Journal of the American College of Cardiology
© 2003 by the American College of Cardiology Foundation
Published by Elsevier Inc.

Vol. 42, No. 12, 2003
ISSN 0735-1097/03/$30.00
doi:10.1016/j.jacc.2003.07.025

Age-Dependent Depression in Circulating Endothelial Progenitor Cells in Patients Undergoing Coronary Artery Bypass Grafting

Robert J. Scheubel, MD,*‡ Holger Zorn,* Rolf-Edgar Silber, MD,* Oliver Kuss, PHD,† Henning Morawietz, PHD,§ Juergen Holtz, MD,‡ Andreas Simm, PHD*

Halle (Saale) and Dresden, Germany

OBJECTIVES The effect of patient age on circulating endothelial progenitor cells (EPCs) and their mobilization during coronary artery bypass grafting (CABG) was assessed.

BACKGROUND The EPCs are able to contribute to reparative neovascularization after tissue ischemia. In experimental models, reparative neovascularization is impaired in senescent animals, but the role of EPCs in this impairment, especially in humans, is unknown.

METHODS In 50 consecutive patients (43 to 80 years old) with stable coronary artery disease undergoing CABG, the numbers of EPCs and the plasma levels of interleukin (IL)-6, IL-8, IL-10, and IL-18, as well as vascular endothelial growth factor (VEGF) and placental growth factor, were determined preoperatively, after coming off bypass, and 6, 12, 24, and 72 h postoperatively.

RESULTS Preoperative values of EPCs were lowered with increasing age, similar to the lowering of plasma VEGF levels. These age-associated decreases could not be explained by differences in atherosclerotic risk factors or cardiac function. Bypass surgery induced a rapid mobilization in EPCs, IL-6, IL-8, IL-10, and VEGF, with a peak 6 h postoperatively. Persistently lower levels of EPCs and VEGF throughout the observation period were observed in patients >69 years old, which could not be explained by differences in the operative procedure or inflammatory IL activation.

CONCLUSIONS Despite a significant increase in EPCs and release of cytochemokines during CABG, age is a major limiting factor for mobilization of EPCs. Further studies are necessary to improve the strategies for mobilization, ex vivo expansion, and re-transplantation of EPCs in aging patients. (J Am Coll Cardiol 2003;42:2073–80) © 2003 by the American College of Cardiology Foundation

Coronary artery bypass grafting (CABG) or percutaneous transluminal coronary angioplasty (PTCA) is used to reconstitute flow into post-stenotic, chronically underperfused myocardium. This post-stenotic myocardium consists of connective tissue scars, dying cardiomyocytes, and hypo-

See page 2081

active or chronically hibernating myocardium with microvascular disturbances due to microthrombi and decreased capillary density (1). When bulk flow is successfully reconstituted by CABG or PTCA, the gradual recovery of microcirculation is considered as decisive for the recovery of mechanical function of surviving post-stenotic myocardium (2).

Traditionally, neovascularization of disturbed microcirculation was considered to result exclusively from the proliferation, migration, and remodeling of fully differentiated endothelial cells (ECs) derived from pre-existing blood vessels (3). Recently, however, it was demonstrated that circulating, bone marrow-derived endothelial progenitor cells (EPCs) may home to sites of postnatal neovascularization and differentiate into ECs in situ (4), which is called "vasculogenesis" (5). Vascular trauma, as it occurs during surgical procedures, or inflammation leads to a cascade of events that result in the chemoattraction of inflammatory cells or other cell types to the site of injury (6). These blood-borne cells produce pro-angiogenic factors that, in turn, attract other cell types such as circulating EPCs (7). Systemic inflammatory responses have been described after cardiac surgery with cardiopulmonary bypass (CPB) (8). Contact of the blood components with the artificial surface of the extracorporeal circuit, ischemia-reperfusion injury, endotoxemia, and operative trauma are possible causes for this phenomenon (8). Trauma has been considered as the critical stimulus for the mobilization of EPCs and pro-angiogenic vascular endothelial growth factor (VEGF) during CABG (9). It has been speculated that this mobilization may contribute to the revascularization of injured tissue (9), which would be of great clinical relevance for a successful outcome of CABG.

Presently, there is a strong trend to perform CABG in patients of advanced age (10). However, experimental data indicate impaired neoangiogenesis in ischemic tissues and impaired re-endothelialization of vascular lesions as a func-

From the *Department of Cardiothoracic Surgery, †Institute of Epidemiology, Biostatistics, and Informatics, and ‡Institute of Pathophysiology, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany; and the §Department of Vascular Endothelium and Microcirculation, University of Technology, Dresden, Germany. This work was supported by grants from the German Bundesministerium für Bildung und Forschung, or BMBF (NBL 3, FKZ 3/6) and the Deutsche Forschungsgemeinschaft (SFB 598/B1, A5).

Manuscript received March 26, 2003; revised manuscript received July 16, 2003, accepted July 21, 2003.

Abbreviations and Acronyms

APC = allophycocyanin
CABG = coronary artery bypass grafting
CAD = coronary artery disease
CPB = cardiopulmonary bypass
EC = endothelial cell
EPC = endothelial progenitor cell
KDR = kinase insert domain containing receptor
IL = interleukin
PlGF = placental growth factor
PTCA = percutaneous transluminal coronary angioplasty
VEGF = vascular endothelial growth factor

tion of advanced age (11–13). The mechanisms for this age-dependent impairment of vascular repair are largely unknown. Therefore, we analyzed the influence of age on CABG-induced mobilization of EPCs and cytochemokines with angiogenesis-modulating potential in a cohort of consecutive patients with stable coronary artery disease (CAD) scheduled for elective CABG. Probably, several types of endothelial precursor or progenitor cells have angiogenic potential after homing into traumatic tissue (14–18). Therefore, we used two phenotypic markers (CD34 [19] and AC133 or CD133 [20]), which are expressed in all EPC types, but in the case of AC133, not in differentiated ECs (15,17,21). This was done to exclude from our analysis any mature or dying ECs with doubtful angiogenic capacity, potentially released from damaged vessels in old patients.

In this analysis, we demonstrate that the preoperative number of circulating EPCs in patients with stable CAD is reduced with increasing age, together with decreased plasma VEGF levels. During CABG, mobilization of circulating EPCs could be detected in all patients, but this mobilization remained on a persistently lower level in the older patient group, suggesting that the responsiveness for mobilization of EPCs is impaired with age. Optimized strategies for ex vivo expansion of those cells might be especially required in the elderly, if transplantation of these cells into post-stenotic tissue will develop as a future co-therapy to existing interventions of revascularization.

METHODS

After approval by the local ethics committee of the University of Halle-Wittenberg, 50 consecutive patients with angiographically documented one-, two-, or three-vessel

Table 1. Baseline Characteristics and Perioperative Data of Patients

Data	All Patients 43–80 Yrs (n = 50)	Patients <62 Yrs (n = 14)	Patients 62–69 Yrs (n = 19)	Patients >69 Yrs (n = 17)
Risk factors, n (%)				
Male gender	37 (74)	11 (79)	17 (90)	9 (53)
Diabetes mellitus	19 (38)	5 (36)	8 (42)	6 (35)
Hypertension	45 (90)	11 (79)	17 (90)	17 (100)
Nicotine abuse	22 (44)	11 (79)	7 (37)	4 (24)
Hyperlipoproteinemia	42 (84)	11 (79)	17 (90)	14 (82)
LVEF (%)	62.1 ± 2.0	61.2 ± 4.6	63.4 ± 2.3	61.4 ± 3.9
NYHA class, n (%)				
I	5 (10)	2 (14)	2 (11)	1 (6)
II	37 (74)	11 (79)	14 (74)	12 (71)
III	6 (12)	1 (7)	2 (11)	3 (18)
IV	2 (4)	0	1 (5)	1 (6)
Operative data				
CPB time (min)	105 ± 5	107 ± 7	98 ± 6	112 ± 11
Cross-clamping time (min)	58 ± 3	61 ± 4	53 ± 4	60 ± 5
No. of grafts	3.4 ± 0.1	3.4 ± 0.2	3.6 ± 0.1	3.2 ± 0.2
EPCs				
EPCs/100,000 lymphocytes	41.9 ± 3.7	58.6 ± 9.5	36.2 ± 5.1	34.5 ± 3.4*
EPCs/100 μl blood	79.1 ± 7.3	118.2 ± 14.4	71.0 ± 11.9*	58.8 ± 8.1*
Lymphocytes/leukocytes (%)	32.9 ± 1.4	34.6 ± 3.1	32.5 ± 2.5	32.0 ± 2.1
Leukocytes (×1,000/μl blood)	6.0 ± 0.3	6.4 ± 0.5	6.3 ± 0.6	5.3 ± 0.3
Plasma cytochemokines (pg/ml)				
IL-6	3.5 ± 1.1	2.0 ± 0.7	5.6 ± 2.9	2.5 ± 0.5
IL-8	4.8 ± 0.6	4.1 ± 1.0	5.2 ± 1.4	4.9 ± 0.8
IL-10	3.0 ± 0.7	5.2 ± 1.9	2.7 ± 0.8	1.5 ± 0.5
IL-18	275 ± 20	249 ± 33	264 ± 32	312 ± 39
VEGF	22.1 ± 2.2	33.4 ± 6.0	18.7 ± 1.7*	15.7 ± 2.5*
PlGF	15.7 ± 1.4	17.2 ± 4.1	14.4 ± 1.3	16.1 ± 2.1

Reported significances (*$p < 0.05$) for the respective parameters result from Bonferroni-adjusted *t* tests of comparing age groups <62 and >69 years, as well as <62 and 62 to 69 years. Data are presented as the number (%) of patients or mean value ± SD.
CPB = cardiopulmonary bypass; EPC = endothelial progenitor cells; IL = interleukin; LVEF = left ventricular ejection fraction; NYHA = New York Heart Association; PlGF = placental growth factor; VEGF = vascular endothelial growth factor.

CAD gave written, informed consent and were enrolled in this prospective study. The patient characteristics are summarized in Table 1. Exclusion criteria were as follows: re-operation for CABG; children; pregnancy; coumarin anticoagulant treatment; coagulopathy; liver dysfunction; nephropathy with dialysis; medication with immune-modulating agents such as steroids and anti-inflammatory agents; any history or signs of infectious disease before surgery; re-animation or revision after CABG; and implantation of an intra-aortic balloon pump or ventricular assist device. All patients underwent nonemergent CABG and received the same intravenous anesthesia, consisting of sufentanil (Sufenta, Janssen-Cilag, Neuss, Germany), propofol (Disoprivan, AstraZeneca, Wedel, Germany), and midazolam (Dormicum, Roche, Grenzach-Wyhlen, Germany) under a standardized protocol. The CPB circuit consisted of roller pumps (Stoeckert, München, Germany), a membrane oxygenator (CML Duo, Cobe, Arvada, Colorado), a hard-shell venous reservoir (Cobe), and a 43-μm arterial filter (Cobe Sentry). Full-dose heparin (350 U/kg; Liquemin N 25000, Hoffmann-La Roche, Grenzach-Wyhlen, Germany) was applied before cannulation of the ascending aorta and right atrium for installation of CPB. Aprotinin (Trasylol, Bayer, Leverkusen, Germany) was given at dosage of 3×10^6 U to all patients. Induction of heart arrest was performed by the use of cold crystalloid cardioplegia (Bretschneider-HTK, Köhler, Alsbach, Germany), intermittent cold blood cardioplegia, or intermittent normothermic blood cardioplegia. After coming off bypass, the patient was given protamine (Protamin ICN 1000 I.E./ml, ICN, Frankfurt/Main, Germany) to neutralize the heparin dosage to 100%.

The blood samples were taken in heparinized tubes at six time points for each patient as follows: sample 1, 10 min before induction of anesthesia; sample 2, after coming off bypass; sample 3, in the intensive care unit 6 h after surgery; sample 4, in the ICU 12 h after surgery; sample 5, 24 h after surgery; and sample 6, 72 h after surgery. All blood samples were evaluated by flow cytometry within 24 h or immediately spun at 1,000 g for 15 min. The plasma was separated and frozen at −20°C.

Flow cytometric analysis. A volume of 100 μl peripheral blood was incubated for 15 min with 2 ml of 1× ammonium chloride lysing solution (Becton Dickinson, Heidelberg, Germany). After centrifugation at 500 g, the cells were resuspended in 100 μl buffer (containing 45 ml RPMI-1640 cell culture medium, 5 ml bovine calf serum, and 500 μl 3% NaN_3) and incubated for 30 min in the dark with fluorescein isothiocyanate-labeled monoclonal antibodies against human CD34 (Becton Dickinson) and allophycocyanin (APC)-labeled monoclonal antibodies against human AC133 (Miltenyi Biotec, Bergisch Gladbach, Germany). Isotype-identical antibodies served as controls (Becton Dickinson). After incubation, cells were washed with 2 ml washing solution (Becton Dickinson), centrifuged at 500 g for 5 min, and resuspended in 500 μl washing solution (Becton Dickinson). Before each analysis, 7-amino-actinomycin-D was added as a viability stain. Each analysis included 100,000 events within the lymphocyte gate. Thus, we obtained the number of EPCs per 100,000 lymphocytes (EPC/lymphocytes) and, after adjustment for the number of leukocytes in peripheral blood and the fraction of lymphocytes/leukocytes, the number of EPCs per 100 μl blood (EPC/blood). Representative examples of these flow cytometric analyses are shown in Figures 1a to 1f.

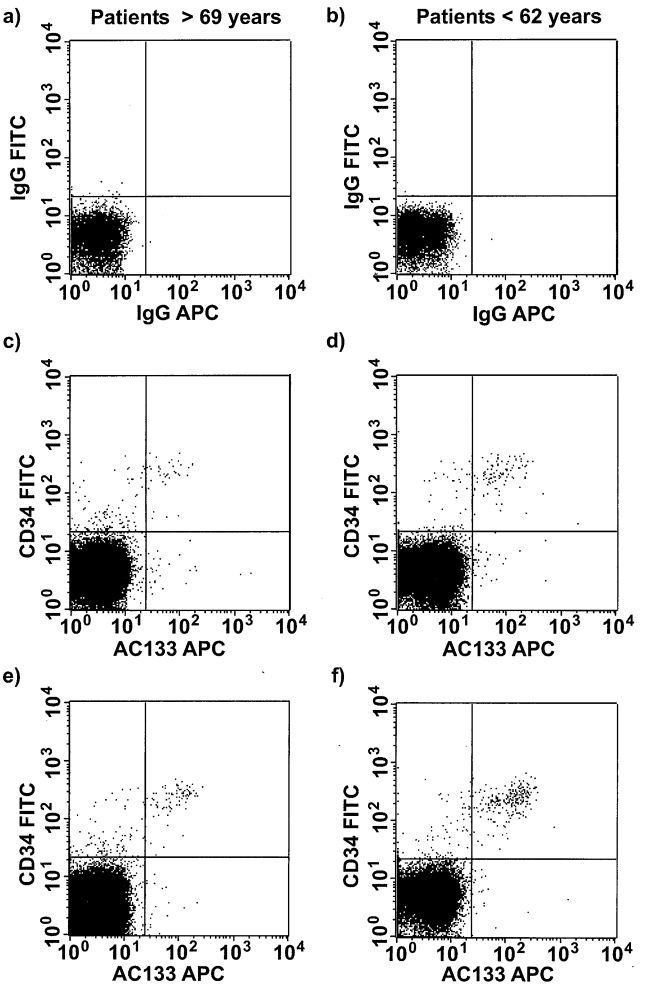

Figure 1. Representative dot blot flow cytometric analysis of the mobilized endothelial progenitor cells/lymphocytes, quantifying the number of $CD34^+$ and $AC133^+$ cells in the older (>69 years) and younger patients (<62 years) preoperatively **(c, d)** and 6 h after surgery **(e, f)**, together with isotype control of older **(a)** and younger patients **(b)**. APC = allophycocyanin; FITC = fluorescein isothiocyanate.

Functional characterization of $CD34^+$ and $AC133^+$ progenitor cells. The endothelial differentiation potential of these progenitor cells was assessed by analyzing the expression of EC-specific markers after incubation for three days in an endothelial growth medium (EGM-2, BioWhittaker, Verviers, Belgium) on fibronectin-coated culture dishes (Becton Dickinson). Human $CD34^+$ cells were isolated from "buffy coats" of donor blood via immunomagnetic CD34 microbead extraction (Miltenyi Biotec), according to the manufacturer's instructions, and analyzed by flow

cytometry for AC133-APC (Miltenyi Biotec), AcLDL-DiI binding (Paesel & Lorei, Hanau, Germany), vascular/endothelial-cadherin-phycoerythrin (Santa Cruz, Heidelberg, Germany), and kinase insert domain containing receptor (KDR) (ReliaTech, Braunschweig, Germany), visualized using APC-conjugated rat anti-mouse monoclonal antibody (Becton Dickinson). Viable double-positive CD34/AC133 cells had a mean fluorescence due to AcLDL binding of 30.0 relative units (rU), which increased to 100.2 rU after 3 days of differentiation in EGM-2, a mean fluorescence of VE-cadherin of 3.7, which increased to 32.7 rU, and a mean florescence of KDR of 65.4 rU, which increased to 457.3 rU. This differentiation potential of $CD34^+/AC133^+$ progenitor cells toward an endothelial phenotype, documented representatively, justifies their denomination as EPCs. However, the functional characterization of $CD34^+/AC133^+$ cells was not performed in all investigated samples of patients. As the true differentiation of $CD34^+/AC133^+$ cells toward an endothelial phenotype can be proved only in vivo, our investigation was an analysis of $CD34^+/AC133^+$ progenitor cells, which should have the potential to differentiate toward an endothelial phenotype.

Plasma VEGF, placental growth factor (PlGF), interleukin (IL)-6, IL-8, IL-10, and IL-18 levels. Quantitative determination of the cytokine plasma levels of patients was performed in duplicate by a highly sensitive enzyme-linked immunosorbent assay (R&D Systems, Wiesbaden, Germany), according to the manufacturer's instructions. Samples were analyzed in a FLUOstar OPTIMA (BMG, Offenburg, Germany).

Statistical analysis. For statistical analysis, standard methods were used, as indicated in the figure legends and tables—for example, Pearson's correlation coefficient (Fig. 2) (see Results section), repeated measures analysis of variance (ANOVA) models (Figs. 3, 4, and 5), ordinary ANOVA models (Fig. 5), linear regression (Table 2) (see Results section), and *t* tests (Table 1). Pairwise comparisons were Bonferroni-adjusted to avoid spurious significances. Calculations were performed by SAS version 8e (SAS Institute Inc., Cary, North Carolina).

RESULTS

Patients. The characteristics of the 50 consecutive patients with elective CABG (median age 65 years, range 43 to 80 years) are presented in Table 1, together with basal circulating EPC values and plasma cytochemokine levels. The patients were classified into three age groups: <62, 62 to 69, and >69 years old. In patients of advanced age, the number of circulating EPCs/lymphocytes, as well as EPCs/blood, and VEGF plasma levels were significantly reduced. These values showed a negative correlation with age (Figs. 2a to 2c). Furthermore, there was an age-independent correlation of VEGF with EPCs/lymphocytes ($p = 0.002$) and with EPCs/blood ($p = 0.001$). Basal EPC values did not correlate with other risk factors (Table 2), with the cumulative number of risk factors, or with any other plasma cytochemokine level. The association between age and EPC values persisted even after adjustment for risk factors in a linear regression model (Table 2).

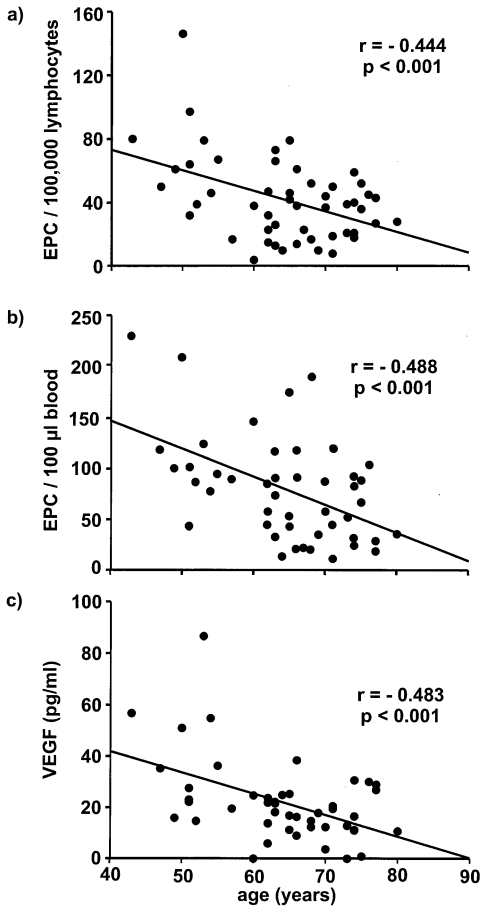

Figure 2. Age-dependent decrease of preoperative endothelial progenitor cells (EPC) values **(a, b)** and plasma vascular endothelial growth factor (VEGF) level **(c)** in patients undergoing coronary artery bypass graft surgery. The Pearson correlation coefficient and p values of the corresponding significance test of the respective values are indicated.

Mobilization of EPCs and induction of cytochemokines by CABG. After CPB, there was a biphasic response of EPC values (Fig. 3). The first phase consisted of an increase in EPCs/lymphocytes, 1.5-fold at the end of CPB and 2-fold 6 h later, relative to the preoperative baseline value, respectively (Fig. 3a). Concomitantly, we observed a two-fold increase in leukocytosis (Fig. 3b) and a progressive decline in the fraction of lymphocytes/leukocytes (Fig. 3c). These alterations resulted in a two-fold increase in circulating EPCs/blood at the end of CPB, which returned to basal levels 6 h later (Fig. 3d). The EPCs/lymphocytes transiently returned to or below preoperative levels one day after surgery (Fig. 3a), whereas the fraction of lymphocytes/leukocytes and circulating EPCs/blood declined below baseline levels (Figs. 3c and 3d). The second phase of increase in EPCs/lymphocytes occurred three days later (Fig. 3a), whereas the leukocytosis remained elevated throughout this period (Fig. 3b).

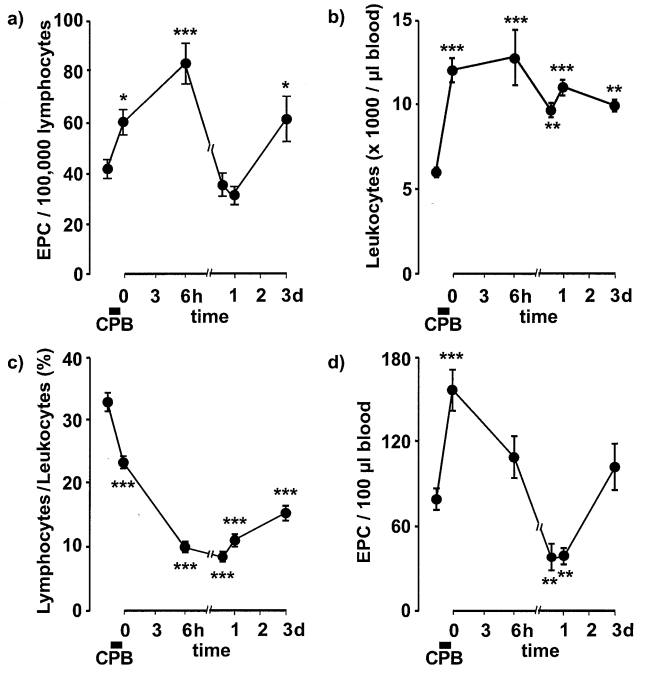

Figure 3. Mobilization of endothelial progenitor cells (EPC) and leukocytes by coronary artery bypass graft surgery. Reported significances (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) were calculated by Bonferroni-adjusted pairwise comparisons with the preoperative level within a repeated measurement analysis of variance model for the respective parameter at five different time points. CPB = cardiopulmonary bypass.

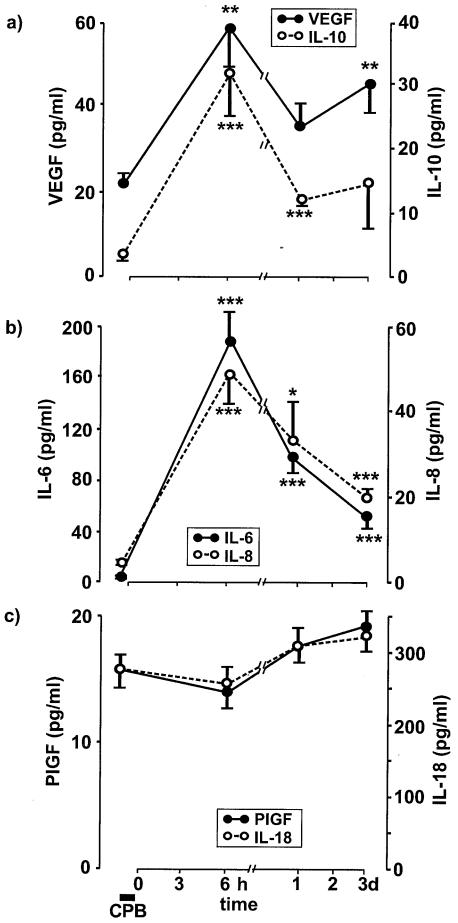

Figure 4. Induction of cytochemokines by coronary artery bypass graft surgery. Reported significances (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) were calculated by Bonferroni-adjusted pairwise comparisons with the preoperative level within a repeated measurement analysis of variance model for the respective parameter at three different time points. IL = interleukin; PlGF = placental growth factor; VEGF = vascular endothelial growth factor.

An apparently similar biphasic response could be observed for plasma levels of VEGF and IL-10 (Fig. 4a). After a first increase 6 h postoperatively (2.6-fold for VEGF and 10.7-fold for IL-10), both plasma levels declined transiently after one day, but the second phase of increase after 3 days did not reach the level of significance relative to the previous values (Fig. 4a). The pro-inflammatory cytokines IL-6 and IL-8 showed a monophasic response, a 50-fold increase for IL-6, and a 10-fold increase for IL-8 after 6 h (Fig. 4b). In addition, the increase of IL-6 after 6 h correlated significantly with the time on CPB ($p = 0.009$). For PlGF and IL-18, postoperative plasma levels did not change significantly relative to preoperative values (Fig. 4c).

In the first phase of the surgery-induced increase in EPC values, the increase in EPCs/lymphocytes showed a negative age dependency ($r = -0.307$, $p = 0.03$). However, the increase in neither EPCs/blood nor EPCs/lymphocytes correlated with any other risk factor, New York Heart Association classification, or operative data. Furthermore, there was no positive correlation of the increases in EPC values with any of the operation-induced increases in plasma cytochemokines.

In the three age groups, the biphasic response of EPCs/lymphocytes and EPCs/blood was similar, but this response remained at significantly higher levels in the younger group compared with the older patients throughout the observation period (Figs. 5a and 5b). In plasma VEGF, the oldest patient group had a retarded response, with significantly lower values 6 h after the end of CPB, compared with younger patients, and significantly lower values throughout the observation period (Fig. 5c).

DISCUSSION

This study demonstrates that the basal number of circulating EPCs in patients with stable CAD is decreased with increasing age. Furthermore, plasma VEGF levels are reduced with increasing age. This age-associated decrease could not be explained by higher prevalences of other risk factors, such as male gender, diabetes mellitus, hypertension, or hyperlipoproteinemia at older ages (Table 2), nor by any differences in left ventricular function or New York Heart Association classes. The operative trauma of complex cardiac surgery with CPB induced a mobilization in EPCs/lymphocytes and EPCs/blood in all patients, but this mobilization remained on a persistently lower level in the older patient group, which could not be explained by any differences in the operative procedure (time on CPB, cross-clamping time,

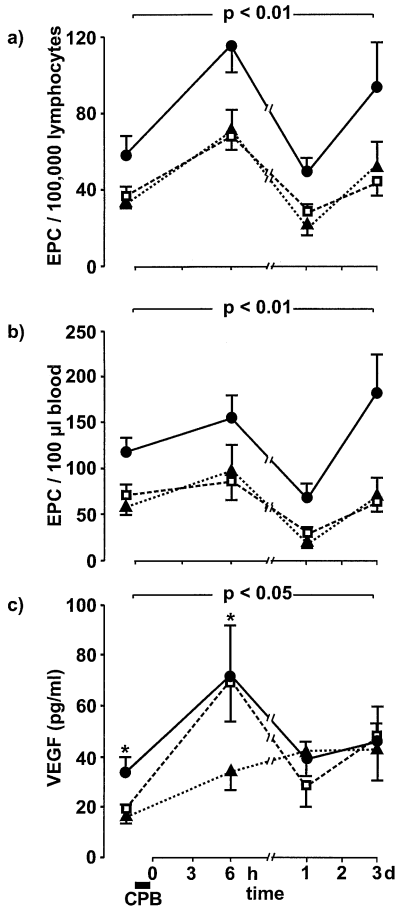

Figure 5. Age dependency of endothelial progenitor cells (EPCs)/lymphocytes **(a)**, EPCs/blood **(b)**, and vascular endothelial growth factor (VEGF) **(c)** after coronary artery bypass graft surgery. The global age effects (EPCs/lymphocytes: $p < 0.01$; EPCs/blood: $p < 0.01$; VEGF: $p < 0.05$) between the two age groups <62 and >69 years were assessed within a repeated measures analysis of variance model also adjusting for time. Reported significances (*$p < 0.05$) for VEGF between the two age groups <62 and >69 years at the time points preoperatively and 6 h postoperatively were calculated by separate (for each time point) analysis of variance models. The three comparisons between the age groups at each time point were Bonferroni-adjusted. **Circles** = <62 years; **squares** = 62 to 69 years; **triangles** = >69 years.

or number of grafts) or in the operation-induced increase in cytochemokines with a reported potency for modulation of angiogenesis (IL-6, IL-8, and IL-10).

To the best of our knowledge, similar age-associated losses in the number of circulating endothelial-related progenitor cells in patients undergoing CABG have not been reported so far. In 45 male subjects without a history of cardiovascular disease, the Framingham risk score and impairment of endothelium-mediated, flow-dependent brachial artery dilation were strong predictors of depressed numbers in circulating progenitor cells with colony-forming capacity (22). In a mixed group of healthy probands and CAD patients, Vasa et al. (23) reported age-associated losses in circulating cells positive for $CD34^+$ and KDR^+, which may include progenitor cells and mobilized ECs (21). In their cohort, smoking was a strong predictor of lowered values in $CD34^+/KDR^+$ cells, independent of age (23). In our patients, self-reported smoking status, which is notoriously unreliable before cardiac surgery, could not be verified by interrogations of spouses or relatives. This may explain why we could not detect an effect of smoking on circulating EPCs.

The reasons for the age-associated losses in circulating EPCs remain unknown at present. In our study, there was an age-independent correlation of circulating EPCs with plasma VEGF levels. Circulating or transplanted EPCs contribute to post-ischemic neovascularization in animal experiments (4,7,24,25) and patients (26,27), and angiogenic factors like VEGF and PlGF are involved in this neovascularization (28–31). In animals, advanced age is associated with attenuated post-ischemic neovascularization and attenuated local induction of VEGF (12,13,32). Similarly, arterial re-endothelialization after vascular trauma is attenuated in old animals in which trauma-induced local VEGF expression is lower and local VEGF supplementation rescues vascular healing (11). Experimental elevation of plasma VEGF in mice by inoculation with adenoviral vectors induced rapid mobilization of endothelial precursor cells (9). Therefore, it is tempting to propose that lowered VEGF levels in our elderly patients are the reason for lowered circulating EPCs. However, this causality remains to be proven for basal steady-state levels, and the cause of depressed circulating VEGF levels in elderly patients remains unknown. In experimental studies, hypoxia-inducible factor-1 stabilization by hypoxia, which mediates hypoxic VEGF expression, is attenuated in cells from old animals (12). This might be relevant for the attenuated and retarded CABG-induced activation of plasma VEGF in older patients (Fig. 5). However, this may be less relevant for the age-associated lowering in basal VEGF levels before surgery.

Local and systemic inflammation by vascular trauma is considered an important contributor of post-ischemic neovascularization (33). In our patients, the operative trauma resulted in a substantial mobilization of cytochemokines with angiogenesis-modulating potential (28,31,34–37), except for IL-18 and PlGF (Fig. 4). These observations are in agreement with previous reports (38–40). Although none of these activated factors could be directly correlated with the individual increase in EPCs during and after the operation in our patients, it is reasonable to assume that the complex spectrum of inflammatory activation is contributing to the mobilization of surgery-induced EPCs. Similar conclusions have been derived from observations on transient mobilization of $KDR^+/AC133^+$ cells in patients after burns or CABG (9). The kinetics of mobilization in that study differed somewhat from our observations, but the two studies are not directly comparable owing to differences in progenitor cell analysis (9). It is remarkable that the substantial inflammatory activation during surgery in our study could not abolish age-associated differences in EPC levels.

The decline in the fraction of lymphocytes/leukocytes after CPB down to one-quarter that of the baseline value at 12 h after CPB (Fig. 3c) most likely reflects substantial

Table 2. Bivariate and Multiple Analyses of Preoperative Levels and Risk Factors

	Bivariate Analysis		Multiple Analysis	
Risk Factors	EPCs/100,000 Lymphocytes	EPCs/100 μl Blood	EPCs/100,000 Lymphocytes	EPCs/100 μl Blood
Age	**0.0012**	**0.0006**	**0.02**	**0.04**
VEGF	**<0.0001**	**0.0012**	**0.005**	0.07
Male gender	0.74	0.97	0.66	0.71
Diabetes mellitus	0.35	0.43	0.17	0.86
Hypertension	0.55	0.80	0.73	0.45
Nicotine abuse	0.28	0.28	**0.007**	0.72
Hyperlipoproteinemia	0.68	0.83	0.27	0.71

Boldface indicates two comparisons between age groups <62 and >69 years, as well as <62 and 62 to 69 years. The influence of risk factors on preoperative values of endothelial progenitor cell (EPC) numbers on bivariate analysis was assessed by linear regression; the simultaneous influence of risk factors on preoperative values of EPC numbers was assessed by multiple linear regression, including all risk factors, as well as age and vascular endothelial growth factor (VEGF), as co-variates. Data are reported as p values.

homing of lymphocytes into tissues in response to the systemic inflammatory activation induced by CPB. Homing must also contribute to the decline of circulating EPCs/blood during this time (Fig. 3d). Therefore, circulating EPC levels underestimate the amount of EPC mobilization. However, quantification of lymphocyte or EPC homing could not be obtained in our patients.

Application of different populations of EPCs or other mononuclear bone marrow cells improves postischemic organ function and microcirculation in animals and patients (16,24–27,41,42). However, it is not clear whether the surgery-induced mobilization of EPCs is sufficient for such a contribution. Furthermore, it is unclear whether the EPCs in elderly patients have the same angiogenic potential compared with those of younger patients. The EPCs collected from the circulation can be amplified in vitro (24,26). The co-application of amplified EPCs, together with native artery recanalization or bypass grafting, probably will develop as a therapeutic option in the future. The experimental data (12,13,32) suggest that such co-therapy is especially desirable in elderly patients. Our data suggest that mobilization of such cells for therapeutic application might be more difficult with an increasing age of patients.

Conclusions. Application of EPCs might expand the tools for therapeutic revascularization and regeneration. However, this potential future strategy seems to be aggravated in aging patients because of lowered numbers of EPCs and lowered VEGF plasma levels. The inflammatory activation by complex CABG does not offset this age-associated lowering. Therefore, further studies are required for a better understanding of optimized strategies for recruitment, ex vivo expansion, and retransplantation strategies involving EPCs in aging patients.

Acknowledgments

The authors express their appreciation for the cooperation of the patients and staff of the Clinic for Cardiothoracic Surgery in Halle (Saale), Germany. They also thank S. Kahrstedt, M. Stiller, and R. Busath for their excellent technical assistance.

Reprint requests and correspondence: Dr. Robert J. Scheubel, Klinik für Herz- und Thoraxchirurgie, Martin-Luther-Universitaet Halle-Wittenberg, Ernst-Grube-Str. 40, D-06097 Halle (Saale), Germany. E-mail: robert.scheubel@medizin.uni-halle.de.

REFERENCES

1. Heusch G, Schulz R. Hibernating myocardium: a review. J Mol Cell Cardiol 1996;28:2359–72.
2. Heusch G, Schulz R. The biology of myocardial hibernation. Trends Cardiovasc Med 2000;10:108–14.
3. Folkman J, Shing Y. Angiogenesis. J Biol Chem 1992;267:10931–4.
4. Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85:221–8.
5. Risau W, Flamme I. Vasculogenesis. Annu Rev Cell Dev Biol 1995;11:73–91.
6. Nissen NN, Polverini PJ, Koch AE, Volin MV, Gamelli RL, DiPietro LA. Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. Am J Pathol 1998;152:1445–52.
7. Takahashi T, Kalka C, Masuda H, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999;5:434–8.
8. Paparella D, Yau TM, Young E. Cardiopulmonary bypass induced inflammation: pathophysiology and treatment: an update. Eur J Cardiothorac Surg 2002;21:232–44.
9. Gill M, Dias S, Hattori K, et al. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)/AC133(+) endothelial precursor cells. Circ Res 2001;88:167–74.
10. Graham MM, Ghali WA, Faris PD, Galbraith PD, Norris CM, Knudtson ML. Survival after coronary revascularization in the elderly. Circulation 2002;105:2378–84.
11. Gennaro G, Menard C, Michaud SE, Rivard A. Age-dependent impairment of reendothelialization after arterial injury: role of vascular endothelial growth factor. Circulation 2003;107:230–3.
12. Rivard A, Berthou-Soulie L, Principe N, et al. Age-dependent defect in vascular endothelial growth factor expression is associated with reduced hypoxia-inducible factor 1 activity. J Biol Chem 2000;275:29643–7.
13. Rivard A, Fabre JE, Silver M, et al. Age-dependent impairment of angiogenesis. Circulation 1999;99:111–20.
14. Quirici N, Soligo D, Caneva L, Servida F, Bossolasco P, Deliliers GL. Differentiation and expansion of endothelial cells from human bone marrow CD133(+) cells. Br J Haematol 2001;115:186–94.
15. Peichev M, Naiyer AJ, Pereira D, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000;95:952–8.

16. Kawamoto A, Tkebuchava T, Yamaguchi J, et al. Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. Circulation 2003; 107:461–8.
17. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964–7.
18. Rafii S. Circulating endothelial precursors: mystery, reality, and promise. J Clin Invest 2000;105:17–9.
19. Nadali G, de Wynter EA, Testa NG. CD34 cell separation: from basic research to clinical applications. Int J Clin Lab Res 1995;25:121–7.
20. Bhatia M. AC133 expression in human stem cells. Leukemia 2001; 15:1685–8.
21. Walter DH, Dimmeler S. Endothelial progenitor cells: regulation and contribution to adult neovascularization. Herz 2002;27:579–88.
22. 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.
23. 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.
24. 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.
25. Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 2001;7:430–6.
26. 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.
27. Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation 2002;106:1913–8.
28. Carmeliet P, Moons L, Luttun A, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 2001;7:575–83.
29. Hattori K, Dias S, Heissig B, et al. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. J Exp Med 2001;193: 1005–14.
30. Hattori K, Heissig B, Wu Y, et al. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. Nat Med 2002;8:841–9.
31. Luttun A, Tjwa M, Moons L, et al. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. Nat Med 2002;8:831–40.
32. Edelberg JM, Tang L, Hattori K, Lyden D, Rafii S. Young adult bone marrow-derived endothelial precursor cells restore aging-impaired cardiac angiogenic function. Circ Res 2002;90:E89–93.
33. Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. Cardiovasc Res 2002;53:31–47.
34. Wei LH, Kuo ML, Chen CA, et al. Interleukin-6 in cervical cancer: The relationship with vascular endothelial growth factor. Gynecol Oncol 2001;82:49–56.
35. Ferrara N. Vascular endothelial growth factor and the regulation of angiogenesis. Recent Prog Horm Res 2000;55:15–35.
36. Garcia-Hernandez ML, Hernandez-Pando R, Gariglio P, Berumen J. Interleukin-10 promotes B16-melanoma growth by inhibition of macrophage functions and induction of tumor and vascular cell proliferation. Immunology 2002;105:231–43.
37. Park CC, Morel JC, Amin MA, Connors MA, Harlow LA, Koch AE. Evidence of IL-18 as a novel angiogenic mediator. J Immunol 2001;167:1644–53.
38. Diegeler A, Doll N, Rauch T, et al. Humoral immune response during coronary artery bypass grafting: a comparison of limited approach, 'off-pump' technique, and conventional cardiopulmonary bypass. Circulation 2000;102 Suppl III:III95–100.
39. Franke A, Lante W, Fackeldey V, et al. Proinflammatory and antiinflammatory cytokines after cardiac operation: different cellular sources at different times (with discussion). Ann Thorac Surg 2002; 74:363–71.
40. Wan S, LeClerc JL, Vincent JL. Cytokine responses to cardiopulmonary bypass: lessons learned from cardiac transplantation. Ann Thorac Surg 1997;63:269–76.
41. Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410:701–5.
42. Kawamoto A, Gwon HC, Iwaguro H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001;103:634–7.