Number and Adhesive Properties of Circulating Endothelial Progenitor Cells in Patients With In-Stent Restenosis

Jacob George, Itzhak Herz, Emil Goldstein, Soulico Abashidze, Varda Deutch, Ariel Finkelstein, Yoav Michowitz, Hylton Miller, Gad Keren

Objective—Intact endothelialization machinery is essential to facilitate vessel healing after stent placement and to prevent restenosis. Circulating endothelial progenitor cells (EPC) have been demonstrated in the peripheral blood and shown to display endothelial functional properties, along with the ability to traffic to damaged vasculature. We reasoned that robust in-stent intimal growth could be partially related to impaired endothelialization resulting from reduced circulating EPC number or function.

Methods and Results—Sixteen patients with angiographically-demonstrated in-stent restenosis were compared with patients with a similar clinical presentation that exhibited patent stents (n = 11). Groups were similar with respect to the use of drugs that could potentially influence EPC numbers. Circulating EPC numbers were determined by the colony-forming unit assay, and their phenotype was characterized by endothelial-cell markers. Adhesiveness of EPC from both groups to extracellular matrix and to endothelial cells was also assayed. Patients with in-stent restenosis and with patent stents displayed a similar number of circulating EPC. Fibronectin-binding was compromised in patients with in-stent restenosis as compared with their controls exhibiting patent stents. Patients with diffuse in-stent restenosis exhibited reduced numbers of EPC in comparison with subjects with focal in-stent lesions.

Conclusion—Reduced numbers of circulating EPC in patients with diffuse in-stent restenosis and impaired adhesion of EPC from patients with restenosis provides a potential mechanism mediating the exuberant proliferative process. These markers, if further validated, could provide means of risk stratifying patients for likelihood of developing in-stent restenosis. (Arterioscler Thromb Vasc Biol. 2003;23:e57-e60.)

Key Words: endothelial progenitor cell ■ stem-cell ■ restenosis ■ stent ■ endothelium

In-stent restenosis, even in the modern era of drug-eluting stents, still poses a significant problem because of the large volume of coronary interventions and their expanding indications.1 ‘Traditional’ risk factors that predispose to restenosis have been defined on the basis of medical history (ie, diabetes, renal failure, etc), anatomical variables (longer lesions, smaller vessel diameter, lesion type, etc), and procedural pitfalls (inappropriate stent apposition). However, there are still a considerable number of patients experiencing restenosis without having these predisposing factors, and for which alternative explanations are sought.1

It is well defined that the process of endothelialization of after-balloon trauma and stent placement is associated with intactness of the healing process and negatively correlates with the risk of both subacute thrombosis and restenosis.2 This principle is kept in mind when designing any therapeutic strategy aimed at inhibiting restenosis in an attempt not to compromise the degree of post-stenting endothelialization.

It has recently been observed that endothelial progenitor cells (EPC) can be recovered from a peripheral pool of mononuclear-cells.3 Immunological phenotyping demonstrates that these cells exhibit a number of endothelial-specific cell-surface markers, as well as endothelial properties.4 Circulating EPC numbers were shown to negatively correlate with atherosclerotic risk factors,5,6 be reduced in patients with allograft vasculopathy,7 and display a dysregulated proliferation and adhesion to tumor necrosis-alpha stimulated endothelial-cells in subjects with diabetes.8 Experimental studies employing transfused EPC suggest that they can be integrated into the vascular infrastructure and contribute to angiogenesis and vasculogenesis9,10 as well as protect against atherosclerosis development.11 A recent study also suggests that transfusion of EPC results in reduced intimal thickening in a mouse model, further showing that these cells are capable of trafficking into the vascular injury site.12

We reasoned that the pool of circulating EPC may be inherently deficient or down-regulated in patients exhibiting restenosis or alternatively, functionally compromised, and can thus be possibly employed for risk stratification. We have tested this hypothesis by assessing circulating EPC number...
and adhesion properties in patients with and without restenosis.

**Materials and Methods**

**Study Subjects**

Sixteen patients were found to have in-stent restenosis judged to be responsible for the unstable angina. The other control consecutive 11 patients with unstable angina exhibited a patent stent, yet had a different de novo culprit lesion responsible for the clinical presentation. An informed consent was provided from all participants in accord with the local institutional protocol.

**Isolation of EPC and Colony-Forming Unit Assay**

A 20-mL sample of arterial blood was obtained for the isolation of EPC as previously described. Briefly, peripheral-blood mononuclear cells (PBMC) were isolated by Ficoll density-gradient centrifugation (Sigma). After washings, isolated cells were resuspended in growth medium and plated on dishes coated with human fibronectin (Chemicon). To eliminate initial contamination with mature circulating endothelial-cells, preplating of PBMC onto fibronectin-coated six-well plate was performed (5x10^6/well) for 48 hours, after which nonadherent cells were collected and re-plated onto fibronectin-coated plates for a final evaluation of colony numbers counted at day 7.

**EPC Characterization**

Colonies were assessed for endothelial cell markers at day 7. The following antibodies were used for immunofluorescent and flow-cytometric phenotyping: rabbit polyclonal anti-Tie-2 (C-20), mouse monoclonal anti-flk-1 (A-3), and goat polyclonal anti-CD31 antibody (PECAM-1, M-20), all from Santa-Cruz. Secondary FITC-conjugated antibodies were from Jackson.

We confirmed the nature of endothelial-cell lineage by indirect immunostaining with the use of 1,1’dioctadecyl-3,3,3,3’ditetramethylindocarbocyanine perchlorate–acetylated low-density lipoprotein (DiI-acLDL) and co-staining with BS-1 lectin (both from Sigma).

**EPC Fibroblast Adhesion Assay**

EPC (day 7) from patients with or without restenosis were washed and gently detached with 0.5-mmol/L EDTA in PBS. After centrifugation and resuspension in basal complete medium, identical cell numbers were placed onto fibronectin-coated culture dishes and incubated for 30 minutes at 37°C. Adherent cells were counted by independent blinded investigators.
Incorporation Assay of EPC Into Bovine Aortic Endothelial Cell Monolayer

Day 7 EPC were labeled with the fluorescence marker Dil (molecular probes). Identical numbers of Dil-labeled EPC were incubated with bovine aortic endothelial cell (BAEC) monolayer plated on fibronectin-coated 96 well plates with or without pretreatment with tumor necrosis factor-α (1 ng/mL) for 12 hours. The total numbers of adhesive EPC in each well were counted in a blinded manner.

Statistical Analysis

Clinical variables between groups were compared by the t test. Student’s t-test was employed for comparison of EPC numbers and adhesion. P<0.05 was considered statistically significant.

Results

In order to homogenize both groups of studied patients, only patients presenting with unstable angina and no ST-segment elevation, increased CPK-MB, or Troponin I levels were enrolled. Table 1 provides the data of both study groups and demonstrates the lack of differences with regard to risk factors and drugs, both of which are known to influence circulating EPC. Among patients with restenosis, a focal in-stent lesion (including edge restenosis) was evident in 6 patients, whereas 10 patients exhibited proliferative or diffuse in-stent restenosis. Table 2 describes the profile of subgroups with diffuse/proliferative and focal in-stent restenosis.

Comparative analysis of colony-forming unit (CFU) disclosed similar numbers in patients with and without restenosis (26.5±2.6 versus 25.3±4.8, respectively; Figure 1A). Patients with diffuse in-stent restenosis exhibited a smaller CFU number (24.0±3.9) compared with patients with focal restenosis (30.7±1.7; P<0.05, Figure 1B). Adherence to fibronectin was compromised in patients with restenosis (9.2±2.5 cells/field) as compared with patients exhibiting patent stents (15.3±3.2; P<0.01, Figure 1C) No differences were evident between groups with regard to adherence to tumor necrosis factor-α primed or nonprimed endothelial cells (Figure 1D).

EPC Phenotyping confirmed the presence of double positive cells for acLDL (red) and BS1 (green; Figure 1E). Colonies were strongly positive by immunofluorescence em-

Figure 1. EPC in patients with in-stent restenosis. A, CFU numbers representing circulating EPC in patients with and without restenosis were determined as described. B, CFU numbers in patients with diffuse (n=10) as compared with focal restenosis (n=6). C, Fibronectin adherence assay as described in methods. Y-axis represents cells/x 40 magnification. D, Tumor necrosis factor-α stimulated or non-stimulated endothelial cell binding by EPC. *P<0.05. E, confirmation of EPC phenotype by co-staining with anti-acLDL and anti-BS-1.
ploying antibodies to KDR, CD31, and Tie-2 (data not shown).

Discussion
The role of endothelial-cell integrity after intraluminal injury was illuminated by numerous studies, all of which suggest that a functionally intact endothelialization process is requisite for the inhibition of neointimal growth.1

We report here that patients with or without in-stent restenosis have similar levels of circulating EPC. However, within restenosis patients, the presence of diffuse in-stent lesions defines a subgroup with reduced EPC numbers. This finding may imply that a defective endothelialization could partially account for the tendency to generate robust lesions.

An additional interesting finding in our study is the reduced capacity of circulating EPC from patients with in-stent restenosis to adhere to fibronectin but not to endothelial cell surfaces. This observation, which may be related to a dysregulated integrin synthesis, can also aid in explaining excessive neointimal growth evident in restenotic patients.

The presence of circulating EPC has been thoroughly validated in recent years.4 As these cells display endothelial-cell functional properties on in vitro growth, they are of particular interest in the context of therapeutic angiogenesis and vasculogenesis. Studies in humans have shown that the number of EPC negatively correlate with atherosclerosis risk factors,5,6 yet it cannot be determined whether it is a mere association or a contributory factor in atherogenesis. Additionally, it has been shown that reduced numbers of EPC are present in patients with allograft vasculopathy,7 and that the number and migratory capacity of EPC is hampered in diabetics.8

The design of our study precludes making conclusions as to whether the reduced adherence properties of EPC in subjects with restenosis are causally related to excessive neointimal growth. Yet it may point to an additional ‘risk profile’ for restenosis that could be potentially considered, if prospective studies confirm our observations. Because of the relatively small sample size in our study, we attempted to preselect patients on the basis of a uniform clinical appearance of unstable angina, as tissue ischemia is known to induce EPC mobilization.13 Additionally, statin therapy, also known to upregulate EPC numbers,14,15 was similar in both groups and could not have accounted for the differences in EPC numbers between both groups. It is noteworthy that additional functional properties of EPC, such as their ability to migrate, could have provided equally relevant insight into the mechanisms of neointimal growth in patients with restenosis, yet they were not tested in the present study.

In conclusion, we have found that patients with diffuse in-stent restenosis exhibit reduced numbers of circulating EPC as compared with subjects with focal lesions, and that the adhesion capacity of EPC was compromised in restenosis patients as compared with those with patent stents. This finding could serve to identify patients with high-risk features that predispose to restenosis and may thus be aided in clinical decision-making during and after coronary intervention provided confirmatory studies will be available.

References
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