Noncultured, Autologous, Skeletal Muscle Cells Can Successfully Engraft Into Ovine Myocardium

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Background—There is compelling evidence showing that cellular cardiomyoplasty can improve cardiac function. Considering the potential benefit of using noncultured muscle cells (little time, lower cost, reduced risk of contamination), we investigated the feasibility of grafting cells obtained directly after enzymatic dissociation of skeletal muscle biopsies into ovine myocardium. We hypothesized that those noncultured muscle cells would engraft massively.

Methods and Results—Autologous, intramyocardial skeletal muscle cell implantation was performed in 8 sheep. A skeletal muscle biopsy sample (∼10 g) was explanted from each animal. The sheep were left to recover for ∼3 hours and reanesthetized when the cells were ready for implantation. A left fifth intercostal thoracotomy was performed, and 10 epicardial injections of the muscle preparation (between 10 and 20 million cells) were carried out. All sheep were euthanized 3 weeks after myocardial implantation. Immunohistochemistry was performed with monoclonal antibodies to a fast skeletal isoform of myosin heavy chain. Skeletal myosin heavy-chain expression was detected in all slides at 3 weeks after implantation in 8 of 8 animals, confirming engraftment of skeletal muscle cells. Massive areas of engraftment (from 2 to 9 mm in diameter) or discrete loci were noted within the myocardial wall.

Conclusions—Our results indicate that noncultured skeletal muscle cells can successfully and massively engraft in ovine myocardium. Thus, avoiding the cell culture expansion phase is feasible and could become a promising option for cellular cardiomyoplasty. (Circulation. 2003;107:3088-3092.)

Key Words: heart • cells • transplantation • infarction • muscles

The concept of myogenic cell transplantation into the myocardium, known as cellular cardiomyoplasty (CCM), is based on the contribution of exogenous cells to replace lost or altered cardiomyocytes to restore functional performance of the heart.¹ There is a large body of evidence showing that CCM can improve cardiac function in ischemic heart disease,²–¹¹ as well as dilated cardiomyopathy,¹²–¹⁷ in numerous animal models.

Most research teams have addressed autologous CCM in a 3-phase process: biopsy, ex vivo cell culture/expansion, and surgical or catheter-based cell delivery. Considering the potential benefit of using noncultured muscle cells (little time, lower cost, reduced risk of contamination), we investigated the feasibility of grafting noncultured cells into ovine myocardium.

Methods

Animal Model

The study was approved by the institutional ethics committee for animal research, and all animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996. Autologous, intramyocardial skeletal muscle cell extract implantation was carried out in eight 1-year-old Ile de France sheep weighing 63 to 67 kg (Lycée Agricole et Vinicole, Crézancy, France). Three other animals were injected with control medium.

Skeletal Muscle Biopsy

A skeletal muscle biopsy sample (∼10 g) was explanted from the left posterior femoral biceps of each sheep under sterile conditions. The biopsy tissue was kept in Dulbecco’s modified Eagle’s medium (DMEM, with Glutamax-I, sodium pyruvate, 4500 mg/L glucose, and pyridoxine; GIBCO) at room temperature until mechanical and enzymatic digestions were started. The operative wound was closed in a routine fashion. The animals were left to recover for ∼3 hours and reanesthetized when the cells were ready for implantation.

Muscle Cell Extraction

The explanted skeletal muscle fragments were weighed and washed in DMEM. Adipose tissue and fascia were removed, and the muscle was minced with scissors to a slurry. The muscle fragments were then sedimented in DMEM at 300 rpm for 2 minutes, and the...
supernatant was discarded. To release satellite cells, the muscle fragments were then incubated at 37°C under agitation in 10 mL DMEM supplemented with 0.4% (wt/vol) crude collagenase (type IA, Sigma). After 20 minutes, the fragments were centrifuged at 300 rpm for 2 minutes. The supernatant containing isolated cells was stored in 20% (vol/vol) fetal calf serum–completed DMEM; the pellet was then subjected to 4 more rounds of digestion, as previously described. The extracted cells were then filtered through a 250-μm nylon cell strainer (Polylabo SA).

**Cell Labeling**

For labeling the nuclei, cells were resuspended in 10 mL serum-free DMEM containing 25 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 10 minutes. The cells were rinsed 4 times in DMEM to remove unbound DAPI. We took advantage of the DAPI labeling to estimate the number of mononucleated cells in the preparation with the use of a hemocytometer and a fluorescence microscope. The cell preparation was then resuspended in 1.2 mL serum-free DMEM and kept at 4°C until implantation.

**Cell Implantation Into the Myocardium**

Animals were anesthetized with an injection of 10 mg/kg thiopental IV, followed by isoflurane in 100% oxygen by inhalation. A constant-rate infusion of lidocaine (50 μg·kg⁻¹·min⁻¹) was started immediately after incision. A left fifth intercostal thoracotomy was performed, and the heart was suspended in a pericardial cradle. Landmarks were made with 5/0 polypropylene suture material on the left ventricular free wall. Ten epicardial 100-μL injections of the cell preparation (n=8) or medium (n=3, control animals) were carried

A through D, Specificity of MY32 immunohistochemistry. Upper left, MY32 immunohistochemistry on sheep skeletal muscle. Note typical fast-twitch (dark brown) and slow-twitch aspect of biopsied muscle (bar=25 μm). Upper right, MY32 immunohistochemistry on sheep myocardium with engrafted noncultured cells (bar=25 μm). Lower left, control MY32 immunohistochemistry in normal sheep heart (bar=250 μm). Lower right, control MY32 immunohistochemistry in sheep heart with media injections (bar=250 μm). E, Detection of engrafted skeletal muscle cells with antibody to fast skeletal muscle–specific isoform of myosin heavy chain (MY32). Massive areas of engraftment (from 2 to 9 mm in diameter) or discrete loci were noted within myocardial wall (bar=250 μm). F, Detection of engrafted skeletal muscle cells with antibody to fast skeletal muscle–specific isoform of myosin heavy chain (MY32). Inside myocardial wall, MY32-positive fibers were generally aligned with native cardiomyocytes. Some fibers were intensely marked by MY32 antibody, and some were only faintly marked (bar=500 μm). G, Codetection of engrafted skeletal muscle cells and native cardiomyocytes with antibody to fast skeletal muscle–specific isoform of myosin heavy chain (MY32, brown) and gap junction protein connexin-43 (red). Assay shows absence of connexin-43 within grafted cells or between grafted cells and their microenvironment (bar=100 μm).
out with a 27-gauge infusion set (Vygon). A 0.1 mg/kg IM injection of dexamethasone was performed before closure. The thorax was closed in a routine manner, and the animals were left to recover with the required analgesic regimen after surgery (morphine 0.5 mg/kg IM BID, flunixin 1 mg/kg IM once) and on the following day as needed.

**Cell Culture Controls**

To confirm the presence of muscle precursor cells in the cell preparation, a 100-μL aliquot of the cell suspension was plated in a culture flask containing fetal calf serum–complemented DMEM. The cells were then grown as a control in humid air with 5% CO₂. Three to 7 days after plating, cells were processed for immunodetection of the skeletal muscle–specific regulatory factor MyoD (DAKO), as previously described.¹⁹

**Histology**

All sheep were euthanized 3 weeks after myocardial implantation. Heparin (10,000 IU) and 60 mg/kg sodium pentobarbital were injected intravenously. The heart was exposed through a left thoracotomy. The site of myocardial injury was identified and dissected free of adhesions. The hearts were then perfusion-fixed with 10% formalin for histological evaluation.

Formalin-fixed, paraffin-embedded blocks were processed. Serial 5-μm sections from the harvested area were prepared for conventional hematoxylin and eosin staining and were immunostained with use of an automated immunoperoxidase technique (Ventana Medical Systems) with monoclonal antibodies to a fast skeletal muscle–specific isoform of myosin heavy chain (MY32, Sigma) and to connexin-43 (Sigma), a component of gap junctions.

In brief, deparaffinized sections were blocked for endogenous peroxidase activity and subjected to antigen retrieval. Sections were exposed to a biotinylated anti-mouse secondary antibody. Sections were developed with dianisobenzidine or alkaline phosphatase, counterstained with hematoxylin, and postcounterstained with a bluing reagent. The specificity of MY32 is well known for skeletal muscle fast myosin heavy chain in general and particularly in sheep.¹³,²⁰ We also performed control studies: MY32 immunohistochemistry was always negative in ovine myocardium. Ovine skeletal muscle biopsies, however, were always positive for MY32, displaying the typical aspect of fast (intensely marked) and slow (faintly marked) fibers (see Figure, A through D). Measurement of engraftment areas was performed with a micrometer eyepiece, by assessing the maximal long axis of MY32-positive areas in the myocardial wall.

**Results**

The cell injection procedure was carried out uneventfully in all animals. Ventricular arrhythmia occurred on needle insertion and cell injection but ceased as the needle was withdrawn. Cell counting allowed us to determine that 1.7±0.3×10⁶ mononucleated DAPI-labeled cells were injected per animal. The clinical status of all the animals remained normal until the day of sacrifice. No DAPI marking was found in the explanted hearts, presumably owing to active cellular division leading to a dilution of DAPI. Indeed, in culture controls, DAPI was undetectable as well after 6 to 8 cell population doublings (data not shown).

Control culture flasks were inspected daily. Immunodetection of the skeletal muscle–specific regulatory factor MyoD showed that ≈50% of the cultured cells were skeletal muscle precursor cells. When the cells were left to fuse, all flasks showed numerous myotubes after 1 week of culture.

Skeletal myosin heavy-chain (MY32) expression was detected at 3 weeks after noncultured cell implantation in 8 of 8 animals, thus confirming cell survival and myogenic expression of implanted cells. Massive areas of engraftment (from 2 to 9 mm in diameter) and discrete loci were noted within the myocardial wall (Figure, E). A few isolated muscle cells were also present within the pericardial fat tissue. Inside the myocardial wall, the MY32-positive fibers were generally aligned with native cardiomyocytes. Some of these fibers were intensely marked by the MY32 antibody, and some were only faintly marked (Figure, F). None of them displayed connexin-43, as assayed by immunohistochemistry (Figure, G). Implanted skeletal muscle cells developed organized sarcomeres either with the elongated morphology characteristic of fused, multinucleated myotubes or as mononuclear cells. They had either peripheral nuclei or centrally located nuclei. Replacement fibrosis and areas showing inflammatory, mononuclear cells were also present. Myocardium of control animals implanted with medium alone did not show any MY32 expression.

**Discussion**

The report presented here on a large-animal model draws attention to the interest of noncultured muscle cells as a potential cell source for CCM. A previous report pointed to the potential use of minced muscle preparations instead of cultured cells on a myocardial infarction model in the rat.⁸ There was no improvement in heart function observed after implantation of minced muscle. In contrast, transplantation of cultured myoblasts improved left ventricular ejection fraction at both 1 and 2 months after cell grafting. No details on the histological findings for the minced-muscle group were provided. The conclusion of the article was that it is not possible to bypass the phase of in vitro cell expansion and solely rely on immediate transplantation of extemporaneously minced muscle. Our findings challenge these results, in that they show that muscle cell implantation can be achieved without the in vitro expansion procedure most research teams have used so far. One specific difference between the study by Pouzet et al⁸ and our study is that we did not stop at the muscle slurry phase of the cell preparation but carried out the process to the phase of cell isolation by enzymatic digestion.

As we were preparing this manuscript, a report by Suzuki et al²¹ provided convincing evidence that small numbers of noncultured, skeletal muscle cells can efficiently colonize the rat myocardium. They grafted single fibers, the minimal functional units of skeletal muscle. This technique, unlike our protocol, requires a muscle biopsy from tendon to tendon, which might not be clinically applicable.

There are 3 practical advantages of using noncultured cells as opposed to cultured, satellite cells that are commonly used: (1) Cell culture can be a hazardous procedure (viral infection, prion, bacterial, and fungal contamination)²²; (2) Cell culture generally requires several days or weeks to yield the estimated necessary number of cells for CCM. If being able to graft healthy cells soon after the infarct is important, then...
avoiding the time required for cell culture could definitely be an advantage; and (3) Cell culture is expensive.

In our experience, noncultured, skeletal muscle cell implantation was feasible. Numerous sections presented large areas covered with skeletal myosin heavy-chain (MY32)-positive fibers at 3 weeks after implantation. The MY32-positive cells were DAPI-negative. Considering the small number of cells injected (a maximum of $20 \times 10^6$ cells, with 50% of them being identified as skeletal muscle precursor cells in control cultures) and the extensive engraftment observed on many sections of all hearts, active cellular divisions must have occurred. Cell division probably accounts for dilution of the DAPI blue fluorescence, as was observed in vitro by our group and others after 6 to 8 cell cycles.

The fact that we could not demonstrate the presence of the gap junction protein connexin-43 between grafted cells and the microenvironment most likely means that there is no electrical coupling between grafted myoblasts/myotubes and cardiomyocytes. Although the presence of connexin-43 between skeletal cells and cardiomyocytes in vitro and in vivo was reported by other groups, most publications do not mention the presence of connexin-43 between grafted cells and resident cardiomyocytes.

One limitation of our study is that only the progeny of myosatellite cells (myocytes or myotubes) present in the muscle preparation were detected, because they can be readily distinguished from resident cells. The initial cell preparation is heterogeneous; it contains myoblasts, fibroblasts, adipocytes, peripheral nerve cells, endothelial cells, and smooth muscle cells. Furthermore, cell preparations from sheep skeletal muscle biopsy samples might also carry pluripotent stem cells, as was reported in mice. Determining the fate of noncultured donor cells and whether they could contribute to CCM requires genetic labeling. This issue is currently under investigation by our group.

Our results show that noncultured cells can successfully and massively engraft into ovine myocardium. It is even possible that avoiding the tissue-culture phase improves the efficacy of engraftment into the myocardium, as was previously reported in skeletal muscle. Are noncultured cell muscle preparations more potent than cultured muscle cells? Which method yields the greatest number of implanted cells in vivo? Our study does not specifically address these issues, which would require histological studies that compared cultured and noncultured muscle cells. Although preliminary, this investigation paves the way for further quantitative and functional studies. Ongoing work in our laboratory is focused on the benefit of grafting noncultured cells in dilated and ischemic cardiomyopathy.

In conclusion, our data indicate that avoiding the cell culture expansion phase is feasible and could become a promising option for CCM.

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References


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