BRIEF REPORT

The characterization of cardiac stem cells obtained from patients who have received left ventriculoplasty

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The recent clinical application of c-kit-positive resident cardiac stem cells (CSCs) for severe heart failure patients revealed their remarkable and long-lasting beneficial effects. Ultimately aiming at a combined therapy of left ventriculoplasty and autologous CSC transplantation, we have successfully isolated and cultivated endogenous stem cells from the right atrium and the infarcted left ventricle, respectively, of all subjects in the target population. Three independent parameters, population doubling time, BrdU incorporation, and colony forming ability, each of which indicates the growth property of cultured CSCs, correlated well in every sample. Overall, CSCs derived from both origins possessed a great proliferative potential with a non-significant superiority in the right atrial CSCs. This brief report provides a fundamental basis for regenerative therapy as a potential novel management of ischemic cardiomyopathy, following the ventriculoplasty.

Keywords: cardiac stem cells; c-kit; left ventriculoplasty; growth potential

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Introduction

Surgical intervention for ischemic cardiomyopathy is a major field to be explored, considering the shortage of donors for organ transplantation and the high cost and potential hazards of ventricular assist devices. Among the currently available regimen, the Dor procedure excises the akinetic or dyskinetic scar of the left ventricle, aiming at its reverse remodeling. While this operation shows a promising outcome in the short term [1], long-lasting therapeutic effects are in question [2]. The adult mammalian heart has been considered post-mitotic and terminally differentiated. However, the recent identification of resident human cardiac stem cells (CSCs) [3] has challenged this dogma and enabled us to offer a novel treatment to those suffering from severe congestive failure [4]. Remarkably, a single injection of CSCs was effective for as long as two years, without increasing the major adverse cardiac event rates [5]. Therefore, it is logically attractive to combine left ventriculoplasty and autologous CSC transplantation, in order to treat patients with advanced ischemic cardiomyopathy. In order to formulate a basis for such therapies, the purpose of this study was to investigate the feasibility of CSC isolation from patients receiving the Dor procedure. Additionally, we attempted to characterize and compare CSCs derived from the right atrium (RA) and the left ventricle (LV) in each subject.

Materials and Methods
Tissue samples. Tiny surgical specimens were obtained during the Dor procedure following a written consent from the participants and were used for the isolation of CSCs. This research was approved by the Institutional Review Board at Tokai University (#12I-18).

CSC isolation and culture. Human c-kit positive cells were prepared as reported [4] with a slight modification. In brief, scissors were used to cut a block of cardiac tissue into small pieces of ~1 mm in size. After digesting the sample in 2 mg/ml of collagenase (Serva NB4) for an hour at 37°C with gentle rotation, cells were dissociated in a GentleMACS C tube (Miltenyi Biotec), employing the preset program B_01 for 31 seconds. Isolated small cells were seeded through a 40-µm filter and cultivated in Ham's F12 Nutrient Serum (HyClone), employing the preset program B_01 for 31 seconds. Isolated small cells were seeded through a 40-µm filter and cultivated in Ham’s F12 Nutrient Mixture (HyClone) supplemented with 10% Fetal Bovine Serum (HyClone), 10 µg/l Trafermin (Kaken, Japan), 5 U/l Epoetin alfa (Kyowa Hakko Kirin, Japan), 0.2 mM Glutathione (Tanabe, Japan), and antimicrobial agents. Subsequently, c-kit-positive cells were sorted by means of CD117 MicroBead and MACS separation system (Miltenyi Biotec), following the manufacturer’s instruction.

c-kit staining. Cultured CSCs were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and incubated with Rabbit Anti-Human CD117 (Dako) diluted 40 times with PBS overnight at 4°C. After washing, Rhodamine-conjugated Donkey Anti-Rabbit IgG (Jackson ImmunoResearch), diluted at 1:50 with PBS, were applied and incubated at 37°C for an hour. Nuclei were counterstained with DAPI (Sigma), and Vectashield (Vector Labs) was mounted for observation by a fluorescent microscope BZ-9000 (Keyence, Japan). An aliquot of cells treated simultaneously without the primary antibody served as a negative control. The c-kit positivity was assessed using the ImageJ software.

Population doubling time (PDT) assay. CSCs were seeded at a density of 5,000 cells per 35-mm dish with a 2-mm grid. The cell number in each of the nine squares was counted on four consecutive days. The PDT was calculated from the plot of time versus cell number in a log scale.

BrdU staining. On a 35-mm dish, 50,000 CSCs were plated the day before the one-hour incubation with a medium containing 1x BrdU (Roche). Cells were then immersed in a fixative consisting of 15 mM Glycine and 70% ethanol (pH 2.0) and incubated for half an hour with Anti-BrdU (Roche) diluted by the Incubation Buffer (Roche). After washing, Anti-mouse IgG-FITC (Roche) was applied and kept for half an hour at 37°C, before adding DAPI. BrdU positivity was calculated by the division of the number of BrdU-positive cells over the total cell number.

Clonal assay. Only 1,000 CSCs were seeded on a 100-mm dish so that each cell was well isolated from the others, and the number of formed colonies in the entire dish was counted a week later. A colony was defined as a cluster of 10 cells or more within a magnified field, which went through more than three rounds of division during the observation period. In most cases, a colony held ~30 cells.

Data presentation and statistical analyses. Results are reported as the mean ± SD. All analyses were performed using SPSS Statistics Version 21 (IBM). The distribution of each parameter was evaluated by the Shapiro-Wilk method prior to the analysis. The Student’s t-test was employed to compare the data of CSCs obtained from the RA and LV, except for the PDT where the Wilcoxon signed-rank test was applied. P < 0.05 was considered significant.

Results

Characteristics of the study population

As summarized in Table 1, 10 patients within the age range of 53 to 77 underwent the Dor procedure and participated in this study. They have a history of old myocardial infarct and received a coronary artery bypass grafting (CABG) simultaneously with the left ventriculoplasty.

CSC preparation and the specificity of the isolation

Human cardiac cells were obtained from the resected tissues of the RA and LV, separately. Following a week of cell culture, CSCs were selected by the c-kit antigen. The specificity of the sorting was evaluated in all samples by immunocytochemistry and fluorescent microscopy. As shown in Fig. 1, more than 80% of the cells were positive for c-kit using our method. Importantly, we were able to isolate and expand CSCs of every patient to a clinically relevant magnitude, i.e. more than one million in number, utilizing small surgical specimens from the RA and LV, respectively.
Comparison between right atrial and left ventricular CSCs

Cultured CSCs, at a passage between 4 and 6, were used to evaluate three parameters reflecting their proliferative activity: population doubling time (PDT), BrdU positivity, and their ability to form monoclonal colonies. As summarized in Fig. 2, these values correlated well with one another (P < 0.05 for 1/PDT vs. BrdU and P < 0.01 for 1/PDT vs. clone number), suggesting the validity and relevance of these mutually independent measurements. Basically, CSCs derived from both regions showed a great proliferative capability. Between them, however, CSCs obtained from the RA tended to possess a higher growth potential than those from the LV (Fig. 3).

Discussion

In this study, we have demonstrated the feasibility of endogenous stem cell preparation from patients receiving the Dor procedure. Regarding the proliferative potential, represented by the PDT, BrdU positivity, and monoclonal colony-forming ability, the right atrial CSCs tended to be superior to those originated from the LV. In fact, recent studies indicated that the RA contains more c-kit-positive CSCs than the LV of adult patients suffering from various cardiac diseases [6,7] as well as of pediatric patients with cardiomyopathy [8], implying a steady distribution of CSCs throughout our life. Moreover, one could reasonably assume that CSCs stored near the damaged left ventricular myocardium of our study population divided more frequently to participate in spontaneous regeneration [9] and thereby acquired senescent phenotype sooner than those in other regions. Also, it should be noted that the CSCs isolated from the right atrial appendage had been utilized for autologous transplantation with great success in a recent clinical trial [4,5].

Regarding the availability of CSCs under various clinical conditions, previous studies indicated that the patient’s age negatively correlates with the number [10,11] and quality [12] of c-kit-positive cells isolated from cardiac tissue samples. Also, the female heart was reportedly associated with a higher number of CSCs [6]. Regarding pathological profiles, some studies suggested that CSCs declined in the presence of coronary heart disease [11], whereas others found increased c-kit-positive cells in the failing heart [6,13]. Such a discrepancy could be due to the variance in the stage of the disease and/or the nature of the harvested tissue specimen. Generally speaking, it may be reasonable to speculate that CSCs derived from patients with an episode of myocardial infarct [14] and/or those suffering from advanced heart failure [12] have impaired proliferative capacity. Importantly, however, this dysfunctional alteration does not occur uniformly in all cells [12,15]; CSCs with preserved growth reserve are still present even at the terminal stage of the organ [12,16]. In fact, we were able to obtain and expand c-kit-positive CSCs from every sample, regardless of age and comorbidities, providing the basis for a potential hybrid therapy employing ventriculoplasty followed by stem cell

<table>
<thead>
<tr>
<th>Age</th>
<th>Male/Female</th>
<th>Smoke/never smoke</th>
<th>BMI (kg/m²)</th>
<th>BNP (pg/ml)</th>
<th>HbA1c (%)</th>
<th>RA (mg)</th>
<th>LV (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>65.1 ± 9.1</td>
<td>6 / 4</td>
<td>4 / 6</td>
<td>23.5 ± 4.9</td>
<td>628.8 ± 634.4</td>
<td>6.96 ± 1.13</td>
<td>116.4 ± 79.7</td>
<td>733.5 ± 791.1</td>
</tr>
</tbody>
</table>

Table 1. The characteristics of the study population (n = 10). Showing the mean ± SD. BMI; body mass index, BNP; brain natriuretic peptide, RA; weight of right atrial tissue sample, LV; weight of left ventricular tissue sample.
transplantation.

In our current research, we did not recognize a significant correlation between the proliferative potential of CSCs and the corresponding clinical data including age, gender, history of smoking, medication, BMI, serum BNP, HbA1c level, and the weight of the tissue specimen. Patients without a history of smoking tended to retain CSCs with a greater growth potential, though (for BrdU, 15.5 ± 6.3% vs. 12.1 ± 5.1%, P ≈ 0.2). The lack of significance might be due to the small sample size and/or the relative homogeneity of the system. Interestingly, D’Amario et al. recently investigated the relationship between the growth kinetics of c-kit-positive CSCs and the clinical outcome of coronary bypass surgery; they revealed that a decline in CSC proliferation is coupled with a lower ventricular function during the follow-up period after revascularization [17]. Therefore, the property of CSCs we observed might serve as a biomarker predicting the evolution of cardiomyopathy, rather than a reflection of the patient’s condition upon operation. Further studies will be required to elucidate this important and clinically relevant issue.

In conclusion, we have successfully prepared CSCs from subjects undergoing left ventriculoplasty due to ischemic cardiomyopathy. Our data provides vital information for the potential clinical use of autologous CSCs for these patients.

Conflict of interests

The authors declare that there is no conflicting interest regarding this study.

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References