Endovascular biopsy: Strategy for analyzing gene expression profiles of individual endothelial cells obtained from human vessels

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Abstract

\textbf{Purpose}—To develop a strategy of achieving targeted collection of endothelial cells (ECs) by endovascular methods and analyzing the gene expression profiles of collected single ECs.

\textbf{Methods and results}—134 ECs and 37 leukocytes were collected from four patients’ intra-iliac artery endovascular guide wires by fluorescence activated cell sorting (FACS) and analyzed by single-cell quantitative RT-PCR for expression profile of 48 genes. Compared to CD45\textsuperscript{+} leukocytes, the ECs expressed higher levels ($p < 0.05$) of EC surface markers used on FACS and other EC related genes. The gene expression profile showed that these isolated ECs fell into two clusters, A and B, that differentially expressed 19 genes related to angiogenesis, inflammation and extracellular matrix remodeling, with cluster B ECs have demonstrating similarities to senescent or aging ECs.

\textbf{Conclusion}—Combination of endovascular device sampling, FACS and single-cell quantitative RT-PCR is a feasible method for analyzing EC gene expression profile in vascular lesions.
Keywords
Targeted endothelial cell sampling; Single cell quantitative RT-PCR; Gene expression of artery endothelial cells

1. Introduction
Gene expression studies of patient-derived endothelial cells (ECs) provide important information regarding the pathogenesis of many vascular diseases [1–3], in and outside of the central nervous system. Several groups have reported EC enrichment and identification from endovascular guide wires by 2 EC separation methods: micropipette picking-up [4] or CD146 antibody-conjugated magnetic beads [5–7], both followed by either traditional gene expression assays like bulk mRNA reverse transcription (RT) PCR which analyzes RNA extracted from a pool of ECs [4,5], quantitative RT-PCR [6] or quantitative immunofluorescence [7–14]. Because of the limitation set by these conventional methods, only a few (up to 3 or 4) genes can be analyzed. Due to the complexity and heterogeneity of ECs [15], these studies have incurred uncertainty and controversy regarding the purity and functionality of the ECs studied.

Although DNA microarray studies of ECs separated from tissue can provide high throughput EC gene expression information and have indicated that heterogeneity of endothelium exists among different tissues or diseases [15,16], this technique needs bulk mRNA extracted from at least thousands of ECs, numbers difficult to attain using endovascular EC sampling methods. Furthermore, DNA microarray can only analyze gene expression patterns of a group of ECs and not each individual EC. A more complete picture of individual EC functional condition in specific environments needs an assay, which can analyze the expression profile of multiple genes in individual ECs.

Recently we reported that EC candidates attached on guide wires can be collected by fluorescence activated cell sorting (FACS) and laser capture microdissection. The quality of mRNA extracted from the ECs is sufficient for analysis of gene expression using quantitative RT-PCR [17]. Single-cell quantitative RT-PCR combined with high-throughput microfluidic array technology facilitates detection of gene expression profiles of up to 96 genes in 96 individual cells simultaneously [18,19]. Therefore, it is a powerful high throughput tool to characterize gene expression of individual cells.

In this study, we demonstrated that combination of FACS and high throughput microfluidic single-cell quantitative RT-PCR is an efficient and powerful method for analyzing the changes of EC gene expression profiles in vascular lesions.

2. Material and methods

2.1. Case selection and EC harvest
Samples were collected from four patients undergoing routine catheter angiography for assessment of cerebrovascular pathology. The patients provided written consent for the procedure inclusive of the collection and study of tissues for research purposes standard on
surgical consent forms. ECs were obtained by inserting a 0.038-inch diameter coaxial curved stainless steel guide wire (Cook Inc., Bloomington, IN) into the right iliac artery as part of routine arterial access. Wires are directed under fluoroscopic visualization so a short (<5 cm) segment of vessel may be specifically contacted. The cells attached on the wires were dislodged by vortexing and centrifuging in a dissociation buffer (Gibco, Grand Island, NY). After lysing RBC by ACK Lysing Buffer (Gibco, Grand Island, NY), and centrifuged at 1500 rpm, the pellets were re-suspended in FACS buffer for incubation of antibodies and sorting. Experiment design is shown in Fig. 1.

2.2. EC candidate identification and sorting on FACS

Single EC candidates were identified and sorted by a protocol of seven fluorescently-conjugated monoclonal antibodies on FACS that we described in our previous study [17]. LIVE/DEAD Fixable Dead Cell Stain (Life Technologies, Carlsbad, CA) was used to exclude the dead cells. The antibody information is listed in Table 1. After staining the dislodged cells with these seven antibodies and the Amine Aqua Reactive Dye (AmCyan channel), the debris, doublets and dead cells were excluded before subsequent procedures (Fig. 2). After excluding CD45+ leucocytes, CD11b+ myeloid cells and CD42b+ platelets by three negative gates, the remaining cells were gated by four EC specific surface markers, CD31, CD34, CD105 and CD146. Cells positive for the 4 EC surface markers were collected as EC candidates. CD45+ leucocytes were also collected and used as control. EC candidates and leukocytes were sorted into 96 well plates on a FACS Aria II (BD Biosciences, San Jose, CA) with 100 nm nozzle using single cell sort mode.

2.3. Reverse Transcription and cDNA pre-amplification

Reverse transcription and cDNA pre-amplification were carried out on a PCR thermocycler. Briefly, each EC candidate was sorted directly into one well with 9 μL reverse transcription-specific target amplification (RT-STA) buffer on the 96-well plates (Eppendorf, Hauppauge, NY). The RT-STA buffer contained 5 μL CellsDirect 2× Reaction Mix (Life Technologies, Carlsbad, CA), 0.2 μL SuperScript III RT Platinum Taq Mix (Life Technologies, Carlsbad, CA), 2.8 μL nuclease free water and 1 μL 10× primer mixture (500 nM) that contained a mix of 48 pairs of primers specific to genes listed in Table 2. The primers were custom designed and all expand introns to minimize the genomic DNA fraction (Fluidigm, South San Francisco, CA). The Fluidigm Assay IDs listed in Table 2 can be used to obtain primer sequences.

The samples were incubated at 50 °C for 15 min for the reverse transcription, 95 °C for 2 min for inactivating reverse transcriptase and activating Taq polymerase, then subjected to 18 PCR cycles (95 °C 15 sec then 60 °C for 4 min for each cycle) for specific targets amplification (STA). To remove the unincorporated primers for best results, each sample was then mixed with 3.6 μL exonuclease treatment buffer composed of 2.52 μL water, 0.36 μL 10× Exonuclease I reaction buffer and 0.72 μL 20 units/μL Exonuclease I (New England BioLabs, Ipswich, MA), incubated at 37 °C for 30 min for digestion and 80 °C for 15 min to inactivate the exonuclease.
2.4. Quantitative RT-PCR

48.48 nanofluidic chips and a BioMark HD system (Fluidigm, South San Francisco, CA) were used. Briefly, each pre-amplified cDNA sample was diluted by 5 fold in TE Buffer (TEKnova, Hollister, CA). Then, 2.25 μL diluted samples were mixed with 2.5 μL 2× SsoFast EvaGreen Supermix with Low ROX (Bio-Rad, Hercules, CA) and 0.25 μL 20× DNA Binding Dye Sample Loading Reagent (Fluidigm, South San Francisco, CA). The pre-mix samples (5 μL each) were loaded into the 48 sample inlets on the 48.48 Dynamic Array (Fluidigm, South San Francisco, CA), which had been primed with control line fluid (Fluidigm, South San Francisco, CA) on IFC Controller MX (Fluidigm, South San Francisco, CA). Assay Mix (5 μl) containing 2.5 μL 2× Assay Loading Reagent (Fluidigm, South San Francisco, CA), 2.25 μL 1× DNA suspension buffer (TEKnova, Hollister, CA) and 0.25 μL primer set (100 μM) were added to the 48 assay inlets on the 48.48 nanofluidic chip (Fluidigm, South San Francisco, CA). After loading both pre-mixed samples and the assay mixtures to the nanochip by IFC Controller MX (Fluidigm, South San Francisco, CA), the chip was loaded into the BioMark HD system (Fluidigm, South San Francisco, CA) for PCR through 35 cycles of 5 sec at 96 °C and 20 sec at 60 °C after a hot start phase of 60 sec at 95 °C. Fluorescence in the EvaGreen channel was detected and collected by a CCD camera placed above the chip and 6-carboxy-X-rhodamine (ROX) intensity was used as normalization.

2.5. Data collection and analysis

Quantitative RT-PCR data of ECs and leukocytes obtained from 4 subjects were analyzed together. Fluidigm quantitative RT-PCR Analysis software (Fluidigm, South San Francisco, CA) was used to process RT-PCR data obtained by Biomark HD system and calculate Ct values. Ct values were further processed in the R statistical language using algorithms provided by SINGuLAR Analysis Toolset 3.5 (Fluidigm, South San Francisco, CA). All Raw Ct values were normalized to the assumed detection Ct level of 24 following the recommendation from this manual. Ct values were converted to relative expression levels using methods described previously [20]. The assumed minimum value of genes without expression was set as 10% lower than the lowest recorded reading. Euclidean distance metric and complete linkage function were used to build the Hierarchical clustering. Mean-centered data were used for principal components analysis (PCA) to avoid bias caused by highly expressed genes.

3. Results

3.1. Selection of genes for profiling EC gene expression

Based on previous EC function studies [21,22], we selected three groups of genes to characterize ECs in this study. They are 19 angiogenesis-related genes, 13 inflammation-related genes and 12 extra-cellular matrix (ECM) remodeling-related genes. To confirm the identity of ECs isolated by FACS, six EC specific marker genes and one vascular smooth muscle cell marker gene (α-actin) were included. We also included the four EC-marker genes and CD45 that were used for FACS selection of EC candidates and leukocytes (Table 2).
3.2. The gene expression profile of EC candidates is distinctively different from that of LCs

A total of 134 EC candidates and 37 leukocytes (LCs) were collected by FACS through the gating strategy we described previously [17] and shown in Fig. 2. Among these ECs, 64 (48%) expressed three EC markers CD31, CD34 and CD105, and 30 (22%) expressed four EC markers CD31, CD34, CD105 and CD146. Furthermore, we compared gene expression profiles of EC candidates and LCs. Among the 11 marker genes, eight were differentially expressed between the ECs and LCs (Fig. 3). Among the five marker genes used in FACS, the expression of the LC marker CD45 ($p = 1.2 \times 10^{-27}$) was significantly higher in LCs than ECs, and the expression of EC markers, CD31 ($p = 0.017$), CD34 ($p = 3.1 \times 10^{-5}$) and CD105 ($p = 6.3 \times 10^{-7}$) were significantly higher in the ECs than LCs. The expression of CD146 showed a trend toward higher in ECs than in LCs ($p = 0.15$). In addition, compared to LCs, ECs expressed higher levels of the other four EC specific genes, VEGFR1 ($p = 1.3 \times 10^{-8}$), vWF ($p = 2.3 \times 10^{-7}$), Tie1 ($p = 1.3 \times 10^{-5}$) and THBD ($p = 0.013$). These data indicate that the EC candidates isolated by FACS were indeed ECs.

3.3. Two EC clusters were identified based on gene expression profile

Unsupervised hierarchical clustering separated the 134 ECs into two distinctive clusters according to their expression pattern of the 48 selected functional genes (Fig. 4a). 69 ECs were in cluster A and 65 in cluster B. Principal component analysis (PCA) also showed two distinct populations and was consistent with hierarchical clustering. Only three cluster B cells identified by heat map-based hierarchical clustering were grouped with cluster A cells in PCA, and two cluster A cells were grouped with cluster B cells (Fig. 4b). The correlation of single cell gene expression and different biological donors was also analyzed by PCA. The 2D PCA (Fig. 5a) showed that the ECs from different donors did not overlap and showed no distinguishable cluster. The PCA scree plot (Fig. 5b) showed the contribution of first 10 PCs, which suggested the PC1 which identifies the two clusters gives much more contribution than other PCs.

3.5. Differential gene expression of the 2 EC subsets

Further analysis showed that seven out of the 19 angiogenesis-related genes were differentially expressed (Fig. 6a Left) by cluster A and B. Among them, five were higher in cluster B [vWF ($p < 7.1 \times 10^{-20}$), CD105 ($p < 9.0 \times 10^{-18}$), TIE1 ($p < 1.1 \times 10^{-13}$), CAT ($p < 7.8 \times 10^{-12}$) and EPHB4 ($p < 9.7 \times 10^{-11}$)], two were higher in cluster A [VEGFA ($p < 1.2 \times 10^{-4}$) and TGFβ1 ($p < 0.023$)]. Compared to cluster A, cluster B express higher levels of seven out of 13 inflammation-related genes (Fig. 6a Middle), [CD34 ($p < 9.0 \times 10^{-39}$), P-selectin ($p < 9.0 \times 10^{-22}$), CD31 ($p < 1.2 \times 10^{-9}$), CD146 ($p < 3.1 \times 10^{-7}$), VCAM-1 ($p < 1.4 \times 10^{-4}$), COX2 ($p < 0.008$) and ICAM-1 ($p < 0.028$)], as well as five out of the 12 ECM remodeling-related genes, were also expressed higher by cluster B than cluster A cells (Fig. 6a Right), [MMP2 ($p < 5.0 \times 10^{-27}$), PAI-1 ($p < 1.7 \times 10^{-15}$), FN1 ($p < 1.1 \times 10^{-15}$), TIMP1 ($p < 2.9 \times 10^{-11}$) and TIMP2 ($p < 9.5 \times 10^{-6}$)].

4. Discussion

In this study we demonstrated an innovative strategy for analyzing gene expression profiles of ECs collected from vessels on a single cell level. ECs are collected from endovascular
guide wires through FACS. Single cell gene expression is analyzed using high throughput microfluidic quantitative RT-PCR. This method could be used to analyze the changes of EC gene expression at single cell level in vascular lesions. A total of 48 genes in four categories (cell-marker, angiogenesis, inflammation and ECM) were analyzed in this study. Two distinctive ECs clusters were identify from ECs collected from normal iliac arteries, suggesting ECs in normal vessel are heterogeneous.

Researchers who study ECs collected using endovascular techniques encounter a paradox that more EC marker genes need to be detected to identify and characterize the collected ECs, while the EC number harvested from such samples is often too small for such multiple marker detection. The traditional assays such as bulk RT-PCR, real-time RT-PCR or quantitative immunocytochemistry can only detect the mRNA transcription or protein expression of up to 3–4 EC functional genes, which are not enough for this purpose. The combination of single EC sorting and high throughput microfluidic quantitative RT-PCR allowed us to check EC identity through analyzing the expression of both the EC specific markers and the expression of functional genes in individual cells simultaneously. Moreover, because this microfluidic quantitative RT-PCR array technology has 96 gene slots, researchers have much more flexibility to expand the functional genes studied to help characterize ECs in varying disease conditions. This technique also presents a sound basis for comparing EC sampling and characterization data from different research centers.

Based on gene expression profiles, two distinctive clusters were identified in ECs collected from normal iliac arteries. A likely explanation is that ECs in normal conditions undergo turnover. The two EC clusters represent ECs at different functional stages, for example healthy and senescent. ECs are a stable cell type with an average turnover rate of about three years [23]. Senescent endothelium has been reported to have decreased expression of angiogenesis and proliferation genes, attenuated production of dilating factors and increased expression of contracting factors, increased oxidative stress, increased production of leukocyte adhesion-related cytokines or inflammation-related cytokines, and increased apoptosis [23]. Although not typical, cluster B ECs showed an expression pattern reminiscent of senescence-related genes like those of previous studies on EC aging and senescence. These gene expression changes include attenuated gene expression of VEGF and TGFβ1 [24], enhanced expression of CD105 (also a EC proliferative marker) [25], COX2 [26,27], catalase [28], VCAM1 and ICAM1 [29], and TIMP2 [30]. Therefore cluster B cells could represent more mature or aged ECs. It is also of interest that cluster B ECs showed enhanced EPHB4 expression compared to cluster A. Although EphB4 is commonly considered a marker for ECs from veins, there is also data indicating EphB4 is expressed on both normal arteries and veins [31]. This gives more supportive evidence that caution should be used when this marker is used to identify venous ECs. We also ran a comparable volume (50ul) blood from each of the same patients on FACS and found no ECs. So, it is unlikely that these ECs came from veins by circulation and attached to the wire.

A noticeable phenomenon in this study is that only a quarter of the FACS sorted ECs expressed the four markers used for sorting and half expressed three. This indicated that FACS sorting cannot give a 100% pure population and a possible solution for this issue is the use of FACs machines with Index sorting capabilities. Several limitations of our study
must also be considered. First, the patients selected for cell collection were not matched for their respective diseases necessitating angiography, demographic and co-morbid conditions. Given the small scale and exploratory nature of the study, controlling for such confounders proved difficult. Despite the absence of such analysis, when ECs were analyzed as it related to their patient origin, we noted no significant differences by either PCA or hierarchical clustering. Second, our choice of target genes for microfluidic quantitative RT-PCR was based on literature searches, introducing unavoidable bias. A more objective selection of target genes may be possible by analysis of previous microarray data on ECs. Such analyses are not possible on such small numbers of cells available, though emerging single cell mRNA sequencing may give an unbiased view of the global gene expression and ultimately identify new genes for study. Lastly, EC gene expression profile analysis was based on fewer than 200 ECs from four patients. Further studies of single EC gene expression and transcriptional regulation based on more ECs separated using endovascular cell collection techniques are necessary to investigate differential gene expression in ECs at different vasculature loci and in various vascular lesions.

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References


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Experimental design. Cells were dislodged from the guide wire (1) and were stained by antibodies specific for different cell surface markers (2). Individual ECs were sorted into 96-well plates by FACS (3). Specific gene cDNAs were pre-amplified by thermocycler (4). Quantitative RT-PCR was performed on Biomark HD system (Fluidigm, South San Francisco, CA) (5). Data were collected and analyzed by quantitative RT-PCR analysis software (Fluidigm, South San Francisco, CA) (6).
Fig. 2.
FACS gating strategy for EC collection. Seven cell surface markers and one viability marker were used to gate the EC candidates. Cells were first gated to exclude debris, doublets and dead cells identified by positive Aqua Amine stain. After gating on the viable single cells, the leukocytes (CD45\(^+\)), macrophages (CD11b\(^+\)) and platelets (CD42b\(^+\)) were eliminated. EC candidates were first selected by CD31 and CD34, and then CD105 and CD146.
Fig. 3.  
Differential gene expression of ECs and LCs. (a) Violin plots showed the expression of 11 cell-marker genes are different in the ECs (Red) compared with LCs (Green). The gene name is indicated on top of each violin plot and the value on Y-axle represents the gene expression level in the binary logarithm (log₂) value. (b) Bar graph shows the values of differential gene expression by fold change of the binary logarithm (log₂) in ECs relative to LCs (*p < 0.05; ***p < 0.001). Gene symbols are used in the figures and corresponding gene names can be found in Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 4.
Two EC clusters were identified by gene expression profiles. (a) Heat map and hierarchical clustering separated the 134 ECs into 2 major clusters, A (n = 69, green triangle) and B (n = 65, Red circle), based on their expression pattern of the 48 selected genes. (b) 3D PCA plots confirmed the segregation of these two clusters. Cluster A is annotated by green dots and B by Red dots. Gene symbols are used in the figures and corresponding gene names can be found in Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 5.
Two clusters identification is stronger identifiers than donor origin. (a) 2D PCA of the 134 ECs from 4 different donors based on their gene expression profile indicated no clear cluster separation among donors. (b) PCA scree plot of the first 10 PCs suggested the PC1 which identifies the two clusters gives much more contribution to the whole variance than other PCs.
**Fig. 6.**
Differential gene expression of the two EC clusters. (a) Violin plots. Three functional gene groups are included, 19 angiogenesis-related genes (Left), 13 inflammation-related genes (Middle) and 12 ECM remodeling genes (Right) of cluster A (green) and cluster B (Red). The gene name is indicated on top of each violin plot and the value on Y-axle represents the gene expression level in the binary logarithm (log₂) value. (b) Bar graph shows the magnitude of differential gene expression by fold change of the binary logarithm (log₂) value in cluster B relative to A (*p < 0.05; **p < 0.01; ***p < 0.001). Gene symbols are used in the figures and corresponding gene names can be found in Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Table 1

Fluorescently conjugated monoclonal antibodies used for EC candidate identification on FACS.

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<sup>a</sup> Genes used in FACS.

<sup>b</sup> Primer sequences for microfluidic qPCR can be traced by these company assay IDs.