Detection of Circulating Endothelial Cells Via a Microfluidic Disk

Ken-Chao Chen,1 Tai-Ping Lee,2 Yu-Cheng Pan,1 Chi-Ling Chiang,3 Chen-Lin Chen,1 Yao-Hsu Yang,4 Bor-Luen Chiang,2 Hsinyu Lee,3 and Andrew M. Wo1*

BACKGROUND: Circulating endothelial cells (CECs) in the blood are rare but have been shown to be associated with various diseases. With the ratio of CECs to peripheral blood mononuclear cells (PBMCs) less than 1 part per thousand, their separation and detection from PBMCs is challenging. We present a means of detecting CECs from PBMCs via an economical microfluidic disk with a model cell system (HUVECs in PBMCs), along with demonstration of its efficacy clinically.

METHODS: To enrich these rare cells, we used immunomagnetic beads and a tailor-made magnet on the disk. CEC-simulating human umbilical vein endothelial cells (HUVECs), as target cells, were stained with primary antibody on anti-PE magnetic beads. PBMCs served as nontarget cells and were labeled with anti-CD45-FITC antibody.

RESULTS: When hundreds of HUVECs were mixed in 106 PBMCs, 95% of spiked HUVECs were detected. This yield also held for 60 HUVEC in 104 PBMCs. We compared data from flow cytometry with that from the disk: CEC counts in 50 μL blood from patients with systemic lupus erythematosus were 61.1 (21.5), significantly higher (P < 0.01) than those of healthy donors, 31.2 (13.3).

CONCLUSIONS: The count of CECs is a suitable marker for symptoms of systemic lupus erythematosus. The microfluidic disk system should be a viable platform for detection of CECs.

The presence of circulating endothelial cells (CECs) in blood is an important issue in biological and clinical studies. Reports have indicated that the amount of CECs can serve as an indicator of the health of blood vessels, and this finding has been leveraged to monitor cardiovascular events (1, 2) and vascular disease (3, 4). Nozaki et al. (1) presented an assessment of endothelial dysfunction by plasma concentrations of endothelium-derived microparticles. Their assessment can independently predict future cardiovascular events in patients at high risk for coronary heart disease. Elshal et al. (4) reported that an increased number of CECs observed in patients with systemic lupus erythematosus (SLE) was associated with the active phase of the disease and may serve as a marker of widespread endothelial injury. Researchers have also evaluated CECs as biomarkers for the efficacy of anticancer/antiangiogenic treatments (5–9). Dome et al. (6) reported that CECs’ presence in increased numbers reflects vascular injury. Along with endothelial progenitor and proangiogenic hematopoietic cells, CECs may play a synergistic role with angiogenesis. Such efforts have demonstrated their value in breast (10), prostate (11), and rectal (12) cancers. Trace amounts of CECs are present among a large number of blood cells. Hence, isolation and detection of these rare cells present a technical challenge.

Efficient purification and detection of CECs are critical to derive their clinical significance (13). The numbers of CECs have usually been obtained by use of immunobeads or flow cytometry. Roughly 10 diseases have been identified based on the numbers of CECs. For cardiovascular and infectious diseases, the numbers of CECs are 3–10 times higher than controls. For vasculitis and lupus, CECs number from 10 to more than 100, or 2–10 times that of controls. For transplantation and cancer, the numbers of CECs are 4–5 times that of controls (13). Thus, increased CEC counts associate with undesirable health status.

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Nonstandard abbreviations: CEC, circulating endothelial cell; SLE, systemic lupus erythematosus; PBMC, peripheral blood mononuclear cell; HUVEC, human umbilical vein endothelial cell; PE, phycoerythrin; FITC, fluorescein isothiocyanate.
Currently, there are a number of approaches to quantify CECs. In most cases, researchers count CECs by fluorescence microscopy (2), analyze the cell mixture with flow cytometry (14, 15), or combine either of these with ELISA (10). A consensus protocol based on CD146-driven immunomagnetic isolation, representing the experience of 3 major centers (16), has been generally adopted (5). Duda et al. (17) proposed a cytometry protocol for phenotypic identification and enumeration of CECs and other cells in the blood, which has been adopted (6). Overall, the counts of CECs reach good agreement among different studies (3). Although flow cytometry is capable of high throughput and multiparametric analysis, capital and operational costs might prohibit its usage in resource-limited regions.

The microfluidic disk is an effective way to achieve integrated analysis systems in a laboratory setting with a small amount of sample (18). Other advantages of the disk-based platform include ease of fluid transport, straightforward valve designs, a disposable batch-like process, and a large range of sample volumes (19). Use of the microfluidic disk for enumeration of rare cells with negative selection configuration has been demonstrated (20).

We describe here a microfluidic disk incorporating immunomagnetic-based separation with direct immunofluorophore for detection in the same region of the disk. To evaluate the performance of the disk, we also analyzed peripheral blood mononuclear cells (PBMCs) from healthy donors with spiked human umbilical vein endothelial cells (HUVECs) via flow cytometry for comparison. CEC counts in 50 μL blood from patients with SLE were compared to those of healthy donors.

**Materials and Methods**

Experimental aspects described in this section include disk design, preparation of cell mixture, disk operation, and data collection from patients. Details of how we achieved high trapping force by using multistage magnets, plus details the flow cytometry are provided in the Supplemental Data file, which accompanies the online version of this article at www.clinchem.org/content/vol57/issue4. Further details of disk and experimental setup have been published (19).

**Disk Design**

Overarching design considerations for the disk are 2-fold: (1) securely trap the maximum number of rare cells while minimizing the shear stress on the trapped cells, and (2) minimize the time required for the entire procedure. The shear stress arises owing to background cells and the flow of the medium passing the stationary/trapped cells. The minimum time is desirable for rapid disk operation.

Toward these ends, we applied design considerations for the 3 main microfluidic components: inlet reservoirs, connecting channels, and waste reservoirs (Fig. 1). The inlet reservoir houses the mixture of rare and background cells and also traps rare cells via the multistage magnet located directly above the disk. Ten identical sectors on an entire disk (12-cm diameter) were incorporated to maximize the number of tests per spin, with each sector consisting of a complete set of microfluidic components. Centrifugal force propelled nontrapped cells and fluid radially outward toward the waste reservoir via the connecting channels. Ease of observation via fluorescence microscope was considered in the design process.

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**Fig. 1.** The microfluidic disk and schematic of cell trapping. (A), Layout of one sector of the disk: inlet reservoir (region a) for containing cell sample and collecting target cells for observation, connecting channel (region c, area in light green), and waste reservoir (region b). (B), HUVECs labeled and trapped to magnet in region a.
Microfluidic Disk Detection of Circulating Endothelial Cells

PREPARATION OF CELL MIXTURE
HUVECs were cultured in gelatin (1%)-V coated tissue culture dishes or flasks (Falcon Labware, Becton Dickinson) in medium 199 (Gibco BRL, Life Technologies) supplemented with 20% fetal calf serum (Gibco BRL), 4 mM L-glutamine, 30 μg/mL endothelial cell growth supplement (Collaborative Research), 14 IU/mL heparin (Leo Pharmaceutical Products), and antibiotics at 37°C under an atmosphere of 5% CO2. PBMCs obtained from the peripheral blood of healthy donor volunteers were purified by Ficoll-Paque (GE Healthcare Life Sciences) and labeled with anti-CD45-fluorescein isothiocyanate (FITC) antibody (555482, BD Pharmingen). We applied a double-step immunomagnetic binding to HUVECs, representing rare cells. HUVECs were stained with human anti-CD146-phycocerythrin (PE) antibody (550315, BD Pharmingen) and bound with BD IMag™ anti-PE magnetic particles (557899, BD Pharmingen). PE and FITC are 2 common fluorophores with red and green colors, respectively. We used a running buffer, composed of pH 7.2 PBS, 0.5% BSA, and 2 mM EDTA, for washing out excessive dye and suspending cells.

COLLECTION AND ANALYSIS OF PATIENT SPECIMENS
CECs were defined as nucleated cells larger than 10 μm and of the CD146+/CD45− phenotype. Peripheral blood samples were obtained with nontraumatic venipuncture (use of tourniquet, selection of a large vein, and avoidance of needle movements). We discarded the first 2 mL of blood drawn to avoid contamination by endothelial cells from venipuncture. We discarded 4 μL FcR blocking agent, antihuman CD45-FITC, and antihuman CD146-PE to 50 μL whole blood in EDTA anticoagulant, mixed thoroughly, and incubated at 4°C away from light for 1 h. We washed the sample with running buffer and added 20 μL magnetic particles conjugated with anti-PE mAb (BD IMag Anti-PE Particles; BD Biosciences) for 30 min at room temperature. The sample was loaded into the microfluidic disk and the disk rotated according to the rotational protocol described in the next section. We then counted CECs (CD146+/CD45−) by fluorescence microscopy.

We analyzed the results, expressed as mean (SD), using Graphpad Prism 4 software. Statistical significance was determined by the Mann–Whitney test. The study was approved by the local Research Ethics Committee, and signed consent was obtained from all participants.

DISK OPERATION
We loaded 50 μL cell suspension in the inlet reservoir. After placing the magnet on top of the disk, the immunomagnetically bound cells were captured to the top of the reservoir by magnetic trapping force. Disk rotation was started at 150 rpm and gradually increased to about 250 rpm to purge residual liquid, within 5 min. Meanwhile, most of the fluid, along with the magnetically unlabeled cells, flowed to the waste reservoir. A rotational speed of 250 rpm for 3 min was usually required to clear the remaining fluid in the inlet reservoir. Finally, the microfluidic disk was flipped upside down and observed directly with a microscope (IX-71, Olympus). Target cells were identified and counted in the microfluidic disk within 15 min.

RESULTS
Results are presented over an extensive range of HUVEC concentrations, with consideration of both yield (percentage of detected cells over spiked cells) and variation of yield. We performed comparison studies with the BD FACSCanto II flow cytometer and demonstrated the disk’s usage with SLE patients vs healthy donors.

FLOW CYTOMETRY
Fig. 2 shows density plots of the mixture of HUVECs in 10^6 PBMCs. The spiked HUVEC count was categorized into 3 groups: 0, 20, and 200 cells, with each group containing 3 replicates of tests. PBMCs were CD45+CD146− and in large quantity, and the strong signals in quadrant IV reflect this fact. HUVECs are CD45+CD146+ and in small quantity, so data in quadrant II should correspond to the HUVECs. As spiked HUVEC count increased for the 3 groups, the events in quadrant II (Fig. 2A to Fig. 2C) became more prominent.

Table 1 shows flow cytometric data for different numbers of spiked HUVECs mixed into 10^6 PBMCs. Values of spiked and detected HUVEC numbers shown include mean (SD), with corresponding CV. The yield was represented in both mean yield and CV of the yield (CVyield). The mean yield is the ratio of detected cell number over the spiked cell count in percentage. Thus, CVyield was calculated from the square root of the sum of squares of both the CV of the detected numbers (CVdetected) and the CV of the spiked number (CVspiked), i.e., CVyield = (21). The CV from the yield accounts for the variation from both spiked and detected cells. In contrast, some calculation accounts only for the variation from detection. However, bad control on the number of spiked cells causes large variation on yield. In reality, both the preparation of sample and the robustness of detection influence the net yield. The stock of 20 cells was produced by diluting 200-cell stock 10 times. Hence, the spiked number of 200-cells was calculated by multiplying 10 to the average of 20 cells. The SD of 200 cells was also calculated (22). The de-
tected numbers are all larger than corresponding spiked numbers.

YIELD OF HUVECs

Table 2 presents tabulated data from the disk’s results over a range of spiked numbers of HUVECs in $10^6$ PBMCs. For a large number of spiked cells, e.g., cases of 60, 180, and 360 HUVEC cells, both the $CV_{\text{spiked}}$ and $CV_{\text{detected}}$ were less than or around 15%, with the uncertainties in yield ($CV_{\text{yield}}$) also below or around 15%. In the cases of 10 and 20 spiked cells, most CVs were larger than 15%.

Fig. 3A presents the number of detected HUVECs after disk operation and detected HUVECs via flow cytometer, both against spiked cells. In disk tests with 1 million PBMCs as surrounding cells, the spiked HUVEC count was categorized into 5 groups: 10, 20, 60, 180, and 360 cells. Each test contained 3 replicates. Results show a linear trend over the 3 orders of magnitude of HUVEC range in the full logarithmic scale plotted. Furthermore, the counts of HUVECs from flow cytometry were used as a reference to evaluate the performance of the disk.

Linear regression was used to fit the data points. Results show that $R^2$ values were all $>0.998$. The strong linearity in the data indicated that the mean yield was independent of the number of spiked HUVECs. Data from the regression analysis suggest that the detected and spike numbers of HUVECs are positively proportional. This proportional relationship implies that the number of spiked HUVECs could be determined from the arbitrary number of detected HUVECs divided by 0.97, at least over the range of spiked cells tested.

For flow cytometry data, 3 sets of spiked HUVECs in PBMCs were tested: 0, approximately 20, and approximately 200 cells (owing to logarithmic scale used in the coordinate, the 0 HUVEC data set is at negative infinity, thus not shown in Fig. 3A). The regression result shows that the amount of detected HUVECs for flow cytometry is higher than that for the disk, and the slope of linear regression for flow cytometry is $>1$. The fact that the mean yield of approximately 300 HUVECs is closer to 1 than for the lower spiked HUVECs suggests that flow cytometry produced less accurate results at lower cell counts. Besides, the $y$-intercept of flow cytometry was higher than that of the disk, indicating that the noise level of flow cytometry was higher than that of the disk.

Fig. 3B presents the yield for 60 spiked HUVECs in $10^6$, $10^5$, and $10^4$ PBMC counts. Results indicate that the mean yield was distinctly around 95% and varied only slightly with PBMC counts. The $CV_{\text{yield}}$ for a cell mixture is noted by the error bar for each case. The differences among the average yields were within the error bars, by comparing any 2 cases for different PBMC numbers. The volume fractions of $10^6$, $10^5$, and $10^4$ PBMCs in 60 $\mu$L liquid are roughly 1.7%, 0.17%, and 0.017%, respectively. The data suggest that the

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**Table 1.** Flow cytometer data for different numbers of spiked HUVECs mixed into $10^5$ PBMCs.

<table>
<thead>
<tr>
<th></th>
<th>0 cells</th>
<th>20 cells</th>
<th>200 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVECs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spiked</td>
<td>0</td>
<td>23.8 (5.9)</td>
<td>238.3</td>
</tr>
<tr>
<td>Detected</td>
<td>1.0 (1.0)</td>
<td>34.7 (5.9)</td>
<td>272.0 (81.6)</td>
</tr>
<tr>
<td>$CV_{\text{spiked}}$</td>
<td>%</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>$CV_{\text{detected}}$</td>
<td>%</td>
<td>100</td>
<td>17</td>
</tr>
</tbody>
</table>

Data are mean (SD) unless noted otherwise. All the detected numbers are larger than corresponding spiked numbers. Even subtracting the base level (detected count for 0 spiked case), the modified detected numbers are still around twice as much as spiked numbers.
mean yield is virtually identical for the various compositions of cell mixtures. Fig. 3A and B therefore demonstrate that results from the disk produced a mean yield that is independent of both the number of HUVECs and the PBMC count.

SLE PATIENTS’ CEC DATA

Fig. 4 shows the detected numbers of CECs of healthy controls (n = 10) and SLE patients (n = 10). The controls’ CEC counts ranged from 10 to 50, with a mean (SD) of 31.2 (13.3), whereas the SLE group’s CECs number ranged from 39 to 103, with a mean (SD) of 61.1 (21.5).

Discussion

Results from the disk platform show an average of 95% recovered cells over a wide range of HUVEC counts. The overall robustness of the disk in handling CECs appears to be sound. The approach might also be viable to study other cells in blood by using different immunological properties. For example, by the difference in immunological phenotype of CECs and endothelial progenitor cells in terms of CD45, CD133, or CD117 (23), we might be able to distinguish these 2 kinds of cells out of CD146 cells.

Comparison of results from the disk and flow cytometry deserves further discussion. First, examination of the CV data proved insightful. Two key points are noteworthy: (1) a failure of the CV to describe count variation exists for low numbers of spiked cells, and (2) the level of CV is an indication of the robustness of the method used. The number of spiked cells played a vital role in the significance of CV values. For small numbers of spiked cells, the underlying cause for the large CV of detected number and uncertainty of yield are less than or around 15%, except for 10- and 20-cell cases.

Table 2. Microfluidic disk data for different numbers of spiked HUVECs mixed into 10^6 PBMCs.

<table>
<thead>
<tr>
<th>HUVECs</th>
<th>10 cells</th>
<th>20 cells</th>
<th>60 cells</th>
<th>180 cells</th>
<th>360 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiked</td>
<td>11.0 (2.6)</td>
<td>24.7 (4.7)</td>
<td>62.1 (6.5)</td>
<td>180.0 (6.6)</td>
<td>328.7 (25.7)</td>
</tr>
<tr>
<td>Detected</td>
<td>10.0 (2.6)</td>
<td>23.3 (2.1)</td>
<td>61.7 (5.5)</td>
<td>182.7 (12.7)</td>
<td>316.0 (49.3)</td>
</tr>
<tr>
<td>CV_{spiked}</td>
<td>24</td>
<td>19</td>
<td>11</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>CV_{detected}</td>
<td>26</td>
<td>9</td>
<td>9</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>Yield (CV_{yield})</td>
<td>91 (36)</td>
<td>95 (21)</td>
<td>99 (14)</td>
<td>101 (8)</td>
<td>96 (17)</td>
</tr>
</tbody>
</table>

Data are mean (SD) unless noted otherwise. Yields are around 100% and CV of detected number and uncertainty of yield are less than or around 15%, except for 10- and 20-cell cases.

Fig. 3. Detected number and yield of HUVECs in PBMCs over a range of cell counts. (A), Number of detected HUVECs by disk and flow cytometer from the mixtures of HUVECs and PBMCs. SDs of both spiked and detected cell count are marked on each HUVF data point. (B), Average yields from disk for 60 HUVECs in 10^4 to 10^6 PBMCs.
Part of cell number variation, due to loss during cell mixture handling, would be unavoidable. Perhaps for the level of target cell number \( \times 10^6 \), SD alone should be a more appropriate parameter than CV. The fact that all CV detected were >15% shows that the count variation from flow cytometry (Table 1) is larger than that of the disk (Table 2). Therefore, we infer that the enumeration of CEC by disks is more robust than by flow cytometer in the model system.

Furthermore, in the flow cytometry result of Fig. 2, the identity of some cells, such as those in quadrant I and III, is not as certain as in isolated HUVECs or PBMCs. Some researchers reported that certain lymphocytes express CD146 (12, 24, 25). Thus, quadrant I dots may represent random clusters of such lymphocytes. Besides, cells in quadrant III, claiming 10%–15% of total population, have weak signal in both colors, and were believed to be the PBMCs with low intensity of FITC.

In flow cytometry, careful optimization of labeling of each fluorophore was required to properly present double-staining data. Otherwise, the intensity of anti-CD146-PE could overwhelm that of anti-CD45-FITC so that the overlapped FITC signal could not be compensated for by software. This would result in only 1 color that can be analyzed. In contrast to flow cytometry, the requirement of balanced labeling is not as strict in tests using the disk.

Both false-positive signals and cell loss in flow cytometry contributed to the yield. One may guess that cell loss in flow cytometry would be apparent, for it has longer fluid conduit compared to the disk. However, judging from the fact that the detected number was greater than the spiked number (Table 1), a false-positive signal seemed to be dominant.

Disk results (Fig. 4) show that the level of CECs for both SLE patients and controls are more than 20 times of that presented in Elshal et al. (4). There are many factors that contributed to this difference. In their study, Elshal et al. analyzed PBMCs from 1 mL blood with flow cytometry. In comparison, we processed 50 \( \mu L \) whole blood with our disks and enumerated CECs by fluorescence microscopy. PBMC enrichment and the precision of flow cytometry down to the level of hundreds should contribute to lower CEC levels.

In conclusion, a microfluidic disk was designed to enable separation and detection of rare cells. The capture of CECs, removal of large amounts of surrounding cells, and detection of target cells were all performed on-disk. The yield was very high and remained near-constant over a wide range of cell counts tested. Using the disk, we enumerated CECs in patients with SLE and found that they were significantly higher than those for healthy donors, suggesting that CEC count is a viable marker for symptoms of SLE.

**Fig. 4.** Detected number of CECs in 50 \( \mu L \) whole blood from 10 healthy donors and 10 SLE patients. The bars denote the averages of 31.2 CECs for healthy donors and 61.1 for SLE patients, respectively. **\( P < 0.01 \), meaning the difference of CEC numbers for 2 groups is significant.**

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**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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