Circulating Apoptotic Progenitor Cells
A Novel Biomarker in Patients With Acute Coronary Syndromes

Shmuel Schwartzenberg, Varda Deutsch, Sofia Maysel-Auslender, Sarina Kissil, Gad Keren, Jacob George

Background—Progenitor CD34 cells are capable of differentiating into endothelial cells and play a role in neoangiogenesis. Circulating CD34⁺ cells and endothelial progenitor cells are increased in acute coronary syndrome (ACS) patients possibly because of peripheral mobilization. We tested the hypothesis that circulating apoptotic progenitors are detectable in healthy subjects and altered in ACS patients.

Methods and Results—Peripheral blood mononuclear cells were isolated by Ficoll density gradient from 53 patients with ACS undergoing coronary angiography and 27 healthy subjects. Apoptosis in progenitor CD34⁺ cells was assessed using the Annexin V-PE/7-AAD detection kit, and fluorescence-activated cell sorter analysis was performed with triple staining for CD34, annexin-V, and 7-AAD. The percentage of apoptotic CD34⁺ progenitors was determined in the 2 subject groups and correlated with clinical characteristics. The percentage of apoptotic CD34⁺ progenitor cells was significantly increased in patients with ACS as compared with healthy subjects and was associated with the extent of coronary stenosis by angiography. There was no significant correlation between apoptotic progenitor CD34⁺ cells and the other parameters that we examined (age, smoking, hypertension, hyperlipidemia, diabetes mellitus, ejection fraction, creatinine levels, or taking any of the various medications, including beta blockers, thiazides, angiotensin-converting enzyme inhibitors, ARBs, calcium blockers, nitrates, or statins).

Conclusion—We established for the first time to our knowledge an assay to detect circulating apoptotic progenitor cells using fluorescein isothiocyanate–anti-CD34 MAb, annexin V-PE, and 7-AAD and found that apoptotic CD34⁺ cells are increased in ACS patients and in patients with more extensive coronary artery disease. This novel assay may shed new light on the factors governing the hemeostasis of progenitor CD34⁺ cells. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: ●●●

CD34⁺ bone marrow progenitor stem cells comprise only 1.5% of marrow mononuclear cells, with minor numbers of circulating cells in the peripheral blood. These cells contain precursors for all lymphohematopoietic lineages, as evidenced by the finding that CD34⁺ cells purified from marrow can reconstitute hematopoiesis of primates, humans, or mice undergoing autologous marrow reinfusion after myeloablative therapy.¹ Recent studies have shown that expression of the CD34 surface antigen characterizes not only hematopoietic progenitor cells but also endothelial progenitor cells (EPCs) and mature endothelial cells.²

In 1997, Asahara et al³ isolated a circulating angioblast (later referred to as EPCs) from human peripheral blood of adults, with a potential to differentiate in vitro into endothelial cells and to contribute to neangiogenesis after tissue ischemia. Shi et al⁴ were the first to show that a subset of transplanted bone marrow-derived CD34⁺ hematopoietic precursor cells participated in the endothelialization in a canine model of vascular graft healing, and thus have the capacity to transform eventually into mature endothelial cells. Expression of the stem cell marker CD34 is also found on a lower level on mature endothelial cells, and the search for more specific stem cell markers led to the discovery of CD133, which is expressed on immature stem cells but whose expression is lost during the differentiation to mature endothelial cells.⁵

EPCs are a heterogeneous group of cells that can be characterized by the expression of surface markers, such as CD34, CD133, and VEGFR-2 (KDR or Flk-1) in various combinations and, currently, precise phenotype definition is lacking.⁶ Circulating numbers of EPCs have been shown to negatively correlate with risk factors for atherosclerosis and with disorders associated with vascular dysfunction.⁷,⁸ Acute coronary syndrome (ACS) is associated with elevated numbers of circulating EPCs, suggesting that these cells are possibly...
mobilized in an attempt to participate in vessel repair after severe ischemia. Similarly, bone marrow-derived progenitor CD34+ cell numbers have been shown to be increased in ACS and acute myocardial infarction.

Endothelial cell damage and apoptosis is associated with the release of small membrane particles, which are called endothelial microparticles. Elevated endothelial microparticles have been described in conditions of severe endothelial damage, including ACS and after myocardial infarction, and indicate increased apoptosis of endothelial cells. Furthermore, in patients with coronary artery disease, the number of circulating endothelial microparticles positively correlate with the severity of coronary endothelial dysfunction.

Because bone marrow-derived CD34+ stem cells and EPCs are mobilized toward the peripheral blood in acute myocardial ischemia, we hypothesized that this condition may be associated also with their increased apoptosis resulting in a similar elevation of circulating apoptotic CD34+ cell levels. In this study, we identified, for the first time to our knowledge, the presence of a unique, yet uncharacterized, population of apoptotic progenitors and evaluated their numbers in patients with ACS.

Materials and Methods

Study Subjects

We studied a total of 53 patients with acute coronary syndrome who underwent coronary angiography and 27 healthy subjects, aged 25 to 60 years (median age, 35), including 13 males and 14 females. The Table summarizes demographic and clinical characteristics of the patient population. Institutional ethics committee approved the study and informed consent was obtained from all patients.

Preparation of Blood Samples

Peripheral blood mononuclear cells were isolated from 30 mL of freshly drawn heparinized blood using Isopaque-Ficoll (Amer- sham Biosciences, Buckinghamshire, United Kingdom) gradient centrifugation.

Flow Cytometry Evaluation of Early Apoptotic Progenitor CD34+ Cells

Apoptosis in progenitor CD34+ cells was assessed using Southern-Biotech ApoScreen Annexin V Apoptosis detection kit (Annexin V-PE, 7-AAD solution, and Annexin V binding buffer). In apoptotic cells, the membrane phospholipid phosphatidylserine is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing phosphatidylserine to the external cellular environment. Annexin V is a 36 kDa Ca2+-dependent phospholipid-binding protein that has a high affinity for phosphatidylserine, and binds to cells with exposed phosphatidylserine. Annexin V-PE staining precedes the loss of membrane integrity, which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with Annexin V-PE is used in conjunction with 7-AAD, which is a vital dye to identify early apoptotic cells before morphological changes associated with apoptosis have occurred and before membrane integrity has been lost. Cells that are viable are Annexin V-PE and 7-AAD–negative; cells that are in early apoptosis are Annexin V-PE–positive and 7-AAD–negative; and cells that are in late apoptosis or already dead are both Annexin V-PE–positive and 7-AAD–positive.

The percentage of apoptotic CD34+ progenitor cells (out of total circulating progenitor CD34+ cells) was assayed by staining peripheral blood mononuclear cells for 3 color fluorescence-activated cell sorter analysis using fluorescein isothiocyanate-anti-CD34 MAb (IQ products), annexin V-PE, and 7-AAD (SouthernBiotech). After Ficoll gradient separation, peripheral blood mononuclear cells were washed with phosphate-buffered saline, and 10⁶ cells were stained with fluorescein isothiocyanate–anti-CD34 MAb for 30 minutes at 4°C in 100 μL fluorescence-activated cell sorter staining buffer (phosphate-buffered saline and 2% fetal calf serum). The cells were washed again with phosphate-buffered saline and resuspended in 100 μL of Annexin V binding buffer and incubated with 10 μL of Annexin V-PE for 15 minutes at 4°C. Without washing, 380 μL of cold binding buffer and 10 μL of 7-AAD solution were added and 80 000 cells were acquired by flow cytometry (FACSCalibur; Becton Dickinson) and analyzed by CellQuest software (BD Bioscience). All analyses and readings were made by technicians that were blinded to the study questions. Intra-assay variability was never >10%, whereas interassay variability did not exceed 15% (when similar patients had samples performed within the same day).

Statistical Analysis

Comparison between apoptotic progenitor cells in healthy subjects and patients with ACS was performed using 1-way analysis of

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n=53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic data</td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>36/17</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>65.3 (41–82)</td>
</tr>
<tr>
<td>Current daily smoker</td>
<td>22 (41%)</td>
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<tr>
<td>Comorbidities</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>28 (53%)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>9 (17%)</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>26 (49%)</td>
</tr>
<tr>
<td>Drug treatment</td>
<td></td>
</tr>
<tr>
<td>Statin</td>
<td>12 (23%)</td>
</tr>
<tr>
<td>Beta blocker</td>
<td>13 (24%)</td>
</tr>
<tr>
<td>Angiotensin converting enzyme inhibitor</td>
<td>11 (21%)</td>
</tr>
<tr>
<td>Angiotensin II blocker</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Calcium antagonist</td>
<td>7 (13%)</td>
</tr>
<tr>
<td>Thiazide</td>
<td>5 (9%)</td>
</tr>
<tr>
<td>Nitrate</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>No. of vessels involved</td>
<td></td>
</tr>
<tr>
<td>Single vessel</td>
<td>21 (40%)</td>
</tr>
<tr>
<td>Multivessel</td>
<td>32 (60%)</td>
</tr>
</tbody>
</table>

*Left ventricle ejection fraction was estimated by 2-dimensional echocardiography performed within 24 hours of the ACS event.*
variance with age as covariant. Linear regression analysis was applied to the data to study the significance of the independent variables (age, smoking, hypertension, hyperlipidemia, diabetes mellitus, ejection fraction, creatinine levels, or taking any of the various medications, including beta blockers, thiazides, angiotensin-converting enzyme inhibitors, ARBs, calcium blockers, nitrates, or statins, and the extent of coronary artery stenosis) on apoptotic progenitor CD34⁺ cells. Analysis was performed using the SAS for windows version 9.1 program (Chicago, Ill). Level of significance was set at \( P \leq 0.05 \).

Results

Progenitor CD34⁺ cells were gated from the standard saline citrate (SSC)/CD34 dot plot according to the Milan protocol\(^{20,21}\) as shown in Figure 1A. Thereafter, apoptotic percentage of progenitor CD34⁺ cells was determined by fluorescence-activated cell sorter analysis of annexin V/7-AAD staining as shown in Figure 1B. Early apoptotic cells were defined as Annexin V-PE–positive and 7-AAD–negative, and thus consist of the population lying in the lower right quadrant in Figure 1B. The percentage of apoptotic CD34⁺ progenitor cells ±SEM (out of total circulating progenitor CD34⁺ cells) was significantly higher in the patients (24.5±3.8%) than in the control group (12.3±2.1%; \( P<0.05 \)), as shown in Figure 2. Interestingly, we found that the percentage of apoptotic progenitor CD34⁺ cells correlated with the number of coronary vessels displaying significant stenosis (Figure 3A) and did not correlate with the presence of diabetes (Figure 3B). We did not find any significant correlation between apoptotic progenitor CD34⁺ cells and the other parameters that we examined (age, smoking, hypertension, hyperlipidemia, diabetes mellitus, ejection fraction, creatinine levels, or taking any of the various medications, including beta blockers, thiazides, angiotensin-converting enzyme inhibitors, ARBs, calcium blockers, or statins).

Discussion

Previous studies have shown that ACS results in mobilization of progenitor cells, including endothelial progenitor cells, as well as with an increase in circulating endothelial microparticles representing mature endothelial cell damage and apoptosis. In this study, we have established an assay in which apoptotic progenitor cells were identified and quantified in the peripheral blood. We have found that apoptotic progenitor CD34⁺ cells are increased in ACS patients in comparison with healthy volunteers. Although control subjects and ACS patients were not age-matched, when multivariate statistical analysis was performed, age was not found to be an independent influencing factor on the percentage of apoptotic CD34⁺ progenitor cells in the patient group. Furthermore, we have

Figure 1. Representative flow cytometric dot plots. SSC/CD34 scatter (A) and flow cytometric evaluation of progenitor CD34⁺ apoptosis percentage (B). Dots in the lower right quadrant represent early apoptotic CD34⁺ cells.

Figure 2. Early apoptotic progenitor CD34⁺ percentage (out of total circulating progenitor CD34⁺ cells) in control (left scatter) and patient (right scatter).
Apoptosis is indeed a major determinant of atherosclerotic plaque vulnerability and thus a key player in ACS induction.\textsuperscript{24,23} Thus, our findings can be explained by increased pro-apoptotic mediators in ischemic heart disease including reactive oxygen species,\textsuperscript{24,25} oxidized low-density lipoprotein,\textsuperscript{25,26} high levels of nitric oxide,\textsuperscript{27,28} and inflammatory cytokines produced by the activated macrophages and T-lymphocytes (including tumor necrosis factor-\(\alpha\), interferon-\(\gamma\), and IL-1\(\beta\)).\textsuperscript{21} In particular, oxidative stress and cigarette smoke extracts were found to increase Fas ligand and caspase-3–mediated endothelial cell apoptosis.\textsuperscript{29,30}

Regardless of whether apoptotic CD34\textsuperscript{+} cells represent a predisposition for plaque destabilization or are byproducts of increased oxidative stress, the finding and identification of this cell population shed new light on the mechanisms governing progenitor cell turnover. It should be mentioned, however, that the relatively small number of circulating CD34\textsuperscript{+} cells still poses a question of the true functional importance of these cells. In several recent studies,\textsuperscript{7–11} small numbers (in the range of 0.01\% of peripheral mononuclear cells) of circulating endothelial progenitors have been associated with reduced angiogenic capacity. These reports support the importance of even trivial numbers of progenitors, yet additional research should lend proof to this assumption.

In summary, we describe a novel assay to detect and quantify circulating apoptotic CD34\textsuperscript{+} cells in healthy subjects and in patients with ACS. This assay may help elucidate the factors that control peripheral progenitor cell hemostasis.

Disclosures
None.

References


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