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Role of Endothelial Progenitor Cells in Restenosis and Progression of Coronary Atherosclerosis After Percutaneous Coronary Intervention

A Prospective Study

Francesco Pelliccia, MD, PhD,*‡ Cinzia Cianfrocca, MD,*‡ Giuseppe Rosano, MD, PhD,† Giuseppe Mercuro, MD,‡ Giulio Speciale, MD,* Vincenzo Pasceri, MD, PhD*

Rome and Cagliari, Italy

Objectives We prospectively investigated the relationship of circulating endothelial progenitor cells at time of percutaneous coronary intervention to the subsequent development of in-stent restenosis or progression of coronary atherosclerosis.

Background Endothelial progenitor cells provide an endogenous repair mechanism of the dysfunctional endothelium and therefore can play a pathogenic role in coronary atherosclerosis.

Methods We studied 155 consecutive stable angina patients (92 men, age 60 ± 11 years). All patients had flow cytometry the day before elective percutaneous coronary intervention in order to derive subpopulations of endothelial progenitor cells. A control group of 20 normal subjects was considered for comparison.

Results At 8-month control angiography, 30 patients showed in-stent restenosis (restenosis group), 22 patients showed progression of coronary atherosclerosis (progression group), whereas the remaining 103 patients had neither in-stent restenosis nor progression of coronary atherosclerosis (stable group). Comparison of the 3 groups did not show any difference in risk factors, cardiac morphology and function, extension of coronary artery disease, and treatment. Absolute numbers of CD34+/KDR+/CD45– cells (i.e., progenitors of endothelial lineage) measured in the restenosis group (1.41 ± 0.64 cells/μl) were significantly higher than in the progression, stable, and control groups (1.03 ± 0.53 cells/μl, 1.07 ± 0.46 cells/μl, and 0.95 ± 0.44 cells/μl, respectively, $p < 0.05$). Similarly, CD133+/KDR+/CD45– cells (i.e., progenitors of endothelial cells at an earlier stage) were significantly higher in the restenosis (0.63 ± 0.23 cells/μl) compared with progression, stable, and control groups (0.33 ± 0.19 cells/μl, 0.41 ± 0.32 cells/μl, and 0.36 ± 0.15 cells/μl, respectively, $p < 0.001$). Also, numbers of CD14+/CD45+ cells (i.e., which have a role in angiogenesis via a paracrine effect) were significantly different among the restenosis, progression, stable, and control groups (0.72 ± 0.56 cells/μl vs. 0.51 ± 0.52 cells/μl vs. 0.28 ± 0.54 cells/μl vs. 0.62 ± 0.67 cells/μl, respectively, $p < 0.05$), whereas CD105+/CD45–/CD34– cells (i.e., which have a receptor for transforming growth factor-beta) were similar among groups.

Conclusions Patients with restenosis have higher numbers of subpopulations of endothelial progenitor cells that incorporate into endothelial cells or play a role in arteriogenesis compared with controls and patients with either progression of coronary atherosclerosis or stable disease. (J Am Coll Cardiol Intv 2010;3:78–86) © 2010 by the American College of Cardiology Foundation

From the *Department of Cardiovascular Diseases, San Filippo Neri Hospital, Rome, Italy; †Department of Medical Sciences, Istituto di Ricovero Cura a Carrettere Scientifico San Raffaele Pisana, Rome, Italy; and the ‡Department of Cardiovascular and Neurologic Diseases, University of Cagliari, Cagliari, Italy.

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Research on stem cells has identified a population of bone marrow–derived cells, called circulating endothelial progenitor cells (EPCs), that incorporate into sites of neovascularization and are home to sites of endothelial denudation thus contributing to the maintenance of vascular homeostasis (1). Although extensive work has been conducted to verify if EPCs impairment plays a key role in coronary atherogenesis (2), it is still matter of debate if the extension and severity of coronary artery disease are associated with reduced (3) or increased (4) numbers of EPCs, as it remains unclear if these cells exert favorable or unfavorable effects at sites of

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percutaneous coronary intervention (PCI) (5–7). One should consider, however, that most previous investigations have been hampered by discordant definitions of EPCs and by different timing of EPCs sampling (8–10), thus determining much uncertainty on the role of EPCs in restenosis and atherosclerosis progression (10). Furthermore, development of de novo lesions and post-PCI restenosis, which are pathophysiologically dissimilar (5), have not been examined concomitantly and serially over time.

Accordingly, the aim of this study was to carry out the first prospective assessment of the significance of subpopulations of circulating EPCs in the subsequent occurrence of restenosis or progression of coronary atherosclerosis after PCI. To this end, a pool of EPCs subtypes that are suggested to play some role in atherosclerosis was measured in a homogenous population of candidates to PCI. At variance with previous work, counts of EPCs were obtained in baseline conditions before PCI in order to avoid the confounding effect that the procedure exerts on EPCs (8,9).

Methods

Patient population. All patients who underwent an elective and successful single or multivessel PCI between July 1, 2005, and June 30, 2006, were considered for the study. Inclusion criteria were presence of typical stable effort angina, positive stress test, and indication for PCI at coronary angiography. Patients were suitable candidates only if complete revascularization of clinically important stenoses were feasible by PCI, and if they underwent 8-month control angiography. Exclusion criteria were: 1) in-hospital death after PCI; 2) myocardial infarction during follow-up to exclude potential subacute stent thrombosis (11); 3) unstable angina; 4) any increase in creatine kinase-myocardial band, troponin I, myoglobin, or liver enzymes above upper normal limit before PCI; 5) left ventricular ejection fraction ≤30%; 6) renal failure with creatinine ≥2 mg/dl; and 7) treatment with statins at referral. Also, patients were excluded if they had recent surgery, immunological disease, immunosuppresion/depression, or organ transplantation. Patients with a history of coronary artery bypass grafting and patients with homozygotic familial hypercholesterolemia were excluded, because, in general, such individuals have diffuse disease that complicates the angiographic measurement of nondilated vessel segments (12). Patients who had a drug-eluting stent were also excluded to avoid the effects that medications can exert on intimal hyperplasia and endothelial healing (6,7). Finally, all patients agreed to perform an 8-month follow-up angiography and gave informed, written consent to participate to the study. Thus, 160 patients (95 men, age 61 ± 12 years) fulfilling the inclusion criteria could be evaluated for this study. Our institutional committee on human research approved the study.

Control group. We selected a cohort of healthy controls comparable with the study population in terms of age, sex, and body mass index. Controls were referred for suspicion of angina pectoris and underwent a complete diagnostic work-up, including echocardiography, stress test, and coronary angiography. None of the controls had cardiac or noncardiac diseases, such as malignancy, inflammatory disease, and kidney disease.

Percutaneous coronary angioplasty and adjunct drugs. According to our standard protocol, all patients without contraindications were pre-treated with aspirin 100 mg/day and ticlopidine 250 mg twice a day at least 3 days before PCI or with clopidogrel 300 mg at least 6 h before the procedure. After PCI, the protocol-mandated antiplatelet therapy consisted of aspirin 100 mg/day indefinitely and ticlopidine 250 mg twice a day or clopidogrel 75 mg/day for 1 month. Other medications such as beta-blockers, statins, and angiotensin-converting enzyme inhibitors were given as appropriate.

Abbreviations and Acronyms

EPC = endothelial progenitor cell

PCI = percutaneous coronary intervention

Blood samples and flow cytometry. Peripheral blood samples were taken after a 14-h overnight period the day before PCI. C-reactive protein was measured by a immunoturbodimetric assay (Hitachi 912, Roche Diagnostic, Basel, Switzerland) with a sensitivity of 0.1 mg/l, with an intra- and interassay variation of <5%. The blood for stem cell assessment was kept at room temperature until analysis within <2 h after drawing. Mononuclear cells were isolated from peripheral venous blood by Ficoll density gradient centrifugation (Histopaque-1077, Sigma-Aldrich, Munich, Germany) and prepared for fluorescent-activated cell sorting. A panel of monoclonal antibodies was used: anti-CD45 (HI30 clone, Becton Dickinson, San Jose, California), anti-CD34 (8G12 clone, Becton Dickinson), anti-CD133 (AC133 clone, Miltenyi Biotec, Auburn, California), anti-CD105 (endoglin, NI-3A1 clone, Ancell, Bayport, Minnesota), anti-CD14 (M5E2 clone, Becton Dickinson), and allophycocyanin antivascular endothelial growth factor re-

ceptor 2 (KDR, R&D Systems, Minneapolis, Minnesota). Monoclonal antibodies were conjugated with peridinin chlorophyll protein, R-phycoerythrin, and fluorescein isothiocyanate. The frequency of peripheral blood cells positive for these reagents was determined by a scatter-fluorescence dot-plot analysis stained with the different reagents. Fluorescence isotypes immunoglobulin G1- and G2a-matched antibodies were used as controls (Becton Dickinson). Cells were analyzed using sequential gating strategies that conformed to International Society of Hematotherapy and Graft Engineering criteria to enumerate total number and subsets of circulating CD45− or CD45+ cells (13). Also, we gated CD34+ and CD133+ peripheral blood cells in the mononuclear cell fraction and then examined the resulting population for the dual expression of KDR.

Circulating EPCs were depicted by the lack of expression of CD45 (i.e., a leukocyte/monocyte antigen), and by the simultaneous expression of KDR (i.e., a marker of endothelial lineage) and CD34 (i.e., a hematopoietic stem cell marker that is also expressed on mature microvascular endothelial cells) (14) or CD133 (i.e., a more immature hematopoietic stem cell marker that is absent on mature endothelial cells and monocytic cells (15). Accordingly, after gating on the CD45 area, the resulting populations of cells identified by the dual expression of KDR and CD34 (CD45−/CD34+/KDR+) or CD133 (CD45−/CD133+/KDR+) were evaluated (3,14). Also, analytical gates were used to enumerate subsets of circulating CD45−/CD34− cells that expressed the antigen CD105 (16). Finally, circulating CD45+ leukocytes that expressed the monocytic marker CD14 were assessed (1). These cells are derived from monocytes/macrophages and cannot be considered early EPCs (1). They are positive for both the leukocyte antigens CD45 and CD14 but are negative for the antigens CD34 and CD133 (10). Noteworthy, they have no proliferation potential but play an important role in angiogenesis and arteriogenesis via a paracrine effect (17). Flow cytometric analysis was performed with a fluorescent-activated cell sorter Calibur laser flow cytometer (FACS Calibur, Becton Dickinson). Data were processed using the Cell-Quest software (Becton Dickinson). The number of EPCs was expressed as the absolute number of cells per 1 μl as whole blood. All measurements were derived by 1 operator (G.M.) who was blind to the patients' status.

To assess the reproducibility of EPCs measurements, circulating EPCs were measured twice in the first 20 consecutive patients from 2 separate blood samples drawn 1 day apart before PCI.

Quantitative coronary angiography. Analysis of coronary angiograms obtained before (baseline) and after PCI, as well as at 8-month follow-up control was performed by the core laboratory Ricerche Orientate alla Malattia Aterosclerotica (ROMA). The operators who performed the evaluation were unaware of the study protocol, the patient characteristics, and the stent type used. Digital angiograms were analyzed offline with the use of an automated edge-detection system (Cardiovascular Medical System, MEDIS Imaging Systems, Leiden, the Netherlands) (18). All measurements were performed on cineangiograms recorded after intracoronary nitroglycerin administration (18–20). All visible lesions, including wall irregularities, were analyzed on the angiograms. Multiple lesions within 1 coronary artery segment were considered distinct whenever separated by a visually smooth arterial wall. The same single, worst-view projection was used at all time points. The contrast-filled nontapered catheter tip was used for calibration, and the reference diameter was measured by interpolation. At baseline, all nondilated segments >2 mm in diameter with a >20% but <100% diameter stenosis were measured (12,18). The angiographic measurements included reference vessel diameter, minimal lumen diameter, diameter stenosis, and lesion length. For stented tracts, angiographic measurements were made both in the stent and in the stented segment (defined as the whole stented tract plus the 5-mm edges proximal and distal to the stent) during diastole. Acute gain was defined as the difference between minimal lumen diameters at the end of PCI and at baseline. Late lumen loss was calculated as the difference in minimal lumen diameter measured after PCI and at follow-up. Binary angiographic restenosis was defined as diameter stenosis ≥50% at follow-up angiography.

Definitions. At baseline and follow-up coronary angiograms, quantitative coronary analysis allowed identification of arteries with significant flow-limiting lesion as defined by a >50% diameter stenosis of the lumen diameter. Procedural success of PCI was defined as a decrease of stenosis ≥20% with a <30% residual narrowing. At follow-up angiograms, progression of coronary artery disease was defined as new lesion or worsening of a previous lesion. A new lesion was considered to have developed when the stenosis was only present at the 8-month coronary angiography, as shown by a retrospective measurement of the corresponding segment at baseline. Conversely, a lesion was defined as progressive when a not significant lesion detected at baseline angiography showed a change in minimal lumen diameter >0.4 mm associated with a change in diameter stenosis >20% (18–20). Stable coronary atherosclerosis was defined when the minimal lumen diameter of any pre-existing lesion did not worsen by ≥0.4 mm or increased by >20% percent diameter stenosis and/or there was no development of a lesion reducing minimal lumen diameter by ≥0.04 mm or determining a percent diameter stenosis >20% (16,18,19). Binary angiographic restenosis was defined as diameter stenosis >50% in the in-segment area (including the stent area and 5-mm segments proximal and distal to the stent edges) at follow-up angiography (21).

Patients with both restenosis and progression of coronary artery disease were included in the restenosis group.

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 (CD34+/CD45−, CD133+/CD45−, CD34+/KDR+, CD133+/KDR+ did not show normal distribution and were analyzed by nonparametric tests). Chi-square or Fisher exact tests, when appropriate, were used to compare differences between categorical variables, respectively. Comparisons between multiple groups were performed by analysis of variance and multiple comparisons were done by *t* test with Bonferroni correction in case of statistical significance. Not normally distributed continuous variables were compared by Kruskal-Wallis and Mann-Whitney *U* tests. Correlation between 2 variables was assessed with Pearson correlation coefficient. Differences were considered statistically significant if the p value was <0.05. Analyses were performed with the S-Plus statistical package (Mathsoft Inc., Seattle, Washington). The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Patient population. After the index PCI, of the 160 patients who were prospectively included in the study, 1 patient died while in-hospital, 1 patient had ST-segment elevation myocardial infarction, and 3 patients experienced non–ST-segment elevation myocardial infarction. These 5 patients were excluded from further analysis as they could not undergo follow-up coronary angiography. Of note, no significant difference was found in levels of EPCs between those patients with major adverse cardiac events and the remaining patients, although the number of events was very small. In the other 155 patients (92 men, age 60 ± 11 years), 8-month control angiography showed significant binary in-stent restenosis in 30 patients (restenosis group) and progression of coronary atherosclerosis in ≥1 segments with no evidence of in-stent restenosis in 22 patients (progression group). Three patients showed both restenosis and progression of coronary artery disease and were included in the restenosis group according to pre-specified definition. The remaining 103 patients had neither in-stent restenosis nor progression of coronary atherosclerosis (stable group). The control group consisted of 20 normal subjects (13 men, mean age 61 ± 9 years) with atypical chest pain and normal coronary angiography.

Baseline clinical and angiographic features. The 3 groups of patients were well matched with respect to age, sex, and cardiovascular risk factors, and were similar with regard to clinical presentation (all patients had stable angina by study design), left ventricular function, renal function, and medical therapy at time of PCI (Table 1). Comparison of C-reactive protein levels did not show any significant difference among the 3 groups of patients. No differences were noted among groups both in the PCI sites and in the nonintervened lesions, as coronary anatomy, number of diseased vessels, number of significant coronary stenoses, lesion types, and stenosis characteristics (i.e., minimal lumen diameter, length, and severity) were similar among the 3 groups (Table 2). Specifically, there were no significant differences in lesion severity or lesion location among the 3 groups.

Table 1. Main Clinical Features in the 3 Groups of Patients

	Restenosis Group (n = 30)	Progression Group (n = 22)	Stable Group (n = 103)	p Value
Men	18 (60%)	14 (64%)	60 (58%)	NS
Women	12 (40%)	8 (36%)	33 (32%)	NS
Age, yrs	62 ± 10	60 ± 8	59 ± 9	NS
Risk factors				
Diabetes mellitus	5 (17%)	3 (14%)	14 (14%)	NS
Systemic hypertension	15 (50%)	12 (54%)	46 (45%)	NS
Hypercholesterolemia	14 (45%)	11 (50%)	45 (44%)	NS
Current smoker	7 (23%)	6 (27%)	28 (27%)	NS
Family history of coronary disease	7 (23%)	5 (23%)	27 (26%)	NS
Previous MI	7 (23%)	4 (18%)	23 (22%)	NS
Clinical findings				
CCS anginal class I or II	18 (59%)	14 (63%)	70 (68%)	NS
CCS anginal class III or IV	12 (41%)	8 (36%)	33 (32%)	NS
LV ejection fraction, %	56 ± 10	53 ± 11	50 ± 12	NS
Creatinine, mg/dl	1.0 ± 0.3	1.1 ± 0.4	1.2 ± 0.5	NS
C-reactive protein, mg/l	1.87 ± 1.77	1.63 ± 1.65	1.71 ± 1.59	NS
Drug therapy at discharge				
Aspirin	28 (93%)	20 (91%)	94 (91%)	NS
Beta-blockers	22 (73%)	15 (68%)	79 (77%)	NS
ACE inhibitors	10 (33%)	8 (36%)	46 (45%)	NS
Calcium antagonist	14 (47%)	9 (41%)	37 (36%)	NS
Lipid-lowering drug	29 (97%)	22 (100%)	94 (91%)	NS

Data are expressed as n (%) or mean ± SD.
ACE = angiotensin-converting enzyme; CCS = Canadian Cardiovascular Society; LV = left ventricular; MI = myocardial infarction.

Procedural characteristics and outcome. Procedural characteristics (including total ischemia time), type of stents, diameter and length of implanted stents were similar among the 3 groups of patients (Table 2). There were no in-hospital major complications (death or need for urgent revascularization).

Follow-up quantitative coronary angiography. All patients underwent the 8-month follow-up coronary angiography. Findings of quantitative coronary angiography at sites of previous PCI are shown in Table 3. A significant progression of coronary atherosclerosis in the non-PCI lesions was

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Table 2. Quantitative Coronary Angiographic Findings at Baseline and Procedural Characteristics in the 3 Groups of Patients

	Restenosis Group (n = 30)	Progression Group (n = 22)	Stable Group (n = 103)	p Value
Nonsignificant coronary lesions				
Number of nonsignificant lesions/patient	4.1 ± 2.9	3.6 ± 3.1	3.9 ± 3.7	NS
Patients with maximal stenosis ≤20%	7 (23%)	6 (27%)	33 (32%)	NS
Patients with maximal stenosis >20%–<50%	23 (77%)	16 (73%)	70 (68%)	NS
Vessels with nonsignificant stenoses				
1-vessel	13 (43%)	10 (45%)	52 (51%)	NS
2- or 3-vessel	17 (57%)	12 (55%)	51 (49%)	NS
Left anterior descending artery	27 (90%)	18 (82%)	88 (85%)	NS
Left circumflex artery	16 (53%)	11 (50%)	42 (41%)	NS
Right coronary artery	12 (41%)	11 (50%)	56 (54%)	NS
Reference vessel diameter, mm	3.03 ± 0.59	2.99 ± 0.41	2.98 ± 0.45	NS
MLD, mm	1.95 ± 0.29	1.94 ± 0.37	1.96 ± 0.35	NS
Diameter stenosis, %	35.6 ± 9.8	35.1 ± 11.2	34.2 ± 10.5	NS
Lesion length, mm	16.9 ± 8.8	14.3 ± 6.9	16.1 ± 7.1	NS
Significant coronary lesions				
Number of significant stenoses/patient	1.26 ± 0.36	1.18 ± 0.43	1.22 ± 0.38	NS
Vessels with ≥1 stenosis >50%				
1-vessel	19 (64%)	15 (68%)	70 (68%)	NS
2- or 3-vessel	11 (37%)	7 (32%)	33 (32%)	NS
Left anterior descending artery	18 (60%)	12 (55%)	66 (64%)	NS
Left circumflex artery	12 (40%)	8 (36%)	32 (31%)	NS
Right coronary artery	15 (50%)	10 (45%)	50 (48%)	NS
MLD, mm	0.56 ± 0.31	0.59 ± 0.23	0.51 ± 0.33	NS
Diameter stenosis, %	81.5 ± 10.1	80.3 ± 8.4	82.8 ± 9.2	NS
Lesion length, mm	14.4 ± 5.5	13.1 ± 4.5	15.9 ± 4.9	NS
PCI characteristics				
Number of stents/patient	1.23 ± 0.42	1.15 ± 0.39	1.21 ± 0.49	NS
Direct stenting	19 (63%)	15 (68%)	75 (73%)	NS
Total stent length, mm	20.03 ± 4.11	18.18 ± 5.55	19.92 ± 4.77	NS
Stent diameter, mm	3.13 ± 0.39	3.05 ± 0.41	3.19 ± 0.44	NS
MLD post-PCI, mm	2.36 ± 0.39	2.29 ± 0.33	2.34 ± 0.43	NS
Acute gain, mm	1.80 ± 0.32	1.70 ± 0.37	1.83 ± 0.36	NS

Data are expressed as mean ± SD or n (%).
MLD = minimal lumen diameter; PCI = percutaneous coronary intervention.

indicated by the development of significant coronary stenoses, coupled with significantly lower minimal lumen diameter and higher stenosis length and stenosis percent diameter. In the progression group, new or progressive disease occurred in untreated vessels in 10 patients (46%), whereas 10 patients (46%) had progression in the same artery but in a separate segment (>5 mm) from the original PCI, and 2 patients (9%) had progression in both treated and untreated vessels. Difference in in-stent luminal loss was minimal between the progression group and the stable group patients.

Blood samples and flow cytometry. Statistical analysis revealed a slight correlation (Pearson correlation coefficient = 0.15) of borderline significance (p = 0.051) between CD34+/KDR+/CD45− cells and CD133+/KDR+/CD45− cells. Of the 20 patients in whom reproducibility of EPCs measurements were assessed at baseline, 14 were later included in the stable group, 3 in the restenosis group, and 3 in the progression group. Statistical analysis disclosed a significant correlation between the 2 assessments of CD34+/KDR+/CD45− cells ($r = 0.82$, $p < 0.001$), CD133+/KDR+/CD45− cells ($r = 0.92$, $p < 0.001$), CD105+/CD45−/CD34− cells ($r = 0.87$, $p < 0.001$), and CD14+/CD45+ cells ($r = 0.90$, $p <$

Table 3. Quantitative Coronary Angiographic Findings at 8-Month Follow-Up Control in the 3 Groups of Patients

	Restenosis Group (n = 30)	Progression Group (n = 22)	Stable Group (n = 103)	ANOVA p Value
Nonstented segments				
Patients with new stenoses >50%	3	22	0	—
Number of new stenoses >50%	3	28	0	—
New stenoses >50%/patient	0.10	1.27	0	—
Type of new stenoses >50%				
De novo lesions/total lesions	1/3	10/28	0	—
Progression of lesions/total lesions	2/3	18/28	0	—
Location of new stenoses >50%				
Left anterior descending artery	2/3	12/28	0	—
Left circumflex artery	0	7/28	0	—
Right coronary artery	1/3	9/28	0	—
Reference vessel diameter, mm	3.12 ± 0.69	3.02 ± 0.47	3.01 ± 0.47	NS
MLD, mm	1.91 ± 0.44	0.55 ± 0.24*	1.89 ± 0.33	NS
Diameter stenosis, %	38.7 ± 6.9	81.8 ± 8.7*	37.2 ± 9.5	NS
Lesion length, mm	13.8 ± 5.7	15.1 ± 4.9	16.9 ± 6.1	NS
Stented segments				
In-segment				
MLD, mm	0.81 ± 0.33†	2.03 ± 0.39	2.07 ± 0.41	<0.001
Diameter stenosis, %	73.3 ± 12.5†	32.1 ± 9.8	30.5 ± 10.1	<0.001
Late lumen loss, mm	1.48 ± 0.18†	0.21 ± 0.12	0.25 ± 0.11	<0.001
In-stent MLD, mm	0.72 ± 0.37†	2.18 ± 0.49	2.14 ± 0.39	<0.001
Diameter stenosis, %	76.3 ± 10.4†	27.8 ± 7.1	28.9 ± 8.5	<0.001
Late lumen loss, mm	1.64 ± 0.21†	0.11 ± 0.09‡	0.20 ± 0.10	<0.001
Degree of restenosis				
Patients with restenosis ≤20%	0 (0%)†	16 (73%)	67 (65%)	<0.0001
Patients with restenosis >20%–<50%	0 (0%)†	6 (27%)	36 (35%)	<0.0001
Patients with binary restenosis	30 (100%)†	0 (0%)	0 (0%)	<0.0001

Data are expressed as n, mean ± SD, or n (%). *p < 0.05 for comparison between progression group versus restenosis and stable groups. †p < 0.05 for comparison between restenosis group versus progression and stable groups. ‡p < 0.05 for comparison between progression group versus stable group.
ANOVA = analysis of variance; other abbreviations as in Table 2.

0.001). Also, there were no differences between the first and the second assessments in mean ±SD and range of CD34+/KDR+/CD45− cells (1.49 ± 0.54 cells/μl vs. 1.37 ± 0.49 cells/μl, 0.55 to 2.12 cells/μl vs. 0.45 to 2.03 cells/μl, respectively), CD133+/KDR+/CD45− cells (0.69 ± 0.13 cells/μl vs. 0.58 ± 0.25 cells/μl, 0.23 to 0.98 cells/μl vs. 0.27 to 0.91 cells/μl, respectively), CD105+/CD45−/CD34− cells (1.61 ± 0.59 cells/μl vs. 1.82 ± 0.48 cells/μl, 0.81 to 2.53 cells/μl vs. 0.77 to 2.67 cells/μl, respectively) and CD14+/CD45+ cells (0.59 ± 0.42 cells/μl vs. 0.79 ± 0.48 cells/μl, 0.22 to 1.23 cells/μl vs. 0.18 to 1.11 cells/μl), respectively.

Subpopulations of EPCs in the study groups. Stem cell analysis (Table 4) showed that patients and control groups had similar white cell count and mononuclear cells. The absolute numbers of CD34+/KDR+/CD45− cells measured in the restenosis group (1.41 ± 0.64 cells/μl) were significantly ($p < 0.05$) higher than in the progression, stable, and control groups (1.03 ± 0.53 cells/μl, 1.07 ± 0.46 cells/μl, and 0.95 ± 0.44 cells/μl, respectively). Similarly, CD133+/KDR+/CD45− cells were significantly ($p < 0.001$) higher in the restenosis (0.63 ± 0.23 cells/μl) compared with progression, stable, and control groups (0.33 ± 0.19 cells/μl, 0.41 ± 0.32 cells/μl, and 0.36 ± 0.15 cells/μl, respectively). No significant difference in CD34+ and CD133+ subpopulations were found at comparison between progression or stable groups and control subjects. Also, CD105+/CD45−/CD34− cells were similar between patients and control groups. Finally, numbers of CD14+/CD45+ cells were significantly ($p < 0.05$) different at statistical comparison among groups, being significantly higher in patients with restenosis than in stable patients (0.72 ± 0.56 cells/μl vs. 0.28 ± 0.54 cells/μl, $p < 0.05$) at statistical comparison between groups.

Discussion

The results of our study indicate that the subpopulations of circulating stem/progenitor cells assessed at time of PCI are different in patients with subsequent restenosis as compared with patients who experience progression of coronary atherosclerosis, those who have a stable disease, and control subjects. Interestingly, progression of coronary artery disease was not associated with different levels of EPCs compared with patients with stable disease or even normal controls, whereas patients with restenosis had higher levels of circulating EPCs.

The relation of EPCs to atherosclerosis progression. The development of atherosclerotic lesions involves injury to the endothelium, activation of platelets, adhesion of leucocytes, and migration and proliferation of vascular smooth muscle cells (12). For this reason, a role for EPCs in atherogenesis has been advocated (2,22). The results of our study, conversely, demonstrate that there is no significant association between the number of EPCs and the subsequent development of de novo coronary stenoses or progression of previously nonsignificant coronary lesions. Indeed, subpopulations of EPCs measured at time of the index PCI did not differ between those patients who showed progressive disease and those who had neither progression of coronary atherosclerosis nor in-stent restenosis. These findings differ from previous work on the putative role of EPCs in atherogenesis, which have described positive or negative relations of EPCs to coronary artery disease progression (22), extension (3), severity (23), and outcome (24). One should consider, however, that all previous clinical studies have been cross-sectional, and none has investigated prospectively the evolution of subclinical coronary atherosclerosis (11). Our study, conversely, has permitted a serial assessment

Table 4. Endothelial Progenitor Cells in the 3 Groups of Patients and Control Subjects

	Restenosis Group (n = 30)	Progression Group (n = 22)	Stable Group (n = 103)	Control Group (n = 20)	ANOVA p Value
White cells, 10^3/ml	6.75 ± 1.21	6.95 ± 0.91	6.51 ± 1.41	6.76 ± 1.30	NS
Monocytes, 10^3/ml	0.54 ± 0.27	0.56 ± 0.21	0.53 ± 0.27	0.59 ± 0.21	NS
CD34+/CD45–, cells/μl	3.55 ± 2.33	3.67 ± 3.15	3.11 ± 2.17	3.06 ± 2.10	NS
CD133+/CD45–, cells/μl	2.41 ± 2.07	2.91 ± 2.12	2.14 ± 1.98	2.28 ± 1.87	NS
CD34+/KDR+, cells/μl	4.15 ± 2.88	3.94 ± 2.15	4.21 ± 2.75	3.69 ± 2.11	NS
CD133+/KDR+, cells/μl	2.87 ± 2.18	2.44 ± 2.29	2.38 ± 1.96	2.33 ± 1.84	NS
CD34+/KDR+/CD45–, cells/μl	1.41 ± 0.64*	1.03 ± 0.53	1.07 ± 0.46	0.95 ± 0.44	<0.05
CD133+/KDR+/CD45–, cells/μl	0.63 ± 0.23*	0.33 ± 0.19	0.41 ± 0.32	0.36 ± 0.15	<0.001
CD105+/CD45–/CD34–, cells/μl	1.70 ± 0.66	1.61 ± 0.72	1.88 ± 0.94	1.92 ± 0.97	NS
CD14+/CD45+, cells/μl	0.72 ± 0.56†	0.51 ± 0.52	0.28 ± 0.54‡	0.62 ± 0.67	<0.05

Data are expressed as mean ± SD. *$p < 0.05$ for comparison between restenosis group versus progression, stable, and control groups. †$p < 0.05$ for comparison between restenosis group versus stable group. ‡$p < 0.05$ for comparison between stable group versus control group.
ANOVA = analysis of variance.

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of the coronary atherosclerotic process with quantitative coronary angiography (25). Apart from methodological considerations, there are alternative explanations for our findings. Multiple studies have demonstrated an association between the number and function of circulating EPCs and risk factors for coronary artery disease, such as age, physical training, smoking, diabetes, hypertension, and dyslipidemia (3), yet it cannot be determined whether it is a mere association or a contributory factor in atherogenesis (4). Our results are in agreement with these previous observations, as they question the hypothesis that circulating EPCs are causally related per se to coronary atherogenesis.

Interestingly, we did not find any significant difference in levels of EPCs between study population and normal controls. This finding is at variance with previous investigations that have shown a difference between normal subjects and patients with coronary artery disease (10,23). The peculiar design and inclusion criteria of our study might constitute possible explanations for this discrepancy.

The relation of EPCs to in-stent restenosis. In our study, patients who subsequently experienced in-stent restenosis had higher levels of circulating CD34, CD133, and CD14 positive cells with respect to the progression, stable, and control groups. Progression of coronary atherosclerosis and in-stent restenosis do not share common histological features, with the latter being caused only by exuberant smooth muscle cells hyperplasia without any significant lipid or foam cell component (5). Indeed, there is the possibility that mobilized bone marrow progenitors differentiate into vascular smooth muscle cells and therefore aggravate the severity of restenosis (26).

The potential association between angiographic restenosis of bare-metal stents and EPCs has been described in 2 previous studies that evaluated only the population of cells that expressed the hematopoietic stem cell marker CD34. Schober et al. (6) reported that post-PCI CD34+ cell counts were increased in patients with restenosis but decreased in those without restenosis, and Inoue et al. (7) found that the increase in CD34+ cells after bare-metal stenting was more striking in patients with restenosis than without restenosis. Our findings confirm and expand these observations and demonstrate that the outcome of PCI is associated with the baseline pattern of EPCs, as we found higher levels of circulating CD34+ and CD133+ cells in those patients who subsequently experienced restenosis. The importance of our findings is underscored by the minimal overlap of numbers of EPCs counts between the restenosis group and the stable, progression, and control groups. Unfortunately, we were unable to establish if the increased number of EPCs resulted in their homing and migration into coronary stents. One should consider, however, that previous human and animal models have described engraftment of EPCs in in-stent neointima. Specifically, previous investigators have noted unexpectedly high expression of c-kit (27) and CD34 (28) in tissue from human atherectomy specimens of in-stent neointima. Thus, one might speculate that an abnormal engraftment of CD34+ and CD133+ EPCs causing excessive intima proliferation and in-stent restenosis may occur particularly in those patients who have greater levels of EPCs at time of PCI.

Our results indicate also a link between stem/progenitor cells that promote the development of new blood vessels and the damage healing after angioplasty (29). Indeed, CD14+/CD45+ monocyte-derived cells have the ability to enhance neovascularization in experimental models with the release of growth factors, such as vascular endothelial growth factor, as recently shown by Sieveking et al. (17). These so-called circulating angiogenic cells were found to be positively correlated with the presence and severity of coronary artery disease by Guven et al. (10), who therefore hypothesized that these cells play a role in the progression or stabilization of vascular disease. Our finding that baseline numbers of CD14+/CD45+ cells were higher in patients with subsequent restenosis suggests also that excessive circulating angiogenic cells may contribute to the pathogenesis of in-stent restenosis, perhaps because they trigger redundant re-endothelialization.

Study limitations. For the purpose of this study, we included only candidates to PCI, and therefore we are unable to draw a definite conclusion on the general relationship between EPCs and coronary artery disease. A limitation of this study lies in the fact that the numbers of patients in each group were relatively small. Also, differences in the number of each subtype of EPCs were relatively narrow, though significant differences between restenosis and no-restenosis groups were found. However, use of strict statistical analysis with Bonferroni correction and the evidence that 3 EPC subpopulations showed a similar behavior (all increased in the restenosis group) make very unlikely that our findings were due to chance.

A major limit of our study is the lack of functional studies of EPCs. Unfortunately, isolation of significant quantities of cells for functional assays could not be possible in the majority of our patients and therefore we could not perform functional studies as previously done (4). Also, a limitation of flow cytometric analysis was that we did not assess what percentage of CD133+ cells were also CD34+. We did not establish if the increased number of EPCs resulted 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. Also, 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 vascular endothelial growth factor

levels and mobilize cells into peripheral blood (4). However, it is unlikely that myocardial ischemia affected our results because all patients had flow-limiting coronary stenoses before revascularization. Although we used well-established, quantitative angiographic analytic methods, angiography is known to underestimate the extent of coronary atherosclerosis, especially in comparison to intravascular ultrasonography. We were unable to acquire routinely intravascular ultrasound data, and therefore we cannot exclude a role for negative vascular remodeling at the non-PCI sites of native coronary atherosclerosis. However, it has been recently demonstrated that quantitative coronary angiography and intravascular ultrasound measures of lumen size correlate very well (25). We did not perform a multivariate analysis for confounders of restenosis because of the relatively small sample size of patients with in-stent restenosis. Although pharmacologic agents (in particular statins) may affect numbers of EPCs (30), it is unlikely that differences in EPCs levels among groups were caused by drug treatment, as medications were similar in the 3 groups of patients and those patients who were on statins at referral were not included in the study. The short-term angiographic follow-up (8 months) may have limited the ability to detect a role for EPCs in long-term evolution of coronary atherosclerosis.

Conclusions

Our study indicates that patients with restenosis have higher numbers of subpopulations of EPCs that incorporate into endothelial cells or play a role in arteriogenesis compared with controls and patients with either progression of coronary atherosclerosis or stable disease. Therefore, further studies are needed to identify the mechanism or the cause-effect relationship of EPCs in restenosis.

Reprint requests and correspondence: Dr. Vincenzo Pasceri, Cardiovascular Department, San Filippo Neri Hospital, Via G. Martinotti 20, 00135 Rome, Italy. E-mail: vpasceri@hotmail.com.

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