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ORIGINAL ARTICLE
Cardiovascular Intervention

Endothelial Progenitor Cells Predict Long-Term Prognosis in Patients With Stable Angina Treated With Percutaneous Coronary Intervention

– Five-Year Follow-up of the PROCREATION Study –

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Background: The association between endothelial progenitor cells (EPCs) at the time of percutaneous coronary intervention (PCI) and the subsequent long-term clinical outcome remains undefined. To address this issue, a pre-specified analysis of the PROgenitor Cells role in Restenosis and progression of coronary ATherosclerosis after percutaneous coronary intervention (PROCREATION) study was done.

Methods and Results: A total of 155 patients with stable angina treated with PCI had flow cytometry before PCI. Patients had a 5-year follow-up. Primary outcome was the composite of major adverse cardiac or cerebrovascular events (MACCE), that is, death, stroke, myocardial infarction, and revascularization. During follow-up, MACCE occurred in 65 of 155 patients (42%). There were no significant differences in clinical and angiographic variables between patients with or without MACCE, apart from a different extent of coronary atherosclerosis. The incidence of MACCE increased significantly over tertiles of CD34+/KDR+/CD45– cells and CD133+/KDR+/CD45– cells, with rates of 25%, 39%, and 69% (P=0.0001), and 26%, 44%, and 59% (P=0.003), respectively. On multivariate analysis it was estimated that the increase in CD34+/KDR+/CD45– cells was associated with a 35% higher risk for MACCE (hazard ratio [HR], 1.75; 95% confidence interval [CI]: 1.07–1.99; P=0.001), and the increase in CD133+/KDR+/CD45– cells was associated with a 25% higher risk for MACCE (HR, 1.35; 95% CI: 1.01–1.74; P=0.03).

Conclusions: Assessment of subpopulations of circulating EPCs in patients with stable angina treated with PCI can improve characterization of long-term prognosis (ClinicalTrials.gov: NCT01575431). (*Circ J* 2013; **77:** 1728–1735)

Key Words: Endothelial progenitor cells; Ischemic heart disease; Prognosis

Endothelial progenitor cells (EPCs) are a heterologous population of bone marrow-derived cells that play a key role in maintaining homeostasis of the endothelium.[1] In pathologic states, EPCs home to areas of endothelial injury, replace damaged endothelium, and participate in neovascularization.[2] Although extensive experimental work has been carried out,[3–6] it remains a matter of debate as to whether the number of EPCs is reduced or increased in patients with angiographic evidence of coronary artery disease (CAD).[7,8] Similarly, studies on the link between EPCs and subsequent clinical outcome have yielded conflicting results.[9,10] One should consider, however, that many previous studies have dealt with the entire population of EPCs,[9] whereas there is now growing evidence that among circulating cells there are subsets with distinct marker phenotypes and potentially distinct roles.[11] In addition, no previous study has specifically focused on the relation of EPCs to the subsequent long-term outcome of stable patients with CAD treated with percutaneous coronary intervention (PCI).

To address this issue, we performed a pre-specified subanalysis of the 5-year follow-up of patients originally included in the PROgenitor Cells role in Restenosis and progression of coronary ATherosclerosis after percutaneous coronary intervention (PROCREATION) study, a prospective investigation in a large, homogenous group of consecutive candidates for PCI.[12] The original study was designed to carry out the first

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Table 1. Main Clinical Features vs. Outcome

	Uneventful outcome (n=90)	Primary endpoint (n=65)	P-value
Men	58 (64)	34 (53)	NS
Women	40 (44)	23 (35)	NS
Age (years)	62±11	60±9	NS
Risk factors			
Diabetes mellitus	12 (13)	10 (15)	NS
Systemic hypertension	43 (48)	30 (46)	NS
Hypercholesterolemia	41 (45)	29 (44)	NS
Current smoker	25 (28)	16 (25)	NS
Family history	23 (26)	16 (25)	NS
Previous MI	19 (21)	15 (23)	NS
Clinical findings			
CCS angina class I or II	65 (72)	37 (57)	
CCS angina class III or IV	25 (28)	28 (43)	NS
LV ejection fraction (%)	54±10	50±11	NS
Creatinine (mg/dl)	1.0±0.4	1.2±0.5	NS
Drug therapy at discharge			
Aspirin	86 (96)	56 (86)	NS
β-blockers	68 (76)	48 (74)	NS
ACEI	35 (39)	29 (36)	NS
ARBs	25 (28)	19 (29)	NS
Calcium antagonists	34 (38)	26 (41)	NS
Lipid lowering drugs	88 (97)	57 (88)	NS

Data given as n (%) or mean±SD. ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CCS, Canadian Cardiovascular Society; LV, left ventricular; MI, myocardial infarction.

prospective assessment of the significance of subpopulations of circulating EPCs in the subsequent occurrence of restenosis or progression of coronary atherosclerosis 8 months after PCI.[12]

Methods

Subjects

All patients who underwent an elective and successful single or multivessel PCI with bare metal stent (BMS) implantation between 1 July 2005 and 30 June 2006 were considered for the PROCREATION study. The detailed study protocol has been described in the main publication.[12] Briefly, inclusion criteria were presence of typical stable effort angina, positive stress test, and indication for PCI with BMS implantation at coronary angiography. Exclusion criteria included in-hospital death after PCI, myocardial infarction (MI) during follow-up to exclude potential subacute stent thrombosis,[13] unstable angina, any increase in CK-MB, troponin I, myoglobin or liver enzymes above upper normal limit prior to PCI, left ventricular ejection fraction <30%, renal failure with creatinine >2 mg/dl, or treatment with statins at referral. A total of 155 patients (92 men; mean age, 60±11 years) fulfilling the inclusion criteria were evaluated for this study. The study protocol complies with the Declaration of Helsinki, and was approved by the Institutional Review Board of the San Filippo Neri Hospital, Rome, Italy. Written informed consent was obtained from all participating patients. The PROCREATION study is registered at ClinicalTrials.gov (Identifier: NCT01575431).

Follow-up and Medication

According to the PCI protocol, all patients started clopidogrel (300-mg loading dose) and atorvastatin (80-mg loading dose) the night before PCI. At discharge, patients were prescribed antiplatelet therapy (aspirin 100 mg/day indefinitely and clopidogrel 75 mg/day for 1 month). Other medications included β-blockers, statins, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers (ARB) as appropriate. All patients underwent 8-month control angiography and were then evaluated at 1-year intervals up to 5 years.

Blood Samples and Flow Cytometry

Details of the laboratory measurements in patients and controls have been reported elsewhere.[12] Briefly, peripheral blood samples were taken after a 14-h overnight fast, the day before PCI. The blood for stem cell assessment was kept at room temperature until analysis within <2 h after drawing. A panel of monoclonal antibodies was used: anti-CD45 (HI30 clone; Becton Dickinson, San Jose, CA, USA), anti-CD34 (8G12 clone; Becton Dickinson), anti-CD133 (AC133 clone; Miltenyi Biotec, Auburn, CA, USA), anti-CD 105 (endoglin, NI-3A1 clone; Ancell, Bayport, MN, USA), anti-CD 14 (M5E2 clone; Becton Dickinson), and allophycocyanin-anti vascular endothelial growth factor (VEGF)-receptor 2 (KDR; R&D Systems, Minneapolis, MN, USA). Cells were analyzed using sequential gating strategies that conformed to International Society of Hematotherapy and Graft Engineering criteria.[13] The following subpopulations of EPCs were identified: CD34+/KDR+/CD45– cells (ie, progenitors of endothelial lineage),[14] and CD133+/KDR+/CD45– cells (ie, progenitors of endothelial cells at an earlier stage),[15] CD105+/CD45–/CD34– cells (ie, which have a receptor for transforming growth factor-β),[16] and CD14+/CD45+ cells (ie, which have a role in angiogenesis via a paracrine effect).[17] Flow cytometry was performed with a fluorescent-activated cell sorter Calibur laser flow cytometer (FACS Calibur; Becton Dickinson). Data were processed using

Table 2. Baseline CAG and Procedural Characteristics vs. Outcome

	Uneventful outcome (n=90)	Primary endpoint (n=65)	P-value
Angiographic findings			
1-vessel CAD	67 (74)	35 (54)	
2- or 3-vessel CAD	23 (26)	30 (46)	0.013
Vessels with ≥70% stenosis			
LAD	56 (62)	40 (61)	NS
LCx	31 (34)	21 (32)	NS
RCA	48 (53)	23 (35)	NS
PCI characteristics			
No. stents/patient	1.18±0.33	1.25±0.45	NS
Total stent length (mm)	18.76±5.55	22.53±6.91	NS
Stent diameter (mm)	3.03±0.51	3.15±0.49	NS

Data given as n (%) or mean±SD. CAD, coronary artery disease; CAG, coronary angiography; LAD, left anterior descending artery; LCx, left circumflex artery; PCI, percutaneous coronary intervention; RCA, right coronary artery. Other abbreviations as in Table 1.

Table 3. EPC Distribution vs. Outcome

	Uneventful outcome (n=90)	Primary endpoint (n=65)	Control group (n=20)	P-value
White cells (10^3/ml)	6.58±1.31	6.68±1.17	6.76±1.30	NS
Monocytes (10^3/ml)	0.53±0.29	0.55±0.31	0.59±0.21	NS
CD34+/CD45– (cells/μl)	3.20±2.11	3.31±2.82	3.06±2.10	NS
CD133+/CD45– (cells/μl)	2.25±1.96	2.37±2.15	2.28±1.87	NS
CD34+/KDR+ (cells/μl)	4.11±2.01	4.35±2.23	3.69±2.11	NS
CD133+/KDR+ (cells/μl)	2.35±2.09	2.66±2.19	2.33±1.8	NS
CD34+/KDR+/CD45– (cells/μl)	1.01±0.61	1.29±0.44	0.95±0.44	0.004
CD133+/KDR+/CD45– (cells/μl)	0.34±0.31	0.58±0.46	0.36±0.1	0.0001
CD105+/CD45–/CD34– (cells/μl)	1.89±0.81	1.69±0.69	1.92±0.97	NS
CD14+/CD45+ (cells/μl)	0.36±0.41	0.45±0.49	0.62±0.67	NS

Data given as mean±SD. EPC, endothelial progenitor cell.

the Cell-Quest software (Becton Dickinson). The number of EPCs was expressed as the absolute number of cells per 1 μl as whole blood.

Definitions

The primary outcome of this study was the composite of major adverse cardiac or cerebrovascular events (MACCE) at 5 years of follow-up. MACCE included all-cause death, stroke, MI, and any repeat revascularization. Secondary endpoints were the individual components of primary outcome and a composite endpoint consisting of all-cause death, stroke, and MI. Stroke was defined as a focal neurological deficit of central origin lasting <72 h resulting in permanent brain damage or body impairment. MI was defined in relation to intervention status according to the Universal definition.

Statistical Analysis

Data are presented as mean±SD for continuous variables or frequency percentages for categorical variables. Kolmogorov-Smirnov testing was applied to assess normality of distribution for continuous variables. chi-square test, or Fisher's exact test, when appropriate, were used to compare differences between categorical variables, respectively. Non-normally distributed continuous variables were compared using Kruskal-Wallis test and Mann-Whitney U-test. In analyses of every endpoint, follow-up continued until the date of an endpoint event, death, emigration, or 5 years after the index PCI, whichever came first. Subgroup analysis of separate EPC cohorts (ie, low, intermediate, and high) were performed. Crude MACCE incidence rates were estimated by EPC tertiles, in order to stratify patients into low-, medium- and high-risk groups. The cumulative incidence of MACCE across EPC tertiles was examined using a Kaplan-Meier-like method while accounting for competing risk for death. Multiple logistic regression analysis was used to assess the association of CD34+/KDR+/CD45– cells and CD133+/KDR+/CD45– cells with incident MACCE events after controlling for possible confounding variables (ie, age, sex, body mass index, left ventricular function).

All analyses were performed with S-Plus (Mathsoft, Seattle, WA, USA). Two-sided $P<0.05$ was considered statistically significant.

Results

Primary and Secondary Outcomes

The follow-up had an average duration of 51±10 months. No patients were lost to follow-up. The predefined composite primary endpoint (all-cause death, stroke, MI, and any repeat revascularization) occurred in 65 out of the 155 patients (42%). Of the 16 all-cause deaths, 9 were defined as cardiovascular death (5 were due to acute MI, 2 to acute cardiac failure, and

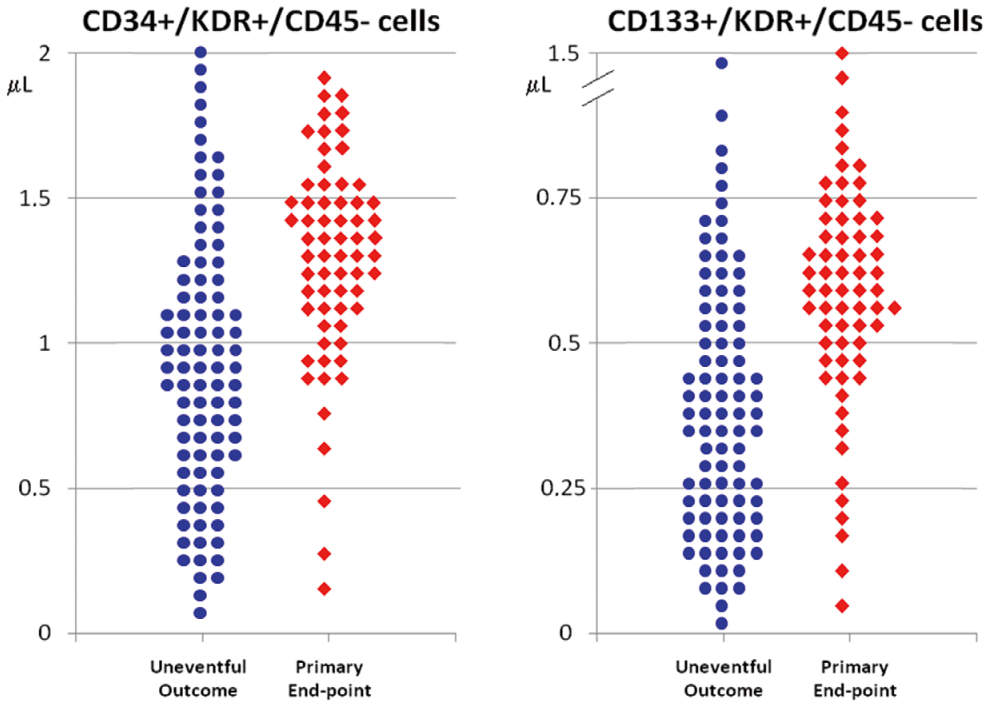

Figure 1. Distribution patterns of CD34+/KDR+/CD45– cells and CD133+/KDR+/CD45– cells vs. outcome: uneventful 5-year follow-up (uneventful outcome) or occurrence of major adverse cardiac or cerebrovascular events (primary endpoint).

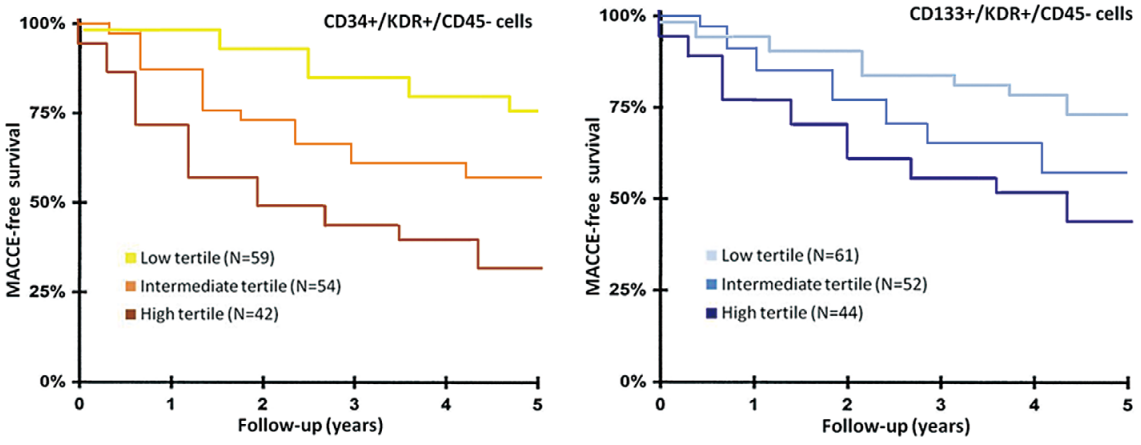

Figure 2. Kaplan-Meier 5-year major adverse cardiac or cerebrovascular event (MACCE)-free survival according to (**Left**) tertiles of CD34+/KDR+/CD45– cells and (**Right**) CD133+/KDR+/CD45– cells.

2 were sudden), and 7 were defined as non-cardiac (2 due to acute hemorrhage, 1 to complications after femoral fracture, 1 to pulmonary embolism, 2 to carcinoma, and 1 to worsening chronic renal failure). The 2 strokes that occurred during follow-up were ischemic. There were also 6 cases of acute MI. Repeat revascularization occurred in 41 patients, 37 of whom had repeat PCI and 5 had coronary artery bypass grafting.

Baseline Clinical, Angiographic, and Procedural Characteristics

The 2 groups of patients had similar age, sex, and cardiovascular risk factors, and did not have different clinical presentation (all patients had stable angina by study design), left ventricular function, renal function, and medical therapy at time of PCI (**Table 1**). The MACCE group had a greater extent of coronary atherosclerosis, with more patients having 2- or 3-ves-

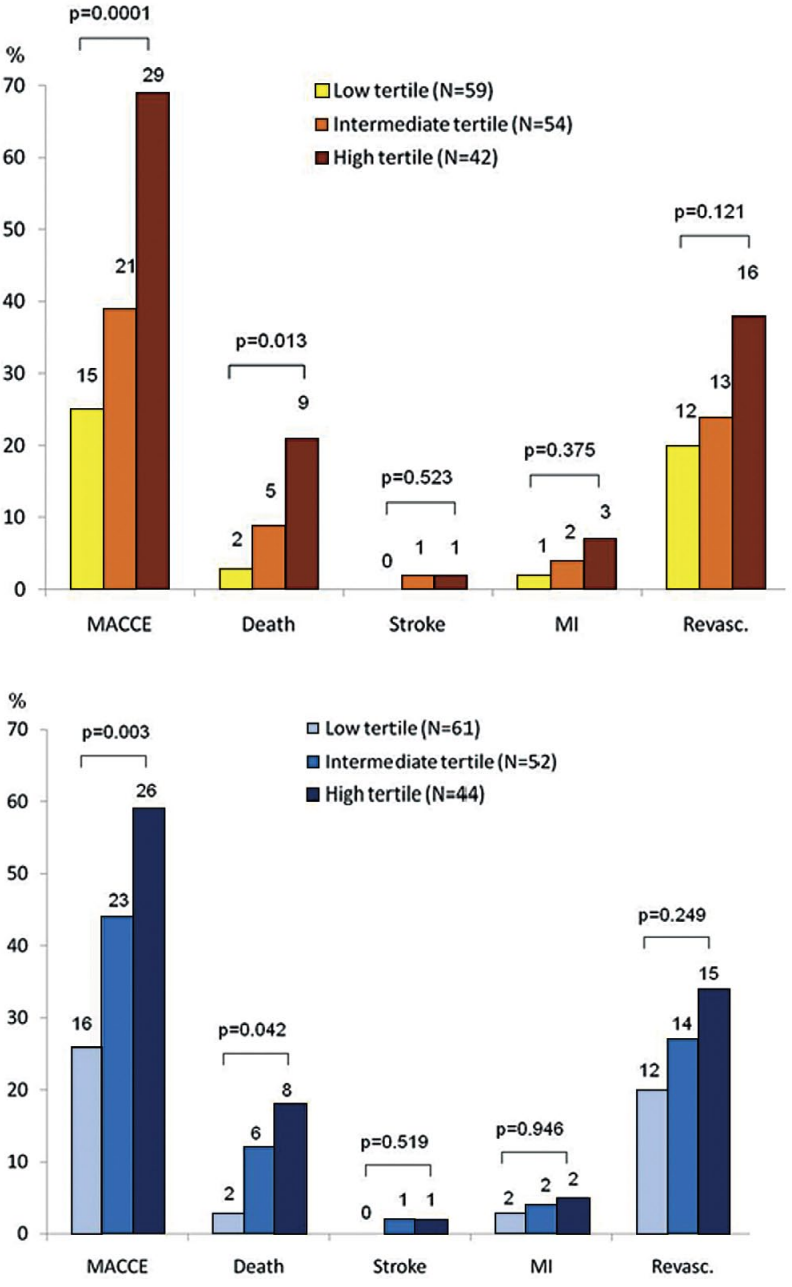

Figure 3. Outcome vs. tertiles of (**Upper**) CD34+/ KDR+/CD45– cells and (**Lower**) CD133+/KDR+/CD45– cells. MACCE, major adverse cardiac or cerebrovascular events; revasc., revascularization.

sel disease than patients without MACCE. PCI characteristics were not different between the 2 groups (**Table 2**). According to the inclusion criteria, all patients had the index PCI with BMS implantation and there were no in-hospital major complications (death or need for urgent revascularization).

Blood Samples and Flow Cytometry

Stem cell analysis (**Table 3**) showed that the 2 groups had similar white cell count, and mononuclear cells. The absolute numbers of CD34+/KDR+/CD45– cells measured in the MACCE group were significantly higher than in patients with favorable outcome (1.01±0.61 vs. 1.29±0.44 cells/μl, respectively; P=0.004; **Figure 1**). Similarly, CD133+/KDR+/CD45– cells were significantly higher in the MACCE group than in patients with uneventful outcome (0.34±0.31 and 0.58±0.46 cells/μl, respectively; P=0.0001; **Figure 1**). Patients with good outcome and the MACCE group had similar numbers of CD105+/CD45–/CD34– cells and CD14+/CD45+ cells.

Multivariate Analysis

According to EPC tertiles, CD34+/KDR+/CD45– cells and CD133+/KDR+/CD45– cells were defined as low in 59 and 61 patients, respectively, intermediate in 54 and 52 patients, respectively, and high in 42 and 44 patients, respectively. The crude MACCE incidence rate increased significantly with CD34+/KDR+/CD45– cell and CD133+/KDR+/CD45– cell tertiles, with rates of 25%, 39%, and 69% (P=0.0001), and 26%, 44%, and 59% (P=0.003), respectively, in tertiles 1–3

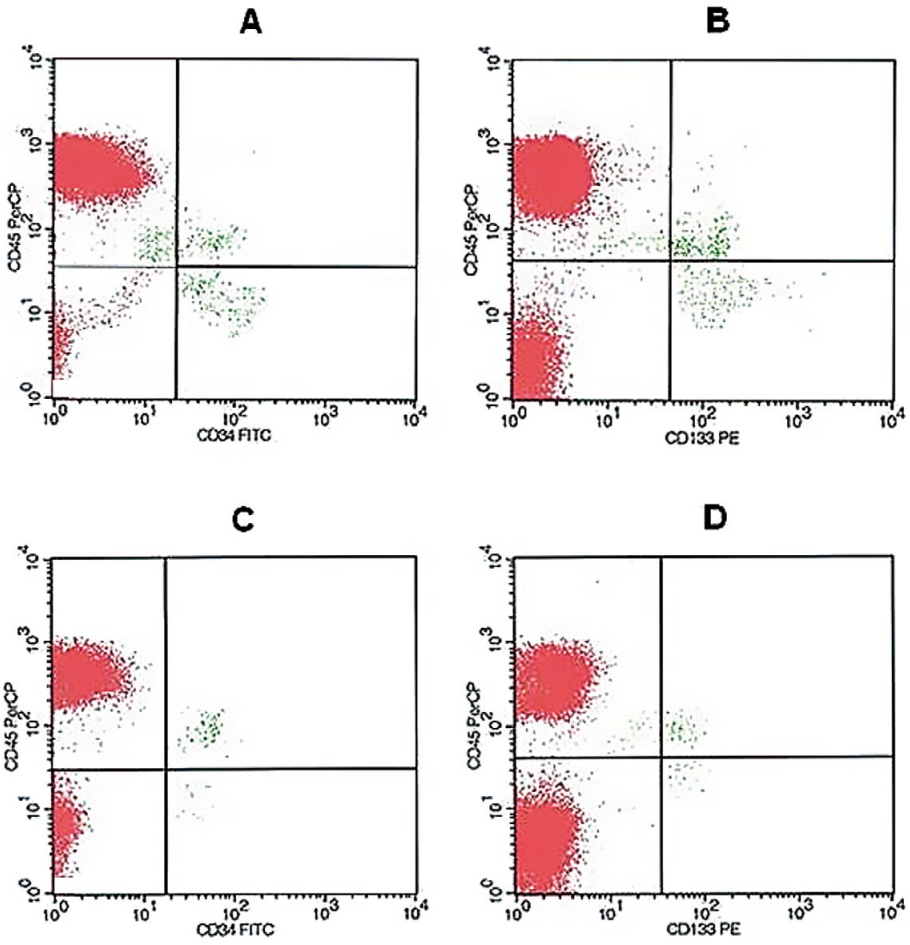

Figure 4. Representative cytograms of CD34+/KDR+/CD45– cells (ie, CD34+/KDR+ cells negative for CD45) and CD133+/KDR+/CD45– cells (ie, CD34+/KDR+ cells negative for CD45) from (**A**,**B**) a patient who died suddenly 32 months after index hospitalization, and (**C**,**D**) a patient with an uneventful 5-year follow-up. (**A**,**B**) The patient who died had an increased number of circulating CD34+/KDR+/CD45– cells and CD133+/KDR+/CD45– cells, as compared with (**C**,**D**) the patient with a benign outcome.

(Figure 2). Breaking down MACCE into separate endpoints, there were significant differences among groups in the endpoint of all-cause death (P=0.013 for CD34+/KDR+/CD45– cells and P=0.042 for CD133+/KDR+/CD45– cells), whereas the other endpoints stroke, MI, and repeat revascularization were comparable either in CD34+/KDR+/CD45– cells or in CD133+/KDR+/CD45– cells (Figure 3). On multivariate analysis it was estimated that the increases in log CD34+/KDR+/CD45– cells was associated with a 35% increased risk for MACCE (hazard ratio [HR], 1.75; 95% confidence interval [CI]: 1.07–1.99; P=0.001), and that the increases in log CD133+/KDR+/CD45– cells was associated with a 25% increased risk for MACCE (HR, 1.35; 95% CI: 1.01–1.74; P=0.03; Figure 4).

Discussion

Predictive Role of EPCs

The present results indicate that high levels of circulating EPCs at the time of PCI is associated with an increased incidence of MACCE at 5 years. In the last decade, the extensive research on EPCs has led to the general belief that circulating EPCs have a protective role, and that their alterations mirror and predict disease progression and future cardiovascular events.[11] Since the pivotal work by Werner et al, increasing levels of CD34+KDR+ EPCs have been associated with a decreased risk of death from cardiovascular causes in the short-term.[9] The present results, conversely, demonstrate that patients who have evidence at the time of PCI of increased numbers of those circulating EPCs that are progenitors of endothelial lineage, have an increased incidence of a cardiovascular or cerebrovascular event in the long-term.

In an attempt to reconcile the discordance between the present findings and those of older investigations, it should be considered that most previous studies have been hampered by their retrospective nature, the lack of uniform criteria to precisely identify EPCs, and the short-term follow-up. Research has focused mainly on cells positive only for the endothelial markers CD34, an adhesion molecule expressed on hematopoietic stem cells,[14] and CD133, a surface antigen that is not expressed as mature endothelium,[15] without further differentiating their properties. As a consequence, poor data exist on the predictive role of the subsets of stem/progenitor cells positive

for KDR and negative for the CD45 antigen, and have therefore endothelial progenitor capacity.[18] For instance, the aforementioned investigation by Werner et al took into consideration only the CD34+/KDR+ cells, without differentiating them on the basis of the CD45 antigen, and evaluated only the 1-year occurrence of cardiovascular events.[9]

Pathophysiologic Link Between EPCs and Outcome

Apart from methodologic differences in EPC characterization, the present results contribute to the understanding of the pathophysiologic significance of EPCs.

In vitro experiments have shown that EPCs are released from the bone marrow in response to ischemia or endothelial injury with the aim of repairing damaged regions either by producing angiogenic cytokines[19] or by differentiating into endothelial cells.[20] These experimental findings are in agreement with the recent observation that numbers of EPCs are increased in patients with higher cardiovascular risk,[21] who are therefore more prone to develop CAD and have clinical manifestations of the disease. Accordingly, it is conceivable that the numbers of EPC subtypes might predict the subsequent prognosis, similarly to what CD14++CD16+ monocytes have clearly been shown to do.[22,23] Development of atherosclerosis poses a threat to endothelial integrity by inducing vascular oxidative stress and a transient prothrombotic stimulus. It has been shown in healthy subjects that circulating EPC numbers increase following a vascular injury,[24] thereby supporting the conjecture that the generation of reactive oxygen species may trigger EPC release. This concept is supported by the results of 2 recent studies that have found that angiographic restenosis of BMS after PCI is associated with an increase in circulating CD34+/KDR+ cells.[12,25] These findings are not in line with those investigators, who maintained that high levels of EPCs have a protective role.[26] One should consider, however, that it has never been established in vivo if an increased number of EPCs results in their homing and migration into coronary arteries. It is not possible to know if mobilized bone marrow progenitors really differentiate into vascular smooth muscle cells or endothelial cells within coronary segments. In the present study the long-term outcome of stable angina patients who undergo PCI was associated with the baseline finding of increased levels of circulating EPCs, because higher levels of circulating CD34+/KDR+/CD45– cells and CD133+/KDR+/CD45– cells were found at the time of index PCI in those patients who subsequently experienced an adverse event. Thus, the more pronounced release of circulating EPCs might be considered a mechanism of defense in an attempt to compensate for more aggressive pathogenetic factors of atherosclerosis.

Study Limitations

Although we reported on one of the largest groups of stable patients with CAD in whom circulating EPCs have been consecutively measured, a limitation of this study lies in the fact that the number of patients was relatively small and therefore the strength of statistical significance was relatively weak. The methods used to assess EPCs still lack standardization and this might therefore have affected the results.[11] Specifically, circulating EPCs were reflected by the lack of expression of CD45. It should be noted, however, that some circulating (hem)angioblasts (including EPCs) are not CD45 negative. We cannot rule out the possibility that the increase in CD34+ and CD133+ cells was caused by tissue ischemia, which can, per se, contribute to raise VEGF levels and mobilize cells into peripheral blood.[27] It is unlikely, however, that myocardial ischemia affected the results because all patients had flow-limiting coronary stenoses prior to revascularization. Also, the study suffered from the limitation that EPCs were assessed only at time of referral and were not re-evaluated during the follow-up period. Previous investigations have shown a link between inflammation and angiogenesis,[28] but we are not able to make any comment on the complex interplay among vascular inflammation and EPCs because we did not assess inflammatory parameters in the present patients. Another limit of the study was the lack of functional studies of EPCs. Unfortunately, we were unable to isolate significant quantities of cells for functional assays in the present patients and therefore we could not perform functional studies as previously done by others.[27,29] Although several pharmacologic agents, such as statins,[30,31] clopidogrel,[32] and angiotensin II receptor antagonists[33–35] may affect number and function of EPCs, it is unlikely that differences in EPC levels among groups were caused by drug treatment. Indeed, no patient was on statins at the time of blood withdrawal for stem cell assessment, and all other medications were similar in the 2 groups of patients both at referral and during the 5-year follow-up period.

Conclusions

Long-term follow-up data of the PROCREATION study shows that assessment of subpopulations of circulating EPCs in patients with stable angina treated with PCI can improve characterization of long-term prognosis. These findings further support the pathophysiologic role of stem cells in the clinical aspect of coronary atherosclerosis, and suggest the possibility of using EPCs as biomarkers for the prediction of cardiovascular outcome.

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