



Clinical research

Circulating endothelial progenitor cells in patients with unstable angina: association with systemic inflammation

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KEYWORDS

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Background Endothelial progenitor cells (EPC) are present in peripheral blood and can develop a functional endothelial phenotype. The number and function of circulating EPCs are altered in atherosclerosis, diabetes, and after myocardial infarction and EPCs have been shown to promote postnatal angiogenesis and vasculogenesis. We investigated the number and adhesive properties of EPCs from patients with unstable angina and no evidence of cardiac necrosis.

Methods and results Patients were selected with unstable angina ($n = 29$) and no evidence of cardiac necrosis, and controls with stable angina ($n = 12$) and atherosclerotic risk factors, medication use, and coronary vessel involvement similar to patients. Circulating EPC numbers were determined by colony-forming unit assay and their adhesive properties were evaluated by EPC capacity to bind immobilised fibronectin. High-sensitivity C-reactive protein (hsCRP) was determined in all patients.

Circulating EPCs were significantly increased in patients with unstable as compared with stable angina (24.5 ± 2.6 vs. 13.3 ± 2.9 , respectively). Seven unstable angina patients followed up for 3 months after clinical stabilisation exhibited a reduction of close to 50% in circulating EPC numbers. The adhesive capacity of EPCs from patients with unstable and stable angina did not differ. A positive correlation was found between systemic CRP levels and circulating EPC numbers, but not their adhesive capacity.

Conclusion Patients with unstable angina and no evidence of cardiac necrosis exhibited increased circulating EPCs. Systemic inflammation, in addition to recognised growth factors, could play a role in the peripheral mobilisation of EPCs in patients with anginal syndromes.

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Introduction

Atherosclerosis has been envisioned as a process with an inflammatory phenotype.^{1,2} Phenotype alterations can

result in plaque disruption and acute coronary events.³ Since a dysfunctional endothelium plays a major role in atherosclerosis, replenishment of a damaged endothelium is likely to have a beneficial influence.

Endothelial precursors capable of transforming into mature, functional endothelial cells can be isolated from peripheral mononuclear cells in circulation.^{4,5} The number of circulating endothelial progenitor cells (EPC) has been shown to correlate negatively with established risk

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factors for atherosclerosis,^{6,7} supporting a possible role of depletion of the pool of endothelial precursors in lesion formation. However, it appears that not only may the number of circulating EPCs be important, but also their adhesive properties, which give them the ability to adhere to damaged surfaces. Interestingly, it has been shown that EPCs from diabetic patients are compromised in their adhesion to tumour necrosis factor alpha-primed endothelial cells.⁸ The potential benefit of EPC transfusion is evident from studies that have demonstrated that EPCs are integrated into the vascular infrastructure and contribute to angiogenesis and vasculogenesis,^{9,10} and protect against atherosclerosis development¹¹ and neointimal formation in mice.¹²

Several lines of evidence obtained in experimental animals support the role of tissue ischaemia in facilitating EPC mobilisation to the peripheral pool. Vascular endothelial growth factor (VEGF) has been suggested as a principal promoter in this respect.¹³ A study conducted recently in patients with myocardial infarction has shown that mobilisation of EPCs to peripheral circulation occurs and is correlated with VEGF serum levels.¹⁴

In the current study we tested the hypothesis that the number of EPCs is increased in patients with unstable angina and no evidence of myocardial necrosis as compared with patients with stable coronary heart disease. We also evaluated EPC adhesive properties comparatively in patients with stable and unstable angina.

Materials and methods

Study subjects

We studied a total of 29 patients with unstable angina and no ST-segment elevation. Twelve consecutive patients with stable angina pectoris with a similar age, atherosclerotic risk profile, and use of medication were selected randomly from the cardiology clinic. All patients underwent coronary angiography in the past 6 months and the extent of coronary artery disease was determined (see below). This group of stable angina patients was selected as the control group because it has been shown that risk factors for atherosclerosis correlate negatively with EPC numbers.^{6,7} Thus, it was necessary to evaluate a control group with a similar profile of atherosclerotic risk factors and medications to exclude the potential influence of these factors on EPC numbers.

The definition of unstable angina required chest discomfort lasting at least 15 min within the 24 h preceding hospitalisation and representing a change in the usual pattern of angina. The diagnosis of unstable angina also required new or dynamic ST-wave or T-wave changes in at least two contiguous ECG leads. Myocardial infarction was ruled out as troponin I levels were below the 99th percentile 6–12 h after the onset of chest pain.

Sample size was estimated on the basis of a previous study showing a significant increase in circulating EPC numbers in patients with acute myocardial infarction.¹⁴ Assuming that the magnitude of the effect would be lower, we enlarged the size of the patient and control samples by approximately 50%.

The extent of coronary artery disease in a patient was determined by the presence of three-vessel or left main coronary

trunk disease and by the “coronary artery greater than 50 (CAGE \geq 50) score”. The CAGE \geq 50 score was defined as the total number of segments with \geq 50% stenosis.¹⁵

Isolation of EPCs and colony-forming unit assay

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density-gradient centrifugation (Sigma) from 20-ml blood samples and EPC-CFU was assayed after two platings and a 9-day culture on fibronectin-coated, 24-well plates, as described.^{7,16} Colonies were counted manually in a minimum of 3 wells by observers who were unaware of the patients’ clinical data. Results were expressed as CFU/well in four separate wells from the same patient.

Confirmation of CFU phenotype

For phenotyping of endothelial characteristics of colony-forming unit (CFU), the following antibodies were used in immunofluorescence and flow cytometric analysis: rabbit polyclonal anti-Tie-2 (C-20), mouse monoclonal anti-flk-1(A-3), and goat polyclonal anti-CD31 (PECAM-1, M-20), all from Santa Cruz. Endothelial cell lineage was further confirmed by indirect immunostaining with the use of 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate-acetylated low-density lipoprotein (Dil-acLDL) and co-staining with BS-1 lectin (Sigma).

Fibronectin adhesion assay

EPCs (day 7) from patients with stable and unstable angina were washed with PBS and gently detached with 0.5 mmol/l EDTA in PBS. After centrifugation and resuspension in basal complete medium supplemented with 5% FCS, identical cell numbers were placed on fibronectin-coated culture dishes and incubated for 30 min at 37 °C.^{8,16} Adherent cells were counted by independent blinded investigators.

High-sensitivity CRP concentrations

The assay for hsCRP was conducted according to the manufacturer’s instructions (Dade Behring Inc).

VEGF plasma concentrations

Levels of serum VEGF were determined by ELISA according to the manufacturer’s instructions (BioSource).

Statistical analysis

Clinical variables were compared between groups using the chi-square test. Student’s *t*-test (two-sided, unpaired) was used to compare EPC numbers and adhesion, as all respective data showed a normal distribution of all the variables tested. The two-sided Spearman test was used to analyse the correlation of CRP with EPC-CFU and fibronectin adherence as data were not normally distributed. All data are presented as mean \pm SEM. $P < 0.05$ was considered statistically significant.

Results

As risk factors for atherosclerosis and statin therapy have been shown to influence the pool of peripheral EPCs,^{6,7,17} we confirmed the absence of any difference between patients with stable and unstable angina in the athero-

Table 1 Clinical and angiographic profile of patients

	Unstable angina (n = 29)	Stable angina (n = 12)	P
Male/female	23/6	11/1	0.21
Age (yr)	64.2 ± 12	63.1 ± 10.8	0.96
LVEF (%)	57 ± 12.7	56.3 ± 13.9	0.96
CAD extent (n × vessels)	1.89 ± 0.9	1.75 ± 0.96	0.93
CAGE >50%	5.1 ± 0.6	4.9 ± 0.9	0.86
Risk factors			
Hypertension, n (%)	22 (75)	9 (75)	1.0
Treated diabetes, n (%)	13 (44)	5 (41)	0.91
Smoking			
Current smoker, n (%)	9 (31)	3 (25)	0.87
Past smoker, n (%)	9 (31)	4 (33)	0.95
Hyperlipidaemia, n (%)	25 (86)	10 (83)	0.94
Family history, n (%)	8 (27)	2 (16)	0.88
Medications			
Beta-blockers, n (%)	22 (75)	10 (83)	0.91
ACEI, n (%)	16 (55)	8 (66)	0.84
ARB, n (%)	0 (0)	0 (0)	
Aspirin, n (%)	29 (100)	12 (100)	1.0
Statins, n (%)	20 (69)	8 (66)	0.95
Calcium blocker, n (%)	5 (17)	3 (25)	0.90
Nitrates, n (%)	19 (65)	7 (58)	0.92
Diuretics, n (%)	9 (31)	2 (16)	0.75
Clopidogrel, n (%)	6 (20)	1 (8)	0.58
Hypoglycaemics, n (%)	13 (44)	5 (41)	0.91

LVEF: left ventricular ejection function; CAD: coronary artery disease; ACEI: angiotensin-converting enzyme inhibitors; ARB: angiotensin receptor blocker.

sclerosis profile and medication use (Table 1). A similar degree of coronary atherosclerosis, as determined by two separate methods (see Methods) and coronary angiography, was present in both groups (Table 1).

We then determined whether unstable angina syndrome with no myocardial necrosis (confirmed by the absence of cardiac enzyme elevation) was associated with changes in EPC numbers, as assessed by CFU counts. As shown in Fig. 1(a), patients with unstable angina had significantly greater numbers of EPC-CFUs (24.5 ± 2.6 /well) compared with patients with stable angina (13.3 ± 2.9 /well; $p = 0.0065$).

We then followed up seven randomly selected unstable-angina patients, all of whom were asymptomatic for three months, and repeated the evaluation of circulating EPC-CFU numbers. Three months after symptom resolution, the number of EPCs was significantly reduced (by a mean of $47 \pm 9\%$) as compared to mean baseline counts in patients with unstable angina (Fig. 1(b)). The baseline CRP levels of these seven patients had been 4.8 ± 0.8 mg/l and decreased to 2.5 ± 0.5 ($p < 0.05$) mg/l at 3 months follow-up.

In five randomly selected patients with stable angina who were followed up for 3 months, no significant differences were evident between baseline (mean CFU/well of 15 ± 3.1) and follow-up values (mean CFU/well of 13.4 ± 4.4 , $p = 0.78$, Fig. 1(b)). When the mean change in the CFU count of each patient from baseline to follow-up was compared between the two groups using the Student *t*-test, a significant difference was

found between patients with unstable (mean change of 16.1 CFU/well) and stable angina (mean change of 1.6 CFU/well; $p < 0.05$).

Next, we compared the functional properties of circulating EPCs from both patient groups. Adherence to extracellular matrix and cultured endothelial cells is a property that may indicate the *in vivo* potency of EPCs, in addition to mere numbers. We found that the number of EPCs adhering to fibronectin-coated surfaces was similar in patients with unstable (12.6 ± 2.4 cells/field) and stable angina (10.3 ± 2.7 cells/field; $p = 0.58$, Fig. 1(c)).

Immunofluorescence studies to confirm endothelial CFU phenotype disclosed robust staining of KDR, Tie2, and CD31, but not of the control isotype-matched antibodies (Fig. 2(a)). Further confirmation was obtained by proving double positivity for Dil-acLDL and BS-1 (Fig. 2(b)).

To investigate the association between systemic inflammation and EPC mobilisation, we evaluated hsCRP levels. Serum CRP levels were indeed increased in unstable angina as compared to stable patients (5.6 ± 0.3 mg/l vs. 1.9 ± 0.3 mg/l, $p < 0.001$). EPC-CFU numbers, but not their capacity to adhere to fibronectin, positively correlated with CRP levels ($r = 0.3$, $p < 0.05$, CI: -0.06 to 0.6 ; Fig. 3).

Mean VEGF serum levels did not differ between stable (56 ± 9 pg/ml) and unstable angina (47 ± 12 pg/ml) patients, nor did they correlate with CFU numbers (data not shown).

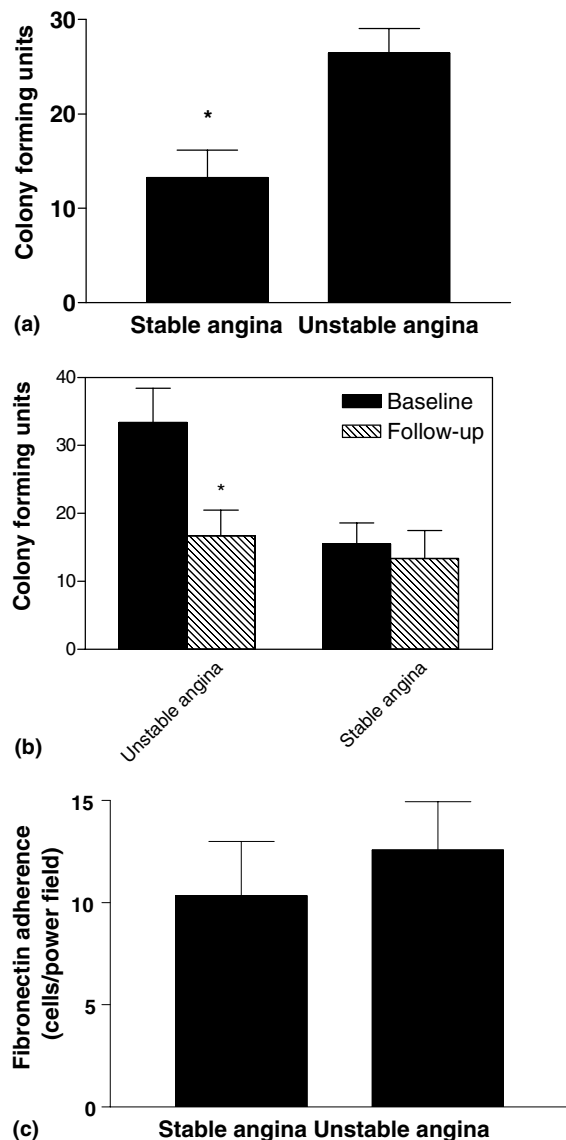


Fig. 1 EPC number and function in patients with angina pectoris. Comparative analysis of circulating EPC numbers by the CFU method (a). Comparative analysis of EPC numbers in seven unstable and five stable angina patients at baseline and 3 months after clinical stabilisation (b) * $p < 0.05$. (c) Adherence to fibronectin in both groups (γ -cells/ $40\times$ magnification). Bars represent mean \pm SEM.

Discussion

Recent data show that EPCs can be recovered from PBMC and could potentially be harnessed for therapeutic angiogenesis and vasculogenesis.^{9,10} Studies in patient subgroups also show that circulating EPC numbers are either altered or their functional properties are impaired, an observation that may account in part for some pathogenic aspects of these disorders.

Tissue ischaemia has been shown to trigger EPC mobilisation in experimental animals – an effect accounted for by VEGF. In humans, the influence of ischaemia has been investigated only in a group of patients with ST-elevation myocardial infarction, where an occluded

artery was demonstrated and significant cardiac necrosis was evident. Although EPC numbers were increased in these patients with myocardial infarction, the control group consisted of 8 healthy subjects and it is likely that the atherosclerotic risk profile and statin use differed between groups. Herein, we investigated patients with unstable and stable angina, both of which had similar risk factor profiles and medication use (Table 1). Additionally, the atherosclerotic “burden” in terms of coronary vessel involvement was similar in both groups and the absence of cardiac necrosis was confirmed by the absence of elevated CPK and troponin levels. We have found that levels of circulating EPCs were significantly higher in unstable compared with stable angina patients, suggesting that necrosis due to infarction is not requisite for their peripheral mobilisation. Since the groups were not very large, we assessed EPC counts in seven unstable angina patients after clinical stabilisation for 3 months, which disclosed a reduction of CFU numbers that approached 50%. No significant change was evident in a comparative group of five patients with stable angina with regard to EPC counts after 3 months of follow up. Despite these results, the small groups do not allow for definite conclusions to be drawn as to whether unstable angina was solely responsible for the increased number of circulating EPCs in these patients.

Recent advances in our understanding of how inflammation contributes to the pathogenesis of vulnerable plaque, accounting for the majority of cases of unstable angina,^{1–3} prompted us to evaluate the association between an inflammatory marker and EPC numbers. CRP has emerged as the leading inflammatory marker, with a powerful predictive value in a variety of vascular disorders¹⁸ and perhaps a contributory pathogenic effect.¹⁹ Interestingly, we found that despite the relatively small sample size, a positive correlation was evident between CRP levels and circulating EPC numbers, suggesting that a systemic inflammatory state in patients with anginal syndromes could potentially facilitate EPC mobilisation, in addition to the known effects of endogenous VEGF,^{13–14,20} which failed to correlate with EPC counts in our study. In a very recent study, Heeschen et al.²⁰ reported a positive correlation between EPC numbers with VEGF and erythropoietin but not with CRP levels. However, their patients were selected mainly on the basis of the presence of coronary artery disease and the aim was not to compare stable with unstable angina.

Another question raised in the current study was whether the functional properties of EPC are altered in patients with unstable angina. The fibronectin-binding assay evaluated the ability of EPCs to adhere to extracellular matrix surfaces.^{8,16} It is presumed that dysfunctional EPCs lacking the ability to properly attach to matrix or to endothelial cells may compromise angiogenesis and vasculogenesis. Indeed, diabetic patients⁸ and patients with in-stent restenosis¹⁶ have been shown to exhibit defective EPC adhesive properties, which may play a role in the pathogenesis of these syndromes. In the current study we failed to observe a change in the adherence capacity of EPC in patients with unstable angina. This finding suggests that triggers of plaque destabilisa-

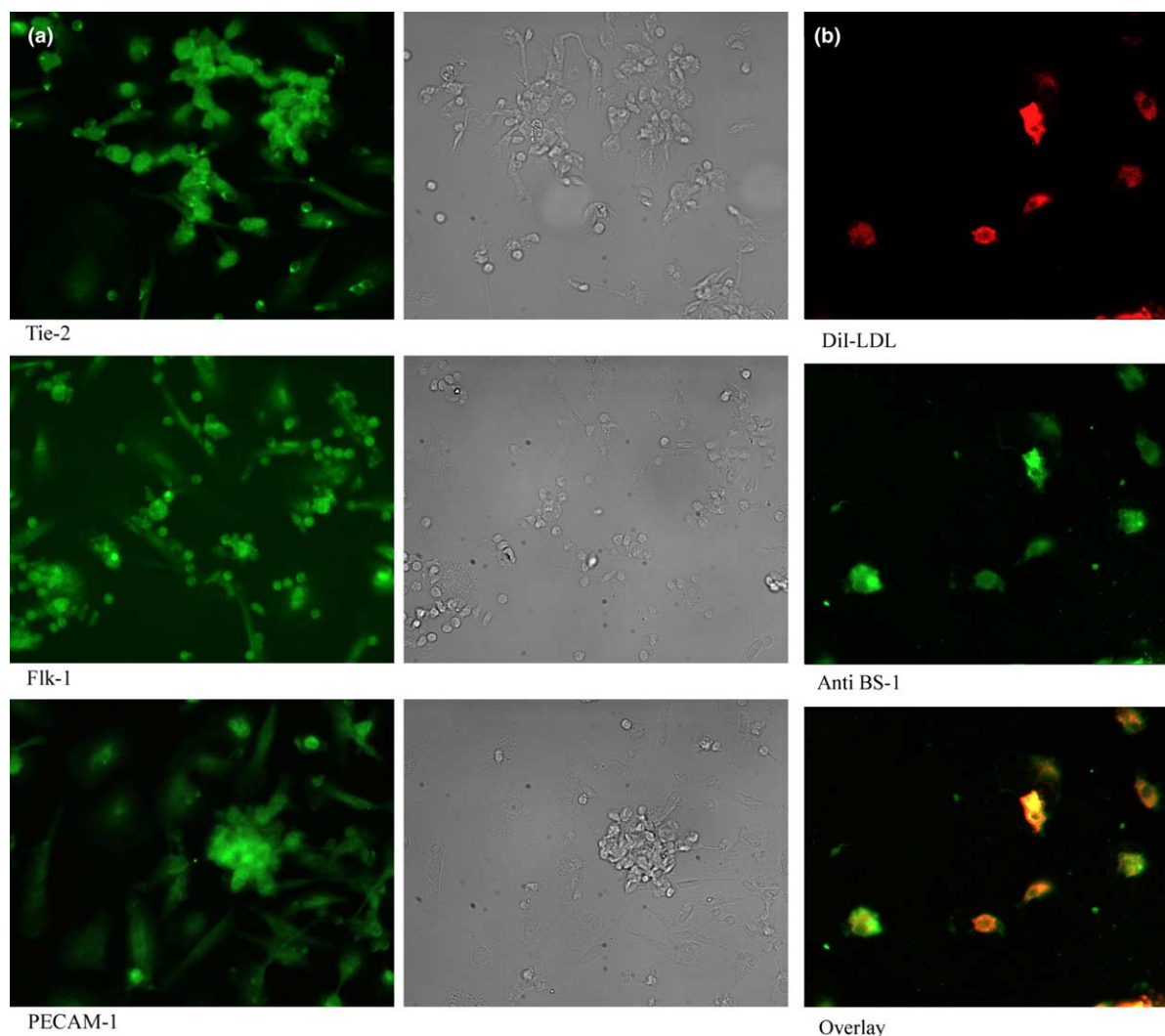


Fig. 2 EPC phenotyping. (a) Confirmation of EPC phenotype by immunofluorescence staining with the indicated antibodies. (b) Validation of double positivity for Dil-acLDL and BS-1 in EPC evaluated at day 4.

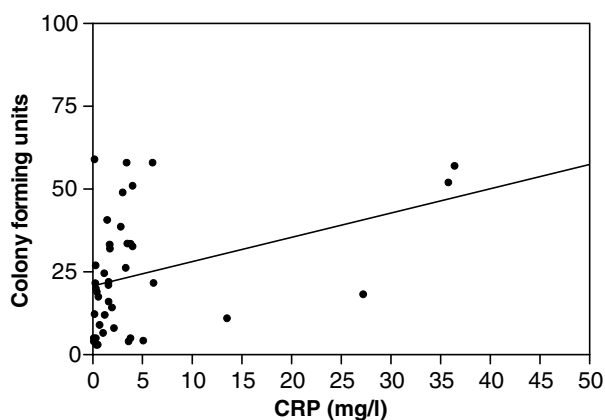


Fig. 3 Correlation between CRP levels and CFU counts in patients with coronary artery disease (stable and unstable angina). $r = 0.29$; $p < 0.05$.

tion (inflammation, for example) do not necessarily alter EPC adherence pattern, despite the changes observed in the number of circulating EPCs.

It should be emphasised that since this study had a limited number of patients, the capacity of statistical analysis to exclude bias in the selection of the two study groups was limited.

In conclusion, we found that patients with unstable angina showed an increased pool of circulating EPCs, but no impairment of their adhesive properties compared with subjects with stable atherosclerotic disease. The inflammatory marker CRP correlated positively with EPC-CFU numbers, suggesting that inflammation, in addition to growth-factor secretion, can promote endothelial precursor mobilisation.

References

1. Glass CK, Witztum JL. Atherosclerosis. The road ahead. *Cell* 2001;104:503–16.
2. Libby P. Inflammation in atherosclerosis. *Nature* 2002;420:868–74.
3. Casscells W, Naghavi M, Willerson JT. Vulnerable atherosclerotic plaque: a multifocal disease. *Circulation* 2003;107:2072–5.

4. Asahara T, Murohara T, Sullivan A et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;**275**:964–7.
5. Hristov M, Erl W, Weber PC. Endothelial progenitor cells: mobilization, differentiation, and homing. *Arterioscler Thromb Vasc Biol* 2003;**23**:1185–9.
6. Vasa M, Fichtlscherer S, Aicher A et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001;**89**:E1–7.
7. Hill JM, Zalos G, Halcox JP et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003;**348**:593–600.
8. Tepper OM, Galiano RD, Capla JM et al. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* 2002;**26**(106):2781–6.
9. Kalka C, Masuda H, Takahashi T et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci USA* 2000;**97**:3422–7.
10. Assmus B, Schachinger V, Teupe C et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation* 2002;**106**:3009–17.
11. Rauscher FM, Goldschmidt-Clermont PJ, Davis BH et al. Aging, progenitor cell exhaustion, and atherosclerosis. *Circulation* 2003;**108**:457–63.
12. Werner N, Junk S, Laufs U et al. Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. *Circ Res* 2003;**93**:e17–24.
13. Asahara T, Takahashi T, Masuda H et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J* 1999;**18**:3964–72.
14. Shintani S, Murohara T, Ikeda H et al. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* 2001;**103**:2776–9.
15. Emond M, Mock MB, Davis KB et al. Long-term survival of medically treated patients in the coronary artery surgery study (CASS) registry. *Circulation* 1994;**90**:2645–57.
16. George J, Herz I, Goldstein E et al. Number and adhesive properties of circulating endothelial progenitor cells in patients with in-stent restenosis. *Arterioscler Thromb Vasc Biol* 2003;**23**:e57–60.
17. Llevadot J, Murasawa S, Kureishi Y et al. HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells. *J Clin Invest* 2001;**108**:399–405.
18. Ridker PM. Clinical application of C-reactive protein for cardiovascular disease detection and prevention. *Circulation* 2003;**107**:363–9.
19. Yeh ET, Willerson JT. Coming of age of C-reactive protein: using inflammation markers in cardiology. *Circulation* 2003;**107**:370–1.
20. Heeschen C, Aicher A, Lehmann R et al. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood* 2003;**102**:1340–6.