Hellenic Society of Cardiology (2017) 58, 98—102

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.journals.elsevier.com/hellenic-journal-of-cardiology/

LETTER TO THE EDITOR

Effect of 2,4-Dinitrophenol preconditioning on the expression levels of mesenchymal markers in neonatal cardiac progenitors

KEYWORDS
Preconditioning;
Mesenchymal;
Cardiomyocytes;
Progenitor;
Adhesion

1. Introduction

The discovery of resident cardiac stem cells has destabilized the paradigm that the adult heart is a postmitotic organ without regenerative potential.[1] Several types of cardiac stem cells in adult heart have been identified using the following markers: c-kit, Sca-1, LIM/homeodomain transcription factor islet-1 (isl-1) positive cells; side population (SP) cells that express ATP-binding cassette transporter Abcg2[2] and CD34 positive epicardial cells have also been identified.[3] Several studies have shown that cardiac progenitors also express mesenchymal markers (e.g., homing associated cell adhesion molecule or HCAM (CD44), Thy-1 (CD90), integrin β1(CD29), and endoglin SH-2 (CD105)) that are routinely used to identify bone marrow-derived mesenchymal stem cells (MSCs). These cardiac-derived MSCs also coexpress the stem cell marker c-kit and show angiomyogenic differentiation with reduced adipogenic and osteogenic gene expression more often than bone marrow-derived MSCs.[4] In addition to characterizing cells, the roles of these markers have not been evaluated in cardiac precursors. These mesenchymal markers belong to the class of adhesion proteins that generate cell-cell and cell-matrix adhesions and perform essential functions of stem cell/progenitor niches, such as cell maintenance, proliferation and differentiation. Blocking studies against the surface receptors integrin β1 and CD44 resulted in reduced bone marrow-derived mesenchymal stem cell migration, engraftment and differentiation into cardiomyocytes, suggesting their possible role in cardiomyogenesis.[5,6] The activated leukocyte cell adhesion molecule (ALCAM or CD166) is another mesenchymal marker expressed on embryonic cardiac progenitors that plays a role in cardiac differentiation during embryogenesis.[7] The oxygen level in stem cell physiological niches is another important factor that plays a critical role in controlling stem cell proliferation and fate determination. The approach of preconditioning or preculturing stem cells in low oxygen levels reduces cell death and increases expression of cardiomyogenic and angiogenic markers, thereby enhancing the effectiveness of stem cell therapy.[8]

These observations led us to hypothesize that hypoxic preconditioning may increase the expression of various cell surface glycoproteins and adhesion molecules that contribute to engraftment and movement of cardiac progenitors across the extracellular matrix to the desired tissues or organs and to differentiation of progenitors into the desired cell type by making cell-cell and cell-matrix adhesions. The present study was conducted to analyze whether DNP preconditioning modulates the expression of cell surface glycoproteins, cell adhesion molecules and the coexpression of mesenchymal markers on neonatal cardiac progenitors. Keeping in mind the role of these molecules in cardiomyogenesis, this study will help to determine if changes in their expression levels occur during ischemic cardiac injury.

2. Methods

2.1. Cell culture

Cardiac progenitors (CPs) were isolated from 1- to 2-day-old neonatal NMRI mice. The excised ventricles were placed in a 0.5% trypsin/EDTA solution at 37°C in a thermal rocker incubator for complete digestion. The cells were cultured in cardiomyocyte growth medium (CGM)

Peer review under responsibility of Hellenic Society of Cardiology.

http://dx.doi.org/10.1016/j.hjc.2017.01.007

containing Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) (1:1) supplemented with 20% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM sodium pyruvate, 6 mM L-glutamine and 1 μg/ml insulin. The cells were incubated at 37°C in a CO_2 incubator and maintained until passage 2.

2.2. Characterization of cardiac progenitors by flow cytometry

CPs were characterized by using primary antibodies against surface epitopes CD34, GATA4, myosin heavy chain, and actin. Cells were stained with Alexa Fluor 546 goat anti-mouse secondary antibody and analyzed by flow cytometer (BD FACSCalibur, Becton Dickinson, San Jose, CA, USA).

2.3. DNP treatment

The cultured CPs were exposed to chemical hypoxia using 2,4-dinitrophenol (DNP). To determine optimal conditions, different concentrations (0.05—2 mM) of DNP were used for 10 minutes. The number of dead cells was counted by a JC-1 cytotoxicity assay (Cayman Chemicals, Ann Arbor, MI, USA) using flow cytometry. The concentration of DNP corresponding with the least cytotoxicity was used in all experiments. Cells were treated with the optimized dose of 0.1 mM DNP in deaerated glucose-free DMEM for 10 min. The medium was replaced with normal DMEM/F12, and the cells were subsequently incubated for 24 hr at 37°C in a humidified chamber with 5% CO_2 for reoxygenation.

2.4. Reverse-Transcription PCR

After 24 hr of reoxygenation, total RNA was extracted from untreated and DNP-treated cardiac progenitors. First-strand cDNA was synthesized and amplified by performing a PCR reaction. Gene primers are shown in Table 1. A densitometeric analysis of PCR bands was carried out. The integrated density of each band was calculated, and band density was normalized to GAPDH.

3. Results

3.1. Isolation, expansion and phenotypic characteristics of CPs

CPs adhered to cell culture flasks after 24 hr of isolation. At passages 1 and 2, the majority of the cells grew larger in size. More striations were seen, and cells were more flat, octagonally shaped and had various cellular projections (Supplementary Fig. S1).

3.2. Identification of CPs

CPs were analyzed for the expression of various epitopes using flow cytometry. More than 60% of the cells showed positive staining after using monoclonal antibodies against CD34 and cardiac proteins including GATA4, myosin heavy chain (MHC) and actin (Fig. 1).

3.3. Effect of DNP preconditioning on CPs

Chemical hypoxia was given to adherent CPs using DNP. Cells were treated with an optimized dose of 0.1 mM DNP for 10 min. During hypoxia, a portion of the cells shrank and lost their connections with neighboring cells, and only a small number of cells became round and detached from the culture flask (Fig. 2b). However, cells regained their normal morphology after 24 hr from when normal conditions were restored (Fig. 2c). JC-1 cytotoxicity assays were not different between normoxic and hypoxic CPs (Supplementary Fig. S2).

3.4. Expression of mesenchymal markers in CPs

Changes in the expression of mesenchymal markers were determined in CPs after DNP treatment and 24 hr of reoxygenation. Except for CD90 expression levels that were significantly increased ($p \leq 0.05$), hypoxic stress did not significantly change the gene expression levels of most mesenchymal markers (i.e., CD29, CD44 and ALCAM) (Fig. 3).

Table 1 Genes, primer sequences, expected product sizes and annealing temperatures.

Genes	Sequences	Annealing temperature (°C)	Product sizes (bp)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	F 5′-GGAAAGCTGTGGCGTGATGG-3′ R 5′-GTAGGCCATGAGGTCCACCA-3′	60	414
Thy-1 (CD90)	F 5′-AATGGAAAAGGGGAATGGAC-3′ R 5′-CACCTGGGCTTCTTCATAGC-3′	59	190
Homing-associated cell adhesion molecule (HCAM; CD44)	F 5′-CCTTCCTTTGCTGTTGCTTC-3′ R 5′-CACCTGGGCTTCTTCATAGC-3′	55	210
Integrin β1 (CD29)	F 5′-AACGCCAGAACAACAATTCC-3′ R 5′-TGAATTGCCACCAGATGTGT-3	56	235
Activated leucocytes cell adhesion molecule (ALCAM)	F 5′-CTTGCACAGCAGAAAACCAA-3′ R 5′-TAGACGACACCAGCAACGAG-3′	56	190

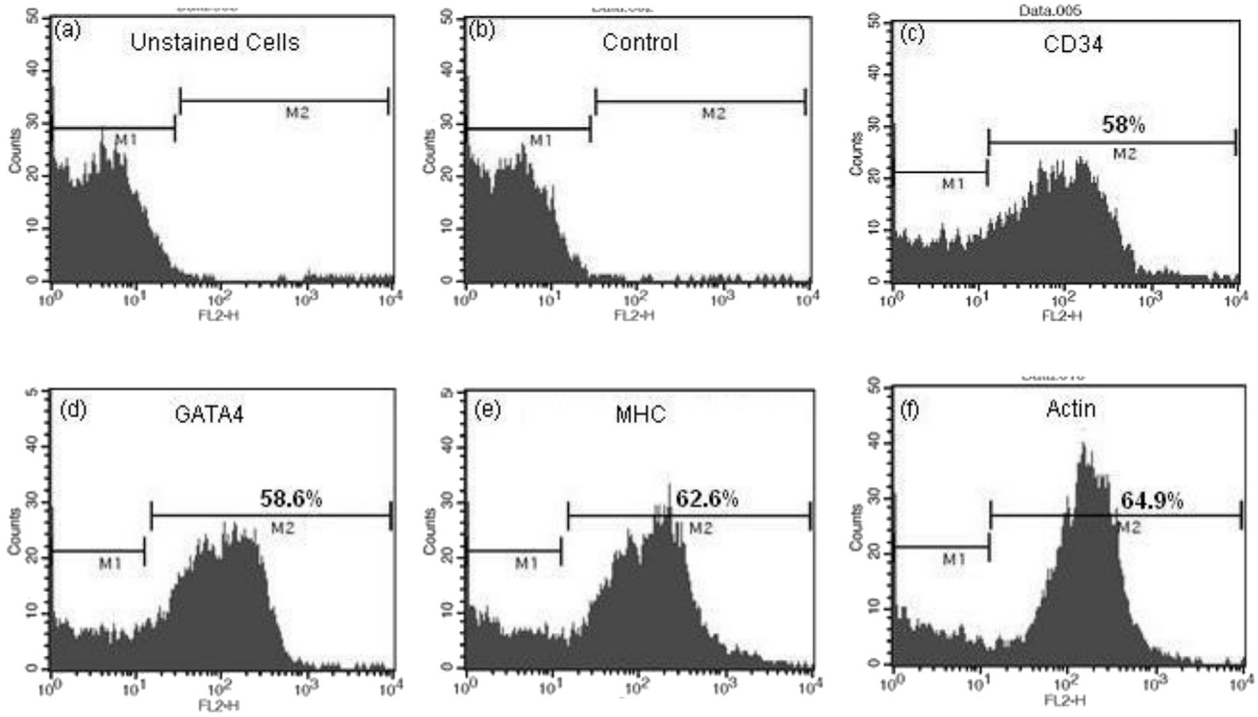

Fig. 1 Characterization of mouse neonatal cardiac progenitors (CPs) using flow cytometry, showing the controls consisting of (a) unstained cells and (b) cells stained only with secondary antibody (Alexa Fluor 546 goat anti-mouse) and experimental cells that were stained with primary antibodies against (c) CD34, (d) GATA4, (e) myosin heavy chain and (f) actin.

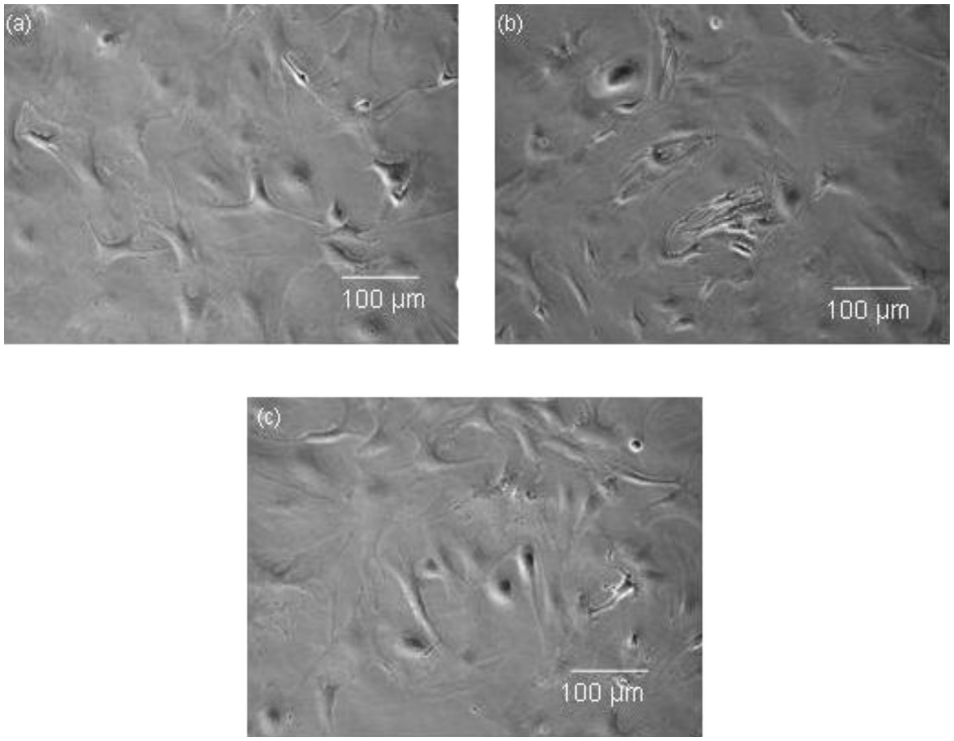

Fig 2 Cardiac progenitors (CPs) showing (a) normal morphology without treatment, (b) shrunken morphology with loss of connections between cells after DNP treatment, and (c) normal morphology after 24 hr of reoxygenation.

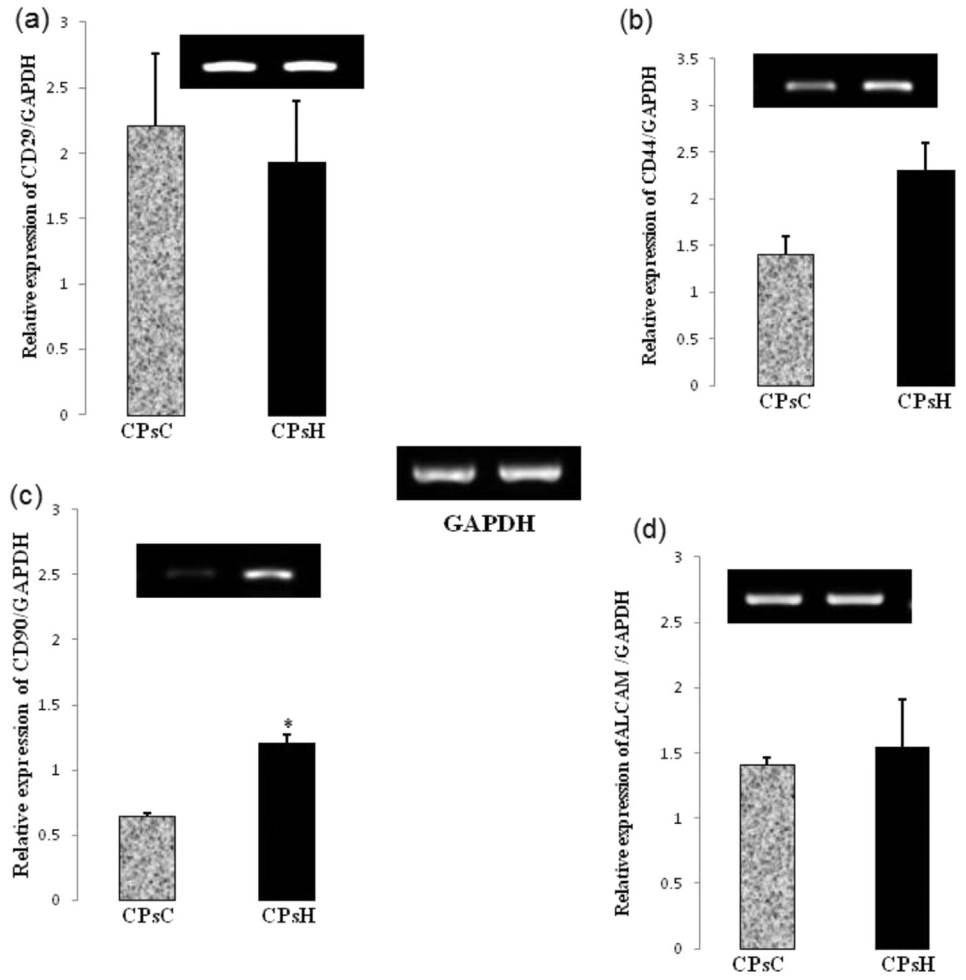

Fig 3 Bar diagrams showing densitometry analysis of the following mesenchymal marker genes in neonatal cardiac progenitors (CPs): (a) CD29, (b) CD44, (c) CD90 and (d) ALCAM in normoxic and hypoxic cardiomyocytes. Representative gel images are also shown. Data are presented as the mean $\pm$ standard error. A value of $p < 0.05$ was considered statistically significant.

4. Discussion

The heart reserves a population of progenitors to have stemness (i.e., they are able to divide and self-renew, differentiate into beating cardiomyocytes and rescue the heart tissue from damage). In the present study, the CPs were isolated from neonatal hearts of NMRI mice. The cells were not fully differentiated and showed the presence of progenitor markers along with cardiac-specific transcription factors and cardiac-specific proteins. Similar results have been observed by Itzhaki-Alfia and coworkers who isolated CPs from adult human heart.[9] The isolated CPs were positive for a hematopoetic marker, CD34, which suggests that they originated from epicardium-derived $CD34^+$ stem cells.[3]

Although the heart is made up of contractile myocytes, smooth muscle cells, endothelial cells, and cardiac stem/progenitors cells, the cardiac fibroblasts and myocytes contribute the most abundant cellular constituents of adult heart. These fibroblast cells were acquired either from blood circulating bone marrow-derived cells or from epicardium-derived cardiac progenitors.[10] The discovery of epicardium-derived CPs supports the hypothesis that cardiac progenitors infiltrate into myocardium and contribute to the development of stem cell niches. Recently, mesenchymal markers have been reported in cardiac progenitors isolated from embryonic, fetal and adult hearts. The expression of mesenchymal markers has been demonstrated in c-kit^+ cardiac progenitors cells, Sca-1^+ cells, cardiosphere-derived cells from human cardiac explants and $CD34^+$ epicardial cells.[3,11–13] In our study, primary neonatal mouse ventricular cardiac progenitors were used to investigate the expression of mesenchymal markers, including CD29, CD44, CD90 and ALCAM.

We analyzed the effects of short-term chemical hypoxia and reoxygenation on the expression of mesenchyme surface glycoproteins and cell adhesion proteins involved in migration and cardiac differentiation of cardiac progenitors. A sublethal hypoxia dose has not been shown to have any detrimental effects on cell morphology. Cells regained a normal morphology with only a few dead cells remaining when cells were reoxygenated for 24 hr. This result suggests that optimized hypoxia was not toxic to the cells. The effect of hypoxic stress on mesenchymal markers

has not been studied to date. We found that hypoxic stress did not effect the expression of CD29, CD44 and ALCAM. However, CD90 expression significantly increased. CD90 is an important regulator of inflammation and wound repair and participates in cell-matrix and cell-cell adhesions during inflammatory events that result in increased migration to inflammatory sites and secretion of various cytokines and growth factors that contribute to healing injured tissue.[14] CD90 also plays an important role in generating the neuronal network during postnatal development and neuronal regeneration.[15] These findings suggest that CD90 may contribute to maintaining a cardiac stem cell pool residing in postnatal heart hypoxic niches and that CD90 is also associated with increased infiltration of cardiac progenitors to repair damaged heart myocardium.

5. Conclusions

Our results suggest that neonatal CPs possess mesenchymal markers that function in cellular maintenance, differentiation and adhesion. CD90 is one of the important mesenchymal markers that may have these roles during times of low oxygen supply to the heart. Future studies should be conducted to determine the role of these mesenchymal markers in the development, maintenance and regeneration of cardiac stem cell niches.

Conflict of interest

None declared.

Acknowledgment

Financial support for this study was provided by the Pakistan Science Foundation, Grant Number: PSF/Res/S-KU/Biotech (92).

Appendix A. Supplementary data

Supplementary data related to this article can be found at doi:10.1016/j.hjc.2017.01.007

References

1. Beltrami AP, UBrbanek K, Kajstura J, et al. Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med*. 2001;344:1750—1757.
2. Barile L, Messina E, Giacomello A, Marbán E. Endogenous cardiac stem cells. *Prog Cardiovasc Dis*. 2007;50(1):31—48.
3. Limana F, Zacheo A, Mocini D, et al. Identification of myocardial and vascular precursor cells in human and mouse epicardium. *Circ Res*. 2007;101:1255—1265.
4. Gambini E, Pompilio G, Biondi A, et al. C-kit+ cardiac progenitors exhibit mesenchymal markers and preferential cardiovascular commitment. *Cardiovasc Res*. 2011;89:362—373.
5. Ip JE, Wu Y, Huang J, Zhang L, Pratt RE, Dzau VJ. Mesenchymal stem cells use integrin β1 Not CXC chemokine receptor 4 for myocardial migration and engraftment. *Mol Biol Cell*. 2007;18:2873—2882.
6. Hirata H, Murakami Y, Miyamoto Y, et al. ALCAM (CD166) is a surface marker for early murine cardiomyocytes. *Cells Tissues Organs*. 2006;184:172—180.
7. Rust W, Balakrishnan T, Zweigerdt R. Cardiomyocyte enrichment from human embryonic stem cell cultures by selection of ALCAM surface expression. *Regen Med*. 2009;4:225—237.
8. Song H, Song BW, Cha MJ, Choi IJ, Hwang KC. Modification of mesenchymal stem cells for cardiac regeneration. *Expert Opin Biol Ther*. 2010;10:309—319.
9. Itzhaki-Alfia A, Leor J, Raanani E, et al. Patient characteristics and cell source determine the number of isolated human cardiac progenitor cells. *Circulation*. 2009;120:2559—2566.
10. Wu SM, Fujiwara Y, Cibulsky SM, et al. Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. *Cell*. 2006;127:1137—1150.
11. Davis DR, Kizana E, Terrovitis J, et al. Isolation and expansion of functionally-competent cardiac progenitor cells directly from heart biopsies. *J Mol Cell Cardiol*. 2010;49:312—321.
12. Smith RR, Barile L, Cho HC, et al. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. *Circulation*. 2007;115:896—908.
13. Naga GV, Tripathy NK, Nityanand S. Fetal cardiac mesenchymal stem cells express embryonal markers and exhibit differentiation into cells of all three germ layers. *World J Stem Cells*. 2013;5:26—33.
14. Saalbach A, Haustein U, Anderegg U. A ligand of human Thy-1 is localized on polymorphonuclear leukocytes and monocytes and mediates the binding to activated Thy-1-positive microvascular endothelial cells and fibroblasts. *J Invest Dermatol*. 2000;115:882—888.
15. Rege TA, Hagood JS. Thy-1 as a regulator of cell—cell and cell—matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer, and fibrosis. *FASEB J*. 2006;20:1045—1054.

Nazia Ahmed, PhD [a]
Irfan Khan, PhD
Sumreen Begum, PhD [b]
Asmat Salim, PhD *
Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan

*Corresponding author. Asmat Salim, Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan.
Fax: +92 21 4819018 19.
E-mail address: asmat.salim@iccs.edu (A. Salim)

4 February 2015
Available online 2 February 2017

[a] Current Address: Nazia Ahmed, Dow University of Health Sciences, Ojha Campus, Gulzar-e-Hijri, Suparco Road, KDA Scheme-33, Karachi, Pakistan.
[b] Current Address. Sumreen Begum, Sindh Institute of Urology and Transplantation, Civil Hospital, Karachi 74200, Pakistan.