Composite Cell Sheets
A Further Step Toward Safe and Effective Myocardial Regeneration by Cardiac Progenitors Derived From Embryonic Stem Cells

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Background—The safety and efficacy of myocardial regeneration using embryonic stem cells are limited by the risk of teratoma and the high rate of cell death.

Methods and Results—To address these issues, we developed a composite construct made of a sheet of adipose tissue–derived stroma cells and embryonic stem cell–derived cardiac progenitors. Ten Rhesus monkeys underwent a transient coronary artery occlusion followed, 2 weeks later, by the open-chest delivery of the composite cell sheet over the infarcted area or a sham operation. The sheet was made of adipose tissue–derived stroma cells grown from a biopsy of autologous adipose tissue and cultured onto temperature-responsive dishes. Allogeneic Rhesus embryonic stem cells were committed to a cardiac lineage and immunomagnetically sorted to yield SSEA-1+ cardiac progenitors, which were then deposited onto the cell sheet. Cyclosporine was given for 2 months until the animals were euthanized. Preimplantation studies showed that the SSEA-1+ progenitors expressed cardiac markers and had lost pluripotency. After 2 months, there was no teratoma in any of the 5 cell-treated monkeys. Analysis of >1500 histological sections showed that the SSEA-1+ cardiac progenitors had differentiated into cardiomyocytes, as evidenced by immunofluorescence and real-time polymerase chain reaction. There were also a robust engraftment of autologous adipose tissue–derived stroma cells and increased angiogenesis compared with the sham animals.

Conclusions—These data collected in a clinically relevant nonhuman primate model show that developmentally restricted SSEA-1+ cardiac progenitors appear to be safe and highlight the benefit of the epicardial delivery of a construct harboring cells with a cardiomyogenic differentiation potential and cells providing them the necessary trophic support. (Circulation. 2010;122[suppl 1]:S118–S123.)

Key Words: heart failure ▪ myocardial infarction ▪ transplantation ▪ cells ▪ stem cells

Clinical trials of cardiac cell therapy have yielded inconsistently positive results and it is now widely agreed that their benefit are due to an increased wall thickness with an attendant reduction in wall stress and/or to the paracrine activation of host-associated cytoprotective signaling pathways.1 Thus, these benefits cannot be ascribed to a true...
myocardial regeneration of the damaged areas. Such a regeneration probably requires the provision of cells endowed with a true cardiomyogenic potential and, in this setting, pluripotent embryonic stem cells (ESC) that can be lineage-directed are particularly appealing. However, a prerequisite for these cells to be functionally effective is that their differentiated lineage-specific progeny be sufficiently purified in vitro to avoid the development of teratoma and can then efficiently engraft and survive after transplantation. Regardless of the cell type, engraftment is also critically dependent on the method of cell delivery. From this standpoint, conventional needle-based intramyocardial injections may be less effective than the epicardial delivery of cellularized biomaterials, among which scaffold-free cell sheets that are prepared onto temperature-responsive dishes and maintain intercellular connections through extracellular matrix adhesion proteins have proven their efficacy in multiple fields of regenerative medicine, including the heart. Finally, survival is influenced by several intermingling factors, among which ischemia plays a central role. Putting these considerations together, we designed a composite cell sheet made of cardiac progenitors derived from nonhuman primate ESC and adipose tissue–derived stroma cells (ADSC) intended to provide new cardiomyocytes and trophic support, respectively. The present study describes the outcomes of these composite cell constructs in a clinically relevant nonhuman primate model of myocardial infarction with safety as the primary end point.

Methods

Animals

Ten Macaca mulatta monkeys weighing an average of 7 kg (6.1 to 9.6) were used in this study, which was approved by our institutional ethics committee and complied with the European legislation on animal care.

Cell Cultures

Primates Embryonic Stem Cells

ORMES-2 cells were cultured on mouse embryonic fibroblasts (MEF) prepared from E14 mouse embryos using KO-DMEM medium (Invitrogen, Burlington, Ontario, Canada) supplemented with β-mercaptoethanol, glutamine, non essential amino acids, 15% KO-SR, and 10 ng/mL fibroblast growth factor (FGF)-2, respectively. Medium was changed every day. Cell colonies were dissociated into single cells every 4 to 5 days using trypsin. Cells were nucleofected with 3 µg of vector pActin–green fluorescent protein (GFP) purified using a Pharmedingen kit and linearized. Cells were then cultured on neomycin-resistant MEF for 2 days and G418 (100 µg/mL) was added for the next 10 days. Colonies were cut with a needle and picked out of the dish to be further cultured and amplified. Clones were genotyped using GFP primers and further amplified.

To commit cells toward a cardiac lineage, they were treated for 4 days with 10 ng/mL bone morphogenetic protein-2 (Wyeth Laboratories, Paris, France) in the presence of 1 µmol/L SU5402 (Calbiochem, Nottingham, United Kingdom), a FGF receptor inhibitor, in RPMI/B27 medium, as previously described. For sorting cardiomyocyte-committed cells, trypsinized cells were incubated for 30 minutes at room temperature with anti-SSEA1 antibody-coated Miltenyi beads (40 µL/106 cells) in D-PBS supplemented with 0.5% (wt/vol) BSA and 2 mmol/L EDTA. Cells were transferred to a L50 Miltenyi cartridge set on the magnet (Miltenyi, Bergisch Gladbach, Germany). Cells were washed 3 times with 3 mL D-PBS/BSA/EDTA and eluted from the column using 3 mL of D-PBS/BSA/EDTA.

Adipose Tissue–Derived Stromal Cells

Adipose tissue cells were isolated from subcutaneous fat pads around the belly at the time of infarction in each primate and cultured as previously described. After enzymatic digestion in a PBS containing 2 mg/mL collagenase medium and filtration, mature adipocytes were separated from the stromal fraction by centrifugation (600g, for 10 minutes). The pellet was resuspended in culture medium and cells were seeded at a density of 10,000 per cm2 in DMEM-F12 medium supplemented with 10% fetal calf serum and maintained in 5% CO2. Six hours after plating, all nonadherent cells were removed by washing. Subconfluent cells, which represent ADSC, were trypsinized and frozen until use, at which time they were thawed and cultured for 8 days before preparation of the cell sheets.

Preparation of Cell Sheets

ADSC suspensions at a density of 4 to 6×106 cells were plated onto thermo-responsive 6 cm2 cell culture dishes (CellSeed, Tokyo, Japan) and kept in a 37°C incubator for 5 days. At this time, plates were cooled to 20°C for 1 hour, which resulted in spontaneous detachment of the confluent cells, thereby yielding a scaffold-free monolayered ADSC graft. SSEA-1+ cardiac progenitors (6×106) were then deposited onto the ADSC sheet for 5 days. In case, scalability of the preparation was tested by coseeding 10×106 SSEA-1+ cardiac progenitors with 21×106 ADSCs in a 10 cm2 dish.

Myocardial Infarction Model

Before infarction, the primates were treated orally for 5 days with amiodarone (100 mg/d) and acebutolol (50 mg twice a day for the last 2 days). Induction of anesthesia was then performed with propofol (3 mg/kg) and sufentanyl (0.5 mg/kg), and maintenance anesthesia was ensured with the same drugs albeit at a lower dose (1 mg/kg and 0.25 mg/kg, respectively). Intravenous atracurium besilate (1 mg/kg) was added whenever required and, in addition, all animals received intravenous heparin (50 IU/kg), intramuscular amoxicillin (20 mg/kg), and lidocaine (1 mg/kg). A myocardial infarction was created by a 120-minute balloon inflation in the left anterior descending artery (n=4), left circumflex coronary artery (n=4), or right coronary artery (n=2), followed by reperfusion and angiographic confirmation of the patency of the revascularized infarct vessel. The occurrence of myocardial necrosis was consistently documented by a postprocedural rise in troponin I values.

Transplantation Experiments

Two weeks after infarction, primates underwent a left thoracotomy. The area of infarction was visually identified and covered with the composite sheet harboring both ADSCs and SSEA-1+ cardiac progenitors, which spontaneously adhered to the epicardial surface without glue or sutures (n=5). This area was then incircled with sutures for subsequent identification. The remaining 3 primates underwent a sham operation. Immunosuppression was achieved with cyclosporine A (Novartis, Rueil-Malmaison, France) given intramuscularly at the dose of 10 mg/kg/d for 3 days, after which it was administered at an initial dose of 150 mg/kg by oral gavage twice a week until the animals were euthanized. Serum drug concentrations were regularly checked by venous punctures performed just before further administration with target levels set at 100 ng/mL.

Assessment of Outcomes

Pathological and Histological Studies

After the primates were euthanized, hearts were excised and separated into 2 halves. One half, encompassing the area of infarction, identified by the previously placed sutures, and the remainder of the heart were fixed in formaldehyde and cut in 7 blocks. Five-micrometer paraffin-embedded hematoxylin and eosin–stained sections were then examined for the presence of teratoma. The second half, which exclusively comprised the infarcted area, was cut into 4 blocks that were snap-frozen at −80°C. Seven-micrometer sections were then obtained for histological analysis. For each heart, 9 to 10 high-power fields were randomly assessed at different section levels. Identific-
tion of the cotransplanted cardiac progenitors and ADSC was based on immunolabeling with antibodies against GFP (Invitrogen, Burlington, Ontario, Canada) and CD90 (BD Biosciences, San Jose, Calif), respectively, using secondary antibodies conjugated with FITC or Texas Red (Vector, Burlingame, Calif). Quantification of capillaries and arterioles was made by counting cells staining positively for CD31 (Dako, Carpentaria, Calif) and smooth muscle actin (Sigma, Saint Louis, Mo), respectively, in ×20 high-power fields and expressed as the number of vessels per high-power field (0.3 mm²). The extent of fibrosis was assessed by computerized planimetry of Sirius red–stained areas and expressed as the ratio between the area of scar tissue (in μm²) to the left ventricular (LV) area. Examinations were performed with a microscope (Leica DML; Leica, Wetzlar, Germany) equipped with a digital camera (Qicam, QImaging, Burnaby, BC, Canada) and a total of >1500 sections were examined for the different histological parameters. qRT-PCR was used to confirm the positive immunostaining for GFP by the presence of GFP transcripts and was performed with Sybr Green mix on a Light Cycler FastStart DNA Master Plus (ROCHE Diagnostic, Mannheim, Germany), using the following primers: EF1 forward: ACGTATACCCGCCCCAAAGTT; EF1 Reverse: AAGTGCTGCTGTCTCAGTG. In addition, the lungs, liver, spleen, pancreas, periaortic lymph nodes, kidneys, testes, vertebrae, and brain were harvested when the animals were killed. The removed organs were sliced and first inspected macroscopically for the presence of a tumor. They were then embedded into paraffin blocks (12 to 14 blocks per animal), and 2 sections per block were finally sampled randomly and processed for further histological examination.

**LV Function**
Pretransplantation and posttransplantation echocardiographic LV ejection fraction was assessed using the biplane Simpson rule. All measurements were made in triplicate and averaged by an investigator blinded to the treatment group.

**Statistics**
Data are summarized using median values [25% percentile; 75% percentile]. The changes in LV function parameters (LV ejection fraction, end-diastolic volume, and end-systolic volume) were compared between the cell-treated and sham groups using Wilcoxon rank-sum test. The numbers of vessels per millimeter squared and the percentages of fibrosis were compared between groups using a mixed model for clustered continuous data, taking into account the intraheart correlation (as multiple slices were used for a single heart). A data transformation (log or sinus or arc sinus) was done to satisfy hypotheses required for the parametric model. Statistical significance was set at the 10% threshold and all statistical analyses were performed with the SAS statistical software version 9.1 (Cary, NC).

**Results**
Fluorescence-activated cell sorting performed 48 hours later after detachment of beads from the cells revealed that the purity of the SSEA-1⁺ cell population reached 95%. These progenitors had lost markers of pluripotency while they had acquired those specific for the cardiac mesoderm, as demonstrated by RT-PCR, immunostaining, epigenetic profiling, and microRNA expression. ADSC were characterized by the expression of the surface markers CD13, CD29, CD34, CD73, and CD90. Two primates died at the time of occlusion of the left anterior descending artery or 1 week later. Among the 8 survivors, 5 were allocated to the treatment group and 3 were sham-operated. During the period of follow-up, 1 of these sham animals (left circumflex occlusion) died 4 weeks after the thoracotomy. There was no death among the 5 cell-treated primates.

A careful examination of the hearts of these cell-treated animals failed to show any teratoma. Likewise, there was no evidence for tumor in any of the peripheral organs that were examined. Consequently, this made irrelevant the use of additional histochemical markers, which are otherwise useful tools for assessing the degree of maturation and differentiation of teratomas. On histological examination, the cardiomyogenic differentiation potential of the SSEA1⁺ progenitors was demonstrated by the presence of clustered GFP⁺ cells indicating expression of the α-actin gene (Figure 1A). To control for the potential artifacts associated with immunostaining, these findings were double-checked by qRT-PCR, which actually confirmed the presence of GFP transcripts in myocardial tissue samples originating from the blocks found positive for immunostaining. A robust engraftment of the ADSC was also documented by linings of CD90⁺ cells whose epicardial distribution spatially overlapped the areas of cell sheet deposition (Figure 1B). Angiogenesis was consistently greater in cell-treated hearts. Thus, the number of CD31⁺ cells was 588/mm² [CI 90%: 247 to 928] and 820/mm² [CI 90%: 564 to 1076] in the sham and treated groups, respectively (Figure 2). Corresponding values for SMA⁺ cells were 24/mm² [CI 90%: 13 to 44] and 42/mm² [CI 90%: 29 to 63], respectively. There was no histological evidence for intramyocardial calcifications or immune response. The extent of fibrosis did not differ between sham and cell-treated primates (15.6% [CI 90%: 0 to 38.4%] and 14.8% [CI 90%: 0.7 to 28.9%], respectively).

The LV ejection fraction tended to be better preserved in the cell-treated group (Supplemental Figure), which also yielded a 2-fold greater reduction in end-systolic LV volume (data not shown). However, the small number of animals in each group clearly prevents any meaningful conclusion to be drawn.

**Discussion**
A positive feature of the cell therapy trials conducted so far in patients with severe ischemic LV dysfunction has been to identify some of the roadblocks that have contributed to their limited success rate. Among them, 3 probably play a critical role: the failure of adult stem cells to adopt a cardiomyocytic phenotype, the high rate of ischemic cell death, and the suboptimal efficiency of cell transfer based on multiple needle injections. The present study has tried to address these issues by the use of nonhuman primate ESC-derived cardiac progenitor cells, the concomitant provision of ADSC endowing an angiogenic potential, and a delivery method intended to optimally preserve the integrity of both the cellular graft and the host myocardium.

**ESC-Derived Cardiac Progenitor Cells**
The failure of adult stem cells to adopt a cardiomyocytic phenotype implies that a true regeneration of the scarred myocardium probably requires the provision of stem cells endowed with an innate cardiomyogenic potential. In this setting, mobilization of a putative pool of endogenous cardiac stem cells is appealing but fraught with the uncertainty regarding the persistence of this population in the aged diseased heart. An alternate approach relies on the external
supply of cardiac-specified stem cells. Although reprogrammed adult cells to a pluripotent cells offer the major advantages of bypassing the ethical and immunologic issues associated with ESC, they still raise major concerns related to the low and inconsistent efficiency of the current reprogramming protocols and to the still limited data on the functionality of cells redifferentiated from this embryonic-like state.11 This limitation does not apply to ESC. Several studies have

Figure 1. A, Clusters of GFP-positive cells 2 months after application of the cell sheet reflecting the cardiac differentiation of the SSEA1 progenitors populating the sheet. Sections were immunostained with an anti-GFP antibody. The left lower quadrant (TRTC) indicates that the GFP labeling is not a result of autofluorescence (magnification ×20). TRTC indicates Texas Red ThioCyanate. B, Clusters of CD90 cells reflecting the persistence of the ADSC 2 months after application of the cell sheet. Sections were immunostained with an anti-CD90 antibody. Note that the lining of CD90 cells spatially matches the epicardial coverage of the infarcted area onto which the cell sheet had been deposited (magnification ×40).

Figure 2. Immunostaining of vessels using an anti-CD31 antibody showing a higher vessel density after application of the composite cell sheet (magnification ×20).
previously demonstrated that experimental transplantation of human ESC into infarcted areas resulted in their differentiation into cardiomyocytes, probably enhanced by local cues and usually associated with an improvement in contractile function and a reduction in remodeling. However, a major limitation of these rodent studies has been the xenogenic setting of the transplantation, which may lead to underestimate the incidence of teratomas. The present study, is, to the best of our knowledge, the first to have tested transplantation of ESC derivatives in a clinically relevant large animal model of allogeneic transplantation. Because a sorted population of developmentally restricted cardiac progenitors is critical for a safe tumor-free outcome, we have developed an antibody-based protocol for isolation of these fate-restricted cells. The SSEA-1 antigen was found a reliable marker of the early differentiation of human ESC, an assumption supported by the finding that the transfer of this developmentally-restricted cell population SSEA1 was not associated with teratoma or extra-cardiac tumor. Clearly, we cannot formally exclude that some residual undifferentiated or poorly differentiated SSEA1− cells did not contaminate our SSEA1+ population. However, their number probably was small because 95% of the cardiac-specified cells were SSEA1+ after sorting and thus likely below the threshold required for teratoma generation.

Adipose Tissue-Derived Stem Cells

It is well established that the efficacy of cell transplantation is dramatically limited by the low rate of cell engraftment. This results from multiple factors among which inflammation, apoptosis, and ischemia caused by poor vascularization of the target areas play a major role. To address this ischemic component of cell death, we cotransplanted cells endowed with an angiogenic potential on the basis of the previously documented benefits of cotransplanting different cell populations. We selected ADSC because they secrete a wide spectrum of growth factors that account for their robust angiogenic effects, demonstrated by the greater number of CD31+ and SMA+ cells in the treated hearts. Furthermore, ADSC are credited for an immune privilege, which could help mitigating rejection of cotransplanted allogeneic cells.

Epicardial Delivery of Composite Cell Sheets

As mentioned above, apoptosis is another contributor to cell death and occurs because anchorage-dependent cells loose the survival signals normally associated with intercellular connections and attachment of cells to the extracellular matrix as a result of the enzymatic digestion required for pretransplantation cell isolation. These observations have led to the development of tridimensional constructs inside which cells can proliferate and secrete extracellular matrix adhesive proteins enhancing their survival. Among different scaffolding options, we selected the cell sheet technology which has already an extensive efficacy record in models of both ischemic and nonischemic cardiomyopathies. The advantages of these cell sheets are the absence of any foreign material, the preservation of cell cohesiveness and the possibility to incorporate different cell populations, such as SSEA1+ cardiac progenitors and ADSC. Overall, our results are line with those of previous studies that have reported successful outcomes after the epicardial delivery of composite constructs harboring cardiomyocytes, endothelial cells, and fibroblasts, most likely because cross talks between these cell populations may synergize trophic effects and ultimately lead to an enhanced survival of the contractile cells of interest.

Study Limitations

Several limitations must be recognized. The cost of primates and the logistical complexity associated with their handling have resulted in a small sample size in which caution is required in the interpretation of outcomes. Furthermore, the high sensitivity of primates to ischemia resulted in that only animals with relatively small infarctions and consequently well-preserved LV functions survived the procedure, thereby leaving little room for showing functional improvements after transplantation of the cell constructs. Third, we used a single sheet incorporating the 2 cell populations, and it is now clear that the benefits of the procedure can be optimized by a multilayered construct, which seems to increase the possibility for cells to migrate in the midmyocardium and establish more robust coupling with the host cardiomyocytes and vessels. Finally, one could argue that mixing the 2 cell populations on the same sheet prevented to assess the respective contribution of each cell type; this could have been theoretically achieved by testing SSEA-1+ alone and ADSC-alone cell sheets but was not technically possible because freshly sorted SSEA-1+ cardiac progenitors require feeder cells, here represented by ADSC, to proliferate and, at this early stage of maturation, still lacked the adhesion proteins that would have allowed them to form a cohesive cell sheet if cultured alone onto the temperature-responsive dish. Despite these limitations, the present study provides encouraging hints of the safety of transplanting lineage-directed and purified ESC-derived SSEA1+ cardiac progenitors. Beyond the specific choice of the contractile cells intended to effect myocardial regeneration, these data also highlight the concept of the epicardial delivery of a construct harboring different cell populations whose effects may synergize for optimizing differentiation, retention, and survival of the cellular graft. As such, this concept might find sound applications during open-chest procedures in patients in need of replenishment of their depleted cardiomyocyte pool.

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Disclosures

All authors have reviewed the manuscript and approved its content. There is no conflict of interest to disclose for any of them.
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Supplemental Material Figure.

Individual values of LV ejection fraction (EF) before and after transplantation. One of the 5 cell-treated animals died during the pre-echocardiography anesthesia and was therefore only available for the histological assessment.