Implantation of Immature Neonatal Cardiac Cells Into the Wall of the Aorta in Rats
A Novel Model for Studying Morphological and Functional Development of Heart Cells in an Extracardiac Environment

Wangde Dai, MD; Sharon L. Hale, BS; Robert A. Kloner, MD, PhD

Background—Morphological and functional development of implanted neonatal cardiac cells in the wall of the abdominal aorta in rats was investigated.

Methods and Results—Cardiomyocytes from neonatal Fischer rats (both sexes) or medium were injected into the wall of the abdominal aorta in female Fischer rats (n=22 in each group). Two or 6 weeks later, the grafted site was exposed and fixed for histological and immunohistological examination. Polymerase chain reaction analysis of the SRY gene to identify male cells was performed in the treated aortas. Seven of 10 cell-treated aortas but none of 10 medium-treated aortas showed spontaneous rhythmic beating at the grafted site after excision of the heart at 2 weeks. Polymerase chain reaction of the SRY gene was positive in 3 cell-treated aortas and none of 3 medium-treated aortas at 6 weeks. Hematoxylin-and-eosin staining showed viable grafts in 9 of 10 aortas at 2 weeks and 9 of 9 aortas at 6 weeks in the cell-treated group but in none of the aortas receiving medium. Neonatal cardiomyocytes in the graft formed compact, longitudinally oriented cardiac muscle bundles and had cross-striations andvascularization. Immunohistochemical staining for sarcomeric actin was positive in 4 of 10 aortas at 2 weeks and 9 of 9 aortas at 6 weeks in the cell group but in none of the aortas in the medium group.

Conclusions—Grafted neonatal cardiomyocytes survive, differentiate, grow, develop a blood supply, and spontaneously contract within the wall of the aorta in rats. (Circulation. 2004;110:324-329.)

Key Words: aorta • grafting • cells • transplantation

Congestive heart failure remains a major cause of morbidity and mortality in the United States.1 There has been recent interest in cellular cardiomypoplasty for therapy of heart failure.2,3 Recently, our research group reported results of studies utilizing fetal or neonatal cardiomyocytes that were injected into the myocardial infarct scar in a rat model.4–7 These engrafted cells grew, differentiated, developed aspects of adult cardiomyocyte phenotype, and survived for at least 6 months when injected into the heart. Importantly, these immature cells contributed to improved left ventricular ejection fraction.5–9 In our most recent study,8 the grafted fetal cells were visible in the scar at 10 months after coronary occlusion. They provided thickness to the scar and improved left ventricular function. These results suggested that cardiomyocytes could be transplanted and could function when injected into the hostile environment of a collagenous scar. Although cellular cardiomypoplasty is considered to be a promising approach for therapy of heart failure, there are still many problems to be addressed before clinical application (for review, see Reffelmann and Kloner3), such as how to increase the survival rate and proliferation and adjust the growth and power output of the grafted cells.

Mechanical stimulation plays an important role in the process of cardiac cell growth.10 The growth of grafted cardiac cells in heart may be influenced by many factors, such as mechanical stimulation, neural factors, hormonal stimuli, and the environment of surrounding heart cells. There is less information regarding the ability of immature cardiac cells to grow, mature, and develop into an adult cardiac phenotype, as well as contrast when these cells are placed into the vasculature outside the heart. Implantation of cardiac cells around the aorta would subject them to pulsatile motion but not the neural, hormonal or other environmental factors of the heart itself. Thus, implantation of cardiac cells around the aorta might serve as a suitable model to study the roles of mechanical stimulation in the morphological development and power output of heart cells in an extracardiac environment in vivo. The present study investigated whether neonatal cardiac cells can survive, differentiate, grow, develop a blood supply, and spontaneously contract within the outer wall of the aorta.
the aorta in rats. As a fatigue-resistant power source, the potential of cardiac cell transplantation to fashion an external auxiliary circulatory pump will also be discussed in detail.

Methods

The experiments were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 85-23, National Academy Press, Washington DC, revised 1996). The Heart Institute in Good Samaritan Hospital is accredited by the American Association for Accreditation of Laboratory Animal Care.

Cell Isolation and Purification

Neonatal ventricular cardiomyocytes were isolated and cultured by previously described methods. In brief, after the atria and great vessels were removed from neonatal (2 days after birth) hearts, the ventricles were minced into small fragments (1 mm³). The minced ventricles were incubated in digestion buffer (100 U/mL collagenase and 0.6 mg/mL pancreatin). The isolated cells were purified by preplating 30 minutes at 37°C as described previously. After preplating, the nonattached cells were plated in 100-mm culture dishes and cultured in the presence of 5% CO2 and 95% air at 37°C for 1 to 2 days.

Surgical Procedure of Retroperitoneal Approach

Female Fischer rats were weighed and anesthetized with intraperitoneal administration of ketamine (7.5 mg/100 g body weight) and xylazine (0.5 mg/100 g body weight). The left lumbar area was shaved and prepared for surgery. Rats were placed in the right lateral decubitus position. A vertical dorsal lumbotomy incision was made along the lateral margin of the erector spine muscle from the margin of the 12th rib to the iliac crest. The peritoneum was exposed after incising the skin and underlying muscle were incised. The left renal artery and aorta exposure were achieved through dissection of the retroperitoneal plane behind the kidney. The site of injection was marked with a 3 mm above the level of the takeoff of the renal arteries. Fascia around the aorta was dissected free at this site with care being taken not to interrupt the renal or mesenteric arteries. After adequate exposure of aorta was achieved, injection of the cells was performed as described below.

Recipient female Fischer rats were divided into 2 groups; those receiving medium only (n=22) and those receiving grafted neonatal cardiomyocytes (n=22, 5x10⁶ cells each). With the aid of a dissecting microscope, base methylcellulose medium in Iscove's modified Dulbecco's medium (Stemcell Technologies Inc), with or without cells, was injected at sites around the circumference of the aorta to form a cuff. A 27-gauge needle attached to an insulin syringe was used, and the needle was inserted directly into the outer wall of the rat aorta to accomplish the injections. Approximately 150 µL of either medium alone or medium plus ~5x10⁶ cells was injected per aorta. Successful injection was typified by a raised pale bleb that extended 2 to 3 mm along the length of the aorta and protruded ~2 mm above the natural surface of the aorta. Injection sites were anterior, left lateral, right lateral, and posterior. The posterior injection was accomplished by twisting the aorta back to front. Injection of the aorta at 4 sites around the circumference ensured an even and homogenous cuff of medium around the aorta. Once injection was completed, the incision was closed by suturing the muscle and stapling the skin. Rats recovered under care and were given Buprenex (0.001 mg/100 g body weight, daily) for 2 days as analgesic.

Evaluation of Contractile Potential

At 2 weeks after surgery, the rats were reanesthetized, and the aorta was reeposed at the site where the cells were implanted. After the "cell cuff" around the aorta was located by careful dissection and the rats had been heparinized (300 U/kg), the aorta was clamped at its origin, and the heart was excised. The graft was examined for spontaneous beating of cardiomyocytes. Beating was documented by video camera. The spontaneous rate of beating was determined by counting the beats per minute on the video recording.

Histological and Immunohistochemical Examination

After the animals were euthanized at 2 or 6 weeks of transplantation, the aortas at the cell graft site were removed, washed in 0.9% NaCl, and fixed in 10% formaldehyde. All of the samples were embedded in paraffin and cut into 5-µm sections for hematoxylin-and-eosin staining and immunohistochemical staining with primary antibody against sarcomeric actin (Dako M0874, 1:75), and some samples were also stained for connexin 43 (ZYMED catalog No.71-0700, 1:50).

Polymerase Chain Reaction for Identifying Y Chromosome

Neonatal cardiomyocytes from rats of either gender were implanted into female rats. The polymerase chain reaction (PCR) amplification was used to identify the Y chromosome to detect survival of implanted male cardiomyocytes. Aortic tissue from the injection site was isolated and frozen in liquid nitrogen. DNA was extracted. PCR was performed with primers that have been designed according to the published partial sequence of the rat SRY gene on the Y chromosome. Three rats in the control and 3 in the cell-injected group were analyzed for PCR.

Statistical Analysis

All results are presented as mean±SD. Differences between groups were compared by paired t test. Statistically significant difference was established at P<0.05.

Results

Of the 44 rats that successfully underwent implantation (22 each in the medium and cardiac cell groups), 10 rats in each group were evaluated for contractile capability and for histological and immunohistochemical examination at 2 weeks. At 6 weeks, 9 rats in each group were studied for histology and immunohistochemistry; and 3 rats from each group were used only for PCR analysis.

Evaluation of Contractile Potential

Contractile capability was determined at 2 weeks in 10 rats from each group. After rats were heparinized, the aorta was clamped at its origin, and the heart was excised, 7 of 10 aortas showed spontaneous rhythmic beating at the site of the neonatal cell grafts (see video clip in the Data Supplement available at http://www.circulationaha.org); none of the 10 aortas in the medium-only group showed spontaneous rhythmic beating. The mean rate of spontaneous beating was 102±23 bpm at 3 minutes after the heart was excised. The beating rate decreased significantly to 68±9 bpm at 7 minutes (P=0.013). There was no correlation between the intrinsic beating rate of the recipient heart versus the aorta.

Histology

Hematoxylin-and-eosin staining showed viable grafts in the outer wall of the cell-treated aortas in 9 of 10 aortas at 2 weeks and 9 of 9 aortas at 6 weeks (Figure 2) and in none of the aortas receiving medium at 2 (n=10) or 6 (n=9) weeks after surgery (Figure 1). Neonatal cardiomyocytes in the graft formed compact, longitudinally oriented cardiac muscle bundles, were differentiated with cross-striations, and had a high degree of vascularization. The cells appeared to have more myofilaments, were thicker, and were more organized at 6
weeks than at 2 weeks (Figures 3 and 4). The majority of the grafted cardiomyocytes were organized into a circular pattern parallel to the aortic layers (Figure 4A). Numerous small vessels and capillaries were observed adjacent to the striated muscle cells. The structure of the aortic wall at the graft site was normal in all samples. No aneurysms were observed either macroscopically or microscopically. Immunohistochemical staining of the aorta for sarcomeric actin was positive in 4 of 10 aortas at 2 weeks (Figure 5) and 9 of 9 aortas at 6 weeks in the cell group (Figure 6) and in none of the aortas in the medium group after surgery; the stain was stronger at 6 weeks than at 2 weeks. The striated cells were easy to identify within the wall of the aorta in contrast to the smooth muscle cells and adventitia of the normal aortic wall. Positive staining of connexin 43 showed that some grafted cardiac cells had already formed gap junctions at 2 or 6 weeks after implantation (Figure 7).

**Survival of Transplanted Cells: PCR Results**

Three aortas taken at 6 weeks after surgery from the medium-only group and 3 from the neonatal cardiomyocyte implantation group were used for detecting the *SRY* gene by PCR. All 3 examined aortas that received neonatal heart cells were positive, confirming the presence of transplanted cells, and all 3 aortas that received medium only were negative.

**Discussion**

The present study demonstrates that neonatal heart cells grafted into an extracardiac location within the outer wall of the aorta in rats mature, grow, develop an adult phenotype, develop a blood supply that allows their survival, and exhibit spontaneous contractions. Naturally, cardiomyocytes are characterized by growth and lose the ability to divide shortly after birth. During normal postnatal growth and maturation, the volume of individual myocardial cells increases 30- to 40-fold. Cardiac cell growth is regulated by many factors, such as hemodynamic, neural, and hormonal stimuli. Mechanical stimulation plays an important role in the process of cardiac cell growth. Vandenburgh et al showed the effect of mechanical stimulation on the morphological alterations of neonatal rat cardiomyocytes cultured in vitro in a computerized mechanical cell stimulator device. The cultured neonatal rat cardiomyocytes under the mechanical stimulation organized into parallel arrays of rod-shaped cells, had increased binucleation, and hypertrophied longitudinally. These morphological alterations were similar to those occurring during in vivo heart growth with hemodynamic loading. Rossi
transplanted newborn mouse hearts into the pinna of the ears of isogeneic adults, an environment without hemodynamic loading. By 2 and 6 months after transplantation, myocardial cell size in the grafted hearts was similar to that observed in newborn mouse cardiac tissue, and the cells became smaller by 12 months after transplantation. Some cells began to atrophy at 6 months, and severely degenerated myocytes were commonly seen at 12 months after transplantation. The explanation for the morphological deterioration of the transplanted newborn mouse hearts in Rossi’s study was that the transplanted hearts were not subjected to hemodynamic load in the pinna of the ear. Hemodynamic overloading or underloading may cause cardiac hypertrophy or atrophy, respectively.16,17 At the time of transplantation into the adult myocardium of rats, these neonatal cells are round, have large spherical nuclei, have minimal contractile apparatus, and do not have visible transcellular sarcomeres.6 By 2 weeks, the cells appear lengthened, the nuclei appear less round and more oblong in shape, and many cells demonstrate readily visible sarcomeres approaching an adult phenotype.6 In the present study, the immature neonatal cardiac cells showed development after implantation into the wall of the abdominal aorta in rats, where the cells were subjected to pulsatile stimulation. By 6 weeks, cells appeared larger and thicker, and they appeared to have more cross-striations. This technique provides a novel experimental model for studies on the mechanism of cardiac cellular growth by mechanical stress in an extracardiac environment in vivo.

Grafted neonatal cardiomyocytes can spontaneously contract within the outer wall of the aorta in rats. Thus, in theory, they could become a potential power source for extra-aortic counterpulsation for the treatment of congestive heart failure. The force that would be needed to contract the aorta during diastole (which would mimic the workings of an intra-aortic balloon counterpulsation device) should be greater than the

Figure 4. Higher magnification of boxed area indicated by yellow arrow in Figure 2A. A, At 6 weeks, grafted cardiomyocytes are organized in circular pattern parallel to aortic layers (yellow arrows). Original magnification, ×200. B and C, Higher magnification of area in Figure 2A; transplanted cardiomyocytes (yellow arrows) show more cross-striations and demonstrate larger fiber size than those at 2 weeks after transplantation. Original magnification, ×400.

Figure 5. Immunohistochemical staining of slices in Figure 3 for sarcomeric actin. Yellow arrows indicate weak brown-stained transplanted cardiomyocytes at 2 weeks after transplantation; red arrows indicate blood vessel. Original magnification, ×400.
force exerted by diastolic blood pressure (blood pressure in rats is \( \approx 19 \pm 2.8/14 \pm 1.6 \text{ mN/mm}^2 \)). The size of the cuff of the cell grafts around the aorta in the present study was approximately the same as a rat papillary muscle, \( \approx 8 \text{ mm in length with a cross-sectional area of } \approx 1 \text{ mm}^2 \) and muscle weight of \( \approx 10 \text{ mg} \), which is the equivalent of \( \approx 200000 \) cardiomyocytes (there are \( \approx 20 \) million cardiomyocytes in 1 g of heart tissue; therefore, 200 000 in 10 mg). The force produced by papillary muscles of rats (more than 20 mN/mm\(^2\)) \(^{19,20} \) is considerably greater than that needed to overcome diastolic pressure. The tension developed by right ventricular myocytes from rats is 39\( \pm 2 \text{ mN/mm}^2 \) measured in single skinned myocytes, \(^{21} \) and in one study, developed tension of rat papillary muscles was as high as 60 mN/mm\(^2\).\(^{18} \) Therefore, in theory, grafted aortic cardiac cells, once fully matured and connected, should be able to sustain enough force to contract the aorta in diastole. At least 200 000 surviving and functioning cardiomyocytes would be needed around the aorta to contract the aorta in diastole.

There had been concern that the functional development of transplanted cardiomyocytes could be impaired if they were placed into the hostile environment of a collagenous scar of a myocardial infarction. In recent years, a number of studies have been performed that show the usefulness of transplanting neonatal or fetal cardiomyocytes directly into infarct scars. Neonatal cardiomyocytes can differentiate toward an adult cardiomyocyte phenotype.\(^{22} \) The grafted neonatal cardiomyocytes can form gap junctions,\(^{9,23} \) but synchronous beating of the graft within the infarcted myocardium in vivo has not been proved because of the confounding motion of the host heart. The cell size of immature cardiomyocytes was small at the early stage after transplantation and was characterized as incomplete differentiation at the level of protein expression.\(^{4,5,24} \) However, cell diameters increased at 8 weeks after transplantation, and cells formed complete sarcomeres, junctional complexes, and abundant mitochondria.\(^{23} \) Li et al\(^{25} \) reported the results of a study in which neonatal cardiomyocytes were injected into the subcutaneous connective tissue of the adult rat hindlimb. Echocardiography demonstrated that engrafted cells formed contracting tissue at 7 days, and fractional shortening of the contractile tissue was 35% at 21 days. The size of the implanted contractile tissue increased 2.4 times for the first 2 weeks in vivo but did not enlarge during the subsequent 3 months after transplantation. In the present study, the implanted neonatal cardiomyocytes in the outer wall of the abdominal aorta continued to increase in size at 6 weeks. Our pilot study clearly shows that the implanted cells have the capability to contract. The study provides a novel experimental model for future work investigating regulation of growth, differentiation, and contractile function of cardiac cells exposed to various stimuli (such as adrenergic stimulation), within an in vivo extracardiac environment. The next steps will be to determine (1) whether these cells can be paced in an in vivo setting to contract during diastole; (2) whether the pressure generated during pacing in diastole is sufficient to augment aortic diastolic pressure and whether relaxation during systole is adequate to provide afterload reduction (much like an intra-aortic balloon pump); and (3)

Figure 6. Higher magnification of boxed area indicated by yellow arrow in Figure 2B; immunohistochemical staining for sarcomeric actin. Original magnification, \( \times 400 \). Transplanted cardiomyocytes showed stronger positive brown staining and typical cross-striations of sarcomere formation (yellow arrows) at 6 weeks after transplantation.
whether they survive, further differentiate and perhaps further enlarge over the long term.

In summary, the present pilot study demonstrated that grafted neonatal cardiomyocytes survived, differentiated, grew, developed a blood supply, and spontaneously contracted within the outer wall of the aorta in rats. The method of implanting immature neonatal cardiomyocytes around the aorta in rats may be used as a novel model for scientific research, such as for studying the relationship between mechanical stimulation and cardiac cell growth, or the response of grafted cardiac cells to various stimuli in an extracardiac environment in vivo. Cardiomyocyte transplantation around the abdominal aorta as a cellular aortomyoplasty may ultimately be a promising approach to fashion an external auxiliary circulatory pump for safe, long-term circulatory support of patients with advanced heart failure.

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References
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