VE-Cadherin<sub>low</sub> α-Smooth Muscle Actin<sup>+</sup> Component of Vascular Progenitor Cells Correlates With the Coronary Artery Gensini Score

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**Background:** Vascular progenitor cells (VPCs) are a heterogeneous population, containing a subpopulation co-expressing both endothelial and smooth muscle phenotypes. This study sought to determine whether the level of this subpopulation correlated with the coronary Gensini score.

**Methods and Results:** VPCs were cultivated in 50 patients undergoing coronary angiography. A subpopulation of VPCs expressed both endothelial (VE-cadherin [VE-Cad]) and smooth-muscle phenotypes (α-smooth muscle actin [α-SMA]). Correlations of the VE-Cad<sup>low</sup>α-SMA<sup>-</sup> VPC level and adhesion molecule expression by VPCs with the Gensini score were investigated. The association between the amount of this subpopulation and the development of intimal hyperplasia (IH) was also estimated in a vascular injury animal model. Both the number of VE-Cad<sup>low</sup>α-SMA<sup>-</sup> VPCs (P=0.002) and the expression level of intracellular adhesion molecule (ICAM)-1 by VPCs (P=0.008) correlated with the Gensini score. However, only the number of VE-Cad<sup>low</sup>α-SMA<sup>-</sup> VPCs correlated with greater IH (r=0.69, P<0.0001).

**Conclusions:** The level of VE-Cad<sup>low</sup>α-SMA<sup>-</sup> VPCs was associated with the severity of coronary atherosclerosis as quantified by the Gensini score. Manipulating this subpopulation may provide a way of attenuating atherosclerosis in the future. (Circ J 2012; 76: 477–484)

**Key Words:** Adhesion molecule; Atherosclerosis; Coronary artery disease; Gensini score; Vascular progenitor cell

Over the past decades, research in atherosclerosis has made substantial progress in the knowledge of the pathophysiological mechanisms and the success of vascular interventions. However, current therapeutic strategies still cannot efficiently control the development of atherosclerosis related to various risk factors, suggesting pathways in the pathogenesis of atherosclerosis that still need to be revealed.

Recent evidence shows that intervention directed at endothelial progenitor cell (EPC) mobilization, homing, and differentiation may provide beneficial effects on post-injury vascular remodeling. However, EPCs represent 1 type of circulating vascular progenitor cell (VPC). Peripheral blood-derived VPCs are a heterogeneous population, including EPCs and smooth-muscle progenitor cells (SMPCs) at the very least. Although no well-established methodology is currently available to separately cultivate EPCs and SMPCs, a differentiation profile for semi-quantification purposes has been developed. Our previous study showed that VPCs contain a subpopulation that co-expresses endothelial and smooth-muscle phenotypes. This subpopulation has the potential to participate in the formation of smooth-muscle-like cells in the hyperplastic intima during vascular remodeling. This causal link was supported by the notion that manipulation of the differentiation of VPCs toward a purely endothelial lineage significantly attenuated intimal hyperplasia (IH). However, the correlation between the level of this subpopulation and the development of atherosclerosis has yet to be elucidated.
Adhesion molecule expression is associated with the adhesion and recruitment of inflammatory cells into activated vascu-
lar tissue, leading to the development of atherosclerotic plaques.\textsuperscript{13,14} We hypothesized that the level of the VPC subpopulation co-
expressing endothelial and smooth-muscle phenotypes, and the level of expression of adhesion molecules by VPCs are both critical components of the VPC profile associated with the Gensini score, a surrogate of coronary atherosclerosis severity.\textsuperscript{15}

**Methods**

**Study Design**

Between February 2010 and January 2011, 50 consecutive patients were enrolled in this study after coronary angiography for diagnosis of coronary artery disease (CAD) causing angina pectoris. Patients with acute coronary syndrome or who were already being treated with statins were excluded. Concurrently, peripheral blood samples were collected in tubes containing EDTA and sent to the hospital laboratory for VPC cultivation and further studies.

At passage 0, VPCs were injected into SCID/NOD mice after femoral artery wire injury to estimate the contribution of VPCs to vascular repair and remodeling in vivo. VPCs were further cultivated to passage 1 to enumerate the distribution of differentiated VPCs by flow cytometry. Correlations of VPC differentiation and adhesion molecule profiles with the Gensini score of the coronary arteries were investigated. This trial was designed and carried out in accordance with the principles of the Declaration of Helsinki and the Ethics Review Board of Chang Gung Memorial Hospital. Written informed consent was given by each patient after the purpose and potential risks of the interventions had been explained.

**Purification of Peripheral Blood Mononuclear Cells (PBMCs)**

Mononuclear cells were isolated from peripheral blood by density gradient centrifugation with Ficoll separating solution (Sigma-Aldrich, St Louis, MO, USA). After resuspension in EGM-2 medium (SingleQuots, Cambrex), roughly 10\(^6\) mononuclear cells/cm\(^2\) were plated on fibronectin-coated dishes or 2-chamber slides. The outgrowth of VPCs was used for the experiments described next.

**Cultivation of VPCs**

To produce VPCs, PBMCs were cultured in EGM-2 medium for 3-weeks.\textsuperscript{15} At passage 1, cells were cultured in EGM-2 medium supplemented with vascular endothelial growth factor (10 ng/ml), platelet-derived growth factor (10 ng/ml), and basic fibroblast growth factor (10 ng/ml).\textsuperscript{16,17} After confluence, cells were subjected to immunofluorescent staining, and the differentiation of VPCs into cells with smooth-muscle or endothelial phenotype was determined by flow cytometry.

**Confocal Microscopy**

Cells and frozen tissues from different experiments were stained with the following primary antibodies: Cy3-conjugated anti-\(\alpha\)-SMA (Sigma), von Willebrand factor (vWF; DAKO, Glostrup, Denmark), CD31 (BD Pharmingen, San Jose, CA, USA), calponin, H-caldesmin (Santa Cruz Biotech, Santa Cruz, CA, USA), or anti-HLA-ABC (Biolegend, San Diego, CA, USA), followed by incubation with FITC-, PE-, or Alexa Fluor 647-conjugated secondary antibodies. Slides were mounted using a Prolong Antifade kit (Molecular Probes, Eugene, OR, USA) and viewed by confocal microscopy (Leica TCS SP2 A OBS, Bergen, Norway). Nuclei were stained with Hoechst 33258 (Sigma, San Jose, CA, USA).

**Fluorescence-Activated Cell Sorter Analysis**

Fluorescence-activated cell sorting (FacScan, Becton Dickinson, Franklin Lakes, NJ, USA) was performed to identify both cell-surface (intercellular adhesion molecule [ICAM]-1, vascular cellular adhesion molecule [VCAM]-1, E-selectin, and VE-cadherin [VE-Cad]) and intracellular antigens (\(\alpha\)-SMA) of differentiated VPCs. Intracellular antigens were exposed using a Cytofix/Cytoperm kit (Pharmingen, San Jose, CA, USA). Amounts of pure \(\alpha\)-SMA\(^+\), VE-Cad\(^+\)\(\alpha\)-SMA\(^-\), VE-Cad\(^-\)\(\alpha\)-SMA\(^+\), and pure VE-Cad\(^+\) VPCs were quantified. In peripheral blood, circulating EPCs were defined as CD34\(^+\)/KDR\(^+\) and AC133\(^+\)/KDR\(^+\) cells.

**Adhesion Assay (Static)**

Human VPCs labeled with quantum dots (Qtracker\textsuperscript{\textregistered} Cell Labeling Kits) and anti-HLA-ABC conjugated with micro-
beads (Miltenyi Biotec) were used to investigate the effect of a magnetic field on cell adhesion. To create an environment mimicking the microenvironment of an injured vessel wall, double-distilled water (ddH\(_2\)O) was used to disrupt confluent mouse endothelial cells (SVEC4-10, purchased from ATCC, Rockville, MD, USA) cultivated in a 35-mm dish. A magnetic field was built up by applying a 5000-Gauss magnet (Amoeba Magnetic, South Africa) beneath the culture dish (Figure S1A). Microbead-labeled VPCs (5\(\times\)10\(^4\)) were plated on top of the disrupted mouse endothelial cells. Compared with the controls without a magnetic field, the effect of the magnetic field on cell adhesion was estimated by applying the magnet for 5 and 15 min. After the magnet was removed, cells were washed with phosphate-buffered saline and fixed. Numbers of adher-
ent cells were randomly counted in 4 areas of each well.

**Cell Rolling and Adhesion Under Continuous Flow (In Vitro)**

For continuous-flow experiments, a closed perfusion chamber (Microslide I, Ibidi, Munich, Germany) was used. The chamber was connected to a computer-controlled setup containing an air pressure pump and a 2-way switching valve (Ibidi pump system 10902), which allowed pumping of 40 ml of cell culture medium from 2 reservoirs in a unidirectional way through the flow channel (which measured 50\(\times\)50\(\times\)0.4 mm; Figure S1B) at a constant rate corresponding to a shear stress of 5–20 dynes/cm\(^2\). The chamber and reservoirs containing the medium were kept in an incubator with 5% CO\(_2\) at 37°C. Under continuous flow, rolling and adhesion of microbead-labeled VPCs were assessed without and with a magnetic field of 1,500–5,000 Gauss. Analysis of cell rolling and adhesion is described next.

**Cell Rolling and Adhesion (In Vivo)**

VPC rolling and adhesion with and without a magnetic field were evaluated in vivo using an intravital fluorescent micro-
scope. Femoral artery injury was performed in C57BL/6 mice as described below (n=5–6 in each group), and the skin was sutured. One day later, mice were anesthetized again, and both the femoral artery and vein were exposed from the bifurcation point of the femoral artery and muscular branch to 5 mm up to the bifurcation point. A catheter (0.20-mm I.D. and 0.36-mm O.D.) was introduced from the left external carotid artery to the descending aorta. Rhodamine-dextran (0.25 mg/ml, 100\(\mu\)l) was injected to visualize the blood flow and localize the site of the artery undergoing imaging using a Leica microscope (20 water immersion objective, \(\times\)10/0.5, Leica) with a 100-W HBO mercury lamp for epi-illumination. For direct visualization of cell rolling and adhesion at the site of vascular injury,
Hoechst 33342 and microbead-labeled VPCs were infused via the catheter. Cell adhesion dynamics were assessed with and without a magnetic field (5,000 Gauss; Figures S1C,D) just to the side of the targeted femoral artery, and results were recorded using a Leica microscope equipped with a Leica monochromator and high-speed CCD (Andor DU897 CCD, Andor Technology). Videos obtained on the CCD during the experiments were analyzed off-line using a digital image-processing system (Matamorph software). Each cell being tracked was followed until it had adhered, detached from the slide or injured vessel, rolled out of the slide or imaged field, or was lost because of technical limitations of the tracking procedure. The rolling velocity was then calculated.

Mouse Femoral Wire Injury and Cell Therapy Model
To test the contribution of VPCs to neointimal formation, male NOD/SCID mice (Jackson Laboratory, Bar Harbor, ME, USA) underwent general anesthesia for transluminal mechanical injury of the left femoral arteries by insertion of a straight wire. Table 1.

<table>
<thead>
<tr>
<th>Mean ± SD or n (%)</th>
<th>Age (years)</th>
<th>Male (%)</th>
<th>Smoking (%)</th>
<th>Hypertension (%)</th>
<th>Diabetes (%)</th>
<th>No. of diseased vessels</th>
<th>Gensini score</th>
<th>Total cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>HDL-cholesterol (mg/dl)</th>
<th>LDL-cholesterol (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60.5±11.7</td>
<td>44 (88)</td>
<td>24 (48)</td>
<td>27 (54)</td>
<td>11 (22)</td>
<td>2.0±1.0</td>
<td>29.8±21.5</td>
<td>181.6±47.1</td>
<td>197.2±258.7</td>
<td>39.5±8.4</td>
<td>105.0±43.3</td>
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</tr>
</tbody>
</table>

CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

**Figure 1.** Heterogeneous population of vascular progenitor cells (VPCs). (A) Colonies of VPCs evident 2 weeks after peripheral blood mononuclear cells were plated. The VPCs have become confluent, and with both a cobblestone and spindle-shape appearance. VPCs were used for studies in an animal model and were also stimulated to differentiate. At passage 1, flow cytometry demonstrated that VPCs differentiated into 4 different subtypes, including VE-Cad^{low}α-SMA^{+}. (B) Portion of VPCs stained positive for CD31 and von Willebrand factor (vWF). (C) Portion of VPCs differentiated into VE-Cad^{−}α-SMA^{+} cells. (D) Subpopulation of VPCs expressing a variety of endothelial and smooth-muscle phenotypes, including vWF, α-SMA, H-caldesmin, and calponin. SMA, smooth muscle actin; VE-Cad, VE-cadherin. Scale bar=50 μm.
spring wire (0.38 mm in diameter; Cook) for >5 mm toward the iliac artery, as previously described. At passage 0, $1 \times 10^4$ microbead-labeled HLA-ABC+VPCs (Miltenyi Biotec) were injected into the abdominal aorta of SCID mice 4 days after the left femoral artery wire injury. A magnet (5,000 Gauss, the same one used for MACS; Miltenyi Biotec) was applied around the left leg for 15 min to assist the microbead-labeled VPCs in adhering to the surface of the injured femoral artery (Figure S1E). Vessels were harvested 24 days after cell injection (28 days after wire injury). IH was assessed by calculating the intima-media (I-M) ratio of the femoral artery. All procedures involving experimental animals were approved by the institutional committee for animal research of Chang Gung Memorial Hospital.

**Gensini Score**

The Gensini scoring system was used to evaluate CAD severity. The Gensini score was calculated for each patient from the coronary arteriogram by assigning a severity score to each coronary stenosis according to the degree of luminal narrowing and its geographic importance. Reduction in the lumen diameter, and the roentgenographic appearance of concentric lesions and eccentric plaques were evaluated (reductions of 25%, 50%, 75%, 90%, 99%, and complete occlusion were given Gensini scores of 1, 2, 4, 8, 16, and 32, respectively). Each principal vascular segment was assigned a multiplier in accordance with the functional significance of the myocardial area supplied by that segment: the left main coronary artery $\times 5$; the proximal segment of left anterior descending coronary artery (LAD) $\times 2.5$; the proximal segment of the circumflex artery $\times 2.5$; the mid-segment of the LAD $\times 1.5$; the right coronary artery, the distal segment of the LAD, the posterolateral artery and the obtuse marginal artery $\times 1$; and others $\times 0.5$. Scoring was performed by 2 observers and averaged.15

### Table 2. Phenotypes of Heterogeneous VPCs (n=50)

<table>
<thead>
<tr>
<th>Phenotypes of different VPCs</th>
<th>Mean ± SD</th>
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<tbody>
<tr>
<td>CD34+KDR+ (%)</td>
<td>0.06±0.07</td>
</tr>
<tr>
<td>AC133+KDR+ (%)</td>
<td>0.04±0.03</td>
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<tr>
<td>VE-Cad+α-SMA- VPC (%)</td>
<td>5.2±7.6</td>
</tr>
<tr>
<td>VE-Cadα-SMA- VPC (%)</td>
<td>6.6±11.6</td>
</tr>
<tr>
<td>VE-Cad+α-SMA- VPC (%)</td>
<td>5.0±5.9</td>
</tr>
<tr>
<td>VE-Cad-α-SMA- VPC (%)</td>
<td>0.8±0.7</td>
</tr>
<tr>
<td>VCAM-1 (%)</td>
<td>9.8±8.0</td>
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<tr>
<td>E-selectin (%)</td>
<td>4.7±5.4</td>
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<tr>
<td>ICAM-1 (%)</td>
<td>34.4±28.4</td>
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</tbody>
</table>

VPCs, vascular progenitor cells; VE-Cad, VE-cadherin; SMA, smooth muscle actin; VCAM, vascular cellular adhesion molecule; ICAM, intercellular adhesion molecule.

### Table 3. Univariate Analysis of Demographic, Biochemical, and Flow Cytometric Variables Related to the Gensini Score of Study Subjects (n=50)

<table>
<thead>
<tr>
<th>Categorical variables</th>
<th>Mean ± SD or Pearson’s correlation coefficient</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
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<tr>
<td>Male (n=44)</td>
<td>29.9±21.5%</td>
<td>0.97</td>
</tr>
<tr>
<td>Female (n=6)</td>
<td>29.5±23.1%</td>
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<tr>
<td>Smoking</td>
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<tr>
<td>Yes (n=24)</td>
<td>32.4±25.4%</td>
<td>0.43</td>
</tr>
<tr>
<td>No (n=26)</td>
<td>27.4±17.3%</td>
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<tr>
<td>Hypertension</td>
<td></td>
<td></td>
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<tr>
<td>Yes (n=27)</td>
<td>30.3±20.4%</td>
<td>0.86</td>
</tr>
<tr>
<td>No (n=23)</td>
<td>29.2±23.2%</td>
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<tr>
<td>Diabetes</td>
<td></td>
<td></td>
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<tr>
<td>Yes (n=11)</td>
<td>37.4±22.0%</td>
<td>0.16</td>
</tr>
<tr>
<td>No (n=39)</td>
<td>27.7±16.5%</td>
<td></td>
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<tr>
<td>Continuous variables</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.048</td>
<td>0.74</td>
</tr>
<tr>
<td>No. of diseased vessels</td>
<td>0.079</td>
<td>0.58</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>0.36</td>
<td>0.01</td>
</tr>
<tr>
<td>No. of circulating endothelial progenitor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+KDR+ (%)</td>
<td>0.17</td>
<td>0.23</td>
</tr>
<tr>
<td>AC133+KDR+ (%)</td>
<td>0.02</td>
<td>0.91</td>
</tr>
<tr>
<td>Profile of differentiated VPCs</td>
<td></td>
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</tr>
<tr>
<td>VE-Cad+α-SMA- VPC (%)</td>
<td>−0.009</td>
<td>0.95</td>
</tr>
<tr>
<td>VE-Cadα-SMA- VPC (%)</td>
<td>−0.005</td>
<td>0.97</td>
</tr>
<tr>
<td>VE-Cad+α-SMA- VPC (%)</td>
<td>0.42</td>
<td>0.002</td>
</tr>
<tr>
<td>VE-Cad-α-SMA- VPC (%)</td>
<td>−0.11</td>
<td>0.45</td>
</tr>
<tr>
<td>VCAM-1 (%)</td>
<td>−0.1</td>
<td>0.57</td>
</tr>
<tr>
<td>E-selectin (%)</td>
<td>0.004</td>
<td>0.98</td>
</tr>
<tr>
<td>ICAM-1 (%)</td>
<td>0.37</td>
<td>0.008</td>
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</table>

Abbreviations see in Table 2.
Statistical Analysis

All continuous data are expressed as the mean±standard deviation (SD), and categorical variables are presented as number (percentage). Univariate analysis was performed using an unpaired t-test or Pearson correlations to determine variables associated with the I-M ratio (in the animal model) or Gensini score. An analysis of variance (ANOVA) followed by Tukey’s post-hoc test was used to compare differences among multiple groups. Multiple linear regression was used to assess the independent effects of relevant factors on the Gensini score after controlling for covariates. The information gathered from study subjects was also evaluated by calculating the appropriate SD and 95% confidence intervals. All statistical analyses were two-sided and performed using SPSS software (v.15.0, SPSS, Chicago, IL, USA). A P value <0.05 was considered significant.

Results

Patients’ Characteristics

Demographic data of the 50 patients enrolled in this study are shown in Table 1. The majority of patients were male (88%).
The average Gensini score was 29.8±21.5.

**Heterogeneous Populations of VPCs**

Colonies of VPCs were evident 2 weeks after PBMNCs were plated. The VPCs became confluent, however, heterogeneously, with some having a cobblestone appearance and some having an elongated morphology (Figure 1A). Although most cells stained positive for CD31 and vWF (Figure 1B), in a subpopulation of VPCs the expression of a variety of smooth-muscle phenotypes was demonstrated, including α-SMA, H-caldesmin, and calponin (Figure 1C). At passage 1 after stimulation with VEGF and PDGF, VPCs were subjected to flow cytometry to assess the levels of endothelial and smooth muscle phenotypes. Flow cytometry showed that VPCs could be differentiated into VE-Cad^low^α-SMA^+^ component in VPCs after differentiation, and ICAM expression by VPCs were significantly associated with higher Gensini scores. However, there was no significant correlation between the Gensini score and other variables such as sex, smoking, hypertension, diabetes mellitus, or numbers of CD34^+^KDR^+^ and AC133^+^KDR^+^ EPCs. In the multivariate analysis, the percentage of VE-Cad^low^α-SMA^+^ in VPCs after differentiation and the LDL-C level were parameters independently associated with the Gensini score; however, the level of ICAM-1 expression by VPCs became insignificant (Table 4).

**Figure 3.** Contribution of vascular progenitor cells (VPCs) to intimal hyperplasia in an animal model. (A) At passage 1, microbead-labeled HLA-ABC^+^ VPCs were injected into the abdominal aorta of SCID mice 4 days after left femoral artery wire injury. A magnetic field created around the injured femoral artery was used to help VPCs adhere to the surface of the injured femoral artery. (B) At 3 weeks after cell injection, HLA-ABC^+^ VPCs are present on the injured vessels, have differentiated into endothelial and smooth-muscle-like cells, and are contributing to intimal hyperplasia. A, adventitia; L, lumen. Arrows indicate internal elastic lamina. (C) The amount of VE-Cad^low^α-SMA^+^ VPCs significantly correlates with the intima-media (I-M) ratio. SMA, smooth muscle actin.

**Correlations of Demographic, Biochemical, and Flow Cytometric Variables With the Gensini Score**

Table 3 demonstrates that higher blood levels of low-density lipoprotein cholesterol (LDL-C), percentage of the VE-Cad^low^α-SMA^+^ component in VPCs after differentiation, and ICAM expression by VPCs were significantly associated with higher Gensini scores. However, there was no significant correlation between the Gensini score and other variables such as sex, smoking, hypertension, diabetes mellitus, or numbers of CD34^+^KDR^+^ and AC133^+^KDR^+^ EPCs. In the multivariate analysis, the percentage of VE-Cad^low^α-SMA^+^ in VPCs after differentiation and the LDL-C level were parameters independently associated with the Gensini score; however, the level of ICAM-1 expression by VPCs became insignificant (Table 4).

**Magnet-Assisted Cell Adhesion and Rolling**

In the static adhesion assay, cell adhesion was estimated by applying a magnetic field of 5,000 Gauss for 5 and 15 min. Compared with the control group, VPC adhesion to disrupted mouse endothelial cells significantly increased after the magnetic field had been applied for 15 min (P<0.01, Figure 2A).

In the flow chamber, the VPC rolling speed was estimated under different shear stress conditions of 5–20 dyne/cm². Compared with the control group, a magnetic field of 5,000 Gauss significantly slowed down the velocity of VPCs, especially under a low flow rate (Figure 2B). In the in vivo model (wire-induced femoral artery injury), a magnetic field of 5,000 Gauss...
also dramatically decreased the velocity of VPCs on the surface of the injured vessel.

**Correlation of the VE-Cad<sup>low/−</sup>α-SMA<sup>+</sup> VPC Level With IH in the Animal Model**

Further investigation was performed to assess the association between the level of VE-Cad<sup>low</sup>α-SMA<sup>+</sup> VPCs and the development of IH in the animal model. At passage 0, VPCs were labeled with microbeads and then injected into the abdominal aorta of SCID mice after femoral artery wire injury (Figure 3A). A magnetic field was created around the injured femoral artery to help VPCs adhere to the surface of the injured femoral artery. As shown in Figure 3B, at 3 weeks after cell injection VPCs were still present on the injured vessels and had differentiated into both endothelial cells (vWF<sup>+</sup>) and α-SMA<sup>+</sup> vWF<sup>−</sup> cells (HLA-ABC<sup>+</sup> indicates injected human cells), contributing to IH. Furthermore, the level of VE-Cad<sup>low</sup>α-SMA<sup>+</sup> VPCs estimated by flow cytometry was significantly correlated with the I-M ratio (r=0.69, P<0.0001) (Figure 3C).

**Discussion**

Our data demonstrate that VPCs are a heterogeneous population. The level of VE-Cad<sup>low</sup>α-SMA<sup>+</sup> VPCs correlated with the Gensini score, a scoring system indicating the severity of coronary atherosclerosis. This finding was also supported by the correlation between the level of VE-Cad<sup>low</sup>α-SMA<sup>+</sup> VPCs and the development of IH after vascular injury in the animal model. Manipulating the fate of VPC differentiation may be a target for attenuating atherosclerosis.

EPCs are considered a potential cell population for therapy of a variety of cardiovascular diseases. However, EPCs are not ready to be used as a clinical therapy because they are a heterogeneous population, and their associated biological functions are yet to be fully clarified. Actually, based on the current methodology of culturing EPCs, the outgrowth cells at least contain EPCs and SMPCs. Under different culture environments and various genetic traits, there are remarkable variations in the component VPCs. For example, the distribution of different VPC subtypes as shown in Figure 2 correlated with coronary risk factors. SMPCs are cultivated more easily in a hypoxic environment. On the other hand, accumulating evidence suggests that EPCs and SMPCs play different roles in spontaneous vascular events and in vascular remodeling after injury. EPCs are able to achieve early re-endothelialization and accelerate angiogenesis after tissue injury. However, SMPCs expedite lesion formation during restenosis, and also serve to stabilize atherosclerotic plaques by producing extracellular matrix proteins. SMPCs appear to be a double-edged sword in the pathogenesis of atherosclerosis. In addition, both EPCs and SMPCs express high levels of ICAM-1. However, only EPCs can be activated by endothelins. Nevertheless, there is no available information about whether the characteristics of these VPC phenotypes are associated with the severity of coronary atherosclerosis.

During the cultivation of VPCs, simulating the microenvironment after vascular injury by adding both VEGF and PDGF, we showed that VPCs differentiate into at least 4 populations. Although the number of patients was limited, the findings demonstrate that the level of VE-Cad<sup>low</sup>α-SMA<sup>+</sup> VPCs is a significant parameter associated with the Gensini score, independent of LDL-C levels. At the present time, whether this VPC component exerts its atherogenic effect directly or indirectly still has to be elucidated. Previously, we showed that the population of VE-Cad<sup>low</sup>α-SMA<sup>+</sup> VPCs has a clear relationship with the development of IH after coronary stenting. In this study using an animal model, we further showed that the in vivo environment guided VPCs to differentiate into mature cells with either a pure endothelial or smooth-muscle phenotype. However, the thickness of the IH was significantly related to the percentage of VE-Cad<sup>low</sup>α-SMA<sup>+</sup> cells among VPCs, lending further support to the notion. These findings are also compatible with a report by Caplice et al demonstrating the existence of circulating SMPCs and showing that circulating SMPCs contribute to smooth-muscle cells in atheromas. Our data also suggest that lowering the number of VE-Cad<sup>low</sup>α-SMA<sup>+</sup> VPCs will attenuate the formation of IH. In the future, determining how to ensure that less VPCs to differentiate to VE-Cad<sup>low</sup>α-SMA<sup>+</sup> VPCs will be an important and clinically relevant issue.

Levels of expressed adhesion molecules are critically associated with the rolling of neutrophils and their adhesion to activated endothelial cells. Provided that VPCs are able to be incorporated into the vasculature undergoing atherosclerosis, a higher level of adhesion molecules expressed by VPCs would supposedly recruit more inflammatory cells to atheromas and hyperplastic neointima. Previously, we showed that both EPCs and SMPCs express significant levels of ICAM-1. However, only EPCs can be activated to express highly functioning ICAM-1. Based on this finding, the amount of EPCs among VPCs is a critical issue. It is not surprising that the relationship between the ICAM-1 level and Gensini score became insignificant after adjusting for the level of VE-Cad<sup>low</sup>α-SMA<sup>+</sup> VPCs in the multivariate analysis model.

**Study Limitations**

To study the role of VPCs in the development of atherosclerosis, ApoE<sup>−/−</sup> mice provide the best microenvironment simulating atherosclerosis. However, to investigate the in vivo differentiation and growth of human VPCs in ApoE<sup>−/−</sup> mice, an immunodeficiency background is an obligatory prerequisite for the mice to be able to immuno-tolerate human cells. So far, ApoE<sup>−/−</sup> nude or ApoE<sup>−/−</sup> SCID/NOD mice are not available. The alternative is to use ApoE<sup>−/−</sup>/RAG2<sup>−/−</sup> mice, which are totally deficient in B and T lymphocytes. Using this animal model, 4 sessions of cell therapy, together with a high-fat diet, are needed to evaluate the contribution of VPCs to atheroma formation. However, with separate injections of VPCs, it is not possible to control the stationary phenotypes expressed by VPCs. Accordingly, we adopted a model of injecting human VPCs once into SCID mice after femoral artery wire injury. By observing the contribution of the heterogeneous VPCs to post-injury vascular remodeling, similar information on their role in atherogenesis can be deduced.

**Conclusion**

VPCs are a heterogeneous population of cells, and some subpopulations may, in fact, be related to the severity of coronary atherosclerosis either directly or indirectly. New innovative strategies need to be developed to guide the differentiation of VPCs away from such subpopulations.

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References


Supplemental Files

Supplemental File 1

Figure S1. (A) For the static adhesion assay, experiments were performed with and without a magnet (arrow) applied beneath the culture dish.

Please find supplemental file(s):