Survival and Function of Bioengineered Cardiac Grafts

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Introduction—Patients with congenital heart disease frequently require graft material for repair of cardiac defects. However, currently available grafts lack growth potential and are noncontractile and thrombogenic. We have developed a viable cardiac graft that contracts spontaneously in tissue culture by seeding cells derived from fetal rat ventricular muscle into a biodegradable material. We report our investigations of the in vitro and in vivo survival and function of this bioengineered cardiac graft.

Methods and Results—A cardiomyocyte-enriched cell inoculum derived from fetal rat ventricular muscle was seeded into a piece of Gelfoam (Upjohn, Ontario, Canada), a biodegradable gelatin mesh, to form the graft. For in vitro studies, growth patterns of the cells within the graft were evaluated by constructing growth curves and by histologic and immunohistologic examination; in vivo studies, the graft was cultured for 7 days and then implanted either into the subcutaneous tissue of adult rat legs or onto myocardial scar tissue in a cryoinjured rat heart. Five weeks later, the graft was studied histologically. The inoculated cells attached to the gelatin mesh and grew in 3 dimensions in tissue culture, forming a beating cardiac graft. In both the subcutaneous tissue and the myocardial scar, blood vessels grew into the graft from the surrounding tissue. The graft implanted into the subcutaneous tissue contracted regularly and spontaneously. When implanted onto myocardial scar tissue, the cells within the graft survived and formed junctions with the recipient heart cells.

Conclusions—Fetal rat ventricular cells can grow 3-dimensionally in a gelatin mesh. The cells in the graft formed cardiac tissue and survived and contracted spontaneously both in tissue culture and after subcutaneous implantation. Future versions of this bioengineered cardiac graft may eventually be used to repair cardiac defects. (Circulation. 1999;100[suppl II]:II-63–II-69.)

Key Words: heart defects, congenital & myocytes & myocardial infarction & myocardial contraction

Congenital heart disease affects approximately 1% of live births.1 Many of the affected children require surgical intervention, which may include patch reconstruction of stenotic lesions or conduit placement for complex congenital lesions.2 Although several graft materials have been available for surgery of congenital heart defects over the last 40 years, their long-term results have been compromised by material-related failure.3 Most significantly, all currently available patch and conduit materials lack growth potential. In addition, they are both noncontractile and thrombogenic.

A viable, autologous, contractile, and less thrombogenic bioengineered tissue graft would be ideal for the surgical repair of congenital cardiac defects. Implanted as a substitute for the wall of a cardiac chamber or an artery, the graft should be able to grow and remodel with the growth of the child. These tissue-engineered grafts have the potential to reduce morbidity and mortality and improve the quality of life of children with congenital heart disease.

This study was designed to create a cell-seeded biodegradable polymer scaffold, describe its initial characterization, and examine its course when implanted as a graft in an animal model. With further development, it may be possible to create a contractile, nonthrombogenic graft that grows and remodels in the same fashion as the cardiac structure which it replaces.

Methods

In Vitro Study

Cell Isolation and Culture

Timed, pregnant, inbred Lewis rats (Charles River, Montreal, Canada) were anesthetized with an intramuscular injection of ketamine (22 mg/kg body weight) and euthanized with an intrathoracic injection of Euthanyl (2 mL/4.25 kg body weight) (MTC Pharmaceuticals). The fetal rat hearts (19 days gestation) were rinsed 3 times in PBS (NaCl 136.9 mmol/L, KCl 2.7 mmol/L, Na2HPO4 8.1 mmol/L, and KH2PO4 1.5 mmol/L, pH 7.4). The blood clots and atria were removed, as were the macroscopically visible blood vessels and connective tissue. The ventricles were rinsed in PBS 3 times and minced into pieces <1 mm3. The tissue was then transferred to a tube containing a mixed enzyme solution (0.2% trypsin, 0.1% collagenase, and 0.02% glucose) in 37°C for 20 minutes. The enzyme solution containing the digested tissue was neutralized with an equal volume of culture medium (Iscove’s modified Dulbecco’s medium), supplemented with 10% fetal bovine serum, 0.1% β-mercaptoethanol (0.1 mmol/L), and 1% penicillin (100U/mL) and streptomycin (100 μg/mL).4–6 Clumps of cells suspended in solution were dispersed by repeated pipetting. The cell
suspension was centrifuged at 580 g for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in culture medium. The cell suspension was then divided into 4- to 6-cell culture dishes (10 cm in diameter), and Iscove’s modified Dulbecco’s medium was added to each of the dishes to a total volume of 11 mL. Cells were cultured at 37°C, in 5% CO₂ and 95% air. Culture medium was changed every 2 days.

Creation of the Cell-Seeded Graft
After isolation, the fetal rat ventricular cells were cultured for 24 hours before use in preparation of the graft. The cell culture medium was discarded and the dishes were rinsed 3 times with PBS. One milliliter of trypsin solution (0.05%) with EDTA (2 μL/ml) was added to each of the dishes. The dishes were placed into the incubator for 2 to 3 minutes; 2 mL of cell culture medium were added to the dishes to stop the digestion. After being pipetted, the suspension was collected in a 50-mL tube. The cell number was determined with a cell counter (Coulter Electronics Ltd). The tube containing the cell suspension was centrifuged for 5 minutes at 580g. After the supernatant was removed, the cell pellet was resuspended in a small volume of culture medium (4×10⁶ cells/mL). The suspension was slowly placed onto the surface of squares of presoaked Gelfoam which were 5 mm in length on each side for the in vitro studies and 15 mm in length on each side for the in vivo studies. After another 30 minutes, 5 mL of culture medium was carefully added along the side of the patch. Two hours later, more medium was added to submerge the patch totally. Grafts were incubated at 37°C, in 5% CO₂ and 95% air, and medium was changed every 2 days. For use as controls, squares of Gelfoam were placed in culture medium without any cell inoculation.

Histologic Studies
Ten days after seeding the cell inoculum onto the Gelfoam, grafts were fixed either with formalin or with tissue fixative for electron microscopy. Three days later, the grafts fixed with formalin were stained with hematoxylin and eosin. Electron microscopic specimen preparations were fixed for at least 6 hours, after which the grafts were cut into 1 mm cubes. Transmission electron microscopic examination was then performed on the sectioned grafts.

Growth of Cells in the Graft
A Gelfoam mesh was cut into 5×5×5 mm cubes, each of which was seeded with an identical aliquot of cells and cultured as described above. Every other day, starting on day 2 and continuing to day 26, the grafts were digested and the cells within the graft counted (n=4 grafts per time point). Each piece of graft was washed with PBS and digested with 7 mL of PBS solution containing 0.1% collagenase (273U/mg, type II), stirring until the Gelfoam was completely digested (about 5 to 8 minutes). For each graft, the cells in the
enzyme solution were counted in duplicate using the cell counter (Coulter Electronics Ltd), and the cell growth curve was then plotted.

In Vivo Study

Myocardial Injury and Repair Using the Cardiomyocyte-Seeded Graft

Twenty-four male inbred Lewis rats, weighing from 325 to 350 g, underwent left ventricular myocardial scar generation by a cryoinjury technique, previously reported from our laboratory. Briefly, the heart was exposed through a left thoracotomy under general anesthesia. A cryoprobe, the temperature of which was maintained by immersion in liquid nitrogen, was used to freeze the left ventricular free wall 15 times. The incision was then closed and the animal was monitored until completely recovered from the anesthesia.

Three weeks after scar generation, the rats were randomly divided into 3 groups (n=8 for each), to undergo implantation of a cell-seeded graft, implantation of an unseeded graft (to determine the effect of the gelatin mesh itself), or a sham operation (to determine baseline function after myocardial scar generation). The hearts were exposed through a midline sternotomy. The grafts, which had been seeded with cells 7 days previously in the experimental group, were sutured to the surface of the scar with 1 suture at each of the 4 corners of the patch. Before the last suture was placed, a scalpel blade was used to score the surface of the scar in a crosshatch pattern, to a depth sufficient to cause minor oozing of blood from the epicardial surface. Close attention was paid to make sure that the top surface of the patch, on which the cells had been seeded, was placed in direct contact with the surface of the scar. In the sham operation group, the procedure was identical except that no Gelfoam patch was sutured to the left ventricular scar. Five weeks after patch implantation, heart function was evaluated using a Langendorff apparatus, a micromanometer, and an intraventricular balloon. Systolic, diastolic, and developed pressures were measured over a range of balloon volumes. The heart was fixed in formalin, in distention at a pressure of 30 mm Hg within the left ventricle, after completion of these studies of ventricular function.

Implantation of the Graft Into the Subcutaneous Tissue

In order to assess graft contractility without the influence of underlying myocardium and myocardial scar, we also implanted cell-seeded grafts into the subcutaneous tissue of adult rats. In 4 of 8 rats from each group receiving a graft (total n=8), 2 small pieces were cut from the large patches, with or without seeded cardiomyocytes, according to the group, and were implanted into the subcutaneous tissue of the groin area, 1 piece on each side. This procedure was performed on the same day that the large graft was sutured onto the surface of the left ventricular scar tissue. Five weeks after implantation, the small grafts were harvested from the groins and fixed in formalin.

Bromodeoxyuridine Preparation and Labeling

Either 24 hours or 7 days (randomly divided within each group) before evaluation of ventricular function, 12.5 mg of bromodeoxyuridine (BrdU) was dissolved in 0.2 mL of dimethyl sulfoxide and 0.3 mL of distilled water. The solution was sterilized using a microfilter (Millipore) and injected subcutaneously.

Figure 3. Electron micrograph of a cell-seeded graft cultured for 10 days. Multiple sarcomeres (S) were noted in cardiomyocytes within the graft, consistent with the rhythmic contraction of grafts observed since the second day in culture. Black strip with Y-shaped tip (arrow) represents the remainder of the Gelfoam mesh.
Morphological and Histological Studies
After fixation in formalin for at least 36 hours, morphological studies were performed as previously described. Briefly, the atria were removed and the heart size, scar size, and heart weight were measured. The ventricles were then sectioned along the long axis in 3-mm slices. Computerized planimetry was used to determine the thickness of the left ventricle, the lengths of both the scar and the left ventricular free wall, and the volume of the left ventricle. The second and the third sections, counted from the apex, along with the graft obtained from the groin area, were stained with hematoxylin and eosin and Brdu.

Results

In Vitro Study
The Gelfoam mesh blocks, seeded with the cell inoculum derived from fetal rat ventricular muscle, formed a cardiac-like tissue in the culture dish. Three to 4 days after seeding, the mesh grafts started beating regularly and spontaneously. The number of cells within the grafts increased progressively during the 26-day period in tissue culture (Figure 1). The number of cells increased slightly in the first 10 days, then rapidly between day 10 and day 20. The cells then stopped growing transiently for 4 days, continuing after day 24. Histologic evaluation showed that the cells grew into the interstices of the grafts (Figure 2). Electron microscopy demonstrated sarcomeres in cardiomyocytes within the grafts (Figure 3). The gelatin mesh did not dissolve in the culture dish. Cell-seeded grafts have survived in tissue culture as long as the 2 month duration of our experiments and continued to contract regularly.

In Vivo Study
Seven days after the cell-seeded grafts were implanted into the subcutaneous tissue of the rats (n=4 rats, 8 grafts), echocardiography was performed and the beating grafts could be clearly identified at the site of implantation. Histologic studies showed that the gelatin material was partially dissolved 5 weeks after subcutaneous implantation. By histologic examination, the density of cells within the grafts was significantly greater at the time of explantation than at the time of implantation. The cells within the graft at the time of explantation histologically resembled cardiomyocytes and appeared to be linked to each other in a pattern resembling normal cardiac tissue (Figure 4). Several blood vessels were also observed within the graft.

Left Ventricular Function
Left ventricular developed pressure, over a range of intraventricular balloon volumes, was best preserved in the sham-operated animals (Figure 7). Rats in which the cell-seeded grafts had been sutured to the myocardial scar tended to have slightly
better ventricular function than rats with unseeded grafts, but the differences were not statistically significant. Left ventricular systolic pressure curves paralleled the developed pressures. Diastolic compliance was not different between the 3 groups.

**Left Ventricular Morphology**

The left ventricles of animals receiving nonseeded grafts (522±58 mm³) were slightly more dilated than the hearts of rats receiving either the cell-seeded grafts (465±58 mm³) or

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**Figure 5.** Graft (G), 5 weeks after being sutured to the surface of left ventricular scar (S). Cells (arrow) had filled interstices of the gelatin mesh and formed tissue resembling myocardium. Graft was adherent to surface of scar. a, hematoxylin-eosin, magnification ×40; b, hematoxylin-eosin, magnification ×100.
sham operation (489±61 mm$^3$); there was no statistically significant difference between the groups.

**Discussion**

The development of a viable, functioning cardiac graft may expand the therapeutic options available to patients with congenital or acquired cardiac defects. We report our initial characterization of the in vitro and in vivo properties of a biodegradable cell-seeded graft. In this series of experiments, we observed that a cell inoculum, derived from fetal rat ventricular muscle, when seeded into a gelatin mesh, proliferated readily in tissue culture over at least 26 days. The presence of a 3D scaffold for the cells resulted in more rapid proliferation than we have previously noted in fetal rat cardiomyocyte cultures grown in a monolayer in a Petri dish. Further studies will be required, however, to determine the cell density at which contact inhibition will result in cessation of cell proliferation within the graft.

The rate at which the gelatin mesh was degraded was dependent on its environment. In tissue culture, in medium that was free of proteolytic enzymes, the mesh did not appreciably degrade over a period of several weeks. When implanted into the subcutaneous tissue of adult rats, mesh grafts underwent significant degradation and absorption over the 5-week duration of our experiments. The mesh grafts sutured onto the epicardial surface of the left ventricular free wall of these rats demonstrated less degradation, perhaps due to more limited contact with native tissue.

We are currently investigating the effect of seeding grafts with multiple cell types, particularly with cell suspensions enriched in cardiomyocytes and endothelial cells. We have noted significantly increased capillary density in rat myocardial scar tissue after transplantation, by needle injection, of endothelial cells (R.-K. Li, MD, PhD, unpublished data, 1998). Seeding with fibroblasts$^8$ may also be required in order to permit secretion of collagen and matrix proteins to maintain structural integrity of the graft as the gelatin mesh slowly degrades.

We observed significant angiogenesis after the cardiomyocyte-seeded grafts were sutured to the left ventricular scar tissue, even without specific co-seeding with endothelial cells. Five weeks after subcutaneous implantation of the cell-seeded grafts, when the grafts were dissected out, medium-size blood vessels, visible to the naked eye, could be
observed leading from the surrounding muscle into the graft. Histologic examination of grafts applied to the left ventricular free wall revealed a modest number of small blood vessels entering the graft from the periphery. However, we did not note significant angiogenesis in the host myocardium and therefore would not expect this technique to induce therapeutically useful angiogenesis in ischemic host myocardium.

Our current graft would likely be too thrombogenic and too porous to be used in direct contact with blood. An endothelialized inner surface on the graft might be necessary to make the graft less permeable, and to prevent activation of the coagulation system leading to graft thrombosis or embolic complications. Transfection of the cultured endothelial cells with constructs permitting long-term low-level expression of tissue plasminogen activator before seeding of the graft matrix may be possible to further reduce thrombogenicity. With this combination of strategies, it may be possible to use this graft material even in low flow, low-pressure environments where currently available synthetic grafts require long-term antiaggregation.

In our study, syngeneic inbred rats were used as both cell donors and graft recipients. There was, therefore, no sign of rejection of the implanted grafts over the course of our study. The use of viable cellular grafts in humans would require either the long-term administration of immunosuppressants or the use of nonantigenic cells. Obtaining an adequate number of nonantigenic or syngeneic cells of the appropriate cell type may be difficult. However, we have been able to obtain a sufficient number of cardiomyocytes and endothelial cells, by percutaneous ventricular septal biopsy with flexible biopettes, to expand in culture and autotransplant in a porcine model of myocardial infarction (R.-K. Li, MD, PhD, unpublished data, 1998). In patients undergoing diagnostic cardiac catheterization, a similar approach may be possible in order to obtain suitable cells with which to seed the graft matrix.

In vivo labeling with BrdU demonstrated ongoing proliferation of the cells within the grafts after implantation onto the left ventricular scar tissue. The long-term growth potential of this type of graft remains, however, to be clarified. The spontaneous, regular contractions of the graft, observed both in vitro and in vivo after implantation into the subcutaneous tissue of rats, showed that this graft retains contractile function. In our rat model of cryoinjury of the left ventricular free wall, we did not, however, note any improvement of ventricular function in hearts receiving either cell-seeded or unseeded grafts. This may have been due to inappropriate sizing of the grafts, because the graft extended beyond the scar tissue onto normal myocardium. This may have impaired the function of the surrounding normal myocardium, resulting in overall depression of global systolic function. Global ventricular diastolic compliance was not different between the groups. We were unable, however, to examine regional ventricular function in this rat model.

Conclusions
Cells derived from fetal rat ventricular muscle, when seeded into a biodegradable gelatin mesh, can grow in 3 dimensions, proliferating to form cardiac-like tissue. Grafts persisted over a 5-week course after implantation either into the subcutaneous tissue or onto the myocardial scar of adult rats. Cells present at the time of graft explantation appeared to have linked with host tissue. Grafts implanted into subcutaneous tissue maintained spontaneous and rhythmic contractility, but the effect of this graft on ventricular function after myocardial scarring remains uncertain. It may in the future be possible to use refinements of this technique to generate grafts which are suitable for the repair of various congenital and acquired cardiac defects.

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