Number and Function of Endothelial Progenitor Cells as a Marker of Severity for Diabetic Vasculopathy

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Objective—Peripheral arterial disease (PAD) is a threatening complication of diabetes. As endothelial progenitor cells (EPCs) are involved in neovasculogenesis and maintenance of vascular homeostasis, their impairment may have a role in the pathogenesis of diabetic vasculopathy. This study aimed to establish whether number and function of EPCs correlate with PAD severity in type 2 diabetic patients.

Methods and Results—EPCs were defined by the expression of CD34, CD133 and KDR, and quantified by flow cytometry in 127 diabetic patients with and without PAD. PAD severity has been assessed as carotid atherosclerosis and clinical stage of leg atherosclerosis obliterans. Diabetic patients with PAD displayed a significant 53% reduction in circulating EPCs versus non-PAD patients, and EPC levels were negatively correlated with the degree of carotid stenosis and the stage of leg claudication. Moreover, the clonogenic and adhesion capacity of cultured EPCs were significantly lower in diabetic patients with PAD versus patients without.

Conclusions—This study demonstrates that EPC decrease is related to PAD severity and that EPC function is altered in diabetic subjects with PAD, strengthening the pathogenetic role of EPC dysregulation in diabetic vasculopathy. EPC count may be considered a novel biological marker of peripheral atherosclerosis in diabetes. **(*Arterioscler Thromb Vasc Biol*. 2006;26:2140-2146.)**

Key Words: stem cells ■ diabetes ■ atherosclerosis ■ endothelium

Diabetic vasculopathy is characterized by high prevalence, early development and rapid progression. Peripheral arterial disease (PAD) is indeed a striking source of morbidity and disability in diabetic subjects and is one leading cause of nontraumatic amputations in western countries.[1] Mechanisms accounting for the aggressiveness of diabetic vasculopathy are poorly understood but may depend on a profoundly impaired collateral vascular development.[2]

Endothelial progenitor cells (EPCs) are a subtype of bone marrow–derived progenitor cells expressing surface antigens of both hematopoietic stem cells and endothelial cells[3]: they are involved in adult neovasculogenesis and maintainance of vascular integrity.[4,5] A growing amount of data suggest that EPCs are altered in clinical conditions characterized by high cardiovascular risk, including type 1 and type 2 diabetes.[6–8] Furthermore, we have demonstrated that circulating EPCs are decreased in subjects with PAD, especially in the presence of diabetes.[9] Therefore, EPC reduction has been hailed as a novel concept in the pathogenesis of diabetic vascular complications. Additionally, recent data have shown that levels of circulating EPC predict cardiovascular events in patients with coronary artery disease.[10,11] However, it is not definitely clear whether EPC alterations have a pathogenic role in the development of cardiovascular disease or simply represent its epiphenomenon. Meanwhile, it has been suggested that EPCs may constitute a novel prototype of cardiovascular biomarkers.[12] Again, strong correlations between EPC alterations and disease severity are still lacking. In fact, if dysfunction of progenitor cells had a causative role in vascular disease, one would expect that patients with more severe disease had more profound EPC decrease or dysfunction.

With this background, we aimed to assess the relationships between number and function of EPCs and the severity of atherosclerotic disease in type 2 diabetic patients.

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Materials and Methods

Patients

The study was approved from the local ethics committee and informed consent was obtained from all subjects. A total of 127 type 2 diabetic subjects, recruited from our Metabolic outpatient clinic and divided into diabetic patients with (n=72) and without (n=55) PAD were included. All patients were screened for other classic risk factors for atherosclerosis: smoking, arterial hypertension, obesity and dyslipidemia. Patients underwent metabolic evaluation including fasting glucose, glycohemoglobin, and lipid profile. Atherosclerosis of carotid vessels was assessed by ultrasonography according to the Society of Radiologists in Ultrasound Consensus Conference.[13] The percentage of vessel obstruction was measured first as a continuous variable, and then classified as stenosis of up to 30%, stenosis of 31% to 50%, stenosis of 51% to 70%, or stenosis of 71% to 100%. Diagnosis of atherosclerotic involvement of the lower extremities was assessed noninvasively, and patients were classified according to the Leriche/Fontaine clinical classification of lower-limb atherosclerosis obliterans and according to the revised version of the recommended standards for reports dealing with lower extremity ischemia.[14] The presence of coronary artery disease (CAD) and diabetic retinopathy were also assessed. For more details on patient selection criteria and evaluation please see the supplemental data, available online at http://atvb.ahajournals.org.

Quantification of Peripheral Blood Progenitor Cells by Flow Cytometry

Peripheral blood progenitor cells were analyzed for the expression of surface antigens with direct 2- or 3-color flow cytometry (fluorescence-activated cell sorter; Calibur, Becton Dickinson Biosciences) as previously reported.[15,16] Progenitor cells were defined by the surface expression of CD34, CD133 and KDR (supplemental Figure I, available online at http://atvb.ahajournals.org). Expression of CD34 and KDR was studied in all subjects, whereas the complete assay, including assessment of CD133 expression, was performed in a subset of 55 (43% of total). For more details on flow cytometry, please see supplemental data.

Cell Culture

EPC isolation and culture were performed as previously described.[17,18] Briefly, peripheral blood mononuclear cells were plated on fibronectin-coated dishes (Becton Dickinson Biosciences) and grown in supplemented endothelial cell growth medium (Clonetics) for 15 days. Clusters of attaching cells were counted every 3 days starting from day 3. For more details on the culture methods, please see supplemental data.

Characterization of Cultured EPCs

At the end of the growth curve, cultured cells were characterized to confirm their endothelial phenotype in 6 randomly selected subjects. Cells were stained with DiI-acetylated low-density lipoproteins (Molecular Probes) and fluorescein isothiocyanate-lectin (Sigma-Aldrich).[19] For further characterization we stained putative EPCs with the endothelial markers von Willebrand factor (vWf), KDR and CD31. To have further methodological confirmation of the endothelial phenotype, cultured EPCs were detached using EDTA and analyzed by flow cytometry for the expression of KDR, CD31, CXCR4 and CD18. For more details on EPC characterization, please see the supplemental data.

Adhesion Assay

Besides studying the clonogenic capacity of patients' EPCs, we also evaluated in vitro the ability of DiI-LDL-labeled EPCs to adhere to a human umbilical vein endothelial cell monolayer, as previously described.[8] For more details on this procedure, please see supplemental data.

In Vivo Functional Characterization of Cultured EPCs

To confirm that isolated cells functionally correspond to EPCs and take part in new vessel growth, CMTMR (5-(and-6)-(4-chloromethyl-benzoyl-amino)-tetramethylrhodamine)–labeled cells were injected into rat ischemic hindlimbs. Muscle capillaries were stained with vWf and nuclei counterstained with Hoescht. Elongated cellular structures double positive for vWf and CMTMR were considered EPC-bearing neovessels. For more details on this procedure, please see supplemental data.

Statistical Analysis

Data are expressed as mean±SEM. All results from flow cytometry are expressed as number of cells/10^6 cytometric events. Differences between 2 or more groups were analyzed using 2-tailed Student *t* test and ANOVA, respectively. The Hochberg procedure was applied to account for α-inflation attributable to multiple testing. The χ^2 test was used for dichotomous variables. Simple linear regression analysis (Pearson *r*) was used to assess correlations between severity of PAD and progenitor cell counts. Spearman ρ was also calculated to assess correlation of progenitor cell numbers with severity of lower extremity atherosclerosis obliterans. Statistical associations between progenitor cell counts and clinical conditions or risk factors were examined by multivariate analysis using multiple linear regression. Statistical association between risk factors or clinical conditions and the presence of PAD was assessed by multiple stepwise logistic regression analysis. The discriminatory capacity of EPC count for the presence of PAD was investigated using the receiver operating characteristic curve: cut-off values were obtained by optimizing the sum of sensitivity and specificity. Statistical significance was accepted at $P \leq 0.05$.

Results

Patients' Characteristics

Subject characteristics are resumed in the Table. As expected, PAD patients had also a higher prevalence of CAD than non-PAD patients.

Characterization of Circulating EPCs by Flow Cytometry

Flow cytometry was used to identify and quantify peripheral blood $CD34^+$ cells and $CD34^+KDR^+$ cells (supplemental Figure IA through IF). On average, 23% of circulating $CD34^+$ cells were KDR^+. The expression of VEGF-R2 (KDR) on $CD34^+$ cell surface has been related to the endothelial differentiation of generic progenitors. In a subset of 55 subjects (43.3% of total) we also determined the expression of CD133, so that 3 subpopulations can be identified as representative of the total circulating EPC pool: $CD34^+CD133^-KDR^+$, $CD34^-CD133^+KDR^+$ and $CD34^+CD133^+KDR^+$ cells (supplemental Figure IG and IH). We show that total $CD34^+KDR^+$ cells are the main constituent of this pool (88.0%), and their level is much more strictly correlated to the total EPC pool (defined as $[CD34^+]$ or $[CD133^+]$ and $[KDR^+]$ cell count) than $CD133^+KDR^+$ and $CD34^+CD133^+KDR^+$ cells ($r=0.98$; 0.46 and 0.55, respectively).

Circulating EPCs Are Reduced in the Presence of PAD

Diabetic patients with either carotid or lower extremity atherosclerosis had a mean 53% reduced level of circulating $CD34^+KDR^+$ cells when compared with diabetic subjects free from PAD (40.9±2.9 versus 87.4±6.9; $P<0.001$). There was also a significant 22% reduction in $CD34^+$ cells and a

Patient Characteristics

Characteristic	Diabetic Patients With PAD	Diabetic Patients Without PAD	*P* Value
	Mean±SEM	Mean±SEM	
No.	72	55	
Age, y	68.8±0.9	68.0±1.6	0.652
Sex M, %	72.6	56.3	0.056
Body mass index, kg/m^2	27.7±0.6	29.3±0.8	0.097
Waist circumference, cm	100.6±1.5	101.2±1.7	0.597
Laboratory testing			
Fasting glucose, mg/dl	194.5±9.8	180.2±11.2	0.339
HbA1c, %	8.4±0.25	8.5±0.37	0.768
Total cholesterol, mg/dl	184.5±5.2	186.6±5.8	0.789
LDL-C, mg/dl	116.9±4.5	112.6±4.8	0.528
HDL-C, mg/dl	45.6±1.8	47.2±1.6	0.525
Triglycerides, mg/dl	135.8±9.6	153.8±13.8	0.271
Clinical History	%	%	
Arterial hypertension	49.3	58.2	0.324
Smoke	22.5	13.2	0.189
Family history	35.2	33.9	0.886
CAD	39.7	14.5	0.002
PAD	100.0	0	...
Carotid, stenosis ≥30%	87.5	0	...
Lower extremity, stage ≥1	77.8	0	...
Diabetic retinopathy	26	18.2	0.298
Medications			
Insulin	45.8	36.4	0.287
Oral Antidiabetics	20.8	27.3	0.394
Metformin	12.5	20.0	0.254
Statins	30.6	25.5	0.531
ACE-Inhibitors/ARBs	63.9	56.4	0.394
Other hypotensive drugs	73.6	40.0	<0.001
Antiaggregants	83.3	70.9	0.094

ACE indicates angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.

41% reduction in CD133$^+$ cells, as determined in a subset of 29 patients with and 26 patients without PAD (Figure 1A). To quantify the discriminatory capacity of progenitor cell count in identifying PAD, we built up a receiver operating characteristic curve, which revealed higher accuracy and sensitivity for CD34$^+$KDR$^+$ than for CD34$^+$ cell count. (Figure 1B).

In order to establish whether risk factors or clinical conditions other than PAD might have explained in part the reduced CD34$^+$KDR$^+$ and CD34$^+$ cell counts, a multiple linear regression analysis was performed: among all clinical conditions, only PAD, but not CAD, was independently associated with CD34$^+$KDR$^+$ cell reduction, whereas among the classic risk factors, increasing age was statistically associated with decreasing CD34$^+$KDR$^+$ cells. On the other hand, retinopathy and male gender were negatively correlated with CD34$^+$ cell count (supplemental Figure II and supplemental Table I, available online at http://atvb.ahajournals.org). Statistical analyses revealed that medications had no influence

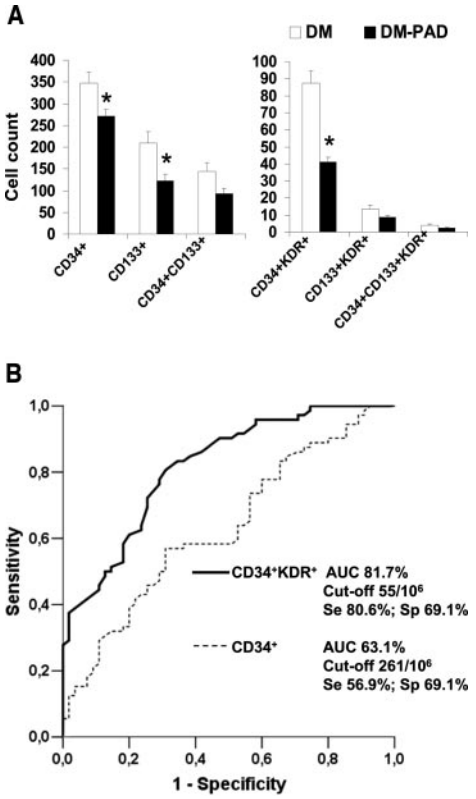

Figure 1. A, Levels of the 6 subpopulations of circulating progenitor cells in patients with and without PAD. *Statistically significant after α-adjustment. B, receiver operating characteristic curve analysis to quantify the discriminatory capacity of CD34$^+$and CD34$^+$KDR$^+$ cell counts in identifying PAD.

on progenitor cell counts (not shown). Moreover, in a multiple stepwise logistic regression analysis, CD34$^+$KDR$^+$ cell count, age and male gender were independently associated with the presence of PAD (supplemental Table II, available online at http://atvb.ahajournals.org).

The Amount of Circulating CD34$^+$KDR$^+$ EPCs Is Negatively Correlated With Severity of Carotid and Lower Extremity Atherosclerosis

CD34$^+$KDR$^+$ cell count was strongly negatively correlated with the extent of carotid atherosclerosis ($r=-0.51$) and with the clinical severity of lower extremity atherosclerosis according to both Leriche/Fontain ($\rho=-0.63$) and Rutherford ($\rho=-0.64$) classifications (Figure 2A and 2B). Remarkably, those correlations remained significant even after adjustment for age, gender, duration of diabetes and HbA1c (supplemental Table III, available online at http://atvb.ahajournals.org). Even if many patients (n=37) had both forms of PAD, carotid and lower extremity atherosclerosis were simultaneously and independently negatively correlated with CD34$^+$KDR$^+$ cells (not shown). However, the presence of both carotid and lower extremity atherosclerosis was not synergically characterized by further CD34$^+$KDR$^+$ cell reduction (Figure 2C).

The correlations between CD34$^+$ cell count and indicators of PAD severity were always weaker than CD34$^+$KDR$^+$ cells and were no more significant after statistically adjustment.

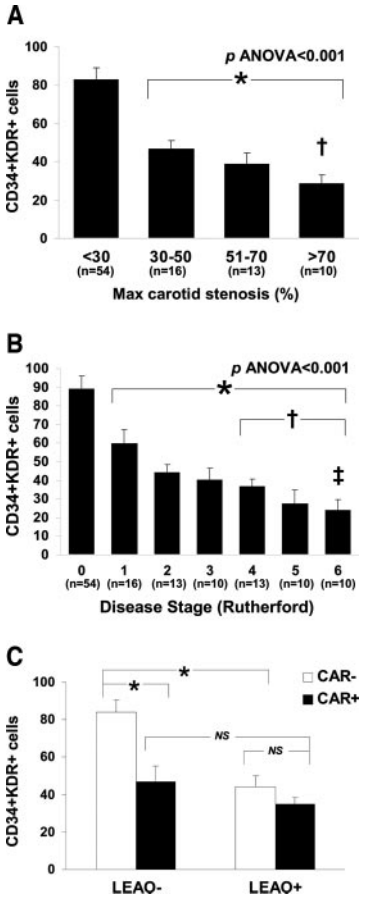

Figure 2. A, Levels of $CD34^+KDR^+$ EPCs in patients divided according to the extent of carotid atherosclerosis. *Statistically significant when compared with the <30% group; †statistically significant when compared with the 30% to 50% group. B, Levels of $CD34^+KDR^+$ EPCs in patients at increasing severity of lower extremity atherosclerosis obliterans according to the Rutherford classification. *Statistically significant when compared with stage 0; †statistically significant when compared with stage 1; ‡statistically significant when compared with stage 2. C, Levels of $CD34^+KDR^+$ EPCs in the presence or absence of >30% carotid stenosis and ≥1 stage leg atherosclerosis. *Statistically significant after α-adjustment. CAR indicates carotid atherosclerosis; LEAO, lower extremity atherosclerosis obliterans.

EPCs Cultured From PAD Patients Display Reduced Clonogenic and Adhesion Capacity

We isolated EPCs using a validated culture method.[18] During growth in endothelial medium, a subset of peripheral blood mononuclear cells form colonies of endothelial cells and after 2 weeks display function and phenotype of endothelial cells: >90% of survived cells bind lectin, uptake LDL and are positive for the surface expression of vWf, CD31 and KDR. In support to these data, flow cytometry analysis showed that 93.5±7.8% of cultured EPCs were KDR^+ and 94.8±4.3 were $CD31^+$. In addition, we show that isolated EPCs were positive for CXCR4 (SDF-1α receptor) and CD18 (intercellular adhesion molecule receptor), which have been shown to be important for EPC function (Figure 3). Finally, we tested in vivo the ability of EPCs to take part in new vessel formation, and show their presence in vascular structures of muscles subjected to IR injury (supplemental Figure III, available online at http://atvb.ahajournals.org), whereas no injected cell was recognized in contralateral nonischemic muscles.

To have a functional demonstration of EPC alterations in vascular complications of type 2 diabetic subjects, we cultured EPCs from 15 patients with and 15 patients without PAD, comparable for age, sex and concomitant risk factors. We show that, from day 3 to 15, peripheral blood from PAD patients gave rise to a lower number of cell clusters than patients without PAD. Specifically, area under curve, which may represent the clonogenic capacity, and cell clusters after 15 days of culture, which represent true outgrown EPCs, were lower in patients with PAD than in patients without PAD (Figure 4A and 4B). Then, we studied the adhesion property of patients' EPCs to a human umbilical vein endothelial cell monolayer and show that EPCs from PAD patients have a 35% reduced capacity to adhere to mature endothelial cells than EPCs from patients without PAD (Figure 4C through 4E). The number of adherent EPCs/high power field was significantly correlated to the number of cell clusters at day 3 (r=0.41; P=0.01).

Discussion

Diabetes mellitus is characterized by a widespread endothelial dysfunction[20] and a 2- to 3-fold increased risk of developing cardiovascular diseases. As previously highlighted by our group, circulating blood cells in diabetes are subjected to many biochemical alterations attributable to the high oxidative stress and the unfavorable vascular environment.[21,22]

The present study demonstrates that number and function of circulating EPCs are profoundly altered in type 2 diabetic patients with PAD compared with diabetic patients without PAD. Moreover, we show strong correlations between circulating EPC levels and the severity of carotid and lower extremity arterial disease.

In this work we have used 2 independent methods to study endothelial progenitors: flow cytometry of fresh blood and ex vivo culture. Flow cytometry is considered the gold standard for quantitative enumeration of EPC, being sensitive, precise and reproducible[23]: using this technique, we have defined EPCs by the surface expression of at least 1 marker of stemness and immaturity (eg, CD34 or CD133) plus the expression of the endothelial marker VEGFR2 (KDR). With the use of 3 surface antigens we have identified 3 subpopulations of undifferentiated progenitor cells ($CD34^+$, $CD133^+$ and $CD34^+CD133^+$ cells) and the 3 corresponding subpopulations of endothelial-committed progenitors ($CD34^+KDR^+$, $CD133^+KDR^+$ and $CD34^+CD133^+KDR^+$ cells) which form the circulating EPC pool. In our study sample, there was a net trend toward decrease of all progenitor cell subpopulations in PAD versus non-PAD patients, but statistical significance was reached only for $CD34^+$, $CD133^+$ and $CD34^+KDR^+$ cell counts after adjusting for multiple testing. This result is consistent with the demonstration that $CD34^+KDR^+$ cell count predicted cardiovascular events in coronary patients.[10,11] Moreover, total $CD34^+KDR^+$ cells are the major (88%) constituent of the circulating EPC pool, whereas $CD133^+KDR^+$ cells are more immature cells, such those

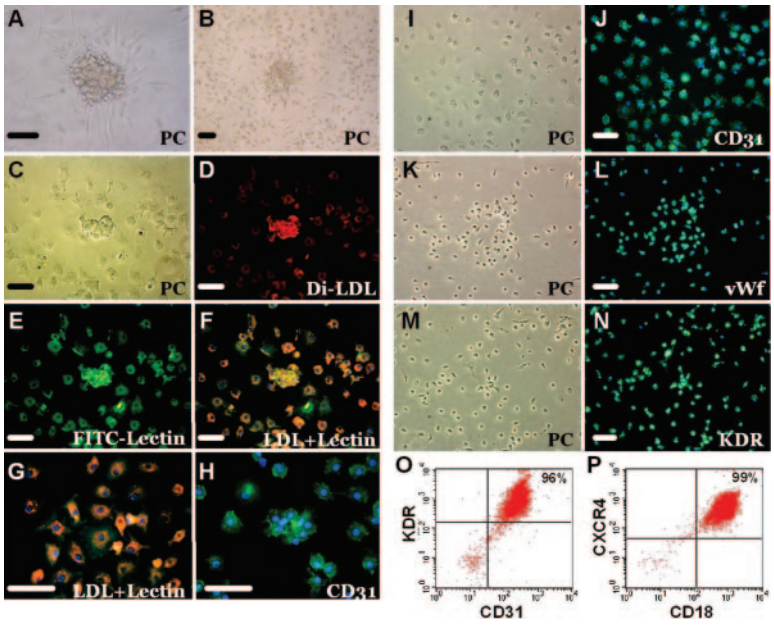

Figure 3. Phase contrast (PC) and fluorescent microscopic photos of representative EPC clusters (A and B), fluorescent staining for DiI-LDL (red) and fluorescein isothiocyanate-lectin (green; C through F, same experiment; and G). PC and corresponding immunofluorescent staining for CD31 (H through J) vWf (K and L) and KDR (M and N). Representative flow cytometry analysis of cultured EPCs for the expression of CD31, KDR (O), CXCR4 and CD18 (P). Bar=50 μm.

recently mobilized from bone marrow,[24] and are much rarer in peripheral blood (12%) in steady-state conditions (ie, without acute bone marrow stimulation). An obvious limitation is that CD133 expression was determined in a subset of patients, thus limiting statistical power. Nonetheless, we would suggest that $CD34^+KDR^+$ is the most appropriate phenotype to identify EPCs, because those cells are more strictly linked to cardiovascular damage, at least in diabetes.

Different from flow cytometry, EPC culture is not fully standardized, as many protocols have been proposed.[19] Moreover, the resulting cell population has been shown to be spurious and its phenotype not entirely defined.[25] In this work, we have used a prolonged culture system to allow for positive selection of the so-called "late outgrowing" EPCs.[18] Putative EPCs have been extensively characterized: >90% of cells have shown to be positive for lectin binding and LDL uptake and for the expression of the endothelial antigens CD31, vWf, and KDR. Remarkably, cultured EPCs were also positive for CXCR4 and CD18 antigens, which have been shown to be fundamental for cell trafficking and recruitment.[26,27] Finally, we have demonstrated that isolated cells are incorporated into vascular structures of rat ischemic hindlimbs. Taken together, these data indicate a high quality of the culture method used to isolate EPCs which allows for showing a 38% decrease in EPC clusters from patients with versus patients without PAD, confirming our flow cytometry data.

Experimental studies have shown that EPCs constitute a circulating pool of cells able to form a patch that actively repairs the denuded or dysfunctional endothelium.[28] Notably, EPC number has been negatively correlated to parameters of endothelial function in humans.[29] As endothelial damage is considered the first step in the development of the atherosclerotic plaque, EPC depression can be causally linked to the atherogenetic process. On the other side, EPCs are also actively recruited at sites of new vessel growth and take part in compensatory angiogenesis in ischemic tissues.[4] The ability to form collaterals represents a crucial response to vascular occlusive diseases, because it determines the severity of residual ischemia, and whether clinical manifestations of atherosclerosis will develop.

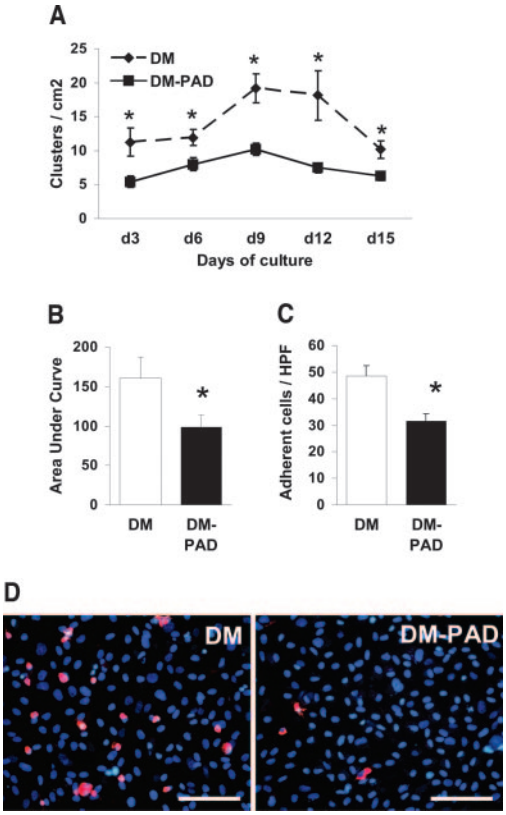

Figure 4. A and B, Number of clusters during 15 day-culture of EPCs from PAD and non-PAD patients and the corresponding area under growth curve. C and D, Representative EPC adhesion assay from a patient without (C) and with (D): EPCs are red stained with DiI-LDL and nuclei of human umbilical vein endothelial cells are stained in blue (bar=100 μm). E, Quantitative evaluation of EPC adhesion in PAD and non-PAD patients.

Consequently, depletion of circulating EPCs may contribute to both endothelial dysfunction, as an early event in the atherogenetic process, and to poor collateralization, as a late event leading to the clinical manifestations of atherosclerosis and cardiovascular disease progression. In this study, carotid stenosis and the stage of lower extremity arterial disease have been considered, respectively, as representative of the anatomic atherosclerotic burden and of the late clinical outcome of local atherosclerosis obliterans.

We report that, in both conditions, $CD34^+KDR^+$ cell count was closely negatively correlated to disease severity: higher degrees of carotid stenosis, as well as worse stages of leg claudication and ischemic lesions were associated with lower levels of $CD34^+KDR^+$ EPCs. Moreover, we have demonstrated that EPCs from PAD patients display a significantly impaired adhesion to mature endothelium, which is a fundamental step for EPC function in both maintenance of endothelial homeostasis and angiogenic processes. Interestingly, in the culture experiments, PAD patients had a lower number of cell clusters as early as day 3, suggesting also impairment in adhesion to extracellular matrix, such as the fibronectin coating culture dishes.

Taken together, these data provide strong evidence for a possible role of EPC reduction in the pathogenesis of diabetic vasculopathy. Although our study was cross-sectional and does not establish cause-effect relationships, we would like to suggest that impaired collateralization leading to the clinical manifestations and complications of atherosclerosis in diabetes may be attributable to decreased and dysfunctional EPCs. Concurrently, increased carotid plaque formation may be related to the depleted reservoir of EPCs, which, also attributable to the reduced adhesiveness to the vessel wall, fail to replace successfully the damaged endothelium. In this light, ways to increase number and improve function of EPCs should be actively pursued as part of the therapeutical armamentarium against diabetic atherosclerotic complications.

It should be noted that many surrogate markers of atherosclerosis have been proposed so far, but most of them, including C-reactive protein, did not show a good correlation with the extent of the atherosclerotic burden.[30] Therefore, EPC count may be proposed as a novel biomarker of diabetic atherosclerotic complications. In agreement with this hypothesis we show that PAD was independently correlated with $CD34^+KDR^+$ cell count and that, conversely, $CD34^+KDR^+$ cell count was independently associated with PAD presence. Consistently, $CD34^+KDR^+$ cell count displayed a high performance in detecting PAD among the entire population of type 2 diabetic subjects.

The present study was designed to explore specifically peripheral rather than coronary atherosclerosis. Not all patients underwent coronary angiography and CAD was defined mainly by clinical and noninvasive instrumental criteria. Therefore, we cannot exclude that some patients classified in the control group had at least some extent of CAD. This may suggest that the difference in EPC levels in patients with versus patients without macrovascular complications is underestimated. However, CAD was associated with a mild reduction in $CD34^+KDR^+$ EPCs ($P=0.03$, not significant after α-adjustment). It should be noted that the literature provides inconsistent data on EPC reduction in stable CAD: in one study,[31] the CAD group included patients with acute coronary syndromes, that are known to be followed by EPC increase; in another study,[32] $CD34^+CD133^+$ cells were not reduced in patients with chronic myocardial ischemia compared with healthy subjects. Moreover, some diabetic patients may display clinical and noninvasive instrumental features of chronic myocardial ischemia in the absence of severe atherosclerotic involvement of epicardial vessels. Indeed, heart disease in diabetes is attributable not only to coronary atherosclerosis, but also to a wide range of biochemical, metabolic and structural microvascular alterations,[33] whereas PAD is the direct consequence of accelerated atherosclerosis in diabetic subjects. These reasons may explain why PAD was more closely associated to $CD34^+KDR^+$ EPC reduction than CAD in this study.

In summary, this study solidly indicates that diabetic vasculopathy is associated with EPC impairment: depletion of the EPC pool and defective adhesive capacity is probably one cause of the aggressive cardiovascular disease in these subjects. Moreover, the strong correlations between EPC reduction and the degree of peripheral atherosclerosis reveal that $CD34^+KDR^+$ EPC count represents a novel prototype of cardiovascular biomarkers and suggest a pathogenetic model by which diabetic vasculopathy worsens as EPCs decrease. We should consider EPC alterations as a therapeutic target for diabetic macroangiopathy.

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Disclosures

None.

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Number and Function of Endothelial Progenitor Cells as a Marker of Severity for Diabetic Vasculopathy

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Fadini et al. Endothelial progenitors in diabetic vasculopathy.

ONLINE DATA SUPPLEMENT

The present data supplement contains the followings:

- Detailed description of materials and methods.
- Tables I, II, III
- Figure I, II, III

Reference citations in this supplement refer to the same references included in the main manuscript.

MATERIALS AND METHODS

Patients. The study was approved from the local ethics committee and informed consent was obtained from all subjects. Type 2 diabetic subjects were recruited from our Metabolic outpatient clinic. The inclusion criterion for the study group was the presence of PAD, meant as carotid stenosis or lower extremity atherosclerosis obliterans. The control group was made up of diabetic subjects free from PAD that were roughly comparable for age, gender and clinical history with patients selected for the presence of PAD. Predefined exclusion criteria were patient's refusal, acute illness or infection, neoplasm, age over eighty, recent surgery or vascular intervention, recent myocardial infarction, unstable angina, hemodialysis, immunosuppression and immunological diseases. According to these criteria, the study involved a total of 127 subjects, divided into diabetic patients with (n=72) and without (n=55) PAD.

All patients were screened for other classical risk factors for atherosclerosis: smoking status, defined as habitual daily use of one or more sigarettes; arterial hypertension, defined by a systolic blood pressure of more than 140 mmHg or a diastolic blood pressure of more than 90 mmHg, or both, on at least two occasions, or by the use of anti-hypertensive drugs; obesity, defined by a body mass index of more than 30 kg/m^2 or by a waist circumference above 102 cm (for men) and 88 cm (for women); dyslipidemia, defined as LDL cholesterol level exceeding 130 mg/dl or triglyceride concentration exceeding 150 mg/dl or low HDL (<50 mg/dl for women and <40 mg/dl for men) or by the use of lipid-lowering drugs. Patients underwent metabolic evaluation including fasting glucose, HbA1c, and lipid profile.

Atherosclerosis of carotid vessels was assessed by bilateral carotid artery ultrasonography, performed by a trained operator who was unaware of the patient's clinical status. Common and internal carotid arteries were evaluated with gray-scale, color Doppler

and spectral Doppler ultrasound in a standardized fashion, according to the Society of Radiologists in Ultrasound Consensus Conference.[13] Briefly, after sagittal and transverse B-mode imaging to assess plaque distribution and morphology, midstream spectral waveforms were obtained at multiple sites along the common and internal carotid arteries. Angle of insonation was maintained less than or equal to 60 degrees and care was taken to position the sample volume within the area of greatest stenosis and to visualize the end of the stenosis; a HDI-5000 SONO CT ultrasound machine (Philips Medical System/ATL S.p.a.) equipped with a Phased array 4-7 MHz transducer was used to perform all tests. Some patients underwent also vascular study by angiography (n=9) or magnetic resonance imaging (n=6) for surgical purposes, which resulted in concordant information, providing internal validation and quality assessment. The percentage of vessel obstruction was measured first as a continuous variable, and then classified as stenosis of up to 30 percent, stenosis of 31 to 50 percent, stenosis of 51 to 70 percent, or stenosis of 71 to 100 percent. When more than one plaque was found, the greatest degree of obstruction was recorded.

Diagnosis of atherosclerotic involvement of the lower extremities was assessed by history of claudication or rest pain, bilateral pulses examination (dorsal pedal, posterior tibial, popliteal, and femoral arteries), ultrasonography performed bilaterally at levels of popliteal and femoral arteries, and, eventually, angiography (n=12). Patients were then classified according to the Leriche/Fontaine clinical classification of lower limb atherosclerosis obliterans and according to the revised version of the recommended standards for reports dealing with lower extremity ischemia.[14]

Patients were considered to have also coronary artery disease in the presence of unambiguous findings suggestive of past myocardial infarction on a resting 12-lead standard electrocardiogram, or a positive ECG at exercise stress-test, or an echocardiography stress-

test positive for inducible ischemia, or evidence of a significant coronary artery stenosis on a coronary angiography, with or without typical chest pain.

The presence of diabetic retinopathy was assessed by an ophthalmologic visit.

All data were collected before the determination of EPCs in peripheral blood.

Quantification of peripheral blood progenitor cells by flow cytometry. Fasting blood samples were processed after one to two hours. Peripheral blood progenitor cells were analyzed for the expression of surface antigens with direct two- or three- color flow cytometry (FACS Calibur, BD Biosciences, Franklin Lakes, NJ) as previously reported.[15,16] Briefly, before staining with specific monoclonal antibodies, cells were treated with fetal calf serum for 10 min, then the samples were washed with buffer containing phosphate-buffered saline and 0.5 percent bovine albumin. Then, 150 µl of peripheral blood were stained with 10 µl of FITC-conjugated anti-human CD34 mAb (BD), 10 µl of PE-conjugated anti-human KDR mAb (R&D Systems, Minneapolis, MN) and 10 µl of APC-conjugated anti-CD133 mAb (Miltenyi Biotech GmbH, Bergisch Gladbach,Germany). Control isotype IgG1 and IgG2a Abs were obtained from BD. The frequency of peripheral blood cells positive for the above reagents was determined by a 2D side scatter-fluorescence dot plot analysis, after appropriate gating, stained with the different reagents (figure I). We gated $CD34^+$ or $CD133^+$ peripheral blood cells in the mononuclear cell fraction and then examined the resulting population for the dual expression of KDR. At the intersection of the CD34 and CD133 gates we identified $CD34^+CD133^+$ cells, while total KDR^+ mononuclear cells were identified separately as cells with high KDR expression and low side scatter. Triple positive cells were identified by the dual expression of KDR and CD34 or CD133 in the CD133 or CD34 gates, respectively. In all subjects expression of CD34 and KDR was studied, while the complete assay, including assessment of CD133 expression, was performed in a subset of 55 (43% of

total, 29 with PAD and 26 without PAD). For FACS analysis, $5x10^5$ cells were acquired and scored using a FACS Calibur analyzer (BD). Data were processed using the Macintosh CELLQuest software program (BD). The instrument set-up was optimized daily by analyzing the expression of peripheral blood lymphocytes labeled with anti-CD4 FITC/CD8 PE/CD3 PECy5/CD45 APC four color combination. The same trained operator, who was blind to the patients' clinical status, performed all the tests throughout the study.

Cell culture. EPC isolation and culture were performed as previously described.[17,18] Briefly, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient (Sigma-Aldrich, St. Louis, MO) and cells were plated on six-well fibronectin-coated dishes (BD) at a density of $6x10^6$ cells per well. Cells were grown in supplemented endothelial cell growth medium (Clonetics, Baltimore, MD) for up to 15 days. Culture medium was changed first on day 4 and then every day until phenotypical characterization was performed. Attaching cells rapidly assume an endothelial-like shape and, starting from day 3 to 6 of culture, cells proliferate in clusters made up of a central core of rounded cells surrounded by radiating spindle-shaped cells. Clusters were counted every three days from day 3 to day 15 of culture in randomly selected microscopic fields by two independent operators who were blind to the patient status. According to previously reported kinetics of cultured endothelial progenitor cells, clusters reached a peak density between 9 and 12 days after plating and then tended to dissolve. It is currently agreed that cultures prolonged for 2 weeks allow positive selection of true endothelial progenitor cells, that should represent the only cell type remaining two weeks after plating.

Characterization of cultured EPCs. At the end of the growth curve, cultured cells were characterized to confirm their endothelial phenotype. There is general agreement that

EPCs display key functional properties of endothelial cells, such as LDL uptake and lectin binding.[19] Therefore, after washing with PBS, cells were incubated at 37°C with 0.5 μg/ml DiI-acetylated low density lipoproteins (DiI-AcLDL, Molecular Probes, Eugene, OR) for 1 hour, followed by dark incubation with 15 mg/ml FITC-conjugated Ulex lectin (Sigma-Aldrich) for 2 hours. Nuclei were stained in blue with Hoechst 3358 (Sigma-Aldrich). EPCs were defined as cells double positive for AcLDL and lectin. As also cultured monocyte/macrophage may share some features of endothelial cells and may uptake LDL, for further characterization we stained putative EPCs with other endothelial markers. Cells were fixed and incubated with anti-von Willebrand Factor (vWF) (Dako Cytomation, Glostrup, Denmark), anti-KDR (also known as VEGFR-2) (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-CD31 (Chemicon International) antibodies and secondary Cy2 anti-rabbit (Chemicon International, Temecula, CA) and TRITC-anti-mouse (Dako Cytomation) antibodies. Positive cells were then visualized under a fluorescent microscope. To have further methodological confirmation of endothelial phenotype, cultured EPCs were detached using EDTA and analyzed by flow cytometry for the expression of KDR (R&D Systems), CD31, CXCR4 and CD18 (BD).

Adhesion assay. We evaluated *in vitro* the EPC functional property to adhere to mature endothelial cells, as previously described.[8] For this purpose, a monolayer of human umbilical vein endothelial cells (HUVECs) was prepared 48 hours before the assay by plating 1×10^5 cells/cm^2 (passage 5 to 8). EPCs were labeled with DiI-LDL as above described and 1×10^5 cells were added to each well and incubated for 2 hours at 37°C. Nonattached cells were gently removed with PBS, and adherent EPCs were fixed with 4% *p*-formaldehyde and counted in 10 random fields.

In vivo functional characterization of cultured EPCs. To provide definite evidence that isolated cells functionally correspond to EPCs and take part in new vessel growth, we employed a model of rat ischemia-reperfusion (IR) injury. Briefly, male Sprangue-Dawley rats (n=3) (Charles River, Wilmington, MA) underwent a 2-hour hind limb IR injury as follows. Animals were anesthetized by intramuscular injection of 700 μg/kg tilethamine hydrochloride-zolazepam hydrochloride mixture (Virbac, Peakhurst, Australia) plus 150 μg/kg subcutaneous xylazine (BIO 98, Bologna, Italy). Distal limb perfusion was monitored with a laser Doppler flowmeter (Periflow, Perimed Italy). A 9 mm-large digit cuff (Perimed, Italy) was placed around the thigh and connected to a standard manometer. The cuff was inflated with air until the laser Doppler recorded biological zero flow and the pressure on the manometer was above 200 mmHg. Perfusion and pressure in the cuff were monitored during the experiment to assure continuous and stable ischemia: additional cuff inflations were performed when pressure fall occasionally below 200 mmHg or perfusion tended to rise. After 2 hours, the cuff was deflated to allow reperfusion. Meanwhile, cultured EPCs were washed with PBS and stained with the orange dye CMTMR (5-(and-6)-(4-chloromethyl-benzoyl-amino)-tetramethylrhodamine) (Molecular Probes) 0.5 μg/ml. Immediately after reperfusion, the tibialis anterior (TA) muscle was exposed and 1×10^6/kg marked EPCs were injected intramuscularly. Animals were killed two weeks after EPC injection and the TA muscles were harvested. Five μm muscle cryosections were stained with anti-vWf antibody (Dako Cytomation) and a secondary goat anti-rabbit Cy2 IgG (Chemicon International) antibody, and then observed under a fluorescent microscope. Nuclei were stained with Hoescht staining. Elongated cellular structures double positive for vWf and CMTMR were considered neovessels made up of EPCs.

TABLES

Table I. Multiple linear regression analysis between progenitor cell counts (dependent variable) and risk factors or clinical conditions (independent variables).

	$CD34^+KDR^+$ cells		**$CD34^+$ cells**	
	β coefficient	*p*	β coefficient	*p*
Risk Factors				
Age	-0.181	0.033	-0.176	0.070
Male Gender	0.102	0.234	-0.186	0.050
Smoke	0.126	0.126	-0.092	0.320
Familiarity	0.064	0.426	-0.049	0.589
Hypercholesterolemia	-0.113	0.160	-0.090	0.316
Obesity	-0.023	0.797	-0.056	0.574
Hypertension	-0.084	0.343	-0.133	0.181
Clinical Conditions				
PAD	-0.588	<0.001	-0.109	0.265
CAD	0.061	0.467	-0.095	0.315
Retinopathy	0.024	0.766	-0.179	0.047
ANOVA *p*	<0.001		0.012	
R^2	0.345		0.179	

Table II. Multiple stepwise logistic regression analysis between the presence of PAD (dependent dichotomous variable) and all covariables (independent variables).

Covariables	In the equation		Not in the equation	
	B (SE)**	***p***	**Score**	***p***
$CD34^+KDR^+$ cells	-0.048 (0.010)	<0.001	-	-
Age	0.091 (0.027)	0.001	-	-
Male Gender	1.788 (0.591)	0.002	-	-
$CD34^+$ cells	-	-	0.005	0.943
Family history	-	-	0.290	0.590
Smoke	-	-	2.985	0.084
Obesity	-	-	1.824	0.177
Hypertension	-	-	1.379	0.240
Dyslipidemia	-	-	0.025	0.875
Blood glucose	-	-	0.457	0.499
HbA1c	-	-	1.047	0.306
Diabetes duration	-	-	0.070	0.791
Retinopathy	-	-	0.337	0.562
CAD	-	-	3.166	0.075

Table III. Linear correlations of $CD34^+$ and $CD34^+KDR^+$ progenitor cells with the extent of carotid atherosclerosis and the clinical stage of leg claudication. * adjusted for age, gender, diabetes duration and HbA1c.

	Carotid atherosclerosis		**Lower extremity atherosclerosis**	
	Maximal stenosis	**R/L average stenosis**	**Leriche/Fontain**	**Rutherford**
$CD34^+$ cells	-0.22 (0.002)	-0.23 (0.011)	-0.23 (0.010)	-0.24 (0.008)
adjusted*	-0.11 (0.266)	-0.12 (0.227)	-0.16 (0.118)	-0.15 (0.131)
$CD34^+KDR^+$ cells	-0.51 (<0.001)	-0.50 (<0.001)	-0.52 (<0.001)	-0.54 (<0.001)
adjusted*	-0.56 (<0.001)	-0.55 (<0.001)	-0.53 (<0.001)	-0.53 (<0.001)

FIGURE I. A-F) Scatter plots illustrating the flow cytometry procedure used to quantify peripheral blood progenitor cells on the basis of the surface expression of CD34, CD133 and KDR. G-H) Relative frequencies of the six subpopulations of circulating progenitor cells.

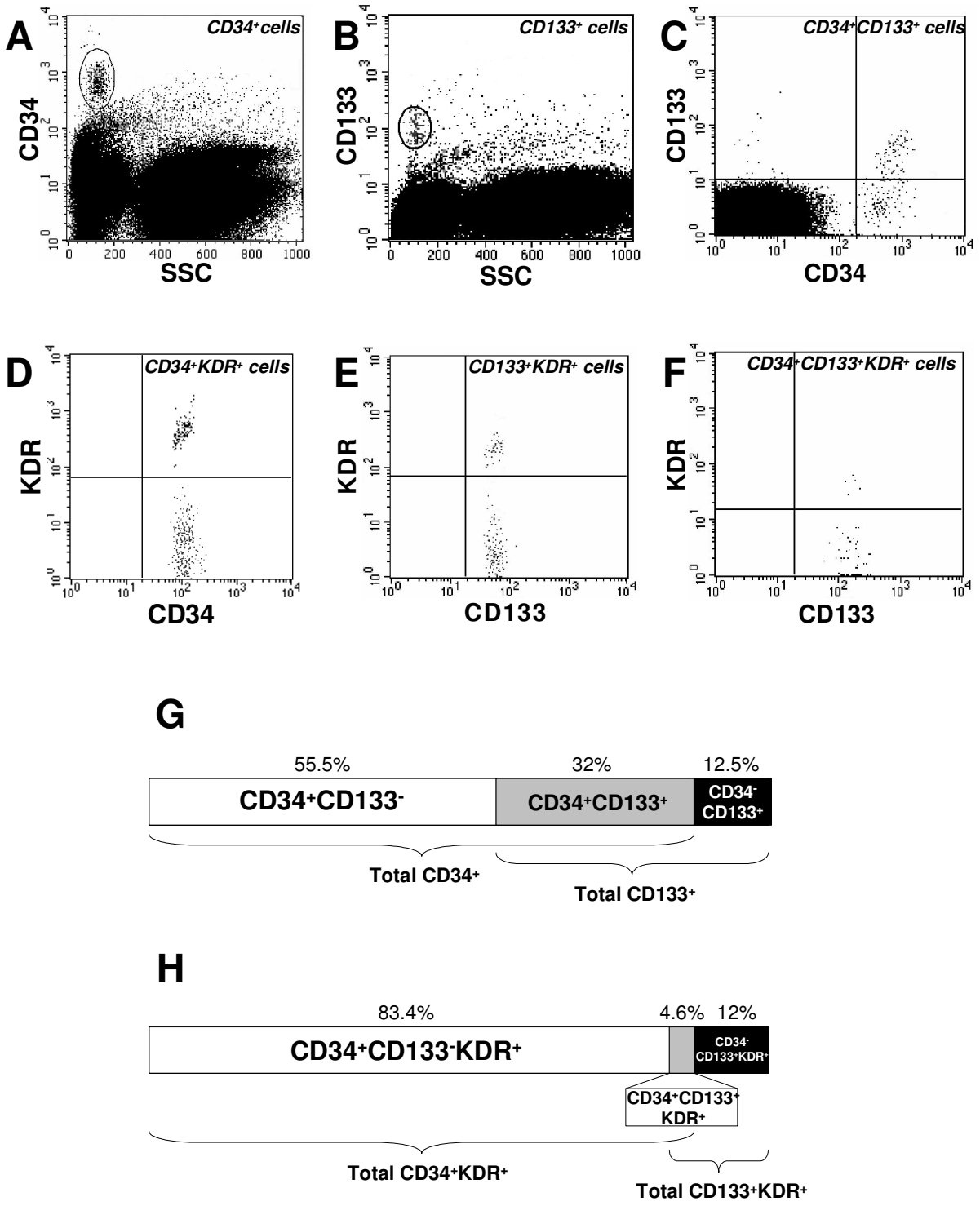

FIGURE II. Levels of $CD34^+$ (A) and CD^+KDR^+ (B) cells in patients divided according to the presence or the absence of risk factors and clinical conditions considered in the study protocol. * statistical significant after α-adjusting.

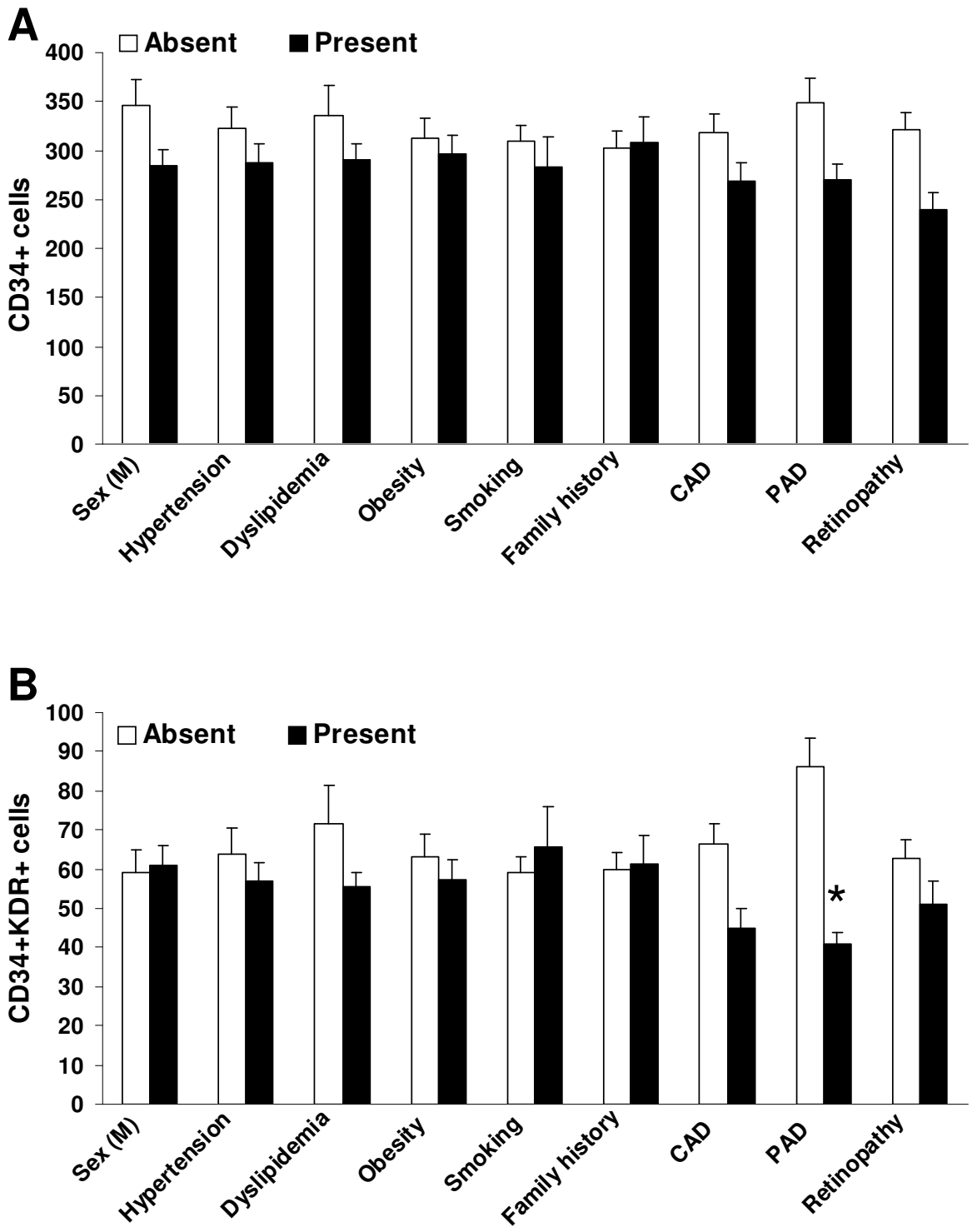

FIGURE III

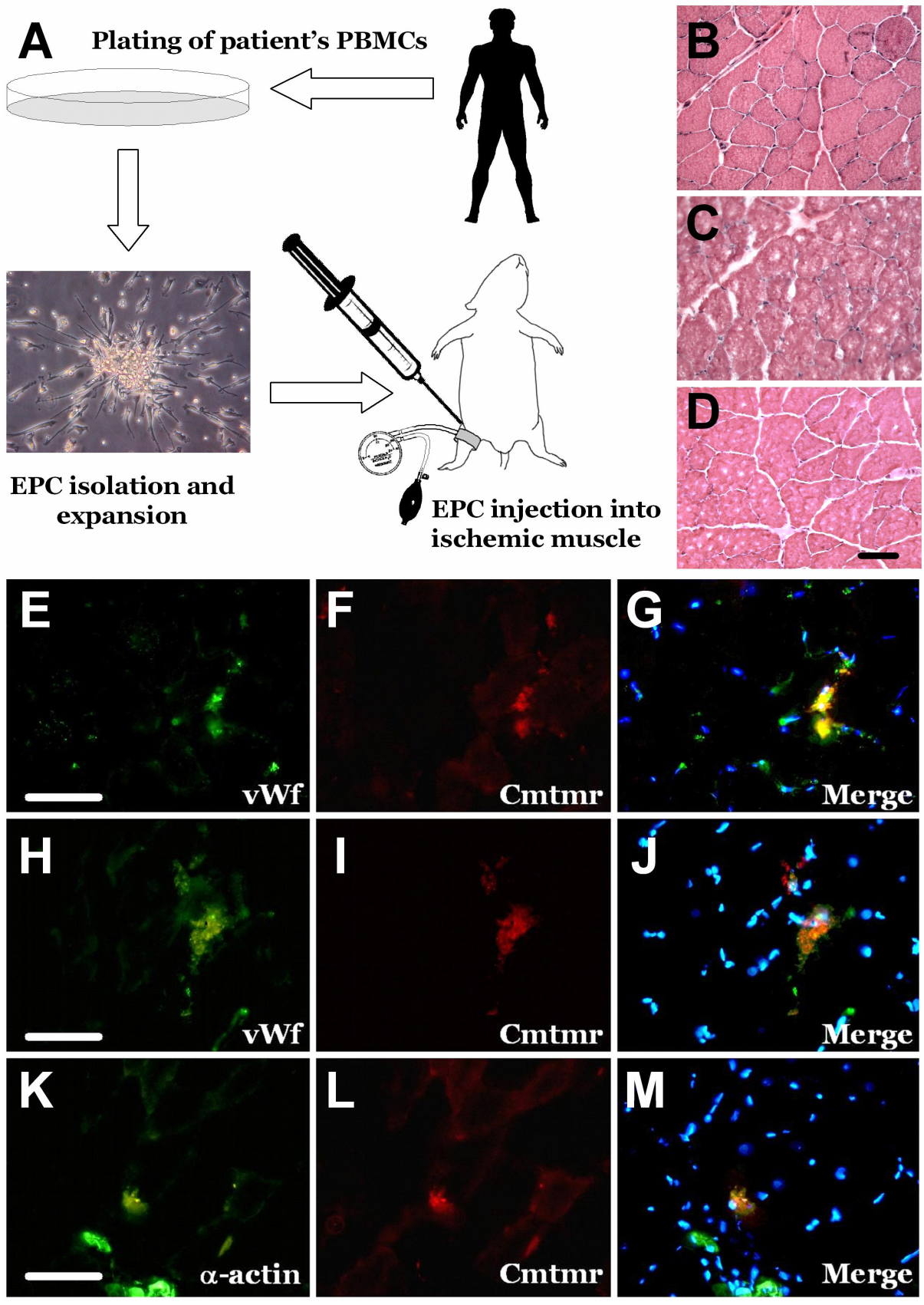
A
Plating of patient's PBMCs
EPC isolation and expansion
EPC injection into ischemic muscle
B
C
D
E
vWf
F
Cmtmr
G
Merge
H
vWf
I
Cmtmr
J
Merge
K
α-actin
L
Cmtmr
M
Merge

Figure III. Illustration of the method used for in vivo evaluation of the angiogenetic properties of isolated EPCs (A). Representative light microscopic photos of tibialis anterior muscle showing extensive fiber disarrangement and interstitial edema at 48 hours after ischemia (C) and attenuated tissue damage at day 14 (D) (baseline represented in B). Immunofluorescent photos of ischemic muscle at day 14 showing vWf^{+} vascular structures bearing Cmtmr-labeled EPCs (E-G and H-J) and α-$actin^{+}$Cmtmr-labeled EPCs (K-M). bar=50 μm.