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Asian Cardiovasc Thorac Ann 2009;17:139-142
DOI: 10.1177/0218492309103292

This information is current as of October 16, 2010

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ORIGINAL ARTICLE

Clinical Determinants of ckit-Positive Cardiac Cell Yield in Coronary Disease

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ABSTRACT

Recent animal studies and clinical trials have reported the scope of heart-resident ckit-positive stem cells in regenerating infarcted myocardium. The determinants of successful isolation of such cells are unknown. The objective of this study was to determine the influence of risk factors for coronary artery disease and disease severity on the successful isolation of ckit-positive cells from right atrial tissue of patients with coronary artery disease. The findings suggest that coronary artery disease and cardiac remodeling in chronic ischemia may not affect the yield of ckit-positive cells from atrial tissue, but a significant negative correlation between the age of the patient and the number of migrated ckit-positive cells was observed. This suggests that in older patients, stem cell isolation from cardiac biopsies may not succeed, and such cells may not be available for cell therapy.

(Asian Cardiovasc Thorac Ann 2009;17:139–42)

KEYWORDS: Adult Stem Cells, Coronary Artery Disease, Risk Factors

INTRODUCTION

Beltrami and colleagues[1] demonstrated the presence of ckit-positive cells in postnatal hearts. Subsequently, they provided evidence that this subpopulation of cells can be isolated from mouse heart, grown *in vitro* in sufficient numbers, and injected into mouse heart to repopulate infarcted regions of the myocardium with cardiocytes and blood vessels.[2] Recently, stem cells have been isolated from endomyocardial biopsies obtained from cardiac transplant patients. These cells are cardiogenic *in vitro* and have been shown to promote cardiac regeneration and improve cardiac function in a nude mouse infarct model. These observations provide hope for adult heart-derived stem cell therapy in patients with ischemia and chronic heart failure. It is not yet clear whether the yield of $ckit^+$ cells from cardiac tissue is influenced by disease severity or risk factors in patients with chronic coronary artery disease. Therefore, we examined the effects of patient characteristics, including coronary artery disease severity and risk factors, on the yield of $ckit^+$ cells from adult human heart.

PATIENTS AND METHODS

The study group comprised 30 patients aged 38–72 years who had undergone coronary artery bypass grafting at our institute (Table 1). The study was approved by the institutional ethics committee, and right atrial samples were obtained from patients after obtaining their informed consent. Cardiovascular risk factors were defined as follows. Smoking was a history of smoking for more than 2 years. Hypertension was repeatedly elevated blood pressure with systolic pressure >140 mm Hg and diastolic pressure >90 mm Hg for more than 1 year. Diabetes was the need for oral anti-diabetic drug therapy or insulin use. Dyslipidemia was

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doi: 10.1177/0218492309103292

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Table 1. **Clinical characteristics of 30 patients undergoing coronary artery bypass**

Variable	% of Patients
Age (years)	54.87 ± 8.9
Male/female	26:4
Risk factors	
Smoking	40%
Hypertension	60%
Diabetes mellitus	70%
Dyslipidemia	53%
Coronary artery disease	
1-vessel	3%
2-vessel	30%
3-vessel	66%
NYHA class	
I	0%
II	83%
III	16%
Mitral regurgitation	
0	26%
1	46%
2	26%
RWMA	46%
Totally blocked vessels	46%
Right coronary artery blockage	23%
Statin	40%
Beta blocker	90%
Aspirin	20%
Ca-channel blocker	16%
Nitrates	80%

NYHA = New York Heart Association, RWMA = regional wall motion abnormality.

defined as low density lipoprotein >130 mg·dL^{-1}, high density lipoprotein <40 mg·dL^{-1}, triglycerides >150 mg·dL^{-1}, and total cholesterol >200 mg·dL^{-1}. The total vascular risk score was calculated for each patient by considering hypertension, diabetes, smoking, and dyslipidemia, as reported earlier.[3] Patients were grouped depending on the presence or absence of individual risk factors as well as according to the severity of coronary artery occlusion (number of affected coronary arteries assessed during coronary angiography), functional class, mitral regurgitation, wall motion abnormalities, total vessel blockage, involvement of the right coronary artery, and intake of drugs. The clinical profile of the study group is given in Table 1.

Cardiac stem cells were isolated by a nonenzymatic method.[4] Right atrial biopsies were collected in ice-cold buffer immediately after surgery, minced, and cultured as explants on 2% gelatin-coated dishes. The cells that migrated from the explants were collected by trypsin-EDTA treatment, suspended in a growth-factor-supplemented medium, and grown in poly-D-lysine-coated culture dishes (BD Biosciences, San Jose, CA, USA). The migrated cells were characterized by the expression of stem cell markers by fluorescent-activated cell sorting (FACS) analysis. The antibodies used to confirm stem cell identity were anti-human CD117 and MDR 1 mouse monoclonals (Santa Cruz Biotechnology, CA, USA). The antibodies were added to collected cells and incubated for 30 min at 4°C. After washing with cold phosphate buffered saline, the cells were incubated with fluorescence marker conjugated anti-mouse secondary antibody (Molecular Probes, Leiden, The Netherlands) for 30 min at 4°C in the dark. The cells were further washed with phosphate buffered saline, fixed with 4% paraformaldehyde, and quantitative analysis was performed on a 4-color multiparameter flow cytometer (FACS Aria; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The cells that migrated from the explants were expanded in a growth-factor-supplemented medium (IMDM + Dulbecco's modified Eagle's Media-Ham's F-12 mix; Sigma-Aldrich, St. Louis, MI, USA) supplemented with 2-mercaptoethanol 0.1 mmol·L^{-1}, epidermal growth factor 10 ng·mL^{-1}, basic fibroblast growth factor 20 ng·mL^{-1} (US Biologicals, Swampscott, MA, USA), 2% B27 serum supplement (Invitrogen, Grand Island, NY, USA), thrombin 40 nmol·L^{-1} (Sigma-Aldrich, St Louis, MI, USA), benzyl penicillin 100 U·mL^{-1}, streptomycin 100 µg·mL^{-1}, and L-glutamine 2 mmol·L^{-1}.[5] After 48 h of culture, cardiospheres were collected and dissociated by trypsin-EDTA treatment. The cells obtained were analyzed by FACS for expression of stem cell markers such as ckit, MDR1, and CD34, as well as cardiac differentiation markers cTNI and MHC (Santa Cruz Biotechnology, CA, USA). The clinical parameters of the patients were analyzed for their role in determining the number of ckit+ cells migrating from the explants.

Data are expressed as mean ± standard deviation. Comparisons of means among groups were performed by one-way analysis of variance. Bivariate correlation and single linear regression were undertaken to assess the relationship between cardiac stem cell counts and age. A p value of 0.05 or less was considered statistically significant.

RESULTS

Small round phase-bright cells were seen migrating from adherent tissue explants grown from right atrial samples collected from all patients in the study. The cells formed cardiospheres when grown in a medium supplemented with growth factors. These cells stained positive for ckit and MDR1 in FACS. However, the number of migrated cells varied significantly among the explant cultures. Cardiosphere-derived cells stained positively for ckit, MDR1, CD34, and the cardiac differentiation markers cTNI and MHC.[4]

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Table 2. **Clinical parameters and results of analysis of variance**

Variables	No. of Patients	Mean	SD	*p* Value
Male	26	7.76	6.42	0.84
Female	4	8.46	6.72	
Smoker	12	6.94	4.55	0.52
Nonsmoker	18	8.47	7.36	
Hypertension	18	8.33	5.6	0.62
No hypertension	12	7.15	7.49	
Diabetes	21	7.52	7.00	0.67
No diabetes	9	8.63	4.72	
Dyslipidemia	16	7.68	5.75	0.87
No dyslipidemia	14	8.06	7.17	
TVRS 1	7	9.36	9.20	
TVRS 2	9	6.64	5.11	0.69
TVRS 3	10	9.26	6.12	
TVRS 4	3	4.27	2.87	
NYHA class II	25	7.96	6.36	0.84
NYHA class III	5	7.32	6.93	
2-vessel disease	9	4.76	4.02	0.15
3-vessel disease	20	9.44	6.83	
RWMA	14	8.90	6.42	0.41
No RWMA	16	6.94	6.33	
Mitral regurgitation grade 0	8	9.33	9.42	
Mitral regurgitation grade 1	14	5.41	3.14	0.12
Mitral regurgitation grade 2	8	10.67	6.00	
Totally blocked vessel	14	6.70	6.13	0.36
No totally blocked vessel	16	8.86	6.54	
RCA involvement	7	6.61	6.05	0.56
No RCA involvement	23	8.23	6.51	
Statin user	12	6.16	5.08	0.23
Nonuser	18	8.99	6.97	
β-blocker user	27	8.52	6.34	0.08
Nonuser	3	1.89	0.75	
Nitrates user	24	6.88	5.12	0.09
Nonuser	6	11.77	9.47	
Ca-channel blocker user	5	3.80	1.93	0.12
Nonuser	25	8.66	6.63	

NYHA = New York Heart Association, RCA = right coronary artery, RWMA = regional wall motion abnormality, SD = standard deviation, TVRS = total vascular risk score.

Cardiovascular risk factors and other clinical parameters did not seem to affect the number of ckit+ cells migrating from explanted atrial tissue samples. The number of migrating cells was also unaffected by any of the drugs taken by the patients (Table 2). Among the various clinical parameters analyzed, only the age of the patient had a statistically significant relationship with the stem cell number ($p \leq 0.05$, $r = -0.419$; Figure 1).

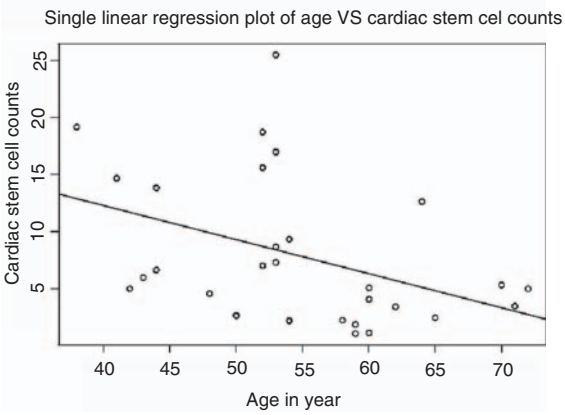

Figure 1. Single linear regression plot of age vs. cardiac stem cell counts.

DISCUSSION

Recent animal studies and clinical trials have reported the scope of heart-resident ckit+ stem cells in regenerating infarcted myocardium. The determinants of successful isolation of such cells are however unknown. The evidence reported here suggests that coronary artery disease and cardiac remodeling in chronic ischemia may not affect the yield of ckit+ cells from atrial tissue. Our findings support current pursuits in developing cardiac regenerative therapy in patients with coronary artery disease, using autologous stem cells isolated and grown from cardiac biopsies.

Embryonic stem cells, bone marrow-derived stem cells, mesenchymal stem cells, skeletal myoblasts, and circulating endothelial progenitor cells have all been assessed for myocardial regeneration, both in animals and humans.[6–9] Recently, it has been demonstrated that ckit+ cells isolated from small myocardial samples (so-called cardiac stem cells) are also possibly capable of efficient myocardial repair.[10] Compared to other cell types, autologous adult cardiac stem cells are considered more efficient in rebuilding damaged myocardium.[11] They have the advantage of being non-immunogenic, and they might not undergo malignant transformation as may happen with cells derived from embryos. Anversa and colleagues[12] showed that stem cells isolated from cardiac biopsies are functionally competent and grow *in vivo* when grafted into a recipient heart, thus indicating the therapeutic possibilities of using cardiac-derived stem cells in myocardial ischemia or end-stage heart disease. Be that as it may, several issues remain to be clarified before the clinical utility of heart-derived stem cells is accepted.[13] Among these is the concern that the population of resident stem cells in cardiac tissue may be affected by the factors responsible for coronary artery disease or myocardial disease. Alterations, if any, in the population of stem cells in the heart would influence the success of stem cell isolation from cardiac biopsies, and thus hamper the clinical utility of such treatment. Our findings embolden the efforts to develop a strategy for myocardial

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regeneration in patients with coronary artery disease, using autologous stem cells isolated from cardiac biopsies.

Our observations are interesting given the contrasting observations on circulating endothelial progenitor cells. There is increasing evidence that patients at risk of coronary artery disease have decreased numbers of circulating progenitor cells, and thus impaired vasculogenesis.[14,15] Cardiovascular risk factors and the number of stenosed coronary arteries also seem to relate to the formation of functional circulating progenitor cells in culture.[16,17] A decline in endothelial progenitor cell number and function with age has been reported.[18] This was attributed to loss of functional telomeres in the absence of telomerase activity, which is a major cause of loss of proliferative capacity in mammalian cells.[19,20] Our data illustrate a similar significant inverse correlation between patient age and cardiac stem cell yield from atrial biopsies. This suggests that in older patients, stem cell isolation from cardiac biopsies may not succeed, and such cells may not be available to them for cell therapy.

We have provided evidence for the first time that the yield of stem cells from cardiac biopsies is not influenced by either disease severity or risk factors for coronary artery disease. Except in older patients, isolation of stem cells from adult heart tissue could provide an avenue for developing regenerative cell therapy in patients with coronary artery disease.

ACKNOWLEDGMENTS

The studies were supported by a grant from the Department of Biotechnology, Government of India. We are thankful to the Director, Sree Chitra Tirunal Institute for Medical Sciences and Technology, for providing the necessary facilities to perform the study. We thank the Medical Records Department of the institute for providing the patients' data.

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