Isolation and Characterization of the SSEA-1+ Progenitor Cells from the Human Embryonic Heart

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Abstract

Background: The optimal stem cell source for regenerative therapy of the failing heart has not been settled. Embryonic stem cells, bone marrow derived cells, skeletal myoblasts, as well as cells derived from adult cardiac biopsies have been explored. Here we investigated the option to generate heart progenitor cells from the early human embryonic heart (hEHPCs) for the treatment of acute myocardial infarction.

Methods: Two hearts (8 and 8.5 gestational weeks) from human abortion material were used for the clonal expansion of hEHPCs after stage specific embryonic antigen 1 (SSEA-1) enrichment by magnetic beads isolation. GFP-transduced hEHPCs were transplanted into 18 SCID mice intramyocardially after induction of myocardial infarction. Hearts were harvested every 72 hours.

Results: In this study we succeeded to isolate clones of hEHPCs with similar characteristics twice from two different early human embryonic hearts (8 and 8.5 gestational weeks) based on SSEA-1, a multipotent stem cell marker. Isolated clones were found to be positive for the multipotent stem cell marker (c-kit), the cardiac progenitors transcription factors GATA4, NKX2.5, TBX5 as well as the endothelial progenitors markers (CD133, CD34 marker). Isolated clones were found to be positive for the multipotent stem cell marker (c-kit), the cardiac progenitors transcription factors GATA4, NKX2.5, TBX5 as well as the endothelial progenitors markers (CD133, CD34 and KDR). After transplantation into the peri-infarcted region they survived up to 12 days, and formed tubule-like structures in the mouse heart.

Conclusions: These data demonstrate that the SSEA-1+ enriched cell population provides a potential basis to find the optimal cardiac progenitor cell population.

Keywords: Human embryonic heart; Progenitor cells; Myocardial infarction

Introduction

Ischemic heart disease is the leading cause of heart failure in the Western world and the prevalence increases with age. Revascularizations by angioplasty or thrombolysis have improved survival of patients with acute myocardial ischemia, but necrotic tissue cannot be restored by such revascularization. But still there is a large population that has already developed heart failure where we treatment options are lacking except for heart transplantation or mechanical assist devices. A future alternative might be heart regeneration, but so far which stem cell to be used has not been settled.

Evidence for a constant turn-over of cardiomyocytes in the adult heart has been reported for rat as well as man [1,2], but the origin of these stem cells or progenitors is unclear. In animal models, transplantation of fetal or neonatal cardiomyocytes into infarcts scar, prevented left ventricular dyskinesis, and improved global ejection fraction [3-5].

Studies done in mice provided evidence that the main lineages of the heart develop from a common cardiovascular progenitor [6,7]. Similarly, human cardiovascular lineages i.e. cardiac, endothelial and vascular smooth muscle cells may be derived from a common progenitor [8].

Goumans et al. [9] suggested that cardiac progenitor cells could be isolated and expanded clonally from human adult or late fetal hearts with great potential to expand and differentiate into cardiomyocytes, endothelial cells and smooth muscle cells. Bilin and co-workers had used the stage-specific embryonic antigen 1 (SSEA-1) to isolate multipotent cardiovascular progenitors [10]. These progenitors were derived from human embryonic stem cells (HESCs) and iPSCs. It would be of great interest to reproduce the achievements reported by Bil et al. using human embryonic cardiac material.

In this study, we were aiming to explore whether the SSEA-1+ enriched cell population from the early human embryonic heart can be a source for clonal identification and expansion of human cardiovascular progenitor cells.

Methods

Isolation of hEHPCs from the fetal heart

Two fetal hearts (8 and 8.5 gestational weeks) were obtained after voluntary abortion. Isolation of the cells were done as described earlier

References

[1-10]
by Smits et al. [11]. Briefly, hearts were mechanically digested into small pieces followed by enzymatic digestion using collagenase enzyme. hEHPCs were then enriched by magnetic cell sorting (Miltenyi Biotec, Sunnyvale, CA, USA) as suggested by Bliin et al. [10], using a cardiac multipotent stem cell marker, SSEA-1-coupled beads, following the manufacturer’s protocol. Isolated cells were plated onto 0.1% gelatin coated 96-well plate at a density of 0.5 cell per well in M199 (Gibco, invitrogen, Carlsbad, CA, USA)/EBM-2 (3:1) medium supplemented with EGM-2 Single Quots (vascular endothelial growth factor, basic fibroblast growth factor, epithelial growth factor, insulin-like growth factor, hydrocortisone, fetal bovine serum, ascorbic acid, heparin and gentamicin) (Clonetics, Lonza, Basel Switzerland). Moreover 10% fetal calf serum, 2% penicillin/streptomycin, 1% MEM nonessential amino acids and just before use, 10 ng/ml basic fibroblast growth factor were added. The wells were analyzed twice weekly for growing colonies.

The present study was approved by the human and animal local ethics committees of the Karolinska Institute at Karolinska University Hospital.

RNA extraction and Real-Time RT-PCR

Total RNA was isolated from hEHPCs using the Pico Pure RNA extraction kit and cDNA was synthesized with the High Capacity cDNA Reverse Transcription kit, both from Applied Biosystems (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. During the RNA extraction process the samples were treated with Dnase (Qiagen, Hilden, Germany) to remove contaminating genomic DNA.

Real-time quantification of cDNA was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). TaqMan Gene Expression Assays for human GAPDH, NXX2.5, TBX5, KDR, c-kit, tropolin T, Islet 1 (Isl1) and GAPDH (Applied biosystems) were used for quantification of gene expression. To visualize the gene expression the PCR products were run on a 2% agarose gel stained with ethidium bromide.

Phenotyping of hEHPCs isolated from fetal hearts

Isolated clones were phenotyped with an array of antibodies to specific markers expressed on endothelial progenitor cells (KDR, CD133, CD34, CD31 and CD105), stem cells markers (c-kit (CD117), hematopoietic cells markers (CD45, CD14) and anti-SSEA-1 using flow cytometry.

Antibodies were purchased from the following companies: anti-KDR (R&D Systems, Abingdon, UK), anti-CD133 (Miltenyi Biotec, Gmbh, Bergisch, Germany). All other antibodies were purchased from Becton and Dickinson, San Jose, CA, USA. Unstained cells were used as controls. The cells were analyzed on a fluorescence-activated flow cytometer (FACSCalibur) and with the help of Cell Quest Software, (Becton Dickinson, San Jose , California, USA).

To detect protein expression of endothelial cells markers vWF (Abcam plc, Cambridge, UK) and VE-cadherin (Becton and Dickinson, San Jose, CA, USA), cardiomyocytes marker tropolin T (Abcam) or smooth muscle α-actin marker (Sigma, Saint Louis, Missouri, USA); hEHPCs were placed on glass slides using cytospin. Immunolabeling was performed using AlexaFluo 568 goat anti-mouse (Invitrogen). The stained cells were mounted with diamidino-2-phenylindole (DAPI). The mounted medium and analyzed by fluorescence microscopy.

Green fluorescent protein lentiviral transduction of hEHPCs

Viral vectors were produced by co-transfection of 3 μg of FUGW [12] (D. Baltimore - California Institute of Technology, Pasadena, CA), 1 μg of pMDG and 2 μg of pCMVΔR8.91 (D. Trono - University of Geneva, Switzerland) into 0.5x10^7 293FT cells in 6-well plates using Fugene 6 transfection reagent (Roche, Mannheim, Germany). The medium was replaced 24 hours post-transfection followed by collection of virus containing supernatants at 48h and 72h. Viral titers were determined by transduction of HeLa cells and supernatants were stored frozen at -80°C until further use.

For transduction, hEHPCs clonally expanded cells were centrifuged at 1000xG for 1 hour at room temperature in the presence of viral supernatant at a multiplicity of infection (MOI) of 3 and polybrene (Sigma) at a concentration of 8 μg/ml. After centrifugation, the viral supernatant was removed; the cells were resuspended in full growth medium and plated.

Functional assays: angiogenesis assay

To study the differentiation capacity of the clonally expanded hEHPCs, cells were added to a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma (matrigel) (Becton Dickinson, San Jose, CA, USA), frequently used for the evaluation of in vitro angiogenesis by cells. Briefly, twenty four-well plates were coated with 250 μl matrigel/well (pre-gelled for 30 minutes at 37°C in 5% CO₂ incubator). The hEHPCs or GFP-transduced hEHPCs were seeded at a density of 5 x 10^4 cells/well and cultured overnight at 37°C in 5% CO₂ incubator. Tubule-like structures formation was examined using a phase-contrast light microscope and fluorescence microscopy.

Transmission electron microscopy

To confirm the differentiation of clonally expanded hEHPCs on matrigel, the formed tubule-like structures were examined using transmission electron microscopy (TEM). Tubules were immediately fixed in 2% glutaraldehyde in 0.1M sodiumcacodylate buffer containing 0.1M sucrose and 3mM CaCl₂, pH 7.4 at room temperature for 30 min. The cells were scraped off with a wooden stick and transferred to Eppendorf tube and further fixed over night in the refrigerator. After fixation cells were rinsed in 0.1 M phosphate buffer containing pH 7.4 and centrifuged. The pellets were then postfixed in 2% osmium tetroxide in 0.1M sodiumcacodylate buffer containing 0.1M phosphate buffer, pH 7.4 at 4°C for 2 hour, dehydrated in ethanol followed by acetone and embedded in LX-112 (Ladd, Burlington, Vermont, USA). Sections were contrasted with uranyl acetate followed by lead citrate and examined in a Leo 906 transmission electron microscope at 80 kV (Leo, Oberkochen, Germany). Digital images were taken by using a Morada digital camera (Olympus Soft Imaging System, Gmbh, Münster, Germany).

Induction of myocardial infarction and hEHPCs transplantation

Acute myocardial infarction was induced in 18 SCID Beige mice (Jackson Laboratories) [13]. Under general anesthesia a left lateral thoracotomy and pericardiotomy were performed, exposing the heart. The coronary left anterior descending artery (LAD) was identified and ligated with a 7-0 polypropylene suture. At the same time 2 x 10⁶ of the clonally expanded hEHPCs were suspended in 15-20μL growth medium and then injected into the myocardium surrounding the infarcted area in one injection. Cells from one clone were transplanted to 8 mice and from the other clone to 10 mice. The thoracotomy was then closed with interrupted sutures of vicryl and Ethilon.

Tracking of transplanted GFP-transduced hEHPCs

After the intracardiac injection of the clonally expanded hEHPCs, one mouse was sacrificed every 72 hours and the heart was harvested.
and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 24 hours at room temperature. The hearts were then rinsed with PBS and were cut in cold PBS using Vibratome (100 µm in thickness). Sections were transferred to slides and mounted with diamidino-2-phenylindole (DAPI) mounting medium and analyzed by confocal laser scanning microscope system (Olympus).

Results

hEHPCs isolated clones

In this study a SSEA-1+ enriched fraction (Figure 1a and b) was used for the identification and expansion of hEHPCs. Two clonal expansions of hEHPCs, isolated from two human embryonic hearts were performed. The phenotype of the colonies was characterized after 4 passages and 30 days of culture from the clonal seeding of cells from the enriched homogenate. The criteria for clonal selection and expansion of hEHPCs clones were high growth potential and small spindle-shaped cells with high nucleus-to-cytoplasm ratio (Figure 1c). The cells showed similar morphology during culturing and were followed up to the 12th passage. hEHPCs expressed the cardiac progenitor transcription factors GATA4, NKX2.5 and TBX5, but did not express Isl1 or mature cardiac marker troponin T as were analyzed by RT-PCR (Figure 1d). Furthermore hEHPCs expressed multipotent stem cell marker (c-kit) and endothelial cell markers (KDR).

Characterization of hEHPCs

Immunophenotyping of hEHPCs showed that isolated clones were positive for endothelial progenitor cell markers (CD133, CD34, KDR, CD31 and CD105 (endoglin)) as well as the multipotent stem cell marker (c-kit) (Figure 2a-f). Although both cell populations were found to highly express the hematopoietic cell–specific surface antigen CD45, they were negative for monocytic cells marker (CD14) (Figure 2g and h). However cells were not found to express SSEA-1 (CD15) (Figure 2i).

Immunocytochemistry showed that cells were positive for the both endothelial cells markers (von Willebrand factor, vWF) and vascular endothelial cadherin (VE-cadherin), but they were negative for the cardiomyocyte marker, troponin T and smooth muscle α-actin markers (Figure 3).

In vitro endothelial differentiation of clonally expanded hEHPCs

Isolated hEHPCs clones were able to form capillary-like structures in matrigel, a characteristic feature of mature endothelial cells. Moreover the formation of these structures was maintained before and after lenti-viral transduction with GFP, indicating that the virus did not adversely affect the transfected cells (Figure 4a and b).

Examination of these capillary-like structures under transmission electron microscopy showed endothelial phenotype where cells formed tight junctions and had a lot of pinocytotic vesicles indicating endothelial cell activity (Figure 4c and d, respectively).

Engraftment of clonally expanded hEHPCs

GFP- transfected hEHPCs were successfully transplanted into the peri-infarct region of 18 SCID mice. In confocal microscopy, GFP-labeled hEHPCs were able to form capillary-like structures six and nine days post-transplant (Figure 5). Cells could be found in mice hearts until day 12 after transplantation, while no cells were found at day 13. The GFP-labelled hEHPCs did not co-stain with troponin T (data not shown), further supporting that the micro environment in this model stimulate differentiation into endothelial cells rather than cardiomyocytes.

Discussion

Cardiac regenerative treatment is a major field of research including its future clinical application. However, the optimal stem cell source...
Figure 2: Representative histogram overlays plot figures for flow-cytometric analysis of cell surface marker expression on clonally isolated cultured hEHPCs. Histogram plots are shown with the control (unstained cells) in dotted line and the specific fluorescent signal in solid line overlay. hEHPCs express CD133, CD34, KDR, CD31, CD117, CD105 and CD45 but do not express CD14 and SSEA-1.

Figure 3: Fluorescence microscopy illustrates that the clonally expanded hEHPCs were positive for endothelial cell markers vWf and VE-cadherin (red) but not for cardiomyocyte marker troponin T or smooth muscle α-actin. Bars indicate 100 µm.
Figure 4: a) Phase-contrast microscopy analysis of hEHPCs show differentiation into tubular structures on matrigel before transduction with GFP lentivirus and b) Fluorescence microscope analysis of hEHPCs differentiated into tubular structures on matrigel after transduction with GFP lentivirus. c) Electron microscopic analysis of isolated hEHPCs, representative pictures of transmission electron microscopy (TEM) of hEHPCs capillary-like structures in matrigel showing attachment between microvillus structures of endothelial like cells resembling tight junctions formation (arrows, scale bar; 0.5µm), and d) TEM image showing an hEHPCs plasma membrane with abundant pinocytotic vesicles (arrows, scale bar; 0.5µm).

Figure 5: Confocal microscopic analysis of SCID mouse heart. Representative images showing in vivo engraftment of GFP lentivirus transduced hEHPCs (green cells) in SCID mouse heart. Nuclei of cells are stained with DAPI, blue in colour. Cells could be traced until day 12 after transplantation. Shown are representative data from two independent experiments after transplanting the two hEHPCs isolated clones. Sections are 100 µm in thickness.
regarding safety, availability and functional engraftment remains unclear. The clinical trials based on bone marrow cell or skeletal myoblast transplantation have so far been discouraging [14,15].

In the present study, we used the early human embryonic heart (gestational week 8 and 8.5) from aborted material as a source for cardiac progenitor cells. When based on SSEA-1 enrichment, the clonally expanded hEHPCs express the early cardiac transcription factors GATA4, NKK2.5 and TBX5 and stem cells marker (c-kit) as well as the endothelial progenitor markers (CD133, CD34 and KDR), suggesting that they are a true progenitor cell population already committed to cardiac-endothelial lineages.

This population demonstrated the capacity to form capillary-like tubules in vitro as well as in vivo up to 12 days after transplantation. In electron microscopy, the clonally expanded hEHPCs showed presence of tight junctions and pinocytotic vesicles suggesting an endothelial phenotype. These results indicate that VEGF in the growth medium preferentially stimulates primary human embryonic SSEA-1+ enriched cell population to differentiate into endothelial progenitors which can be clonally expanded in vitro and derive endothelial structures in the ischemic myocardium of mice. Taken together these hEHPCs seem to have properties which are beneficial for angiogenic treatment. Lack of material and ethical considerations restrict the use of these cells in clinical settings, but they may serve as a template for other stem cells such as embryonic stem cells or IPS cells. From this point of view the hEHPCs are interesting for development of differentiation protocols for derivation of heart progenitors for treatment of ischemic heart disease.

Burlacu [16], showed that in vitro treatment with 5-azacytidine promoted the commitment of bone marrow stromal cells into cells that expressed muscle-specific proteins and genes, but it was not enough to complete the differentiation towards beating cardiomyocytes. In this study we showed that hEHPCs have potentiality to become cardiomyocytes by expressing the cardiac progenitor markers GATA4, NKK2.5 and TBX5, but they did not differentiate in vivo. These data suggest that, hEHPCs may need to be primed first towards cardiomyocytes by using 5-azacytidine or other strategy prior to their transplantation into the failing myocardium. Thus human cardiac stem cells are multipotent and support myocardial regeneration as previously shown in the rat albeit with some differences in surface markers expression [16].

The transient engraftment of the GFP-labelled hEHPCs could be due to the harsh environment in the heart and also that hEHPCs find difficulties to migrate into their appropriate niche in this harsh injury area in the heart. To overcome such problem, hEHPCs could be transplanted together with biodegradable materials. Such studies are under investigation in our laboratory. Bergman et al. [17] succeeded to regenerate bone tissue by using hydrogel in a minimally invasive manner. The formed bone is restricted to the volume of the gel and there are no signs of inflammation or foreign body response and the gel was degraded within four weeks.

In conclusion, these data suggest that the early human embryonic heart contains hEHPCs that can be clonally expanded expressing cardiomyocyte progenitor cell markers and can after implantation into ischemic myocardium differentiated into endothelial progenitor cells forming capillary structures. This cell type might be used as a template for the generation of cardiac progenitor cells from unfractionated stem cell sources.

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References
