date, there is no ideal tongue reconstruction after partial glossectomy to restore the form and function of the native tongue, even when innervated free flaps are used.

Our hypothesis was that delivering large numbers of syngeneic myoblasts partially overcomes fibrosis and may result in improved muscular function of the neotongue.

We have previously shown that myoblast transplantation into hemiglossectomy defects seemed to lead to new muscle formation in syngeneic rats,22,23 but no direct evidence supports the presence of the transplanted myoblast cells in host muscle. The objectives of this study were to locate the transplanted myoblasts by marking the surgically created pocket with ink intraoperatively and to verify the survival of the transplanted muscle cells in the reconstructed tongue by using a fluorescent myoblast label. Finally, to understand the overall effects of delivering myoblasts into a hemiglossectomy pocket, we evaluated muscle regeneration within a hemiglossectomy defect after myoblast transplantation through histologic evaluation.

MATERIALS AND METHODS

Selection of Optimal Dye for Marking the Hemiglossectomy Pocket. Twelve female Lewis rats, weighing 225 to 250 g, were anesthetized with an intramuscular injection of ketamine/medetomidine followed by injection of 0.1 mL of 1% lidocaine with 1:100,000 epinephrine into the left hemitongue for local vasoconstriction. All rats underwent a left-sided anterior hemiglossectomy by means of a lateral approach, with preservation of tongue mucosal and submucosal layers. The limits of the dissection were defined medially by the midline raphe and posteriorly by the circumvallate papillae. The resulting tongue pocket in each group (n = 4) was coated with one of three different surgical dyes, black India ink (Higgins waterproof ink; Eberhard Faber, Inc., Newark, NJ) dilution 1:10, methylene blue (Sigma, St. Louis MO) 1% solution, crystal violet (Sigma) 1% in 10% alcohol, and was left until air-dry. The tongue pocket was filled with collagen gel and prepared as follows: a 1.7 mg/mL Cellagen solution (ICN Pharmaceuticals, Costa Mesa, CA) in Ham’s F10 medium (Sigma) to Matrigel (Collaborative Biomedical Products, Bedford, MA) in a 1:6 vol/vol ratio. The mixture was enriched with 3 ng/mL basic fibroblast growth factor (bFGF) and neutralized to pH 7.1 with sterile 7.5% NaHCO3. The pockets were closed with a running locked 4-0 chromic suture. Animal Care and Use Committee (ACUC) guidelines were strictly followed.

One rat from each group was killed at the following intervals: 2 days and 2, 4, and 6 weeks. Animals were killed under deep anesthesia after harvesting the tongue by transection at the circumvallate papillae, and the tongues were prepared for histologic analysis. The sections were stained with hematoxylin-eosin (H&E) and processed for routine microscopy.

In Vitro Selection of the Optimal Cell Tracker for Labeling Myoblasts.

Myoblast Harvest and Culture. Myoblasts were harvested according to previously described methods.22,23 Paraspinal muscles were dissected from an 18- to 19-day-old Lewis rat fetus, placed in chilled Ca2+, Mg2+, free phosphate-buffered saline (PBS) (Sigma), and minced. The tissue was enzymatically digested by incubation for 30 minutes at 37°C in a dissociating medium containing 1% type II collagenase (Worthington Biochemicals, Lakewood, NJ), 2.4 U/mL Dispase (Boehringer Mannheim, Ingelheim, Germany), and 2.5 mM CaCl2 solution. The material was then triturated with 25-mL and 10-mL pipets, respectively, and strained through a 70-μm filter.
Cells were placed in myoblast growth medium (GM) consisting of Ham’s F-10 Nutrient Mixture (Sigma), 20% fetal bovine serum (Sigma), 3 ng/mL bFGF (Promega Corp, Madison, WI), and 1% glutamine penicillin streptomycin (Sigma). Cells were spun at 600 rpm for 10 minutes and transferred to precoated laminin six-well plates (5 μg/mL in PBS for 24 hours at 37°C) and incubated at 37°C overnight. Plates were visibly inspected for culture purity and seemed to be greater than 90% pure. Formal quantification of culture purity was not performed.

**Labeling of Mononucleated Cells.** Two different cell trackers, chloromethylbenzamido (CM-DiI; Molecular Probes, Carlsbad, CA) and 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes), and one type of fluorescent latex microspheres (Green FLMs; LumaFluor, Naples, FL) were used to label the myoblasts. Cells were labeled according to the standard protocol recommended by the supplier. For CM-DiI labeling, the attached cells in the plate were rinsed twice with PBS and incubated in 0.1, 1, 10, 20 μM CM-DiI solutions for 5 minutes at 37°C and for 15 minutes at 4°C. For CMFDA, the attached cells in the plate were rinsed twice with PBS and incubated in 0.5, 1, 5, 10, 20 μM CMFDA for 30 minutes at 37°C and for 30 minutes at 37°C in growth medium. For FLMs, Green FLMs were added after 12 hours to all rat cultures at a dilution of 1:3000 in growth medium. After 12 hours, the cultures were rinsed three times with Hank’s balanced salt solution (HBSS) and analyzed for labeling efficiency using quantitative fluorescence microscopy.

**Myoblast Transplantation in the Tongue Pocket after Hemiglossectomy.**

**Myoblast Cultures and Cell Labeling.** Myoblasts were labeled with 10 μM CMFDA and FLMs 1:3000, as described previously, and collagen gel was likewise prepared. For preparation of the cell suspension in the collagen gel, myoblasts were allowed to adhere to tissue culture plates for 24 hours, after which they were enzymatically dissociated with 0.05% Ca²⁺ Mg²⁺–free trypsin (Sigma). Trypsin was neutralized with myoblast growth medium, and the cells were centrifuged at 600 rpm for 10 minutes. The pellet was then resuspended in the collagen gel preparation at 4°C.

**Myoblast Constructs.** Muscle constructs were prepared according to the method of Vandenburg et al.²⁴ The collagen gel/myoblast preparation, still in liquid form, was poured into preformed casts and warmed in the incubator for 15 minutes to solidify. Each construct in its cast was placed into a separate well of a six-well tissue culture plate, and the wells were filled with myoblast growth medium. The medium was changed every 2 to 3 days.

**Surgical Implantation.** Twenty-eight female Lewis rats, weighing 225 to 250 g, underwent hemi-glossectomies as previously described. After coating the surgical tongue bed with black India ink 1:10, the tongue pocket was filled with collagen gel in the control group (n = 14) and with labeled myoblast/collagen gel constructs in the experimental group (n = 14). The filled pocket was then closed with a running chromic suture. Two rats from each group were killed immediately, and the remainder were killed 6 weeks after surgical implantation. The tongue tissues were prepared by cryostat section (6 μM thick, −20°C). Thin sections mounted on glass microscope slides were stored at −20°C and protected from light until evaluation for presence and distribution of the labeled cells by fluorescence microscopy. H&E-stained and desmin-stained sections were also prepared and examined using routine light microscopy.

**Evaluation of Myoblast Distribution.** A Nikon Eclipse E600 fluorescence microscope with a green filter was used to detect CMFDA or FLMs at a magnification of 10x. The microscopic field was centered around the highest distribution of labeled cells, and the density was measured with the Metamorph Imaging Software Program; values obtained are reported as the relative area occupied by labeled cells.

**Histologic Evaluation.** Histologic slides of representative muscle sections through the reconstructed hemitongues were reviewed and blindly ranked for the quality of neomuscle regeneration. A single pathologist did this in a semiquantitative fashion. Both degree of fibrosis and degree of inflammation were considered. First, all specimens were inspected to understand the spectrum of inflammation and fibrosis present. Specimens were then ranked as mild, moderate, or severe according to where they fell on the spectrum, with respect to the degree of chronic inflammation (judged by the presence of histiocytes and lymphocytic infiltrate) and fibrosis (judged by...
the degree and thickness of the fibrous, acellular collagen deposition). The amount of desmin-stained muscle presented in the surgical defect area (lined by India ink) was ranked as low, moderate, or high based on similar analysis, with normal muscle as a measure of “high” desmin staining. A desmin rhodamine immunofluorescence technique was also performed to determine the presence of muscle protein within the labeled cells. Histologic scoring data were compared using a Student’s t test analysis, with a p value of <.05 considered significant.

RESULTS

Selection of Optimal Dye for Delineating the Surgical Pocket. Animals receiving methylene blue– and crystal violet–marked pockets had no visible dye present outlining the surgical pocket after day 2. Methylene blue–treated pockets demonstrated soaking into many cell layers, thereby not producing a continuous line or well-defined pocket. India ink–coated pockets were visible throughout the 6-week study period and were seen as discrete lines outlining the surgically created hemiglossectomy pocket (Figure 1). There was no visible inflammatory response involving the dyed area.

In Vitro Selection of the Optimal Cell Tracker for Labeling Myoblasts. Myoblasts were intolerant of CM-DiI at any concentration. The cells appropriately phagocytosed the FLMs at a 1:3000 dilution (Figure 2) and were successfully labeled with CMFDA at all concentrations, although there was a marked decrease in cell survival when labeled with higher concentrations (20 μM) of CMFDA (Figure 3).
Myoblast Transplantation in the Tongue Pocket after Hemiglossectomy. Cells labeled with either 10 \( \mu M \) CMFDA or FLMs were observed in clusters throughout the cross section of all harvested tongue muscle specimens. The fluorescent cells were localized in the pocket and were not spread throughout the muscle (Figure 4). The mean relative numbers of the labeled cells were higher at week 6 than at week 0 (Figure 5). The tongues in the experimental group showed less scar contracture and more similarity to normal tongue macroarchitecture than in the control group (Figure 6).

Histologically, the amount of desmin-stained muscle present in the pocket was statistically significantly higher in the experimental group than in the control group \((p < .05)\) (Figures 7 and 8), but there were no significant differences in the degree of fibrosis or inflammation between the two groups (Figure 8).

DISCUSSION

Transplantation of myoblasts into a tongue defect after hemiglossectomy has been considered as a possible strategy in tongue reconstruction. One of the problems associated with studying muscle cell hybridization in vivo is identification of both the transplanted myoblasts in the host and the muscle fibers resulting from their fusion with host cells. Marking the surgical boundaries of a defect at the time of surgery in a way that could later identify the pocket would be useful to locate the manipulated area within the host tongue. Three different dyes, India ink, methylene blue, and crystal violet, were investigated as to their long-term pocket-staining potential. They were chosen based on literature supporting their minimal or absent inflammatory reaction.\(^{25–27}\) India ink was
Cells can be fluorescently labeled in the tissue constructs with microspheres, Cell Tracker CMFDA, or with CM-DiI. In this study, myoblasts were successfully labeled with either CMFDA or FLMs, but they could not tolerate CM-DiI. CMFDA at its highest noncytotoxic concentration (10 μM) was used to label the transplanted myoblasts to last for 6 weeks. FLMs were used as a comparison, because they were retained within the sarcoplasm of myoblasts and myotubes for several weeks with no cytotoxic effect, and reuptake of the FLMs by host cells is reportedly very rare.

In this study, all of the rats receiving myoblasts contained viable labeled cells on sacrifice.
In addition, movement of the transplanted cells from within the pocket into adjacent host muscle was rare, in agreement with many reports about minimal movement when donor cells derived from primary cultures were injected into preinjured dystrophin-deficient or normal hosts. Histologic evaluation of experimental animals demonstrated a statistically significant increase in desmin-positive cells within the reconstructed area in week 6 compared with week 0. The transplanted myoblasts seemed to survive, multiply, and fuse to form myotubes and myofibers in the host rat tongue, confirmed by the presence of desmin rhodamine immunofluorescence within the labeled cells. This illustrates the improved capability for muscle regeneration in the group receiving transplanted engineered tongue over the control group. Although the degree of fibrosis showed no difference between groups, delivering myoblasts may result in less scar contracture and more functional muscle at later time points.

Our study confirmed the survival of donor cells. The excellent survival of these transplanted cells implies that immunologic rejection of the donor myoblasts by the hosts was not a dominant feature of the healing process. In these experiments, the rats were not only histocompatible but also inbred, and the grafts were not rejected. This agrees with the study of Vilquin et al. The other explanation for our success is that muscle regeneration occurs only in areas that have been revascularized, and rapid capillary formation in hybrid muscular tissues implanted in the body is required for a viable and functioning muscular tissue. Because tongue is one of the most richly vascularized tissues, it is ideally suited for receipt of transplanted cells. Moreover, evidence suggests that muscle fibers will regenerate and incorporate a motor nerve supply if provided adequate nourishment. Our model may provide just such conditions, and future studies are planned to examine this phenomenon.

CONCLUSION

Myoblast transplantation has been considered a potential alternative strategy for head and neck reconstruction. The introduction of myoblast/collagen gel constructs into rat tongue hemi-glossectomy defects increases the amount of regenerating muscle, resulting in less scar contracture. Ultimately, this approach could be used in a manner similar to other currently used clinical tissue engineering applications, in which a biopsy specimen is taken from the patient and cells of interest are isolated, purified, and expanded and then redelivered in a polymer gel at the time of resection and/or reconstruction. For tongue reconstruction, the concept would be that a small muscle biopsy from an extremity could be taken preoperatively and the myoblasts expanded in culture and delivered at the time of surgical ablation and reconstruction.

The fluorescent dye, cell tracker CMFDA, and fluorescent latex microspheres can be used to identify transplanted myoblasts. India ink is an appropriate surgical boundary marker, promoting only modest inflammation, and there is no measurable effect on the survival of the transplanted myoblasts. The technique of inking the surgical cavity and labeling transplanted myoblasts has significant research utility, because it permits locating and identifying transplanted myoblasts in the host muscle and can also be used to evaluate the migration of transplanted cells.

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