Regenerative Potential of Cardiosphere-Derived Cells Expanded From Percutaneous Endomyocardial Biopsy Specimens

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Background—Ex vivo expansion of resident cardiac stem cells, followed by delivery to the heart, may favor regeneration and functional improvement.

Methods and Results—Percutaneous endomyocardial biopsy specimens grown in primary culture developed multicellular clusters known as cardiospheres, which were plated to yield cardiosphere-derived cells (CDCs). CDCs from human biopsy specimens and from comparable porcine samples were examined in vitro for biophysical and cytochemical evidence of cardiogenic differentiation. In addition, human CDCs were injected into the border zone of acute myocardial infarcts in immunodeficient mice. Biopsy specimens from 69 of 70 patients yielded cardiosphere-forming cells. Cardiospheres and CDCs expressed antigenic characteristics of stem cells at each stage of processing, as well as proteins vital for cardiac contractile and electrical function. Human and porcine CDCs cocultured with neonatal rat ventricular myocytes exhibited biophysical signatures characteristic of myocytes, including calcium transients synchronous with those of neighboring myocytes. Human CDCs injected into the border zone of myocardial infarcts engrafted and migrated into the infarct zone. After 20 days, the percentage of viable myocardium within the infarct zone was greater in the CDC-treated group than in the fibroblast-treated control group; likewise, left ventricular ejection fraction was higher in the CDC-treated group.

Conclusions—A method is presented for the isolation of adult human stem cells from endomyocardial biopsy specimens. CDCs are cardiogenic in vitro; they promote cardiac regeneration and improve heart function in a mouse infarct model, which provides motivation for further development for therapeutic applications in patients. (Circulation. 2007;115: &NA;–)

Key Words: cells • biopsy • electrophysiology • myocardial infarction • myocytes

We sought to develop a clinically applicable method for the isolation and expansion of adult stem cells capable of regenerating myocytes and vessels and improving function in the injured heart. Given recent evidence that the adult mammalian heart contains endogenous, cardiac-committed stem cells,1–5 we began with cardiac tissue as our stem cell source, postulating that cardiac-derived cells might be particularly well-suited for myocardial regeneration. Percutaneous endomyocardial biopsy specimens were utilized as a convenient, minimally invasive tissue source.6,7 We began with the observation that cardiac surgical biopsy specimens in culture yield spherical multicellular clusters dubbed “cardiospheres.”8 Cardiospheres resemble neurospheres9 in that they are derived from primary tissue culture and contain many proliferative cells that express stem cell–related antigens, as well as other cells undergoing spontaneous cardiac differentiation.8 We modified the original culture method to improve efficiency and added a postcardiosphere expansion step to obtain reasonable numbers of cells (cardiosphere-derived cells [CDCs]) for transplantation from the small specimens in a timely manner.

Methods

Biopsy Specimen Processing and Cell Culture

Percutaneous right ventricular endomyocardial biopsy specimens were obtained from the septal wall during clinically indicated procedures after informed consent, in an institutional review board–approved protocol. Specimens were processed as described previously8 with several modifications. Samples were cut into fragments from which gross connective tissue was removed, then washed and
partially digested enzymatically. The tissue fragments were cultured as “explants” on dishes coated with fibronectin (Figure 1a, step 2), a procedure which reduced the time to first explant harvest (Figure 1a, step 3) by 1 week in side-by-side comparisons. Explants were also much more likely to remain attached during subsequent harvests when plated on fibronectin. After several days, a layer of stromal-like cells arose from adherent explants over which small, round, phase-bright cells migrated. Once confluent, the cells surrounding the explants were harvested by gentle enzymatic digestion (Figure 1a, step 3). These cardiospheres-forming cells were seeded at 2 to 3×10⁴ cells/mL on poly-D-lysine–coated dishes (Figure 1a, step 4) in cardiosphere medium (see supplementary Table I for composition and additional methodological details). Several days later, cells that remained adherent to the poly-D-lysine–coated dishes were discarded, whereas detached cardiospheres were plated on fibronectin-coated flasks and expanded as monolayers (Figure 1a, step 5). CDCs were subsequently passaged by trypsinization and splitting at a 1:2 ratio. Single cells were counted under phase microscopy with a hemocytometer as cardiospheres-forming cells and during CDC passaging to track cell growth for each specimen. Isolation of the cardiospheres-forming cells was repeated up to 3 more times from the same specimen. Average cumulative growth was calculated in 2-week bins with the raw data collected during that time frame from all specimens.

Porcine specimens were obtained according to a protocol approved by the institutional animal care committee, excised from the right ventricular septum from explanted hearts with a scalpel to acquire small pieces similar to those obtained with a bioptome, and processed in the same manner as human samples. Human dermal fibroblasts served as a live-cell control and were cultured in the medium used for explants and CDCs. Neonatal rat ventricular myocytes (NRVMs) were isolated and cultured as described previously.¹⁰

Flow Cytometry
CDCs were passaged twice as adherent monolayers and then used for flow cytometry experiments with a FACSCalibur flow cytometer.
with CellQuest software (BD Biosciences, San Jose, Calif). The following monoclonal antibodies and similarly conjugated isotype-matched control monoclonal antibodies were used: c-Kit-APC, CD45-PE, CD105-PE, CD90-FITC, CD133-PE, CD34-FITC, CD45-PE, CD331-PE, and an FITC-conjugated lineage cocktail. Gates were established by 7-AAD (7-aminactinomycin D) fluorescence and forward scatter to exclude dead cells. Human dermal fibroblasts were similarly characterized.

**Immunostaining, Immunohistochemistry, and Microscopy**

Cardiospheres were collected for immunostaining when they had reached 100 to 1000 cells in size, fixed in ethanol and acetone, and sectioned in 5-μm slices. Secondary antibodies conjugated with Alexa fluorochromes were used. Confocal fluorescence imaging was performed on an Eclipse TE2000-U (Nikon, Melville, NY) equipped with a krypton/argon laser with UltraVIEW software (PerkinElmer, Boston, Mass).

**Statistical Analysis**

All results are presented as mean±SEM. Significance of differences between any 2 groups was determined by the Student t test. Multiple groups were compared with GB-Stat software with 1-way ANOVA, and group pairs were compared by the Bonferroni-Dunn method. The generalized estimating equation method was used to identify patient parameters that might be independently associated with specimen growth. Exchangeable correlation structure was chosen for the generalized estimating equation analysis. The generalized estimating equation analysis was performed with the use of SAS software. A final value of P<0.05 was considered significant for all analyses. All probability values reported are 2-sided.

The authors had access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results

Heart specimens from patients (single whole or partial biop- tome “bites”) were stored on ice in high-potassium cardioplic solution and processed within 2 hours (Figure 1a, step 1). A typical biopsy fragment, or explant, is shown after mincing and partial enzymatic digestion, on the day it was obtained (Figure 1b) and on days 3 (Figure 1c) and 13 (Figure 1d). Harvesting of cardiosphere-forming cells, the loosely adher- ent cells that spontaneously shed from the explants (Figure 1a, step 3), was initially performed 8 or more days after a specimen was obtained. Typical cardiospheres are shown 2 (Figure 1e) and 12 (Figure 1f) days after harvest, when growth was robust. Cardiospheres were plated for expansion (Figure 1a, step 5) 4 to 28 days after harvest and the resultant CDCs passed at 2- to 7-day intervals thereafter. The typical morphology of CDCs is evident during expansion at passage 2 (Figure 1g). CDCs from 4 randomly selected patients in the present study were karyotyped after 3 or more passages (=6 population doublings and 60 days in culture). All samples were found to be normal.

Specimens (21.0±1.9 mg) were obtained for analysis from a total of 70 patients (59 transplanted, 11 nontransplanted), and 69 of 70 yielded CDCs in large numbers (1.7±0.4 million CDCs within 45±7 days). Figure 1 shows growth curves for most specimens, depicting population doublings from the time of first harvest. Nontransplanted heart failure patient biopsy specimens (Figure 1h) exhibited similar growth rates to those from transplanted patients (Figure 1i). Patient parameters are summarized in the Table. A general- ized estimating equation analysis involving all patient param- eters revealed no strong, independent predictors for high cell yield within either study group.

Human cardiospheres consistently expressed c-Kit, the receptor for stem cell factor, and CD105 (Figure 2a), the regulatory component of the transforming growth factor-β receptor complex important in angiogenesis23 and hematopoi- esis.24 Cells primarily within the cardiosphere core were proliferative, as identified by Ki67 expression (Figure 2b). Cardiospheres also demonstrated widespread expression of the gap junctional protein connexin 43 (Figure 2c). Supplemental Figure 1 shows complementary results in sectioned cardiospheres. CDCs exhibited antigenic and cytochemical similarities to cardiospheres, as well as nuanced differences. Proliferative CDCs frequently expressed α-sarcomeric actin (Figure 2d), and expanded CDCs expressed connexin 43 (Figure 2e), suggestive of their potential to electrically couple to each other (and to myocytes; see Figure 3). After expansion, the vast majority of CDCs after 2 passages were CD105+, with significant pluralities that were c-Kit+, CD90+, CD34+, and CD31+ (Figure 2f). These cells were also largely MDR1+, CD133+, and CD45+, as well as negative for a cocktail of blood lineage markers, which indicates that CDCs are phenotypically distinct from human cardiac stem cells identified in vivo (c-Kit+ MDR1+),1,4 as well as from bone marrow–derived or endothelial progenitor stem cell populations that might populate the heart via the circulation (CD133+ CD34+ CD45+).25 CDCs do contain significant subpopulations that phenotypically resemble mesenchymal cells or fibroblasts (CD105+ CD90+) or endothelial cells (CD34+ CD31+).26 Figure 2g demonstrates that c-Kit+ CDCs were largely distinct from CD90+ and CD31+ CDC subpopu- lations. We hypothesize that the mesenchymal cell subpopu- lation might provide physical or secretory support to the c-Kit+ subpopulation during CDC expansion. Human skin fibroblasts, used as control cells for in vivo experiments, were similarly characterized (Data Supplement, Figure II).

Cardiospheres and CDCs derived from adult human or porcine endomycardial biopsy specimens do not spontane- ously contract. To examine the ability of CDCs to differen- tiate fully into functional myocytes, we utilized an in vitro coculture system. Lentivirally transduced green fluorescence protein–positive CDCs were identified in cocultures with NRVMs. A number of such cocultured porcine CDCs dem- onstrated biophysical features characteristic of cardiac myocytes: calcium transients synchronous with those in neigh- borings myocytes (Figure 3a), spontaneous action potentials (Figure 3b), and fast, inward sodium currents (Figure 3c). Human CDCs in coculture expressed I_K1 (Figure 3d), consistent with a ventricular phenotype,27 and, when transduced with the β-subunit of the L-type calcium channel, I_Ca,L (Figure 3e), which indicates the presence of the pore-forming α-subunit.28 These functional data reflect the ability of CDCs to acquire a cardiomocyte phenotype when cultured with cardiac myocytes in vitro.

Such data may be attributable to cell fusion rather than genuine differentiation of CDCs. To evaluate this possibility, we quantified the ability of human CDCs to undergo cell fusion with NRVMs in vitro. Nuclei were stained with Hoechst (for all nuclei) and a human nuclear antigen (for CDCs); cell borders were identified under white light. The majority of CDCs were mononucleated after 1 to 2 weeks of coculture (Figure 4a), whereas nearly all NRVMs were binucleate. Trinucleated cells were never observed (n=200 CDCs counted) in cocultures performed with 4 different patient samples. Binucleate CDCs could occasionally be identified in coculture (Figure 4a inset, Figure 4b). In such cases, both nuclei were positive for the human nuclear antigen. Mononucleate and binucleate human CDCs in cocul- ture often expressed the cardiac-specific transcription factor Nkx2.5 (Figure 4b), although to a lesser extent than the surrounding or underlying NRVMs (Figure 4d). Both mono- nucleate and binucleate CDCs expressing cardiac troponin I in a striated pattern could be identified in cocultures (Figure 4e–f). These in vitro data argue against cell fusion as a mechanism by which CDCs express cardiac-specific proteins in the presence of myocytes.

CDCs at passage 2 (after ∼4.3 population doublings and 50 days in culture) from 7 different randomly selected patients were transduced with an adenovirus expressing β-galactosidase and utilized for in vivo experiments. Transduc- tion did not alter CDC growth or morphology, and β-galactosidase expression was maintained for at least 20 days in vitro. CDCs, human fibroblasts, or vehicle were injected under direct visualization at 2 peri-infarct sites. Eleven mice were injected with CDCs and monitored for 20 days. Eighteen mice served as controls (7 injected with fibroblasts and 11 with PBS). Figure 5 shows engraftment of CDCs at 0 (panel a), 8 (panel b), and 20 (panels c through f)
days. Figure 5g shows a typical β-galactosidase staining pattern indicating the distribution of injected human cells in the infarct region after 20 days in vivo. Note the band of blue cells infiltrating the infarct zone, which was not apparent in fibroblast-injected mice (Figure 5h) or PBS-injected mice (not shown).

Regeneration was quantified in sections stained with Masson’s trichrome (Figure 6a and b), in which viable tissue binds
fuchsin and stains red, whereas fibrous tissue stains blue. An example of the analysis is depicted in Figure III in the Data Supplement. Total infarct area after 20 days was similar in all groups (60.6 \pm 6.4 CDCs, 76.9 \pm 7.0 fibroblasts, 75.7 \pm 2.7 PBS; units in 10^6 pixels at 10 \times magnification; \( P = \text{NS} \)). Figure 6a, from a CDC-injected heart, shows a number of obvious red regions within the predominantly blue infarct zone; fewer such regions are evident in the fibroblast-injected heart (Figure 6b). CDC-injected mice had a higher fraction of viable fuchsin-positive tissue within the infarct zone (24.9 \pm 1.1\%) than fibroblast-injected mice (17.7 \pm 1.8\%, \( P < 0.01 \)) or PBS-injected mice (13.7 \pm 0.7\%, \( P < 0.01 \)). The differences between the CDC and control groups quantify the myocardial regeneration attributable to CDCs: In absolute terms, viability increased 1.4-fold (versus fibroblasts) or 1.8-fold (versus PBS).

Echocardiograms (eg, Figure 7c through 7f and supplementary movies) performed on day 20 revealed a higher LVEF (Figure 7a) in the CDC-treated group (42.8 \pm 3.3\%) than in either the fibroblast-treated (25.0 \pm 2.0\%, \( P < 0.01 \)) or the PBS-treated (26.0 \pm 1.8\%, \( P < 0.01 \)) group, whereas the 2 control groups were indistinguishable. Baseline LVEF before surgery was similar in all groups (80.4 \pm 2.8 CDC, 78.4 \pm 2.9 fibroblast, 75.4 \pm 1.7 PBS; \( P = \text{NS} \)), and LVEF 2 days after surgery was similar in all groups (45.2 \pm 4.8 CDC, 42.8 \pm 4.3 fibroblast, 46.5 \pm 4.6 PBS; \( P = \text{NS} \)). Left ventricular fractional area showed differences similar to those in LVEF (Figure 7b).

We examined the ability of CDCs to differentiate in vivo via immunostaining with cardiac- and endothelium-specific antigens. CDCs could be found engrafted throughout the infarct region (compare to Figure 5g) and the

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**Figure 3.** Evidence for CDC differentiation. a, Spontaneous intracellular calcium transients recorded from the porcine CDC shown and a representative neighboring NRVM. Fluorescence images correspond to the tracings shown. The CDC of interest is outlined in white. b, Spontaneous action potentials from porcine CDCs recorded with no input current. c, \( I_{Na} \) recordings made after cocultures were washed with palmitoleic acid (40 \( \mu \)mol/L) to uncouple porcine CDCs and the neighboring NRVMs. d, \( I_{K1} \) recorded in a cocultured human CDC. e, \( I_{Ca,L} \) recorded in a cocultured human CDC.
border regions of the mouse heart at 20 days (Figure 8a and 8b), and several CDCs could be identified as differentiated cardiomyocytes (Figure 8c, 8e, 8f, and 8g) and endothelial cells (Figure 8d) within the viable myocardium. Although we did find evidence of cardiogenic differentiation in vivo, the numerous islands of myocardial regeneration within the infarct zone in the CDC-injected mice may reflect not only direct regeneration but also paracrine effects leading to increased mobilization of endogenous cardiac stem cells and/or enhanced survival of existing myocardium after injury. Improvements in global left ventricular function likely reflect a summation of numerous as-yet-undefined CDC-mediated beneficial effects.

Discussion

We have shown the feasibility of generating human cardiospheres and expanding stem cells from routine endomyocardial biopsy specimens. Human and porcine CDCs can differentiate into electrically functional myocytes in vitro. Human CDCs injected into mice lead to myocardial regeneration and functional improvement after infarction.

Distinctions From Prior Work

The present approach differs from previous ones in 2 ways: first, no antigenic selection step is required (Beltrami et al\(^2\) and Oh et al\(^3\)); second, cardiospheres are used not as a final product but rather as the basis for cell expansion (Messina et
al8). The flow cytometric analyses previously reported8 were
done with mouse-derived cells, not human cells. The in vivo
studies previously reported8 used human cells derived from
either atrial or ventricular surgical biopsy specimens from
patients of all ages. We have limited our starting material to
small percutaneous biopsy specimens from adult patients.
Given the regional variations in the density of resident
cardiac stem cells29 and possible age-associated differences,
we sought to demonstrate that cardiospheres can reliably be
generated from endomyocardial right ventricular biopsy spec-
imens from adult patients. We have also extended the in vitro
characterization of cardiosphere-derived cells to include mea-
sures of electrophysiological function. Furthermore, we per-
formed a new in vivo study, demonstrating the regenerative
potential of CDCs, and made the new observation that CDC
transplantation increases the percentage of viable tissue
within the infarct region.

The use of the cardiosphere as a pivotal step is designed to
provide a consistent starting material for cell expansion and
to minimize fibroblast contamination (only nonadherent car-
diospheres are plated to create CDCs). Nevertheless, because
there is no antigenic selection or cell-sorting step, CDCs
include subpopulations of mesenchymal cells and possibly
other cell types, in addition to genuine cardiac progenitor
cells. We speculate that the use of a mixed cell population
may be advantageous for progenitor cell proliferation and
possibly for regenerative efficacy, although the present work
does not establish such superiority. Further studies will be
needed to define the relative advantages and disadvantages of
antigenically purified progenitor cells, CDCs, cardiosphere-
forming cells, and cardiospheres themselves as products for
regenerative therapy.

**Risks and Limitations**

CDC transplantation offers the potential for the use of
perfectly matched autologous cardiogenic cells, but it also
poses certain risks. We did not observe any evidence of
tumors in vivo or sudden death in the CDC-treated animals,
but preclinical and clinical studies will need to evaluate safety
with respect to transformation risk. The use of just 2 passages
(≈5 population doublings) for expansion will minimize the
risk of cancerous transformation of CDCs, a problem that has
been observed in mesenchymal stem cells, but only after >6
passages.30 We have also noted human CDCs to be karyo-
typically normal in 4 of 4 specimens analyzed.

A novel risk of cell transplantation lies in the potential
for cardiac arrhythmias.30–33 CDCs express connexin 43 and
have a demonstrated ability to differentiate into functional
myocytes, which would be expected to promote beneficial
functional integration but also could, in principle, create
tissue heterogeneity in vivo, which highlights the need for
careful assessment of arrhythmogenic risk in large-animal studies and in phase 1 clinical trials.

A practical limitation with the use of autologous cells arises from the delay from tissue harvesting to cell transplantation. Here, we have used only 1 small specimen as the tissue source; multiple biopsies performed specifically for therapeutic purposes could easily allow for the overall cell yield to be scaled upward by 10-fold or more within the same time frame, or for the delay to be abbreviated. Although we have shown that CDCs engraft and have functional benefits in the acute infarct setting, it is not yet clear whether CDCs would be similarly effective if administered to the scarred or healing myocardium several weeks after an infarction. Clinical data available to date with bone marrow cells suggest that delay from the time of infarction may actually be beneficial. Further studies are necessary to determine optimum cell dosing, timing, and means of delivery for CDCs. Another potential target group for CDC therapy is patients with chronic heart failure, for whom efficacy has yet to be tested in preclinical models (although promising results have been reported with transplantation of bone marrow–derived cells in small trials of patients with chronic cardiomyopathy).

Finally, we wish to emphasize that the present work describes a promising new cell therapy strategy but only begins to explore the mechanisms of benefit. The functional improvement and histological evidence for regeneration in CDC-treated postinfarct mice now serve as motivation for establishing how much of the observed benefit is attributable to direct regeneration versus other factors, such as paracrine effects and enhanced angiogenesis. The fact that fibroblasts do not have similar benefits is noteworthy and excludes a nonspecific effect of cell transplantation.

Relationship to Endogenous Repair Capacity
The extensive prevalence of cardiac dysfunction in patients teaches us that the ability of the heart to regenerate itself after injury must be limited. Adult human cardiac stem cells have been shown to respond to a state of cardiac hypertrophy by proliferation and myocardial regeneration and to acute ischemia by mobilization to the injury border zone and subsequent regeneration, but endogenous cells often ultimately succumb to apoptosis. Prospects for enhancing survival, mobilization, proliferation, and subsequent differentiation of cardiac stem cells in vivo are numerous. Ex vivo isolation of cardiac-derived progenitor cells may in fact remove (as-yet-undefined) factors present in vivo that inhibit their proliferation. Our method for ex vivo expansion of cardiac-derived progenitor cells for subsequent autologous transplantation may give the resident and the expanded cell populations the combined ability to mediate myocardial regeneration to an appreciable degree. If so, cardiac stem cell therapy may well change our fundamental approach to the treatment of heart disease.

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Figure 7. Functional improvement. a and b, LVEFs and left ventricular fractional areas for the 3 experimental groups. +P<0.05 relative to CDC group at 20 days. *P<0.01 relative to CDC group at 20 days. #P<0.01 relative to same group at 2 days. c and d, Long-axis views from an echocardiogram performed after 20 days in a CDC-injected mouse. Panel c shows end diastole; panel d shows end systole. Yellow lines trace around the left ventricular area used for the calculation of LVEF and left ventricular fractional area. e and f, Comparable views in a fibroblast-injected mouse.
Figure 8. Differentiation. a and b, Human CDCs engrafted in a mouse heart at 20 days. c, Coexpression of α-sarcomeric actin and β-galactosidase in a CDC engrafted in the noninfarct region of the heart. d, Coexpression of von Willebrand factor and β-galactosidase. e, Connexin 43 expression detected between engrafted CDCs and myocytes. f, Coexpression of myosin heavy chain and β-galactosidase as detected by an X-gal stain. g, MF-20 expression seen in CDCs near the infarct region.

Disclosures

Dr Marbán is a founder and equity holder in Capricor, Inc. R.R. Smith and Dr Abraham are co-inventors on a patent licensed to Capricor, Inc. Capricor provided no funding for the present study. The remaining authors report no conflicts.

References

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CLINICAL PERSPECTIVE

Regenerative therapy by cell transplantation has already reached clinical application with autologous bone marrow–derived cells or skeletal myoblasts. Such cell types are not, however, predestined to become genuine heart tissue. Here, we develop and utilize methods for isolating endogenous cardiac stem cells from percutaneous endomyocardial biopsy specimens. In culture, human cardiac stem cells self-organize into spherical clusters called cardiospheres, which are enriched in “stemness” and which represent the starting material for our ultimate cell product, cardiosphere-derived cells (CDCs). Human and porcine CDCs can differentiate into cardiac myocytes in vitro. For in vivo experiments, we created infarcts in mice with severe combined immunodeficiency. Direct injection of human CDCs into the infarct border zone led to myocardial regeneration by histology and to functional improvement by echocardiography. Our data set the stage for a novel paradigm of autologous therapy with CDCs in postinfarction cardiomyopathy.
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