Factors associated with purity, biological function, and activation potential of endothelial colony-forming cells

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¹Heart Failure Center, Division of Cardiology, Department of Internal Medicine, Chang Gung Memorial Hospital at Keelung, and Chang Gung University College of Medicine, Taoyuan; ²Graduate Institute of Clinical Medical Sciences, Chang Gung University, Taoyuan; ³Institute of Clinical Medicine, National Yang-Ming University, Taipei; and ⁴Department of Medical Research and Education, Cheng Hsin General Hospital, Taipei, Taiwan

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Wang CH, Hsieh IC, Pang JHS, Cherng WJ, Lin SJ, Tung TH, Mei HF. Factors associated with purity, biological function, and activation potential of endothelial colony-forming cells. Am J Physiol Regul Integr Comp Physiol 300: R586–R594, 2011. First published December 15, 2010; doi:10.1152/ajpregu.00450.2010.—Endothelial colony-forming cells (ECFCs) are undergoing extensive investigations to tackle certain deliberating cardiovascular diseases. However, the success of this approach depends on a thorough understanding of ECFC biology. This study sought to determine the factors associated with the purity, biological function, and activation potential of ECFCs. Seventy-three patients with newly diagnosed coronary artery disease (CAD) and 24 controls were studied. ECFCs were cultured for up to 10 passages to investigate changes in and the impact of coronary risk factors on ECFC biological functions and the other factors associated with purity, biological function, and activation potential of ECFCs exhibited higher endothelial phenotype expression and better biological functions, in terms of nitric oxide secretion and tubular formation, but lower activation potentials compared with later passages (P < 0.05). Studies on passage 3 showed that endothelial phenotype expression and biological functions were impaired, and the activation potentials of the ECFCs were significantly upregulated in subjects with coronary risk factors and especially those with CAD (P < 0.05). Furthermore, ECFCs were already activated before inflammatory stimulation in subjects with diabetes mellitus, hypertension, and CAD. Atorvastatin upregulated the endothelial nitric oxide synthase expression of ECFCs in CAD patients (P < 0.01), although not up to the baseline level of controls. In conclusion, the passage number and a variety of coronary risk factors were associated with the purity, biological function, and activation potential of ECFCs. Functional assessments and manipulations of ECFCs need to be pursued in patients with extensive risk factors.

endothelial progenitor cell; risk factor; atherosclerosis; cell therapy

THE DISCOVERY OF CIRCULATING endothelial progenitor cells (EPCs) in 1997 opened up a new era of EPC-based therapies for angiogenesis in critical ischemic tissues (1, 2, 16, 20), postinjury vascular endothelial regeneration (5, 10, 31), and ex vivo tissue engineering (3). Previous studies demonstrated two major types of circulating EPCs, early and late EPCs, also called endothelial colony-forming cells (ECFCs) (9). Although early EPCs enhance angiogenesis by providing a variety of cytokines, cell therapy with highly proliferative autologous ECFCs is a seminal therapeutic option to promote endothelial regeneration in a variety of cardiovascular diseases. Although researchers are enthusiastic about applying ECFCs to therapeutic experiments (35), progress has been substantially limited due to a lack of a thorough characterization and understanding of ECFC biology.

On the other hand, a concept has emerged in recent years that endothelial dysfunction plays a key role in intimal hyperplasia (22), atherosclerotic lesion formation, progression, and eventual rupture (8, 34). Although measures of endothelial function have improved our understanding of the pathophysiology of atherosclerosis and are becoming well-established surrogates of disease activity and risk (34), ideal tests of endothelial function have yet to be established. Similarly to endothelial cells, ECFCs may become activated in response to proinflammatory stimuli (36). This activation potential estimated for patients’ own ECFCs may be relevant to disease outcomes and can possibly be used to tailor individualized therapeutic strategies. Moreover, tissue engineering cardiovascular structures with potentially atherogenic ECFCs may even be detrimental (36). So far, it has yet to be established when ECFCs expanded ex vivo switch from being healthy to atherogenic and whether ECFCs from patients with different risk factors behave differently in their response to proinflammatory stimuli.

Before ECFCs can be extensively applied to clinical therapies, further study is necessary. The present study focused on subjects with coronary artery disease (CAD) and investigated at what passages ECFCs reach a purity high enough for clinical use and have the best biological functions for therapeutic tissue engineering. Finally, we delineated correlations of endothelial phenotype expression, biological function, and activation profiles of ECFCs with the presence of various coronary risk factors.

MATERIALS AND METHODS

Study Design

In total, 141 subjects undergoing coronary angiography were recruited for ECFC cultivation (Supplemental Table 1). (Supplemental data for this article is available online at the American Journal of Physiology-Regulatory, Integrative and Comparative Physiology website.) Patients with acute coronary syndrome, statin use, or concomitant inflammatory or malignant disease were excluded. In 44 (31.2%) subjects, ECFCs could not be cultivated. In the remaining 97 subjects in which ECFCs were grown out, 73 patients were found to have newly angiographically diagnosed CAD. The control group (n = 24) included 12 women and 12 men without significant coronary artery stenosis on angiograms. After angiography was performed, 50
ml of peripheral blood were collected into tubes containing EDTA in the catheterization room and sent to the core laboratory to cultivate the ECFCs. In the first 40 subjects (22 CAD patients and 18 controls), ECFCs were cultured for up to 10 passages to investigate changes in the phenotypes, biological functions, and atherogenic potentials at different passages. This trial was designed and carried out in accordance with the principles of the Declaration of Helsinki and with approval from the Ethics Review Board of Chang Gung Memorial Hospital. Written informed consent was obtained from all subjects.

**Definition of Risk Factors for CAD**

Hypertension was defined as a history of hypertension for >1 yr that required antihypertensive therapy. Smoking was self-reported and was defined as patients revealing a history of smoking more than 2 pack-years and still currently smoking. Hypercholesterolemia was defined as fasting low-density lipoprotein (LDL) cholesterol levels exceeding 130 mg/dl. Diabetes mellitus (DM) was defined as the need for hypoglycemic medication or insulin use. The age was set to ≥60 yr to examine the impact of age on ECFCs. The family history was considered positive if either the study subject’s father or mother had been diagnosed with CAD at or before the age of 55 yr.

**Isolation and Cultivation of Late ECFCs**

Mononuclear cells were isolated from peripheral blood by density gradient centrifugation with Ficoll separating solution (Sigma, St. Louis, MO). After resuspension in EGM-2 medium (containing vascular endothelial growth factor, 10 ng/ml), 1 × 10^6 mononuclear cells/cm² were plated on fibronectin-coated dishes (6 cm in diameter) or two-chamber slides. Nonadherent cells were removed 4 days after cells were plated, and cell medium was replenished every 3–4 days. After mononuclear cells were cultured in EGM-2 for 2 wk, colonies of ECFCs were noted and subcultured at appropriate times (usually at the 3rd week after primary culture). ECFCs were subcultured at 80–90% confluence and examined from the 3rd week after primary culture. This trial was designed and carried out in accordance with the principles of the Declaration of Helsinki and with approval from the Ethics Review Board of Chang Gung Memorial Hospital. Written informed consent was obtained from all subjects.

**Fluorescence-Activated Cell Sorting Analysis**

When confluent, the expression of adhesion molecules by ECFCs was assessed by flow cytometry before and after stimulation with tumor necrosis factor (TNF)-α (2 ng/ml) for 6 h. The estimated adhesion molecules included vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and E-selectin.

**Fluorescence-Activated Cell Sorting Analysis**

Fluorescence-activated cell sorting (FACS; FacScan, Becton Dickinson) was performed to estimate the phenotypes of ECFCs at each passage with phycoerythrin (PE)-conjugated anti-human CD144 (Sero), anti-α-Ki, anti-CD31, and biotin-conjugated anti-human KDR antibodies (eBioscience), with secondary detection by FITC-conjugated streptavidin. α-Smooth muscle actin (α-SMA), an intracellular antigen, was exposed using a Cytofix/Cytoperm kit (Pharmingen, San Jose, CA) and stained with an FITC-conjugated anti-α-SMA antibody (Sigma). Analysis was performed on all cells without gating on a specific population (Supplemental Fig. S1A). Adhesion molecules of ECFCs were estimated with FITC-conjugated anti-human VCAM-1 antibodies (Sero), PE-conjugated ICAM-1, and biotin-conjugated anti-human E-selectin antibodies (eBioscience), with secondary detection by FITC-conjugated streptavidin. Since ICAM-1 expression contained populations with low and high fluorescence intensities, the analysis focused only on the population with a high fluorescence intensity (Supplemental Fig. S2).

**Biological Functions**

**NO assay.** The concentration of total NO (nitrite plus nitrate) secreted by ECFCs was measured using an ELISA kit (R&D Systems, Minneapolis, MN) 24 h after the medium was replaced.

**Tubular formation assay.** A Matrigel tube formation assay was performed to assess in vitro angiogenesis at different passages of ECFCs. Growth factor-reduced Matrigel (Becton Dickinson) was placed in 24-well tissue culture plates (150 μl/well) and allowed to set at 37°C for 30 min, and then 5 × 10^4 ECFCs were added to each well and incubated in EBM-2. Morphological changes were observed and photographed using a phase-contrast microscope at 24 h.

**Confocal Microscopy**

Cells were stained with an anti-von Willebrand factor (vWF) antibody (DAKO) and an anti-CD31 antibody (BD Pharmingen), followed by incubation with FITC- or PE-conjugated secondary antibodies. Slides were mounted using a Prolong Antifade kit (Molecular Probes, Eugene, OR) and observed under a confocal microscope (Leica TCS SP2 AOBS). Nuclei were stained with Hoechst 33258 (Sigma).

**Western blot Analysis**

ECFCs at P3 were cultured with or without atorvastatin (1 μM) treatment for 48 h and lysed with RIPA lysis buffer containing freshly added protease inhibitor cocktail (Sigma) and 500 μM phenylmethylsulfonyl fluoride. Protein concentrations were determined using the dye-binding assay (Bio-Rad, Hercules, CA). Total protein (30 μg) was subjected to electrophoresis on Cosmo PAGE Bis-Tris gels (4–15%; Nacalai USA, San Diego, CA), followed by electrophoretic transfer to polyvinylidene difluoride membranes at 300 V for 1.5 h. Membranes were blocked in 5% dried milk in TBST (15 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 1 h, followed by incubation with a rabbit anti-human endothelial NO synthesize (eNOS) antibody (Cell Signaling Technology) for 1 h at room temperature. After five washes with TBST, membranes were incubated in a horseradish peroxidase-conjugated secondary antibody for 1 h and washed with TBST five times. Immunoblots were visualized using LumiGLO chemiluminescent substrate (Millipore).

**Statistical Analysis**

Data were means ± SE or number (%). Continuous variables were tested for a normal distribution with the Kolmogorov-Smirnov test. Differences between groups were analyzed by t-test. Nonparametric tests were used in case of a nonnormal distribution. Intragroup differences between P2 and every other passage were compared by analysis of variance. Categorical variables were compared by χ² test and Fisher’s exact test. Correlations of coronary risk factors with endothelial phenotype expressions and biological function of ECFCs at P3 were examined by multivariate analysis using a linear regression model. Statistical significance was assumed if the null hypothesis could be rejected at P = 0.05. All statistical analyses were performed using SPSS for Windows 15.0.

**RESULTS**

**Patient Characteristics**

Compared with those who had no ECFC outgrowth, subjects with ECFC outgrowth had a higher number of diseased coronary arteries; however, there were no significant differences in terms of age, sex, number of risk factors, or lipid profiles (Supplemental Table 1). In total, 97 subjects with ECFC outgrowth were studied, including 73 patients with CAD and 24 controls. Patient characteristics are summarized in Table 1. Compared with the controls, patients in the CAD group in-
Table 1. Baseline clinical characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All Subjects</th>
<th>CAD Group</th>
<th>Control Group</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>97</td>
<td>73 (75.3)</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>60.9 ± 1.1</td>
<td>60.6 ± 1.3</td>
<td>61.9 ± 1.7</td>
<td>0.34</td>
</tr>
<tr>
<td>Male sex, no. (%)</td>
<td>73 (75.3)</td>
<td>61 (83.6)</td>
<td>12 (50.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26.9 ± 0.8</td>
<td>26.9 ± 0.5</td>
<td>26.7 ± 1.2</td>
<td>0.83</td>
</tr>
<tr>
<td>Diseased coronary arteries, no. (%)</td>
<td>0</td>
<td>0 (0)</td>
<td>24 (100)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Medical history, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>55 (56.7)</td>
<td>41 (56.2)</td>
<td>14 (58.3)</td>
<td>1.0</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>28 (28.9)</td>
<td>25 (34.2)</td>
<td>3 (12.5)</td>
<td>0.07</td>
</tr>
<tr>
<td>Current smoking</td>
<td>40 (41.2)</td>
<td>35 (47.9)</td>
<td>5 (20.8)</td>
<td>0.03</td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>16 (16.5)</td>
<td>14 (19.2)</td>
<td>2 (8.3)</td>
<td>0.343</td>
</tr>
<tr>
<td>Lipid profile, mg/dl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>182.5 ± 4.9</td>
<td>186.9 ± 5.6</td>
<td>169.0 ± 9.7</td>
<td>0.11</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>113.9 ± 4.5</td>
<td>116.6 ± 5.3</td>
<td>105.6 ± 8.5</td>
<td>0.29</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>35.2 ± 1.1</td>
<td>35.9 ± 1.2</td>
<td>33.0 ± 2.3</td>
<td>0.24</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>177.3 ± 21.0</td>
<td>185.3 ± 27.5</td>
<td>152.9 ± 15.6</td>
<td>0.51</td>
</tr>
<tr>
<td>Current medication, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>82 (84.5)</td>
<td>61 (83.5)</td>
<td>21 (87.5)</td>
<td>0.76</td>
</tr>
<tr>
<td>PPAR-γ agonists</td>
<td>1 (1.0)</td>
<td>1 (1.4)</td>
<td>0 (0)</td>
<td>1.0</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>62 (63.9)</td>
<td>48 (65.8)</td>
<td>14 (58.3)</td>
<td>0.63</td>
</tr>
<tr>
<td>ACE inhibitors/ARB</td>
<td>42 (43.3)</td>
<td>33 (45.2)</td>
<td>9 (37.5)</td>
<td>0.64</td>
</tr>
<tr>
<td>Insulin</td>
<td>1 (1.0)</td>
<td>1 (1.4)</td>
<td>0 (0)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Continuous data are means ± SE; discrete data are number counts with percentages in parentheses. CAD, coronary artery disease; LDL, low-density lipoprotein; HDL, high-density lipoprotein; PPAR, peroxisome proliferator-activated receptor; ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.

Included a higher percentages of males and a current smoking habit. In the first 40 patients (22 CADs and 18 controls), ECFCs were cultivated for up to 10 passages to determine changes in endothelial phenotype expression, biological function, and the activation potential. The preliminary results were used to choose an appropriate passage of ECFCs for further analysis of the entire study population (97 subjects). There were no differences in medications between the CAD and control groups.

Changes in Endothelial Phenotype Expressions on ECFCs During Passaging

Endothelial phenotypes expressed on ECFCs were estimated after P1 (Fig. 1A). Positive controls for the FACS analysis are shown in Supplemental Fig. S1B. To compare the phenotypic changes in the entire cell population between different passages, no gating strategy was used for the FACS analysis. Positive rates of CD31 became higher than 80% at P3, P4, and P5, declined from P6, and were lower than 50% from P9 (Fig. 1B). The expression of CD144, an intercellular junction protein, gradually increased from P2, reached a peak at P5, and gradually declined from P6. At P3–P5, the positive rates of CD31 and CD144 were significantly higher in the controls than in CAD patients. Expressions of KDR, a receptor of VEGF, and c-kit, a marker of hematopoietic stem cells, were higher in early than in late passages. Furthermore, in early passages, positive rates of KDR and c-kit were also higher in the controls than in CAD patients. As demonstrated by FACS, our method of cultivating ECFCs produced different subgroups defined on the basis of forward and side scatter characteristics and expression levels of CD144 and α-SMA (Supplemental Fig. S3, A and B) (32). In addition, a higher number of α-SMA+ cells was correlated with the presence of DM (P = 0.001), CAD (P = 0.041), and an elderly age (P = 0.019).

Changes in Biological Functions: NO Secretion and Tubular Formation

NO secretion and the tubular formation capacity on Matrigel were evaluated at different passages. The amount of NO secreted by ECFCs reached a peak at P3 and then significantly decreased from P5 (Fig. 2A, left). NO secretion became very low in late passages. As demonstrated by Western blotting analysis, eNOS expression was noted only in the CD31+ population (Fig. 2B). After correction for the number of CD31+ cells, differences in the amounts of NO secreted in serial passages remained and were even more remarkable. The tubular formation capacity was functioning well at P3 but deteriorated as the number of passages increased (Fig. 2A).

Factors Related to Endothelial Phenotype Expression and Biological Function

The association of coronary risk factors with endothelial phenotype expression levels and biological functions was investigated. A lower CD31+ rate of ECFCs at P3 was correlated with the male sex and the presence of DM, smoking, and CAD (Fig. 1C, top). A lower level of CD144 expression by ECFCs was related to the male sex, the presence of DM and CAD, and a family history of CAD (Fig. 1C, bottom). Lower NO secretion by ECFCs was correlated with the presence of smoking, hyperlipidemia, and CAD and a family history of CAD (Fig. 2B, left). Western blot analysis showed that eNOS expression levels in ECFCs from CAD patients were lower than those from normal subjects (P < 0.01). Ex vivo incubation with atorvastatin (1 μM) significantly upregulated the expression of...
eNOS protein by ECFCs from patients with CAD ($P < 0.01$), although it was still lower than the expression levels in normal controls at the baseline.

The multivariate analysis revealed that CAD and a family history of CAD were factors independently associated with a lower level of endothelial phenotype expression and NO secretion (Table 2). DM and a family history of CAD were independent factors related to lower endothelial phenotype expression. Smoking was only associated with an impaired NO secretion ability.

**Changes in Adhesion Molecules Expressed by ECFCs**

Before TNF-α stimulation, the expressions of adhesion molecules were estimated at different passages in 40 subjects (Fig. 3). Expression levels of VCAM-1 did not significantly change throughout the passages (Fig. 3, top). Expression levels of E-selectin were low at P2–P5, gradually increased to a peak at P6, and then decreased. Expression levels of ICAM-1 were similar from P2 to P5, significantly increased at P6, and then decreased. After TNF-α stimulation, significant increases from levels before stimulation were seen from P5 for VCAM-1 and E-selectin and from P8 for ICAM-1 compared with P2 (Fig. 3, bottom). In the subgroup analysis, significant increases in the expressions of all three adhesion molecules after TNF-α stimulation were noted in subjects with CAD in the early passages compared with the controls. Examples of staining are shown in Supplemental Fig. S2. Further analysis showed that activation was mainly located in the CD31+ population (Supplemental Fig. S4).

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**Association Between Risk Factors and Adhesion Molecule Expressions by ECFCs**

Further analysis of all 97 subjects was performed to relate coronary risk factors to the expressions of adhesion molecules by ECFCs, which were used as surrogates for endothelial activation and the activation potential. The presence of CAD was associated with a higher ICAM-1 level at the baseline and stronger expressions of all 3 adhesion molecules in response to TNF-α (Table 3). DM and hypertension and high LDL cholesterol were respectively correlated with higher baseline levels of ICAM-1 and E-selectin. A stronger response of E-selectin expression to TNF-α stimulation was noted in subjects ≥60 yr of age and with DM. A stronger response of ICAM-1 expression to TNF-α stimulation was also noted in subjects of male sex and with DM. No risk factor other than the presence of CAD was correlated with baseline levels of VCAM-1 or its response to TNF-α stimulation.
DISCUSSION

Cell Purity

The dawn of understanding ECFCs heralds an era of applying autologous endothelial cell therapy to cardiovascular diseases. However, we and others recently demonstrated that ex vivo-cultured ECFCs may be substantially contaminated by other cell types in peripheral blood, such as circulating mesenchymal stem cells (14, 31), smooth muscle progenitors (25), and inflammatory cells. Our present data revealed that the purity of ECFCs is acceptable for their use in early passages.

A recent report suggested a gross misinterpretation of early EPC phenotypes by uptake of platelet microparticles (23), our study on late EPCs and the increase in expression Table 2. Multivariate analysis examining factors related to endothelial phenotype expression and biological function of ECFCs

<table>
<thead>
<tr>
<th>Variable</th>
<th>CD31</th>
<th>CD144</th>
<th>Nitric Oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-Value</td>
<td>Value</td>
<td>β-Value</td>
</tr>
<tr>
<td>Age ≥60 yr</td>
<td>0.12</td>
<td>0.43</td>
<td>-0.15</td>
</tr>
<tr>
<td>Male sex</td>
<td>-0.38</td>
<td>0.01</td>
<td>0.16</td>
</tr>
<tr>
<td>Hypertension</td>
<td>-0.17</td>
<td>0.36</td>
<td>-0.23</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>-0.22</td>
<td>0.02</td>
<td>-0.39</td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>0.16</td>
<td>0.38</td>
<td>-0.53</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>0.12</td>
<td>0.49</td>
<td>0.25</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.1</td>
<td>0.51</td>
<td>-0.12</td>
</tr>
<tr>
<td>Presence of CAD</td>
<td>-0.7</td>
<td>0.0004</td>
<td>-0.76</td>
</tr>
</tbody>
</table>

Data for endothelial colony-forming cells (ECFCs) are from 97 subjects.
from P2 to P3 preclude this possibility. Notably, low expression levels of these endothelial phenotypes were found in subjects with DM, a male sex, and a family history of or the presence of CAD; this may have been due to impaired differentiation and proliferation capacities or increased heterogeneous populations of circulating cells (17), or because cells from patients with CAD present different surface antigen profiles. Nevertheless, the overall levels were significantly lower than reported in the data published by Lin et al. (18) and Ingram et al. (12), who demonstrated that almost all ECFCs express a variety of endothelial phenotypes. One of the major reasons for this is that no gating strategy was used for the FACS analysis in our study so that we could scientifically and fairly reveal the trend of changes from P2 to P10. The discrepancy also may have been due to the obviously different patient populations between studies and different cell cultivation protocols. The subjects in the studies by Lin et al. and Ingram et al. (18) were relatively younger than our subjects (41.5 ± 0.6 yr in Lin et al. and 22–50 yr in Ingram et al. vs. 60.9 ± 1.1 years in the present study). Ingram et al. removed nonadherent cells 24 h after the cells had been plated. However, the patients, even the controls, in our study were clinically suspected of having CAD and were undergoing coronary angiograms, and they may represent a population in need of cell therapy in the real world.

Previously, Güven et al. (7) demonstrated that higher numbers of circulating EPCs were associated with the presence of significant CAD compared with normal controls. However, ECFCs did not grow out in ~40% of their patients, which is compatible with the 31.2% in our study. Although the number of late EPC colonies was higher in patients with CAD than in control subjects, the present data obviously demonstrate a higher number of other contaminating cells in subjects with coronary risk factors. Cytokines released by ischemic stress probably mobilize progenitors other than ECFCs. Previously, we showed that ECFCs contain a subpopulation of cells coexpressing smooth muscle phenotypes (32). Thus specific purification or pharmaceutical manipulation processes should be conducted before cell implantation (32, 36). It is still not known whether CD31 is a good marker for ECFC purification, since different CD31 positive populations as shown in Supplemental Fig. S3A have not yet been demonstrated to have similar endothelial functions. Our data provide useful references as a basis for future ECFC purification and manipulation.

**Biological Functions**

NO is the key endothelium-derived relaxing factor that plays pivotal roles in maintaining vascular function in a healthy state (30). ECFCs exhibited well-preserved biological functions in early passages as suggested by good NO-secreting and angiogenesis capacity. The impaired biological function of subjects with CAD or risk factors may be related to a lower ECFC purity and ECFC dysfunction. Although our study did not investigate whether pure ECFCs from patients with CAD have the same NO-secreting ability as those from controls, the remarkably low eNOS expression levels in the CAD group

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Fig. 3. Expression of adhesion molecules at different passages. Levels of vascular cell adhesion molecule (VCAM)-1, E-selectin, and intercellular adhesion molecule (ICAM)-1 expressed on ECFCs before and after tumor necrosis factor-α stimulation at different passages in 40 subjects (Total) and separately in subjects with CAD (n = 22) and in controls (n = 18). *P < 0.05 compared with control at P2. ‡P < 0.05 compared with CAD at P2. †P < 0.05 compared with total at P2. ††P < 0.05 compared with controls at the same passage.
suggest an impaired NO-secreting capacity of ECFCs in CAD patients. Assays of purified CD31$^+$ ECFCs may clarify this issue in the future. On the other hand, the relations of coronary risk factors to eNOS expression and the NO secretion of ECFCs support the hypothesis that functional assays of ex vivo-cultured ECFCs in early passages can potentially be used to assess individualized endothelial function, which has been implicated in the pathogenesis of various cardiovascular diseases and is associated with a risk of cardiovascular events (8, 22, 34).

Effects of Risk Factors on the Activation Potential of ECFCs

Over the last decade or so, the concept has emerged that vascular inflammation plays a key role in endothelial dysfunction, and a variety of soluble forms of inflammatory markers have been evaluated as predictors of atherosclerotic lesion formation and outcomes (26). The upregulated expression of adhesion molecules on activated endothelial cells has been used as an indicator of atherogenesis (29). Our data support the use of ECFCs in early passages, again based on their lower adhesion molecule expression levels and activation potential. The higher activation potential in late passages might have been due to aging or endothelial dysfunction in the ex vivo environment, although the exact underlying mechanisms still need to be elucidated. In our study, levels of adhesion molecules expressed on ECFCs were lower than expected, as seen in mature endothelial cells both before and after proinflammatory stimulation. One possible explanation may be related to the heterogeneous population of cells, the “ungated” strategy for the FACS analysis, and the proliferation status. Previously, Luo et al. (19) showed that E-selectin expression is also regulated by a noninflammatory pathway related to the proliferative state of endothelial cells. The confluent state of our ECFCs may partially explain the low E-selectin expression rate.

Relationships linking risk factors to either endothelial dysfunction in experimental studies or to clinical cardiovascular events were also demonstrated in ex vivo-cultured ECFCs at P3 (4, 27, 28, 34). Surprisingly, ECFCs were already activated before inflammatory stimulation in subjects with CAD or various risk factors. This significantly deviates from previous reports and the concept that endothelial activation is only induced on inflammatory stimulation. The deviation can be explained by the enrollment of CAD patients and controls with various risk factors in our study. Most of previous studies recruited subjects much younger than ours, or healthy volunteers (18, 25). Our findings raise the notion, for the first time, that ECFCs are already activated in an in vivo pathological environment. Nevertheless, it should be noted whether the lower purity of CD31$^+$ ECFCs in patients with CAD or risk factors can interfere with the interpretation of adhesion molecule expression and activation by pure ECFCs. However, our data showed that activation was mainly located in the CD31$^+$ population (Supplemental Fig. S.4), suggesting that the differences in activation levels between patients with CAD or risk factors and controls would be even more remarkable if only CD31$^+$ ECFCs were estimated. Future assays of purified CD31$^+$ ECFCs may definitely provide clearer information.

The cross talk between coronary risk factors and the activation potential of ECFCs supports the activation profile of ECFCs at P3 being capable of representing the in vivo health of ECFCs and potentially being used for cell-based risk stratification. This notion discloses the impaired self-repair ability of ECFCs in patients with cardiovascular diseases and is a likely expla-
nation for the divergent results reported in recent clinical trials (2, 13, 22, 24). Modulating the effects of risk factors on ECFCs either in vivo or ex vivo may ameliorate the biological behavior of ECFCs (36).

In line with the effect of hypertension, hyperlipidemia, DM, and aging on ECFCs, previous clinical studies have reported very similar associations of circulating adhesion molecule levels to these risk factors, suggesting activation on endothelial progenitors (6, 11). Previous reports have demonstrated that increased oxidative stress and insulin resistance play a mechanistic role in linking risk factors to endothelial activation (15). Managing oxidative stress and insulin resistance both in vivo and ex vivo appears to be seminal for ECFCs. Ex vivo pretreatment with atorvastatin improved the eNOS expression of ECFCs from CAD patients, although not up to the baseline level of controls as demonstrated in our study, and attenuated the activation levels of ECFCs as demonstrated by others (36). Although statins were shown to attenuate oxidative stress and improve insulin resistance as shown in our previous report (33), the exact mechanisms through which statins exert these beneficial effects still need to be elucidated.

Conclusions

The passage number and a variety of coronary risk factors are associated with the purity, biological function, and activation potential of ex vivo-expanded ECFCs. In the era of cell therapy, potential work of purifying, modifying, and bioengineering ECFCs should focus on early passages.

Prospectives and Significance

This study demonstrates that ex vivo-cultured ECFCs maintained a higher purity from P3 to P5 with a better biological functional capacity and endothelial phenotype expression compared with those of later passages. However, the characteristics necessary for efficient therapeutic applications were impaired in subjects with a variety of coronary risk factors, especially in those with CAD. The biological deficiencies were not only related to cell purity but also associated with ECFC dysfunction. In addition, adopting expression levels of adhesion molecules as surrogates of endothelial activation, ex vivo-cultured ECFCs presented lower activation potentials in early passages compared with late passages. Furthermore, the activation potential at P3 was substantially modulated by the existence of various coronary risk factors, particularly documented CAD. Notably, in high-risk patients, ECFCs were already activated before inflammatory stimulation. ECFCs from patients with CAD also demonstrated impaired eNOS expression, which was improved by ex vivo pretreatment with atorvastatin. Our data raise the possibility that without additional modifications, transplantation of dysfunctional ECFCs may lead to impairment of endothelial functional recovery or may even cause atherosclerosis. Functional assessments and manipulations of ECFCs have to be pursued in patients with extensive coronary risk factors.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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