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Myoblasts Survive Intracardiac Transfer and Divide Further after Transplantation

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ABSTRACT

Objective: Skeletal myoblasts have been proven to survive transplantation into myocardial scar tissue. The objective of this study was to evaluate whether these cells can also be transferred into vital myocardium and maintain their ability for cell division after transplantation. In addition, an intravital fluorescence dye for marking these cells was evaluated.

Material and Methods: Skeletal myoblasts were harvested from male Lewis rats (n = 6) and then expanded in culture. Before implantation, these cells were trypsinized and labeled using an intravital fluorescence dye (PKH-26). Syngenic myoblast transfer to recipient female Lewis rats (n = 36) was used to simulate autologous transplantation. Under general anesthesia, the rats received injections of 10^6 myoblasts via a subxyphoidal approach into the apex of the heart. The animals were then divided into 3 groups (n = 10 each). The animals were sacrificed at several time points, and the hearts were harvested for histologic examination: group A, 7 days postoperatively; group B, 14 days postoperatively; and group C, 28 days postoperatively. An additional group, group D (n = 6), served as a control group; these animals were injected with only cell medium. Corresponding to the study groups, 2 animals of this control group were sacrificed at each time point, and the hearts were explanted. At histological examination, 8-μm sections were investigated to identify surviving stained cells. For further evaluation, the sections were stained using monoclonal antibodies against n-cam, desmin, and α-actin.

Results: No fluorescing cells were found in any hearts of rats in the control group. Surviving fluorescing myoblasts were found in 9 of 10 hearts of groups A and C and 8 of 10 hearts of group B. Labeled myoblasts were located in the intercellular spaces between the myocardial fibers. Fibrotic or inflammatory reactions could not be identified around the injection site in any hearts of the study groups. Immunohistochemical staining results showed that the labeled cells expressed n-cam, desmin, and α-actin. The myoblasts had regained their physiologic structures and had started to form myofibers. In groups B and C, more n-cam–positive cells than labeled cells were found, indicating further cell division.

Conclusions: Intravital fluorescence staining with PKH-26 dye proved to be an easy and reliable method for identifying cells after cellular transplantation. Myoblasts survived an intracardiac transfer, regaining their physiologic structures and maintaining their ability for further cell division.

INTRODUCTION

Skeletal myoblasts can be isolated from skeletal muscle pieces and selectively expanded in culture [Walsh 1990, Cifuentes-Diaz 1993]. Myoblasts have been shown to survive transplantation onto myocardial scar tissue [Chiu 1995, Murry 1996, Greentree 1994, Zibaitis 1994, Atkins 1999, Taylor 1998, Dorfman 1998, Koh 1993, Yoon 1995]. For detection of the transplanted cells, different labeling techniques including virus-mediated transfection and T^3-thymidine markings have been described [Murry 1996, Greentree 1994]. Primary myoblasts, however, undergo a limited number of cell divisions. Because virus-mediated transfection techniques require several selection passages, they reduce the ability of primary myoblast cells to divide further after transplantation. The primary goal of this study was to evaluate an alternative labeling method that uses an intravital fluorescence dye. Another purpose was to demonstrate cell survival within vital myocardium and further cell division after transplantation.

MATERIAL AND METHODS

Myoblasts from inbred Lewis rats were cultured as described previously [Walsh 1990, Cifuentes-Diaz 1993]. In brief, skeletal muscle specimens of approximately 1 cm^3 were minced into small pieces and then trypsinized (Trypsin EDTA; Gibco, Ivitrogen, Carlsbad, CA, USA) and plated onto culture dishes using skeletal muscle growth medium (SGM) (Promocell, Heidelberg, Germany). The cells were passaged before reaching confluence to avoid in vitro differentiation. To confirm that the cultured cells were skeletal

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Table 1. Group Distribution of the Study Animals*

	Group A	Group B	Group C	Group D
n	10	10	10	6
Heart explantation, postoperative d	7	14	28	7, 14, 28 (n = 2 each)

*Groups A, B, and C were study groups; group D served as a control group.

myoblasts, cultured cells were stained immunohistochemically for n-cam. N-cam is a surface antigen specifically expressed by myoblasts [Walsh 1990, Cifuentes-Diaz 1993] that takes part in the development of the neuromuscular junction and therefore allows for identification of myoblasts.

For intravital fluorescence labeling, PKH-26 dye (Sigma Chemical Company, St. Louis, MO, USA) with an excitation maximum at 551 nm was used. The dye was deposited in the membrane of viable cells. The body of labeled cells fluoresced red, whereas the nucleus remained unstained. After cell death, the dye left the cell membrane. Therefore, detected fluorescing cells were regarded as viable.

Skeletal myoblasts were incubated with 10 mL trypsin at 37°C and 5% CO_2 for 3 minutes. Thereafter, they were centrifuged 2 times at 4°C and 1000 U/min for 10 minutes each. After resuspension of the pellet in SGM, the cell number was determined using the Neubauer cell-count chamber. For labeling, 4 μL PKH-26 was added to 1 mL Diluent C (Sigma) and mixed with the resuspended cells for 3 minutes. The reaction was stopped using 1% bovine serum albumin for 1 minute. After 4 mL cell medium (SGM) was added, the suspension was centrifuged again for 10 minutes to wash out any remaining dye. Thereafter, the number of viable cells was evaluated. After the cells were plated for 2 more days, the success of the labeling procedure was confirmed by results of fluorescence microscopy of the cultured cells.

For cell transfer, the cells were trypsinized, centrifuged, and resuspended in SGM. This cell suspension was injected into the myocardium of 30 inbred Lewis rats (mean weight, 381 ± 44 g). For transplantation, the animals were anaesthetized by intramuscular injection with 0.25 mg midazolam and 6 mg ketamin. While breathing was maintained with assisted ventilation, the xyphoid was dissected using a median incision, and the apex of the left ventricle was exposed through the diaphragm. A total of 0.1 mL of the cell suspension containing 10^6 labeled myoblasts was injected into the apex region of the heart. After closing of the incisions and recovery, the animals were divided into 3 groups (Table 1). The groups differed with regard to the end of the study period: group A, 7 days; group B, 14 days; and group C, 28 days. One further group, group D (n = 6), served as a negative control group. Rats in this group received injection of only cell medium. Animals from each group, including 2 animals of the control group, were sacrificed at each time point. The hearts were then explanted and conserved in fluid nitrogen.

After explantation, serial frozen sections of the apex region of the hearts (average thickness, 8 μm) were prepared and surviving labeled cells were detected by scanning with a fluorescence microscope (Axiovert 35; Carl Zeiss, Baden-Wuerttemberg, Germany). For further characterization, differential staining of the sections was performed using antibodies against desmin, α-actin (Chemicon, Hofheim, Germany), and n-cam (a gift from Professor Lochmueller, Gene Centre, Munich). For visualization, an antimouse antibody (Antimouse Ig fluorescein; Chemicon) was used, fluorescing at 495 to 520 nm.

RESULTS

Light microscopy of the culture dishes showed vital and normal configured myoblasts before and after the labeling procedure in all cases (Figure 1A). Fluorescence of the labeled cells on the culture dishes (Figure 1B) proved the cell labeling to be successful. The staining procedure, however, reduced the number of viable cells: 6% to 13% (mean, 8%; n = 30) of the initially labeled cells died during or directly after incubation with PKH-26. Nevertheless, during the 2 days of culturing following the labeling procedure, several myoblasts began dividing. Most cells resulting from these divisions showed only very poor fluorescence, and some cells showed no fluorescence at all. These findings indicated that the dye was not sufficiently transmitted during cell division. Fewer than 2% of the cells, however, were unlabeled. The cultured cells expressed n-cam, as was proven by immunohistochemical staining results (Figure 2).

All animals (study groups, n = 30; control group, n = 6) tolerated the cell transfer without complications. There were no late deaths during the study. Surviving transplanted cells were found in 9 of 10 animals of group A and C and in 8 of 10 animals of group B (Table 2). Labeled cells were found in at least 40 sections of each heart. No fluorescing cells were detected in the hearts of the control group.

The detected cells were distributed within the host myocardium, in the intercellular spaces between the myocardial fibers (Figure 3). No scar formation or inflammatory reaction was seen at the implantation site. Only 2 of the animals in group A (22%) showed slight edema surrounding the transplanted cells, whereas this finding was not seen in any of the animals of the other 2 study groups.

Immunohistochemical staining for n-cam (Figures 4) revealed more n-cam–positive cells than PKH-26–labeled cells. Within these sections, n-cam–positive myotubes were also found. The cells and myotubes were also positive for desmin (Figure 5) and α-actin. The desmin myofibrils, however, did not form structures as highly organized as those within the myocardial tissue surrounding them.

DISCUSSION

Recent studies proved that autologous myoblast transfer could be performed successfully [Koh 1993, Greentree 1994, Zibaitis 1994, Chiu 1995, Yoon 1995, Murry 1996, Atkins 1999, Taylor 1998, Dorfman 1998]. Even short-term functional improvement after cell transplantation was reported [Taylor 1998]. These cells represent an ideal substitute for lost myocardial tissue because they can be easily obtained for

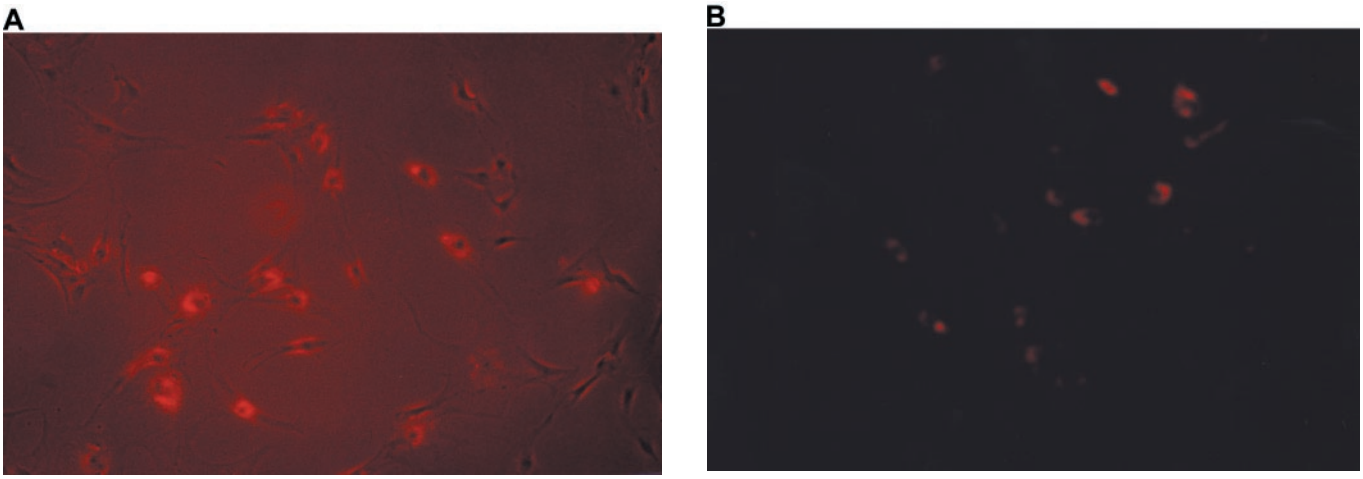

Figure 1. A, Primary skeletal myoblast in culture, combination view using light and fluorescence microscopy (original magnification 100×). Labeled myoblasts (red ◎) can be identified, but rare unlabeled cells can also be seen. B, Same myoblast culture, view through the fluorescence microscope alone at 551 nm. Labeled myoblasts show red fluorescence ◎.

transplantation. Furthermore, immunosuppressive therapy, which is necessary in embryonic cardiac cell transplantation, can be avoided in myoblast transplantation. In all cell transplantation experiments, reliable labeling of the transferred cell is an important issue.

In our experiments, the cells were labeled using a fluorescing dye that is incorporated into the cell membrane. Approximately 8% of the initial cells did not survive the labeling procedure, a rate of cell loss considered acceptable. The success of the labeling procedure was proven by results of subsequent fluorescence microscopic examination of the cell culture dishes. After a resting period of 2 days, the cells were trypsanized and injected without further passaging. The resting period allowed the surviving cells to recover from the labeling procedure so that only vital cells in good condition were injected. Additionally, the reculturing of the cells in fresh growth medium prevented contamination by free fluorescence dye, thus avoiding potential artifacts within the sections. Because labeling using this fluorescence dye did not require selection passages, the cells maintained their capacity for cell division and thus increased in number after the labeling procedure. This preservation of the capacity for cell proliferation is an important advantage of this technique compared to viral-mediated transfection techniques [Greentree 1994, Chiu 1995, Murry 1996]. Primary myoblasts have only limited capacity for cell division. Therefore, the selection passages required for transfection techniques reduce the number of potential cell divisions. Lipid-mediated DNA transfection also requires selection passages [Greentree 1994] and thus has the same disadvantages as viral-mediated transfection techniques.

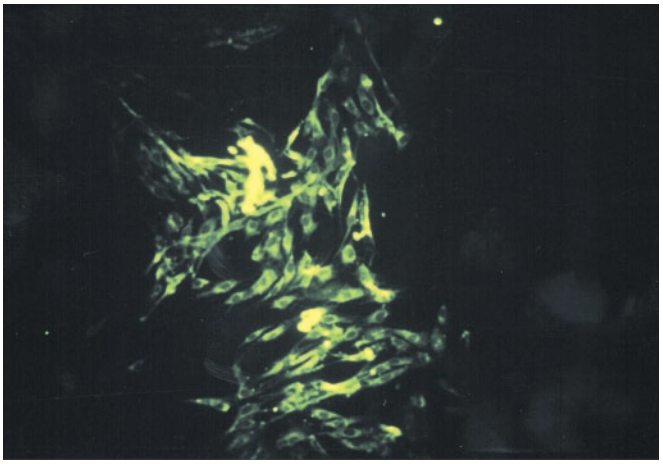

Figure 2. Immunohistochemical staining of cultured myoblasts for n-cam, fluorescence microscope at 495 to 520 nm (original magnification 100×). N-cam is distributed over the cell surface so the nucleus remained unstained.

Because only viable cells kept the fluorescence dye in their cell membrane, this labeling method proved to be very reliable for detection of surviving cells after transplantation. The cell products (for example, green fluorescing protein) could be detected even after cell death, so this method might be advantageous compared to adenovirus-mediated gene transfer of cell markers. Although the labeling technique was primarily successful in all cases, in 4 hearts of the study groups no fluorescing cells could be found. This result might have

Table 2. Results of Cell Transplantation and Immunohistochemical Staining

Explantation date	7 days	14 days	28 days	Controls
Labeled cells found	9/10	8/10	9/10	0/6
Cells n-cam positive	+	++	+++	–
Labeled cells desmin positive	++	++	++	–

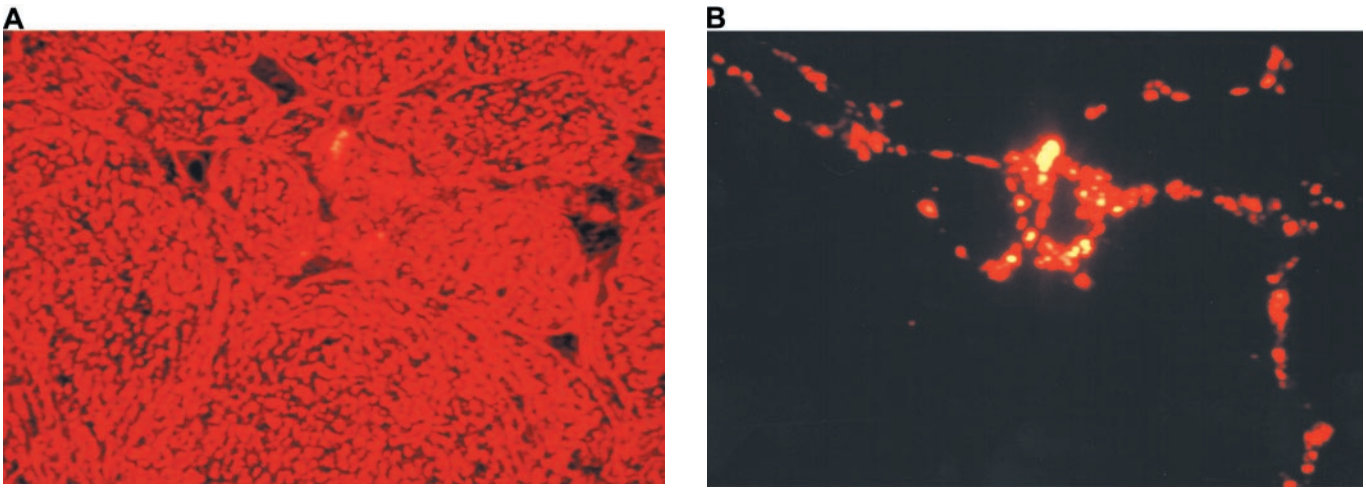

Figure 3. A, Myoblasts 28 days after cell transfer within the host myocardium viewed with both fluorescence and light microscopy to identify the labeled fluorescing cells within the myocardium. Because the intensity of the light microscope dominates, only larger numbers of labeled cells can be identified (yellow ◎). No scar or inflammatory reaction can be seen. B, Same section, viewed with the fluorescence microscope alone. Fluorescing myoblasts lie in the spaces between the myocardial fibers.

been caused by incorrect injection of the cells into the host myocardium: most probably, the needle penetrated the ventricular wall, resulting in injection of the cell suspension into the left ventricle with subsequent dissemination of the cells over the entire body of the animal. Another possible reason might be death of the implanted cells caused by prolonged ischemia after trypsinization. Furthermore, the cell concentration of 10^6 cells per 100 μL was rather high, so the cells could have been affected by a relative lack of oxygen and nutrition supply.

The only disadvantage of the present labeling technique was the dilution of the labeled cells by subsequent cell divisions. In former experiments, transfection with the lac-Z gene before transplantation proved to be a very reliable labeling technique [Greentree 1994, Chiu 1995, Murry 1996], also allowing for detection of cells originating from cell divisions following the labeling procedure. The necessity of having several cell passages for virus-mediated transfection and consecutive selection, however, represents the major disadvantage of these techniques. Alternatively, marking the cells with ^{3}T-thymidine resulted in dilution by cell division and proved to be too toxic for in vivo use [Greentree 1994]. In contrast, no toxic effects of the fluorescence dye were demonstrated in our study.

N-cam is a surface antigen expressed specifically by myoblasts. It is involved in the formation of neuromuscular junctions during muscle development [Walsh 1990, Cifuentes-Diaz 1993], resulting in expression by myoblasts and early myotubes. This antigen could be used for detection of myoblasts and myotubes surrounding the injected labeled cells, because cardiomyocytes did not express n-cam in a relevant quantity. In the sections of hearts explanted after 2 and 4 weeks postoperatively, more n-cam–positive than labeled cells were found. These results indicated further cell division of the transferred myoblasts within the myocardium. The cells also started to form myotubes, the first step in myoblast differentiation [Walsh 1990, Cifuentes-Diaz 1993]. This result confirmed the findings of other groups [Koh 1993, Atkins 1999]: Atkins et al [1999] showed that transplanted myoblasts further divided and formed 2 populations in the recipient. One of these populations maintained the ability for further cell divisions and differentiation into myofibers, whereas a smaller number formed satellite cells. Because these cells serve as a stem cell reserve for further regeneration in skeletal muscles, the transplanted myoblasts could not only

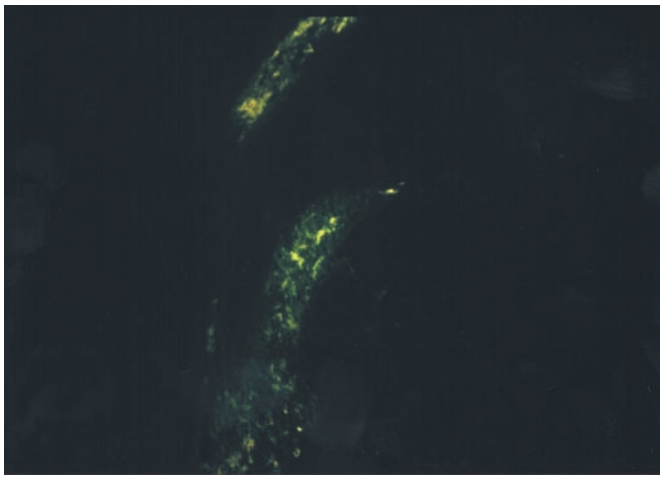

Figure 4. Immunohistochemical staining for n-cam (495-520 nm, original magnification 100×). Myoblasts with strongly positive staining (green ◎) and newly formed myotubes are found around the implantation site. Staining of the surrounding myocardium was completely negative for n-cam. More n-cam–positive than labeled cells were found, indicating further cell division.

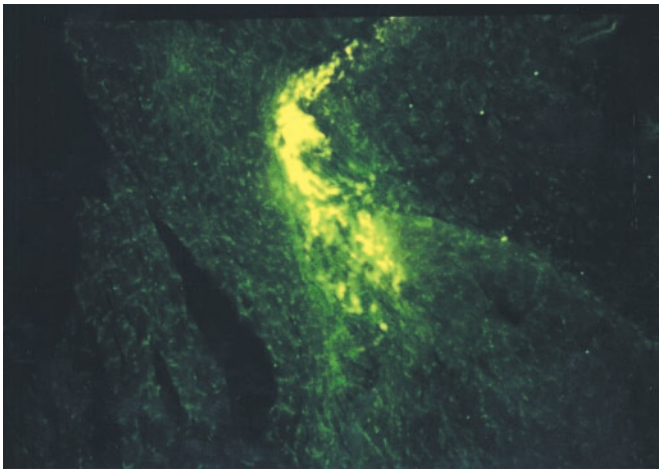

Figure 5. Immunohistochemical staining for desmin (495-520 nm, original magnification 100×). Desmin was expressed within the myocardium as well as within the implanted myoblasts. Myoblast staining for this myofilament was strongly positive, proving that the implanted cells build muscle-specific proteins.

form stable intracardiac grafts but also build a stem cell reservoir within the heart.

Despite these findings, the further differentiation pattern of skeletal myoblasts within the myocardium is not yet clear. Although differentiation of skeletal myoblasts into myocardialike cells that build gap junctions has been reported [Zibaitis 1994, Chiu 1995, Dorfman 1998], Murry et al [1996] reported only differentiation into fatigue-resistant skeletal myofibers. In these experiments the cells were transferred only onto myocardial scar tissue [Koh 1993, Zibaitis 1994, Chiu 1995, Murry 1996, Atkins 1999], and thus they were prevented from direct contact with cardiomyocytes. Direct contact with the myocardium, however, may play an important role in the further differentiation of the transplanted cells. For integration, the cells must have close contact with the myocardium to form tight junctions. In this study, no scar tissue was seen surrounding the transplanted cells. They lay side-to-side with the myocardial cells and even formed myotubes that stained positive for desmin, which is expressed by cardiomyocytes and by skeletal myoblasts, myotubes, and muscle fibers. After immunohistochemical staining, myoblasts and myotubes expressed desmin in large quanties. This desmin expression proved the cells to be of muscular origin and able to form muscle fibers. Within the study period, however, the organization of the cells and myofilaments did not reach the perfect arrangement found within the surrounding cardiomyocyte area.

The positive staining for n-cam and desmin, together with the lack of any scar tissue surrounding the transplanted cells, made integration of the myoblasts within the myocardial tissue likely. These findings proved that skeletal myoblasts can be transplanted into vital myocardium. Additionally, the cells maintained their ability to divide further and started to differentiate into myotubes.

CONCLUSIONS

Labeling myoblasts with PKH-26 intravital fluorescence dye was an uncomplicated and reproducible method that allowed for detection of transplanted myoblasts within the host myocardium for at least 4 weeks. Myoblasts transplanted into vital myocardium maintained the capability for cell division and differentiation.

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