Microarray-Based Characterization of a Colony Assay Used to Investigate Endothelial Progenitor Cells and Relevance to Endothelial Function in Humans

Aditi Desai, Alexander Glaser, Delong Liu, Nalini Raghavachari, Arnon Blum, Gloria Zalos, Margaret Lippincott, J. Philip McCoy, Peter J. Munson, Michael A. Solomon, Robert L. Danner, Richard O. Cannon III

Objective—An assay proposed to quantify endothelial progenitor cell (EPC) colonies in humans was investigated to determine the phenotype of recovered cells and their relevance to in vivo endothelial function.

Methods and Results—Twelve sedentary subjects participating in a worksite wellness program underwent endothelial flow-mediated dilation (FMD) testing of the brachial artery and blood sampling for EPC colony assay. Microarray-based genotypic characterization of colonies showed surface markers consistent with T lymphocyte phenotype, but not with an EPC (CD34, CD133, VEGFR-2) or endothelial (CD146) phenotype. Gene expression patterns more closely matched T lymphocytes (r=0.87) than endothelial cells (r=0.66) in our microarray database. Flow cytometry of colonies confirmed large populations of CD3+CD45+ T cells (>75%) and few CD146+CD45− endothelial cells (<1%). Further, there was no correlation between colony number and the magnitude of FMD (r=−0.1512, P=0.6389). After exercise training, subjects improved FMD, from 6.7±2.0 to 8.7±1.9% (P=0.0043). Colonies also increased (P=0.0210), but without relation to FMD (r=0.1074, P=0.7396). T lymphocyte phenotype persisted after exercise (r=0.87).

Conclusions—Cells in a commonly used EPC colony assay have a gene expression and cell surface marker profile consistent with a predominance of T lymphocytes and have an unclear relevance to endothelial function, either before or after exercise training. (Arterioscler Thromb Vasc Biol. 2009;29:121-127.)

Key Words: exercise • gene expression • endothelial function • endothelial progenitor cell • T lymphocyte
by Hill et al.9 We then repeated gene expression profiling of EPC-CFUs after exercise—which has been reported to independently increase EPCs and to improve endothelial function6–8,22—for 3 months in a worksite wellness program. Our purpose was to determine whether phenotypic characterization of these CFUs and changes in their gene expression patterns would provide insight into endothelial function and EPC differentiation capacity, before and after exercise training.

**Methods**

This study was conducted at the Clinical Center of the National Institutes of Health in employees who reported no previous exercise training and provided written informed consent for participation in this protocol, approved by the institutional review board of the National, Heart, Lung, and Blood Institute.22 Brachial artery flow-mediated dilation (FMD) testing was performed by an experienced technician who also performed the testing in Hill et al.9 Peripheral blood mononuclear cells were cultured for the EPC-CFU assay (Stem Cell Technologies EndCult Liquid Medium Kit) according to the manufacturer’s instructions. All testing was performed 48 hours after exercise to avoid acute effects of exercise on EPC mobilization and endothelial function.

RNA was extracted from EPC-CFUs with an RNAGene isolation kit (Ambion) according to the manufacturer’s directions. T7-based RNA amplification was performed on 10 ng of the isolated total RNA with the Riboamp OA 2-round amplification kit (Arcturus) and subjected to biotin labeling with Affymetrix’s IVT labeling kit according to the manufacturer’s directions (Affymetrix). Fragmented cRNA was hybridized to Affymetrix Human Genome (HG) U133 Plus 2.0 chips, washed, stained on an Affymetrix fluidics station, and scanned with Affymetrix GeneChip scanner.

Affymetrix GeneChip operating software version 1.4 was used to calculate signal intensity and the percent present calls on the amplified transcriptomes.

expression profiles for these 388 probe sets was compared between microarrays to compare gene expression patterns of cells from the EPC-CFU assay with those of T lymphocytes and endothelial cells. Several highly expressed transcripts corresponded to markers consistent with lymphocytes, including CD2, CD3, CD20, CD25, CD38, CD45, CD69, and CD71 (Figure 1A). The consistent with lymphocytes, including CD2, CD3, CD20, CD25, CD38, CD45, CD69, and CD71 (Figure 1A). Theendothelial cell adhesion molecule (CD31) was highly expressed, yet other endothelial markers (CD105, CD144, CD146, and von Willebrand factor [vWF]) were not expressed above median values. EPC markers CD34, CD133, and VEGFR-2, as well as the monocyte/macrophage markers CD14 and CD115, were expressed below median values.

**EPC-CFU Assay Is Primarily Composed of T lymphocytes Before and After Exercise**

To identify the phenotype of cells in the EPC-CFU assay, we examined transcript data for cell identification markers. Several highly expressed transcripts corresponded to markers consistent with lymphocytes, including CD2, CD3, CD20, CD25, CD38, CD45, CD69, and CD71 (Figure 1A). The traditional endothelial marker platelet-endothelial cell adhesion molecule (CD31) was highly expressed, yet other endothelial markers (CD105, CD144, CD146, and von Willebrand factor [vWF]) were not expressed above median values. EPC markers CD34, CD133, and VEGFR-2, as well as the monocyte/macrophage markers CD14 and CD115, were expressed below median values. Inspection of highly expressed transcripts of cell surface markers after 3 months of exercise training showed near-identical patterns compared with baseline transcripts (Figure 1B).

**Comparison of Cells Recovered From the EPC-CFU Assay to Cell-Type–Specific Signatures in a Microarray Database Before Exercise**

To confirm the phenotype of cells in the EPC-CFU assay we performed comparative analysis on transcriptional patterns from EPC-CFUs at baseline with other cells of known phenotype in our database. Preliminary gene expression and principal component clustering analysis suggested a lymphocyte phenotype for EPC-CFUs. We performed regression analysis using a list of 159 T lymphocyte specific genes25 (388 probe sets) on Affymetrix GeneChip HG-U133 Plus 2.0 arrays to compare gene expression patterns of cells from the EPC-CFUs to those of T lymphocytes and endothelial cells. From this analysis, cells from the EPC-CFU assay display a high degree of similarity with CD3+CD45+CD146− resting T lymphocytes (Figure 2A) but a low degree of similarity

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**Table. Test Data for the 12 Participants in a Worksite Exercise Program**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>3 Months</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vital signs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>28.0±6.1</td>
<td>27.8±5.9</td>
<td>0.0855</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>123±12</td>
<td>122±14</td>
<td>0.6085</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>74±5</td>
<td>74±9</td>
<td>0.8296</td>
</tr>
<tr>
<td>Resting heart rate, beats/min</td>
<td>67±8</td>
<td>71±12</td>
<td>0.1774</td>
</tr>
<tr>
<td><strong>Lab values</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>199±35</td>
<td>195±39</td>
<td>0.2736</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>130±36</td>
<td>132±33</td>
<td>0.1116</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>57±17</td>
<td>57±22</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>129±59</td>
<td>132±67</td>
<td>0.7334</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>90±5</td>
<td>89±10</td>
<td>0.7708</td>
</tr>
<tr>
<td>Insulin, μU/mL</td>
<td>12±6</td>
<td>13±7</td>
<td>0.2419</td>
</tr>
</tbody>
</table>

The average age was 50 years (range 32 to 62 years). Seven subjects were on drug treatment for hypertension (4) or hypercholesterolemia (3) for at least 2 months before the study, with normal test values at baseline and no change in treatment during the exercise program. Exercise at the worksite averaged 89±37 minutes per week.

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**Results**

Twelve subjects (Table) completed the program and had RNA of sufficient quantity and quality after amplification for microarray analysis. Seven of the subjects were female, and...
with HUVECs (Figure 2B). We also performed analysis against CD3+CD45+CD146+ activated T lymphocytes ($r=0.87$) and against HMVECs ($r=0.62$), with similar results.

**After Exercise**

Regression analysis of gene expression patterns from EPC-CFUs after exercise training showed persistence of a strong relation with resting T lymphocytes (Figure 2C) and a weak relation with cultured HUVECs (Figure 2D).
relation with endothelial cells (Figure 2D). These data were virtually identical to transcript data at baseline, suggesting that cells in culture after repetitive exercise were unchanged in phenotype.

**EPC-CFUs Are T Lymphocytes by Flow Cytometry**

To determine whether cultured cells were a mixture of T lymphocytes and endothelial cells, flow cytometric analysis of EPC-CFUs from 4 healthy subjects was performed using markers for T lymphocytes (CD3^+^CD45^+^) and endothelial cells (CD146^+^CD45^−^). In all 4 samples, large populations (>75%) of CD3^+^CD45^+^ T lymphocytes were present, with few CD146^+^CD45^−^ endothelial cells (<1%) apparent in samples from any subject (Figure 3).

**Exercise Improves Endothelial Function and EPC-CFU Number**

FMD at baseline averaged 6.7±2.0% and was not associated with EPC-CFU number (r = −0.1512, P = 0.6385). Exercise training for 3 months had no effect on weight, vital signs, or cholesterol levels (Table). Treadmill exercise duration (standard Bruce protocol) increased from 531±71 seconds to 601±95 seconds (P = 0.0005). Brachial artery FMD improved to 8.7±1.9% (P = 0.0043). The number of EPC-CFUs also increased with exercise, from 44±37 to 60±52 colonies.
per well, minimum 4 wells counted ($P=0.0210$), although there was no correlation between increase in colonies and improvement in FMD ($r=0.1074$, $P=0.7396$).

**Differentially Regulated Genes After Exercise**

To identify genes with potential relevance to changes in colony number and endothelial function after exercise training, we selected probe sets either upregulated or downregulated in EPC-CFUs after 3 months of exercise ($P<0.001$, fold change $>1.2$). The cohort of 12 participants exhibited a significant change in 29 probesets representing 25 unique genes. Examination of differentially regulated genes from this analysis revealed 5 upregulated genes and 20 downregulated genes (supplemental Table 1). Gene ontology analysis of these modulated genes identified them to be involved in functions such as signal transduction (C14orf100, CREB1, GNAI3, RAP2C, RIPK3, TBC1D14), metabolic processes (ADIPOR1, ADSL, CRLS1, NDUF10), immune response (IGKC, IGW, PRELID1), translation (EIF4E2, MRPL11, MRPL42), gene expression and transcription (CREB1, E2F6, PRRX1), and transport (GNAI3, NUP160, TMEM1).

**Discussion**

Colonies of cells grown in fibronectin culture assays from peripheral blood mononuclear cells—believed to represent bone marrow–derived EPCs—have been associated with endothelial function in men, reduced in patients with coronary artery disease, and inversely associated with cardiovascular outcomes. However, markers of endothelial phenotype used by several groups to validate these assays—including CD31, CD105, CD144, lectin binding, and DiI-acetyl LDL uptake—are shared by many subsets of mononuclear cells. Because of the uncertainty involved in phenotypic characterization using cellular markers, we considered whether genotypic characterization could serve as a more exact determinant of cellular phenotype. Our data suggest that cells derived from a commercially available EPC-CFU assay, based on the assay used by Hill et al, are primarily T lymphocytes by cell surface marker expression, by flow cytometry, and by transcriptome regression analysis with T lymphocytes. Although gene expression patterns correlated weakly with those of endothelial cells in our database, few endothelial cells (CD146+/CD45−) were observed by flow cytometry.

Marginal expression of the transcript for CD146, a surface marker traditionally present on endothelial cells, was present in our microarray analysis. Flow cytometry of EPC-CFUs showed a small number of CD146+ cells, but the majority coexpressed CD3, suggesting that these cells are T lymphocytes and not endothelial cells. Coexpression of CD146 on lymphocytes is representative of an activated lymphocyte phenotype with increased capacity to bind to endothelial cells and extravasate to sites of inflammation. Blood samples from healthy subjects in this study showed that approximately 2% of CD3+ cells in circulation were also CD146+. Accordingly, cells from our assay likely represent an expansion of this unique population.

Our findings are consistent with reports from Hur et al and Rohde et al, who reported that colonies from mononuclear cells plated on fibronectin are predominately lymphocytes. Rohde et al used the same commercial assay as was used in our study and demonstrated predominance of T cells and monocytes in their colonies by flow cytometric determination of cell surface markers. Of interest, inclusion of both CD2+ lymphocytes and CD14+ monocytes in their starting population of blood mononuclear cells in culture was necessary for colony formation to be observed after five days of culture. Our data extends these findings by using a microarray approach to determining the phenotype of cells in this assay, demonstrating expression of genes encoding surface markers consistent with T lymphocyte phenotype, but not with an EPC (CD34, CD133, VEGFR-2) or endothelial (CD146) phenotype. Gene expression patterns closely matched T lymphocytes in our microarray database. Flow cytometric analysis of cells from colonies was also consistent with T lymphocytes, with few endothelial cells present after 5 days of plating on fibronectin-coated plates. We also investigated whether colonies in this assay may have relevance in subjects participating in a worksite wellness program, the majority of whom had risk factors for impaired endothelial function. Contrary to our original hypothesis and to the report of Hill et al, we found no correlations between EPC-CFUs and FMD at baseline. Several groups have suggested that cells in colonies, despite limited potential of endothelial differentiation, may have paracrine effects on endothelium via secretion of cytokines and growth factors. In this regard, our findings are consistent in part with data of Hur et al, who also reported a predominance of CD3+CD31+ cells in their colony assay. They claimed that these cells represent a unique population of T lymphocytes capable of secreting angiogenic cytokines and facilitating endothelial differentiation.

Exercise has been reported to mobilize bone marrow–derived EPCs into the circulation, with the potential of attachment to arteries in the circulation and replacement of dysfunctional endothelium. Although we saw an increase in EPC-CFUs in our culture assay after exercise, the cells remained predominately T lymphocytes by microarray analysis of cell surface markers and by transcriptome regression analysis with T lymphocytes. Gene expression profiling before and after exercise training revealed 25 differentially regulated genes, in a magnitude of change that has been considered relevant for human microarray studies. Gene ontology analysis of these genes revealed an enrichment of transcripts involved in signal transduction, metabolism, immune response, translation, transcription, and transport. Yet the majority of these genes were downregulated after exercise, thus the relevance to the observed increase in colonies is obscure. Despite absence of correlations with endothelial testing to our study, it remains possible that the observed T lymphocyte population recovered from the EPC-CFU assay has a functional relationship to the endothelium or EPC capacity. Alternatively, the observed improvement in endothelial function by FMD may be independent of these T lymphocytes and may be attributable to more conventional responses. For example, repetitive shear stress attributable to exercise has been shown to increase expression and activity of endothelial nitric oxide synthase (eNOS), the product of which (NO) improves endothelial function, and could account...
for the enhanced dilator response of the brachial artery to shear stress in our study participants.33,34

Although other assays than the one used in our study have been proposed for demonstrating endothelial differentiation potential of blood-derived progenitor cells,39 none to date has been shown to be relevant to endothelial function in humans. Further, the role of bone marrow–derived cells in postnatal angiogenesis or arterial repair has been questioned by some groups.35–39 Animal models of bone marrow transplant and parabiosis, with donor animals genetically engineered to permit cells tracking, have shown few-to-no cells incorporated into vasculature of recipient animals, even with stimulated mobilization and homing by VEGF administration or tumor induction.39 Accordingly, improvement in endothelial function with exercise training may depend more on upregulation of eNOS within endothelium or reparative effects of resident progenitor cells within vasculature rather than bone marrow–derived progenitor cells in the circulation.40–43

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References


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