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Original article

Isolation and expansion of functionally-competent cardiac progenitor cells directly from heart biopsies

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ABSTRACT

The adult heart contains reservoirs of progenitor cells that express embryonic and stem cell-related antigens. While these antigenically-purified cells are promising candidates for autologous cell therapy, clinical application is hampered by their limited abundance and tedious isolation methods. Methods that involve an intermediate cardiosphere-forming step have proven successful and are being tested clinically, but it is unclear whether the cardiosphere step is necessary. Accordingly, we investigated the molecular profile and functional benefit of cells that spontaneously emigrate from cardiac tissue in primary culture. Adult Wistar–Kyoto rat hearts were minced, digested and cultured as separate anatomical regions. Loosely-adherent cells that surround the plated tissue were harvested weekly for a total of five harvests. Genetic lineage tracing demonstrated that a small proportion of the direct outgrowth from cardiac samples originates from myocardial cells. This outgrowth contains sub-populations of cells expressing embryonic (SSEA-1) and stem cell-related antigens (c-Kit, abcg2) that varied with time in culture but not with the cardiac chamber of origin. This direct outgrowth, and its expanded progeny, underwent marked *in vitro* angiogenic/cardiogenic differentiation and cytokine secretion (IGF-1, VGEF). *In vivo* effects included long-term functional benefits as gauged by MRI following cell injection in a rat model of myocardial infarction. Outgrowth cells afforded equivalent functional benefits to cardiosphere-derived cells, which require more processing steps to manufacture. These results provide the basis for a simplified and efficient process to generate autologous cardiac progenitor cells (and mesenchymal supporting cells) to augment clinically-relevant approaches for myocardial repair.

1. Introduction

Historically, the post-natal heart has been viewed as an organ incapable of regeneration. This conclusion was based upon early evidence that cardiogenesis is completed soon after birth [1,2]. However, this dogma has been challenged by evidence that cardiomyocyte replacement occurs throughout adulthood [3,4] and by recognition of the existence of adult heart progenitor cells expressing embryonic (SSEA-1) and/or stem cell-related (abcg2, c-Kit, isl-1, sca-1) antigens [5,6]. Furthermore, several studies have shown that isolated and purified populations of these cardiac progenitor cells (CPCs) are capable of differentiating into cardiac tissue and improving function after a myocardial injury [5,6].

With a view to providing patient-specific therapy, efficient and rapid methods for CPC isolation are highly desirable. Neural stem cells can be expanded from tissue as self-assembling spherical aggregates ("neurospheres") [7]. This technology was applied to the heart to produce spherical progenitor-rich aggregates of cardiac cells, termed cardiospheres [8,9]. Our group advanced this method towards clinical translation by the expansion of cells from human endomyocardial biopsies with an intermediate cardiosphere-forming step to generate cardiosphere-derived cells (CDCs) [10,11]. The rationale for this cardiosphere formation step was to enrich in "stemness", but it is unknown whether this step is actually required. Other groups have employed techniques based on allowing cells to proliferate from the initial sample of cardiac tissue, with positive candidate cell selection (e.g., SSEA-1$^+$, c-Kit$^+$, sca-1$^+$) prior to further *ex vivo* proliferation of these defined sub-populations [6,12,13]. As with cardiospheres, these initially homogenous sub-populations have been shown to contain clonogenic and multipotent cells capable of self-renewal.

This study investigates the ultimate simplification of these culture techniques by focusing on the primary product, that is, the initial cellular outgrowth from cardiac samples without recourse to antigenic sub-selection or cardiosphere expansion. This approach is

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attractive as it would improve production efficiency, limit prospects of culture-acquired phenotypic drift and, as has been demonstrated in mesenchymal stem cells, the risk of cancerous transformation [14]. Accordingly, we profile the regional and temporal patterns of growth, differentiation and gene expression of CPCs cultured directly from myocardial tissue. Additionally, we provide translational relevance by examining the capacity for functional differentiation and post MI functional improvement as compared to those expanded as CDCs.

2. Materials and methods

2.1. Cell culture

Cardiac progenitor cells were cultured from the hearts of adult male Wistar–Kyoto rats (WK; 3.0 ± 0.4 months old) as previously described [10]. In brief, hearts were excised from heparinized rats (1000 U IV) and underwent retrograde perfusion with heparinized PBS to minimize thrombus formation. The heart was then dissected into five different regions (atria, LV-free wall, RV-free wall, septum apex, septum base) and each region was separately cut into fragments less than 1 mm^3, washed and partially digested with collagenase (1 mg/ml). These tissue fragments (termed cardiac explants; Figs. 1(A) and (B)) were cultured on fibronectin (20 μg/ml) coated dishes in cardiac explant media (CEM; Iscove's Modified Dulbecco's Medium, 20% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 2 mmol/l L-glutamine, and 0.1 mmol/l 2-mercaptoethanol). During the first week of growth, a layer of fibroblast-like cells emerge from the cardiac explant (Fig. 1(C)) above which loosely-adherent cells later become suspended (Fig. 1(D)). The cells surrounding the explant (termed cardiac outgrowth) were harvested using mild enzymatic digestion (0.05% trypsin). Cardiac outgrowth could be harvested up to four more times from the same specimen (Fig. 1(A)). For experiments utilizing CDCs, cardiac outgrowth was seeded at 2×10^4 cells/ml on poly-D-lysine coated dishes in cardiosphere growing media (CGM; 35% IMDM/65% DMEM-Ham's F-12, 2% B27, 0.1 mmol/l 2-mercaptoethanol, 10 ng/ml EGF, 20 ng/ml bFGF, 40 nmol/l Cardiotrophin-1, 40 nmol/l thrombin, 100 U/ml pen-strep, 2 mmol/l L-glutamine). Cells that remained adherent to the poly-D-lysine coated dishes were discarded, while detached cardiospheres were plated on fibronectin coated flasks and expanded as monolayers to generate CDCs. Single cells were counted under phase microscopy to track cell growth for each specimen and region.

WK rat dermal fibroblasts served as a negative live-cell control and were cultured as described [15]. Neonatal rat ventricular myocytes (NRVMs) were used in co-culture experiments and were cultured as described [16,17].

The colorimetric WST-8 assay (Cell counting kit 8, Dojindo Molecular Technologies, Inc. Gaithersburg, MD) was used to track CDC, outgrowth and dermal fibroblast proliferation. Population doubling was calculated with first colorimetric assay as the starting population [# of population doublings = log2 (cell number)]. Doubling time was calculated as the difference between the time (t) and cell counts (N) of the starting population (1) and the final population after two weeks in culture (2) [Doubling time = (t2 − t1)log2 / (logN2 − logN1)] [18].

2.2. Genetic cell fate mapping with bi-transgenic MerCreMer-Z/EG mouse cardiomyocytes

Bi-transgenic MerCreMer-Z/EG mice were produced by crossbreeding cardiomyocyte-specific MerCreMer mice [19] and Z/EG mice [20] (Jackson Laboratory) as described previously [21]. The Z/EG reporter mouse carries cytomegalovirus (CMV) enhancer/chicken b-actin promoter driving floxed b-galactosidase and multiple stop codons, followed by eGFP. Animal genotype was verified by standard PCR on tail genomic DNA using the following primers: MerCreMer forward: 5′-ATACCGGAGATCATGCAAGC-3′; MerCreMer backward: 5′-AGGTG-GACCTGATCATGGAG-3′; and ZEG forward: 5′-ACGGCAAGCTGACCCT-GAAG-3′; ZEG backward: 5′-AAGATGGTGCGCTCCTGGAC-3′; internal control forward: 5′-CTAGGCCACAGAATTGAAAGATCT-3′; and internal control backward: 5′-GGATGATGCTAGAATTTCCACCTAC-3′. Double heterozygous bi-transgenic MerCreMer-Z/EG mice were used for the myocyte lineage tracing experiments after induction of Cre recombination for GFP labelling exclusively in cardiomyocytes by 4-OH-Tamoxifen treatment [21]. Tamoxifen-treated bi-transgenic mice were used at the age of 6–10 week and we did not see significant difference in their capability of cell outgrowth. Bi-transgenic hearts underwent the same cell culture as WK rats on 2-well chamber slides with immunostaining and microscopy performed 1 week after plating.

2.3. PCR analysis

Screening for markers of CPC or cardiac identity was performed using RT PCR. One million outgrowth cells were pooled from 4 to 6

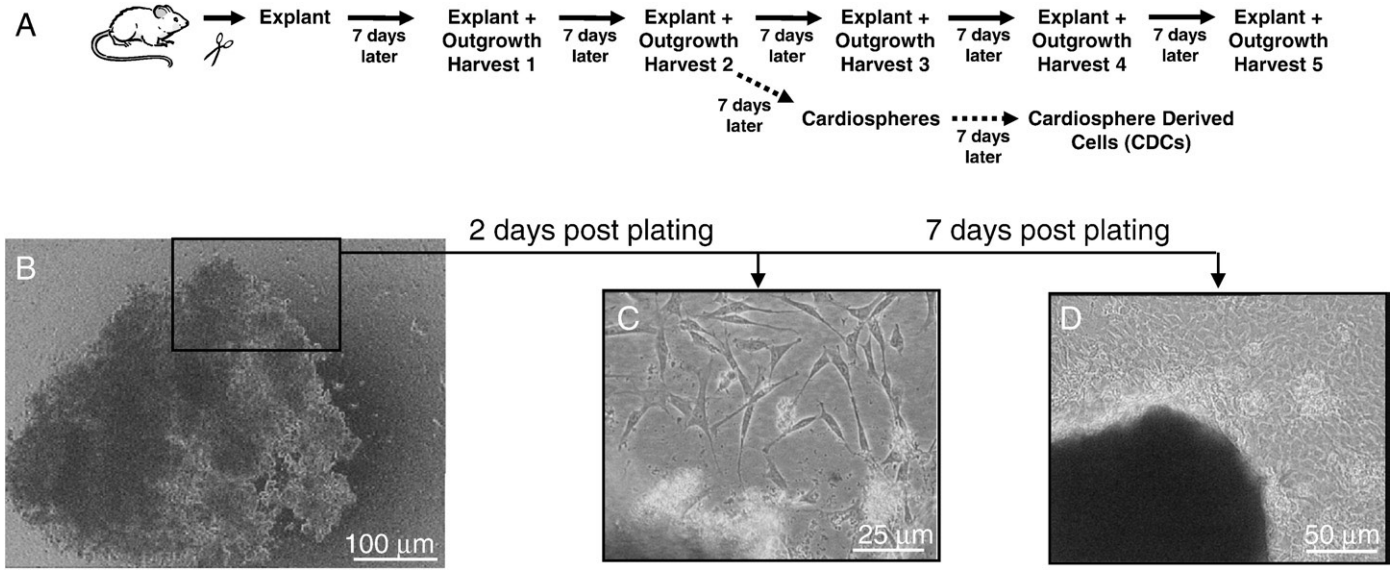

Fig. 1. Specimen processing for cardiac outgrowth, cardiosphere and cardiosphere-derived cell (CDC) expansion. (A) Schematic depiction of the steps involved from tissue harvest and through serial collection of cardiac outgrowth from the plated tissue (referred to as the explant). (B) Example tissue explant immediately following plating. (C) Magnified view of the same tissue explant 2 days later and (D) loosely-adherent cells 7 days after plating.