

Journal of Cardiovascular Disease Research

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Volume 7, Issue 1, Jan-Mar, 2016

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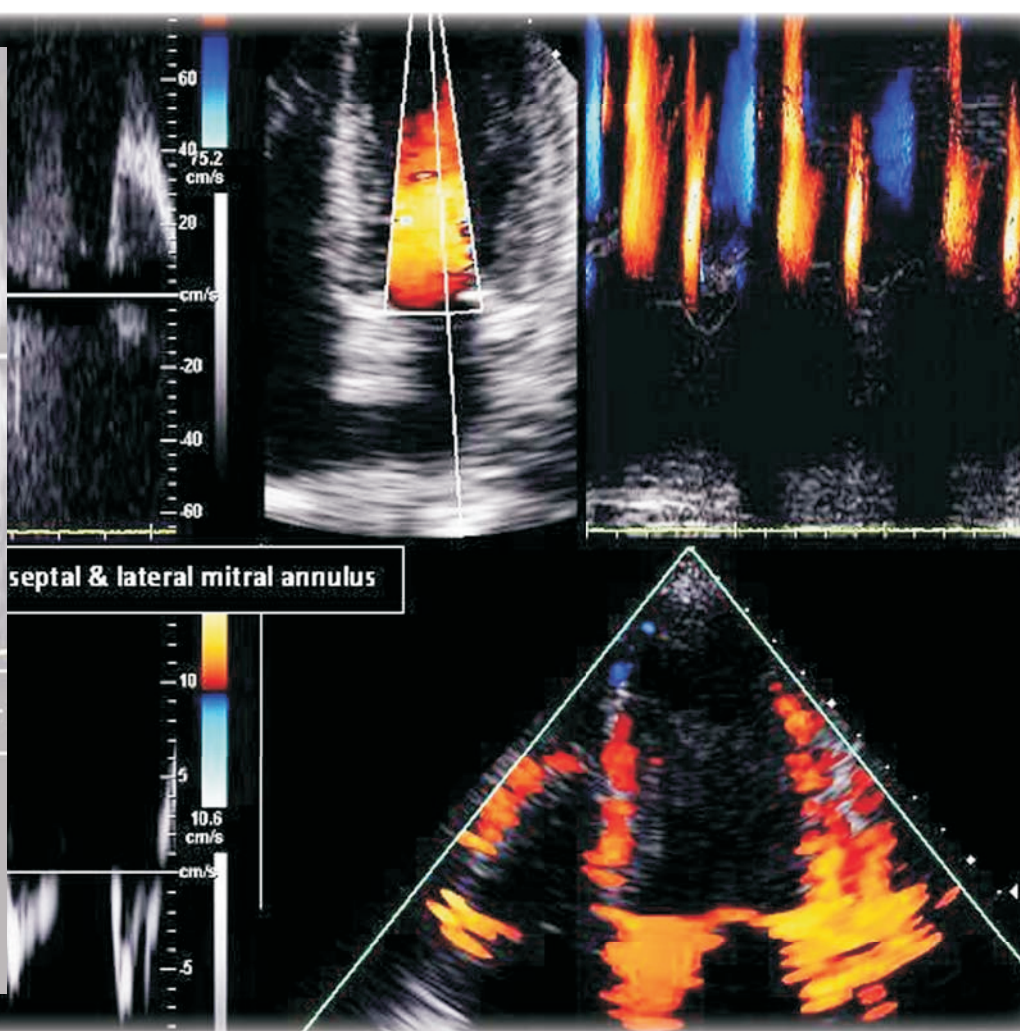
Journal of Cardiovascular Disease Research (J Cardiovasc. Dis. Res.)

[www.jcdronline.org]

[ISSN: Print -0975-3583, Online - 0976-2833]

An official publication of SciBiolMed.Org (www.scibiolmed.org);

it is a double-blind peer-reviewed, open access international circulating professional journal led by a group of research scientists, vascular disease experts and cardiologists coming from North America, Asia and Europe etc.



Indexed and Abstracted in : The journal is indexed with Caspur, Chemical Abstracts, CNKI (China National Knowledge Infrastructure), DOAJ, EBSCO Publishing's Electronic Databases, Expanded Academic ASAP, Genamics JournalSeek, Google Scholar, Health & Wellness Research Center, Health Reference Center Academic, Hinari, Index Copernicus, MANTIS, OpenJGate, PrimoCentral, ProQuest, Scimago Journal Ranking, SCOLAR, SCOPUS, SIIC databases, Summon by Serial Solutions and Ulrich's International Periodical Directory



JCDR - Providing Cutting Edge Research information on Cardiovascular Diseases

Senior International Editor: Dr. Dayi Hu
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Manuscript

A study of Analysis of Endothelial Progenitor cells in Peripheral blood in patients with coronary artery disease

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ABSTRACT

Objective: The study is aimed to analyze endothelial progenitor cells (EPCs) in coronary artery disease and also to identify predictive marker to assess EPCs in the same condition. **Background:** Endothelial progenitor cells (EPC) predict morbidity and mortality in cardiovascular risk. The ideal way to cure atherosclerosis and the subsequent end organ damage is to restore and rejuvenate the dysfunctional vasculature by induction of EPCs. **Methods and Results:** EPCs characterized by CD34 and KDR or CD133 markers still in progress to standardize. Hence, current study demonstrates the marker to identify the EPC in peripheral blood of patients with CAD. Flow cytometry used to quantify EPCs in 25 coronary artery disease as confirmed on angiography patients and 25 control subjects without CAD were prospectively enrolled in the study. To identify predictive EPCs in the peripheral blood, we used various combinations of markers like CD34+KDR+CD133+CD45-. We found that CAD patients presented significantly lower levels of EPCs, which expresses the markers CD34+CD45-KDR+ and CD133+CD45-KDR. EPCs which express CD34+CD45-KDR+ subpopulation shows higher significant change between CAD and control group ($p < 0.001$). **Conclusion:** In conclusion, study indicates that there is a progressive decrease in EPCs levels in CAD patients.

Patients with low EPC counts had a higher incidence for cardiovascular events. The level of circulating EPCs with CD34+CD45-KDR+ expression predicts the occurrence of cardiovascular events and may help to identify patients at increased cardiovascular risk.

Key words: Atherosclerosis, Endothelial progenitor cells, Bone marrow, Vascular endothelial growth factor.

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Submission Date: 19-01-2016; Review completed: 17-02-2016;

Accepted Date: 27-02-2016

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DOI : 10.5530/jcdr.2016.1.4

INTRODUCTION

Atherosclerosis is the leading cause of death in the throughout world. Clinical manifestations like myocardial infarction, stroke, heart failure and peripheral artery disease, resulting in irreversible organ damage. Coronary artery disease results from a chronic inflammatory disease of the vascular wall and leads to vessel occlusion and organ damage.¹ Various serum markers have been identified that predict mortality and morbidity due to cardiovascular cause.²⁻⁵

Current guidelines for the prevention of atherosclerosis focuses in life-style change and reduction in risk factor and to minimize devastating factors like oxygen free radicals and subsequent damage to endothelial cell (EC). Clinical studies suggest that risk factors and a genetic predisposition together induce inflammatory processes leads to cell damage and vessel wall degeneration.⁶⁻⁷ After damage, resident endothelial cells will proliferate to vascular replenishment.⁸ Asahara *et al.* first reported the isolation of circulating angioblast from human peripheral blood of adults, which had EC differentiation in ischemia *in vivo*.⁹ Further it was named as endothelial progenitor cell[EPC]. Origin of this phenotype may be distinguished between EPCs reside in bone marrow (BM) and circulating EPCs. It was also demonstrated that myelomonocytic cells, spleen derived mononuclear cells (MNCs) and cord blood derived MNCs contribute to the pool of EPCs.^{10,11} Experimental studies shows that the systemic application or mobilization of progenitors beneficially influence repair of endothelial cells in atherosclerosis.¹²⁻¹⁸

In previous studies, EPCs characterized by markers CD34 and vascular endothelial growth factor (VEGF) receptor 2 or kinase domain receptor (KDR) and CD133.^{7,10,19,20} Whereas, functional and clonogenic capacity of EPCs evaluated using colony forming unit assays.^{17,21} Yet there are no/

less studies demonstrates the full-fledged markers to identify the EPC in peripheral blood of patients with CAD.

Hence, the current study aimed to analyze the enumeration of endothelial progenitor cells in patients with coronary artery disease by combining various specified markers.

MATERIALS AND METHODS

Study Population

25 coronary artery disease as confirmed on angiography patients were prospectively enrolled in the study between May 2015 and October 2015. Normal healthy persons were taken to analyse same marker as a control group. Study protocol was approved by the ethics committee of NMCH Nellore. Baseline demographics and clinical data were recorded for all patients.

Quantification of EPCs by flow cytometry

Arterial blood collected in 5 ml sodium citrate Tube. Immediately after blood collection, samples were transferred to laboratory and processed between 1-2 hours. Progenitor cells from blood were analyzed by fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) FACScanto II system (Becton Dickinson, US) as previously reported.²²⁻²⁴ Red blood cell deprived from whole blood using RBC lysis solution(Sigma Chemicals, US). After RBC wash, cell count done using haemocytometer. In a FACS tube, 10 µl of FITC-conjugated anti-human CD34 mAb (BD Biosciences, US) and 10 µl of FITC-conjugated anti-human KDR mAb (BD Biosciences, US) and 1x10⁶ cells were mixed followed by kept for incubation at 4°C for 30 minutes in dark. Cells were

washed twice with FACS buffer. The percentage of positivity against each antibody was determined by side-scatter fluorescence dot plot analysis after appropriate gating. Here we gated CD34+ peripheral blood cells and then examined the resulting population for dual expression of KDR marker. For FACS analysis, 5×10^5 cells were acquired and scored using a FACSscan analyzer. Data processed using the Macintosh CELLQuest software program (Becton Dickinson). The same immunofluorescent cell staining was performed with fluorescent conjugated antibody to CXCR4, CD45 and CD133. In our experiment, sub-populations co-expressing various markers were quantified: 1) CD45-CD133+KDR+ cells; 2) CD45-CD34+KDR+ cells; 3) CD45-CD34+CD133+KDR+ triple positive cells; and 4) the subpopulation of CD45-CD34+KDR+CXCR4+ EPCs. For each analysis, a corresponding negative control with IgG-FITC antibody was used.

Statistical analysis

Cell count number expressed as mean \pm standard deviation. Results from flowcytometry are expressed as the number of cells per one million events (10^6). We calculated correlation analyses using Pearson correlation coefficient to identify various cell population count. Comparisons between two or more groups were performed by the unpaired Student's 't' test and analysis of variance (ANOVA) respectively. The chi-square test was used for dichotomous variables. Statistical significance was accepted if p value < 0.05 . Statistical analysis was performed with SPSS for Windows software version 11.5. Data analysis was performed by SPSS version 20 (IBM SPSS Statistics 20, IBM, Munich, Germany).

RESULTS

A total of 50 patients with coronary artery disease as diagnosed on angiography were enrolled. The mean (\pm SD) age of the cases and control were 48.5 ± 8.8 years (range, 32 to 68) and 45.2 ± 10.5 years (range, 35 to 65) respectively. The mean BMI of cases and control were ≥ 25 and 23-26 respectively (Table 1).

Endothelial Progenitor Cell Counts

The total Leukocyte count analysed before flow cytometry, the mean count was 7.26 ± 2.35 in CAD group and 7.50 ± 2.93 in control group. There was no statistical significance $p=0.25$ observed in leucocyte count between two groups.

The mean CD34 count in cases and control group was 195.5 and 398.3 per 10^6 WBC. There was no statistical significance $p=0.065$ observed in mean CD34 count between two groups. The mean CD133 count in cases and control group was 32.5 and 42.2 per 10^6 WBC. There was no statistical significance $p=0.211$ observed in mean CD133 count between two groups (Figure 1).

The CD45-CD34+KDR+ co-expression was observed as mean of 2.5% and 6.5% in cases and control with statistical significance $p<0.001$. CD45-CD34+KDR+CD184+ co-expression was observed as mean of 0.8% and 2.1% in cases and control with statistical significance $p=0.003$. CD45-CD133+CD34+ KDR+ co-expression was observed as mean 0.8% and 1.5% in cases and control with statistical significance $p=0.004$. CD133+KDR+ co-expression was observed as 2.7% and 5.2% in cases and control with statistical significance $p=0.005$. CD133+KDR+CD184+ co-expression was observed as 2.2% and 3.7 in cases and control with statistical significance $p=0.003$ (Figure 2).

There was a higher significant change was observed in the CD34+CD45-KDR+ sub-population among all other sub-expression between cases and control group.

DISCUSSION

Maintenance of structural and functional endothelial integrity is vital to the preservation of healthy vasculature.²⁵ The impairment and insufficient regeneration potential of the endothelial cell monolayer is thought to be critical factor during the progression of atherosclerosis.

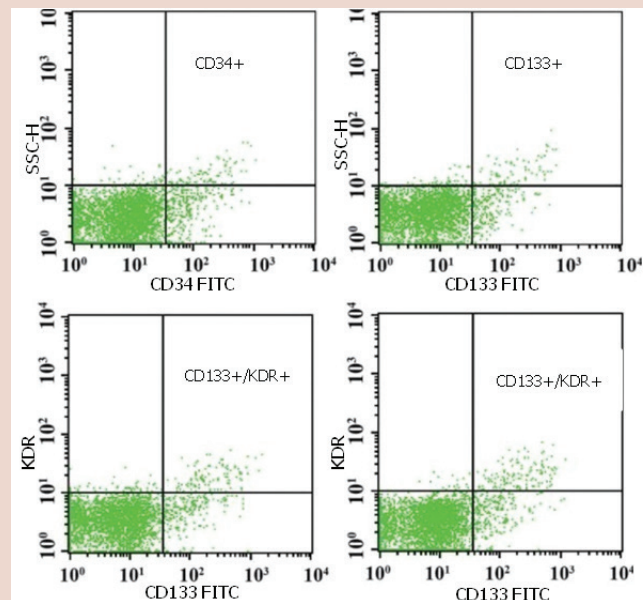


Figure 1: Flow cytometry analysis of peripheral blood cells with CD34, CD133, and KDR markers

Table 1: Comparison of endothelial progenitor cell levels with various markers in CAD and control group

	CAD	Control	p-value
Age	48.5 \pm 8.8	45.2 \pm 10.5	0.24
BMI(Range)	≥ 25	23-26	0.35
Leukocyte count (10^6 per liter)	7.26 \pm 2.35	7.50 \pm 2.93	0.25
CD34+ cells/ 10^6 WBC	195.5 \pm 102.5	398.3 \pm 126.6	0.065
CD133+/ 10^6 WBC	32.5 \pm 18.6	42.2 \pm 19.5	0.211
CD45-CD133+CD34+ KDR+ cells/ 10^6 WBC	0.8 \pm 0.5	1.5 \pm 0.9	0.004
CD45-CD34+KDR+CD184+ cells/ 10^6 WBC	0.8 \pm 0.6	2.1 \pm 0.9	0.003
CD133+KDR+/ 10^6 WBC	2.7 \pm 1.5	5.2 \pm 2.5	0.005
CD133+KDR+CD184+/ 10^6 WBC	2.2 \pm 1.6	3.7 \pm 2.4	0.003
CD45-CD34+KDR+ cells/ 10^6 WBC	2.5 \pm 1.1	6.5 \pm 3.4	<0.001

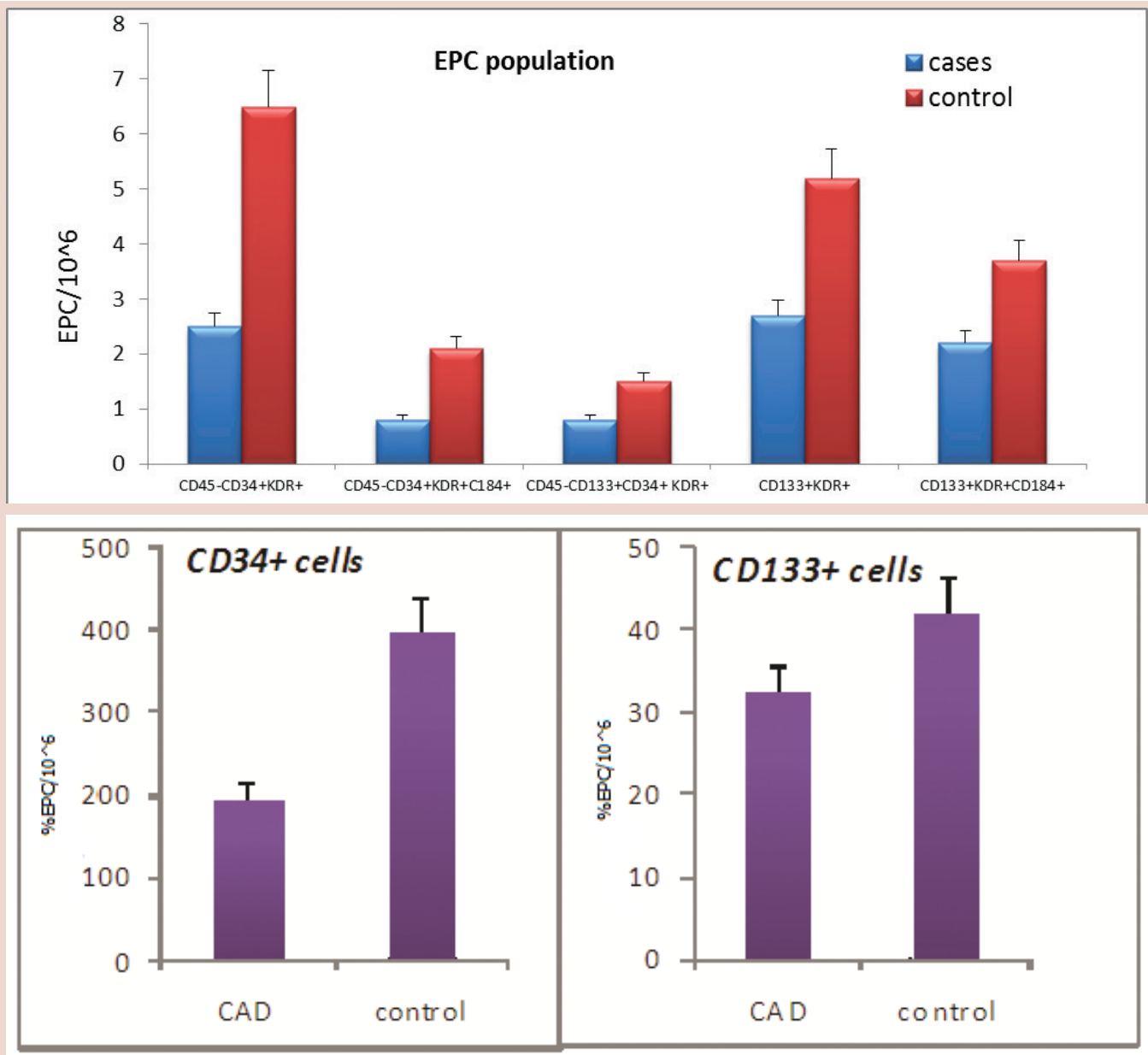


Figure 2: Comparison of EPCs levels in CAD and control subjects. Number of CD45-CD34+KDR+ cells; number of CD45-CD34+KDR+CD184+ cells; number of CD45-CD133+KDR+ cells and number of CD45-CD133+KDR+CD184+ cells. Bars represent standard deviation

Experimental studies suggest that there is an evolving role for EPC in neoangiogenesis and rejuvenation of the endothelial monolayer.^{9,26,27} The presence of immature circulating cells in the peripheral blood has been used in current trials to increase the number of progenitor cells at the site of tissue damage.^{18,28,29} In previous studies, circulating EPCs are determined by a culture method,³⁰⁻³³ but results vary largely on culture conditions. In fact, endothelial cells cultured from peripheral blood corresponds to mature circulating endothelial cells, they do not corresponds to actual circulating EPCs.³⁴ EPCs can be identified by flow cytometry, gold standard method for the quantification of these cells in peripheral blood on the basis of the expression of surface markers.³⁵ In records, there are no unique marker that can be used to identify circulating EPCs. Therefore, we used the combination of various surface markers for EPCs quantification. FACS protocol based upon the detection of CD34 (haematopoietic stem cells), CD133 (immature subset of EPCs, which share more characteristics of stem/progenitor cells), KDR/VEGF-R2 (a typical endothelial

marker), CXCR4/CD184 (a homing marker) and CD45-(exclude myeloid cells).³⁶⁻⁴²

Until now there are few records available to identify these CD combinations to elucidate the mechanism of endothelial damage in coronary artery disease. Peichev *et al.*, showed that CD34 CD133 KDR cell subset may be used to identify and quantify circulating EPCs.⁴³

In the present study, first, it was confirmed that, in CAD patients present dramatically reduced levels of circulating EPCs by comparison with normal subjects. Second, EPCs in this study appears to be positive with various cell surface markers, which co-expression patterns showed varied percentage frequencies in CAD condition when compare to normal subjects.

In the present study, there was no significant change observed in CD34, CD133 markers alone in CAD patients. Whereas, small scale studies suggest that after acute myocardial infarction, the numbers of circulating

CD34+ and CD133+KDR+ endothelial progenitor cells are up-regulated in response to tissue ischemia.^{44,45} AMI present significantly increased numbers of circulating EPCs as compared with control subjects or with patients with stable angina.⁴⁶ It has been shown that circulating EPCs increase immediately after the onset of an AMI, with a subsequent peak at day 5 and a rapid decline thereafter, normalizing within 2 months.^{47,48}

In the present study we found CD45-CD34+KDR+ EPCs were significantly lower in CAD, as compared with individuals without CAD. CD45-CD133+KDR+ EPCs and both subpopulations of CXCR4+ EPCs (CD45-CD34+KDR+CXCR4+ and CD45-CD133+KDR+CXCR4+ cells) were not reduced in CAD patients compared to patients without CAD. There is a higher difference observed in CD45-CD34+KDR+ population between CAD and control groups.

In a prospective study by Werner N *et al.*, showed the number of endothelial progenitor cells with CD34+KDR+ with a mean of 86.3 ± 71.9 . This study demonstrated that a single measurement of CD34+KDR+ endothelial progenitor cells is a useful tool to predict cardiovascular outcomes in patients with coronary artery disease. This finding may suggest that there was an excess of deaths from noncardiovascular causes among patients with increased endothelial progenitor-cell levels.⁴⁹

Whereas a study by António N *et al.*, found that in the acute phase of an acute myocardial infarction patients, diabetic patients presented significantly lower levels of circulating CD45dimCD34+KDR+ and CD45dimCD133+KDR+ EPCs by comparison with nondiabetics, with a parallel decrease in the subpopulations CXCR4+.⁵⁰

Our results suggest that peripheral blood endothelial progenitor cells in patients with coronary artery disease can be used to identify patients at high risk for various adverse cardiac events. These finding supports the notion that progenitor cells with CD45-CD34+KDR+ co-expression plays an important role in pathogenesis of atherosclerosis. Further studies assessing the therapeutic targeting of circulating EPCs are need to prove

the underlying biologic concept of endothelial cell regeneration by circulating EPC in vascular healing.

CONCLUSION

In this study, we measured EPCs by various CD combinations to elucidate the mechanism of endothelial damage in coronary artery disease. CD45-CD34+KDR+ expression shown high significance, which may be useful to estimate endothelial progenitor cells to predict endothelial regeneration capacity in coronary artery disease. It is demonstrated that patients with low numbers of endothelial progenitor cells had increased risk of endothelial damage. Hence experimental studies needs to carry to increase circulating endothelial progenitor cells by conventional management and stem cell therapy to replenish the endothelial monolayer after vascular injury.

ACKNOWLEDGEMENTS

The authors would like to thank the medical and paraclinical staff, students in cardiology department for their valuable assistance in collection of samples. Authors would also like to acknowledge Narayana Medical College & Hospital Management for financial support of this project.

CONFLICT OF INTERST

The author have no conflict of interest.

ABBREVIATION USED

CAD: Coronary artery disease, CD: Cluster of differentiation, KDR: Kinase insert domain protein receptor, EPC: Endothelial progenitor cells CXCR: Chemokine (C-X-C Motif) Receptor, FACS: Fluorescence-activated cell sorting.

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