Characterization of CD45⁻/CD31⁺/CD105⁺ Circulating Cells in the Peripheral Blood of Patients with Gynecologic Malignancies

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Abstract

Purpose: Circulating endothelial cells (CEC) have been widely used as a prognostic biomarker and regarded as a promising strategy for monitoring the response to treatment in several cancers. However, the presence and biologic roles of CECs have remained controversial for decades because technical standards for the identification and quantification of CECs have not been established. Here, we hypothesized that CECs detected by flow cytometry might be monocytes rather than endothelial cells.

Experimental Design: The frequency of representative CEC subsets (i.e., CD45⁻/CD31⁺, CD45⁻/CD31⁻/CD146⁺, CD45⁻/CD31⁻/CD105⁺) was analyzed in the peripheral blood of patients with gynecologic cancer (n = 56) and healthy volunteers (n = 44). CD45⁻/CD31⁺ cells, which are components of CECs, were isolated and the expression of various markers (CD146, CD105, vWF, and CD144 for endothelial cells; CD68 and CD14 for monocytes) was examined by immunocytochemistry.

Results: CD45⁻/CD31⁻/CD105⁺ cells were significantly increased in the peripheral blood of patients with cancer, whereas evaluation of CD45⁻/CD31⁺/CD146⁺ cells was not possible both in patients with cancer and healthy controls due to the limited resolution of the flow cytometry. Immunocytochemistry analyses showed that these CD45⁻/CD31⁻/CD105⁺ cells did not express vWF and CD146 but rather CD144. Furthermore, CD45⁻/CD31⁺/CD105⁺ cells uniformly expressed the monocyte-specific markers CD14 and CD68. These results suggest that CD45⁻/CD31⁻/CD105⁺ cells carry the characteristics of monocytes rather than endothelial cells.

Conclusions: Our data indicate that CD45⁻/CD31⁻/CD105⁺ circulating cells, which are significantly increased in the peripheral blood of patients with gynecologic cancer, are monocytes rather than endothelial cells. Further investigation is required to determine the biologic significance of their presence and function in relation with angiogenesis. Clin Cancer Res; 1–11. ©2013 AACR.

Introduction

Overcoming resistance to therapy is the ultimate goal of the development of novel treatment modalities in cancer (1). The biologic heterogeneity and genetic instability of cancer cells are significant barriers for the design of effective therapies. Therefore, relatively more homogeneous and genetically stable host factors have been suggested as alternative targets (2). Angiogenesis, which is one of the common and crucial steps in the development and progression of solid tumors, is a host-dependent process and, consequently, has been introduced as an attractive target of cancer treatment (3). A significant number of drugs designed to interrupt the establishment of tumor-associated vasculature by neutralizing vasulogenic factors is currently in clinical trials and some of them have been approved for clinical use in patients with cancer (4, 5). However, understanding the mechanisms of angiogenesis and establishing validated markers that accurately reflect the pharmacologic effects of antiangiogenic therapeutics remain major challenges (6).
Circulating endothelial cells (CEC) are recognized as a marker of tumor angiogenesis and a predictor of prognosis, as well as a target for therapy. However, their presence in the peripheral blood of patients with cancer and role in angiogenesis remain controversial. Our results showed that CD45+/CD31+/CD105+ cells were significantly increased, whereas CD45+/CD31-/CD146+ cells were not detected in the peripheral blood of patients with gynecologic cancer. In addition, the expression of monocyte-specific markers such as CD14 and CD68 in CD45+/CD31+/CD105+ cells suggested that they are monocytes rather than endothelial cells. Collectively, our data suggest that the accuracy of conventional flow-cytometric analyses for identifying CECs should be meticulously reevaluated in its technical and biologic aspects. Moreover, further investigation is necessary to establish the biologic significance of the presence of CD45+/CD31+/CD105+ monocytes and their function in relation to angiogenesis.

Because circulating endothelial cells (CEC) are likely to contribute to new vessel formation (7) and their levels in the blood change in response to pro- or antiangiogenic drugs (8–10), the measurement of CECs (total CECs including progenitor cells) has been regarded as a promising strategy for monitoring tumor angiogenesis. Several studies reported a significant increase in the number of CECs in patients with cancer with progressive disease (i.e., lymphoma, breast cancer, renal cancer, etc.; refs. 11–13). In addition, studies have shown that CEC kinetics and viability correlate well with clinical outcomes of patients with cancer undergoing antiangiogenic treatment (14, 15).

However, the presence and role of CECs have been controversial for decades because trials using CECs as a diagnostic parameter or a therapeutic target did not produce consistent results. Moreover, markers that accurately identify CECs have not been established because many circulating cells such as monocytes express many of the same markers as endothelial cells (16). Consequently, the detection and estimation of CECs have remained challenging as they comprise a small proportion of peripheral blood cells, and there is no consensus in the immunophenotype of CEC (17). The application of various combinations of markers or different enumeration techniques to the detection of CECs has produced discrepant results in the amount and immunophenotype of CECs (18–20). Furthermore, certain authors have questioned whether CECs detected by flow cytometry are authentic endothelial cells and their function in angiogenesis, if any (17, 21). Recent studies have suggested that the actual angiogenic cell types incorporated into newly formed vessels are myeloid cells such as monocytes (22–24) rather than CECs and/or their progenitor cells.

Therefore, establishing a method to validate the identity of CECs detected by flow cytometry and to assess their biologic significance is critical before expanding their clinical use.

Considering the angiogenic role of monocytes and the technical hurdles of flow cytometry, we hypothesized that a subset of circulating cells detected by flow cytometry using conventional CEC markers might be monocytes rather than endothelial cells. To show this, we first evaluated the flow-cytometric techniques and markers currently in use and analyzed the frequency of representative CEC subsets (i.e., CD45+/CD31+, CD45+/CD31+/CD146+, CD45+/CD31+/CD105+; refs. 25–28) in the peripheral blood of patients with gynecologic cancer and healthy volunteers. To identify the genuine lineage of those cells, we isolated CD45+/CD31+ cells (a common denominator of CECs) and assessed the expression of various markers for endothelial cells or monocytes by immunocytochemistry.

Materials and Methods

Subjects
Peripheral blood samples (1–2 mL) were collected from 44 healthy donors (12 men and 32 women; age, 28–54 years) and 56 patients with gynecologic cancer including 8 patients with endometrial cancer (age, 39–59 years), 24 with cervical cancer (age, 30–71 years), and 24 with ovarian cancer (age, 23–67 years; Supplementary Table S1). All healthy volunteers were free of any medications and had no cardiovascular disease. The Institutional Review Board at Kwandong University College of Medicine (Seoul, Republic of Korea) approved all protocols, and informed consent was obtained from all subjects.

Antibodies for flow cytometry
The following monoclonal antibodies directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll A protein (PerCP), or allophycocyanin (APC) were used for flow-cytometric analysis: anti-CD31 FITC (WM-59 clone), anti-CD61 FITC (VI-PL2 clone), anti-CD3 PE (SK7 clone), anti-CD19 PE (HB19 clone), anti-CD31 PE (WM-59 clone), anti-CD41a PE (HIP8 clone), anti-CD56 PE (MY31 clone), anti-CD146 PE (PIH12 clone), anti-CD45 PerCP (2D1 clone), and anti-CD14 APC (M5E2 clone). Isotype-matched FITC-, PE-, PerCP-, and APC-conjugated control antibodies were purchased from BD Biosciences. Anti-CD105 PE (SN6 clone) and isotype-matched PE-conjugated control antibodies were purchased from Serotec. Anti-CD31 APC (WM-59 clone) and isotype-matched APC-conjugated control antibodies were purchased from eBioscience.

Preparation of peripheral blood mononuclear cells
Peripheral blood was collected from healthy volunteers and patients with cancer using EDTA as an anticoagulant and processed within several hours after collection as follows: whole blood was diluted 1:1 (vol/vol) with PBS containing 0.5% bovine serum albumin (BSA) and 2 mmol/L EDTA and overlaid onto an equal volume of Ficoll...
Paque (GE healthcare). Samples were centrifuged at 1,800 rpm for 25 minutes at room temperature with no brake. The mononuclear cell layer was carefully collected and washed twice with cold PBS containing 0.5% BSA and 2 mmol/L EDTA at 4°C. Red blood cells were lysed with 0.38% ammonium chloride solution. The final mononuclear cell preparation was resuspended with PBS containing 0.5% BSA and 2 mmol/L EDTA and then subjected to flow-cytometric analysis. The viability of the mononuclear cells used for the analyses was determined by the dye exclusion test, and cells with a viability of 99% or more were used for further experiments.

**Flow cytometry and cell sorting**

Isolated peripheral blood mononuclear cells (PBMC; 10⁷ cells per mL of blood) were pretreated with FcR blocking reagent (Miltenyi Biotec) to block nonspecific antibody binding and incubated on ice for 25 minutes with a panel of monoclonal antibodies (summarized in Table 1). Cells were washed with PBS containing 0.5% BSA and 2 mmol/L EDTA and fixed with 4% paraformaldehyde (PFA; Electron Microscopy Sciences). The antibody-labeled cells were analyzed using a FACS Aria flow cytometer (BD Biosciences) equipped with 2 lasers (488 nm and 633 nm). Data were analyzed with FlowJo software (Tree Star, Inc.) or FACS Diva (BD Biosciences). For the analysis of CEC candidates, at least 100,000 singlet lymphocytes were isolated and the frequencies of CD45⁻/CD31⁺, CD45⁻/CD31⁺/CD146⁺, or CD45⁻/CD31⁺/CD105⁺ cells were analyzed and expressed as a percentage of the singlet lymphocyte population. For sorting of CD45⁻/CD31⁺ cells, PBMCs prepared from patients with cancer were pretreated with FcR blocking reagent and stained with fluorescence-labeled monoclonal antibodies against CD45 and CD31. The cells were fixed with 4% PFA and then sorted with a FACS Aria flow cytometer. A 70-μm nozzle (BD Biosciences), a sheath pressure of 20 to 25 pounds per square inch, and an acquisition rate of 2,000 to 3,000 events per second were used according to the guidelines for FACS Aria users (BD Biosciences).

**Immunofluorescence staining**

Immunocytochemical fluorescence labeling of cells was conducted as previously described (24). Briefly, CD45⁻/CD31⁺ cells isolated by flow cytometry were cyto spun onto glass slides and washed 3 times with PBS for 3 minutes. To stain intracellular antigens, cells were permeabilized with 0.5% Triton X-100 (Sigma Chemical Co.). To prevent cross-reaction with antibodies used to stain cells in the flow-cytometric analysis, Fab-fraction blocking was conducted overnight at 4°C with an antibody from the same host species of antibody, which was a F(ab')₂ fragment from goat anti-mouse immunoglobulin G (IgG) (Jackson Immunoresearch Laboratories) in this study. After the blocking step, cells were stained with the following antibodies: mouse anti-human CD105 monoclonal antibody (mAb; 1:100, Serotec), mouse anti-human CD146 mAb (1:100 dilution, Chemicon), mouse anti-human CD144 mAb (1:100, Reliatech GmbH), mouse anti-human CD68 mAb (1:100 dilution, DAKO), polyclonal rabbit anti-human vWF Ab (1:100 dilution, DAKO), or mouse anti-human CD14 mAb (1:20 dilution, DAKO), followed by labeling with the corresponding secondary antibodies conjugated with FITC or Texas Red. For double-staining experiments,

| Table 1. Antibody panels used for flow-cytometric analysis and isolation of CD45⁻/CD31⁺ cells |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **FITC** | **PE** | **PerCP** | **APC** |
| **For immunotyping** | Controls for compensation | IgG1 | IgG1 | IgG1 | IgG2a |
| | CD31 | IgG1 | IgG1 | IgG2a |
| | IgG1 | CD31 | IgG1 | IgG2a |
| | IgG1 | IgG1 | CD45 | IgG2a |
| | IgG1 | IgG1 | IgG1 | CD14 |
| **Samples** | CD31 | CD3 | CD45 | CD14 |
| | CD31 | CD19 | CD45 | CD14 |
| | CD31 | CD56 | CD45 | CD14 |
| | CD31 | CD146 | CD45 | CD14 |
| **For CEC detection** | Controls for compensation | IgG1 | IgG1 | IgG1 | - |
| | CD31 | IgG1 | IgG1 | - |
| | IgG1 | CD31 | IgG1 | - |
| | IgG1 | IgG1 | CD45 | - |
| | FMO control | CD31 | IgG1 | CD45 | - |
| **Samples** | CD31 | CD146 | CD45 | - |
| | CD31 | CD105 | CD45 | - |
| **For isolation of CD45⁻/CD31⁺ cells** | Controls for compensation | - | - | IgG1 | IgG1 |
| | - | - | IgG1 | CD31 |
| | - | - | CD45 | IgG1 |
| **Sample** | - | - | CD45 | CD31 |
the protein and fragment blocking steps were repeated before treating with the second primary antibody to prevent cross-reaction. Cell nuclei were counterstained with Hoechst 33342 dye (Invitrogen). The slides were then washed 3 times with PBS for 3 minutes each and mounted in Vecta-shield (Vector Laboratories). Images were acquired with a LSM510 Meta DuoScan confocal system (Zeiss).

**Statistical analysis**

The Mann–Whitney U test was used to determine the statistical significance of differences in the frequencies of CEC candidates between the peripheral blood collected from patients with cancer and healthy volunteers. All statistical tests were two-sided. P values less than 0.05 were considered significant.

**Results**

**Establishment of flow cytometry gating strategies for the measurement of circulating endothelial cells**

First, we found that monocytes showed higher levels of autofluorescence than lymphocytes (data not shown), indicating that they should be analyzed separately for fluorescence compensation. In flow-cytometric analysis, the detection of equal levels of autofluorescence in positive and negative populations for each single stain indicates that the fluorescent compensation is appropriate (29). As an initial step to establish the efficient gating strategies for the detection of CECs by flow cytometry, we attempted to determine the subset(s) of PBMCs expressing the CD45+/CD31+ phenotype. PBMCs were plotted according to the forward scatter (FSC) versus side scatter (SSC) profiles and FSClow/SSCmid (Fig. 1A, left), FSChigh/SSCmid (Fig. 1B, left), and FSCmid/SSChigh (data not shown) fractions were gated as lymphocytes, monocytes, or granulocyte subpopulations, respectively. Subpopulations of cells with different CD45 and CD31 expression patterns were further analyzed for the expression of CD45 or lineage-specific markers including CD3 (T lymphocyte), CD14 (monocyte), CD19 (B lymphocyte), and CD56 (NK cells). The FSClow/SSClow subset was mostly composed of CD45+/CD31− cells expressing CD3, CD19, or CD56 antigens (Fig. 1A), whereas the FSChigh/SSCmid subset mainly included CD45+/CD31+ cells expressing the CD14 antigen (Fig. 1B). CD45−/CD31+ cells were detected only in the FSClow/SSClow fraction (Fig. 1A) but neither in the FSChigh/SSCmid (Fig. 1B) nor FSCmid/SSChigh fractions (data not shown).

In the polychromatic flow-cytometric analysis used in the present study, adequate threshold was assessed by fluorescence-minus-one (FMO) gating, which consists of analyzing cells stained with all antibodies except the one being tested (30). To investigate the effects of the gating controls on the actual event frequencies, the flow-cytometric analysis of the singlet lymphocyte fraction was conducted using either the isotype control or the FMO control (Fig. 2A). Because the negative threshold of the FMO control (Fig. 2A, b) was higher than that of the isotype control (Fig. 2A, a), gating with FMO controls could decrease the false-positive event frequencies. In line with the study by Cui and colleagues (31), gating with FMO controls was shown to be a more efficient method to increase the accuracy and specificity of the positive signals in polychromatic flow-cytometric detection of rare events, such as CECs, than the use of isotype controls.

On the basis of these results, we established a gating strategy to determine the frequencies of CECs in PBMCs, as described in Fig. 2B. In brief, cells were stained with a panel of antibodies in parallel with FMO controls. The singlet lymphocyte population was identified on a FSC/SSC plot and subgated onto a bivariate antigen plot to identify CD45+/CD31− cells. These cells were further subgated to identify the corresponding CD146+ or CD105+ subpopulation.

**Flow-cytometric analysis of CECs in PBMCs**

A number of protein markers including CD31, CD34, CD105, CD146, CD144, and VEGF receptor-2 have been used to define CECs. However, there is no truly specific marker to identify CECs because those markers are also expressed in other type of cells (32). A generally accepted definition of CECs is CD45+/CD31+ cells expressing CD146 or CD105 (11), but this definition needs to be modified. According to the gating strategy described above, we examined the frequencies of CD45+/CD31−, CD45+/CD31−/CD146+, and CD45+/CD31−/CD105+ cells in the PBMCs of patients with cancer (n = 56) and healthy volunteers (n = 44) by flow cytometry. The frequency of CD45+/CD31+ cells was significantly higher in the singlet FSClow/SSClow population of patients with cancer (median, 1.365%; range, 0.110–26.85%) than in that of healthy volunteers (median, 0.183%; range, 0.027–3.980%; P < 0.0001) as shown in Fig. 3A. In contrast with a previous report (11), the frequency of CD45+/CD31−/CD146+ cells in healthy volunteers (median, 0%; range, 0–0.003%) and patients with cancer (median, 0.001%; range, 0–0.016%) was lower than the cutoff values of the FMO control group (median, 0.007%; range, 0–0.021%), indicating that estimation of the frequency of those cells is not possible both in cancer patients and healthy controls due to the limited resolution of the flow cytometry. Actually, when the isotype control was used, the frequencies of CD45+/CD31−/CD146+ cells were significantly higher (median, 0.041%; range, 0.007–0.132%; Fig. 2C), underscoring the importance of using FMO controls.

Meanwhile, CD146 expression was detected only in the CD45+/CD31− population of the FSClow/SSClow subpopulation (Fig. 1A). These CD146-positive cells also expressed CD105 (data not shown). These results, together with those of previous studies showing that CD146 is present in a subset of activated T lymphocytes (21, 33), indicate that CD146+ cells in the CD45+/CD31− lymphocyte subpopulation are T lymphocytes.

The frequency of CD45+/CD31−/CD105+ cells in healthy volunteers (median, 0.003%; range, 0–0.027%) showed no significant differences statistically when compared with FMO control (P > 0.05). On the other hand, the
The frequency of CD45+/CD31+/CD105+ cells in patients with cancer (median, 0.012%; range, 0–0.461%) was significantly higher than in healthy volunteers ($P < 0.0001$) as shown in Fig. 3B.

There was no significant statistical difference in the frequency of CD45+/CD31+, CD45+/CD31+/CD146+, and CD45−/CD31+/CD105+ cells between healthy male and female volunteers.
Characterization of anucleated CD45⁻/CD31⁺ cells

To date, several methods have been used to quantify CECs. However, there is currently no consensus on the most accurate markers for their identification. Moreover, CEC quantification methods have not been adequately validated or standardized. For these reasons, there has been a significant variation in the CEC numbers reported, including those of the present study. To determine whether the CEC candidate cells identified by flow cytometry have true endothelial phenotypes, we isolated CD45⁻/CD31⁺ cells from the PBMCs of patients with cancer by FACSAria sorter and examined their morphologic and immunologic characteristics. CD45⁻/CD31⁺ cells (mostly lymphocytes) and CD45⁻/CD31⁺ cells (mostly monocytes) were also isolated and used as controls.

Two different populations of CD45⁻/CD31⁺ cells were observed by confocal microscopy and scanning electron microscopy, anucleated cells (2–6 μm in diameter), which were smaller than lymphocytes, and nucleated cells (8–10 μm in diameter; data not shown). Strijbos and colleagues reported that the vast majority of CD45⁻/CD31⁺ cells are, in fact, large platelets rather than endothelial cells (17). To investigate the possibility of false-positive results of the flow-cytometric quantification of CECs, we assessed CD45⁻/CD31⁺ cells for the expression of platelet markers such as CD41 and CD61 by flow cytometry and found that most CD45⁻/CD31⁺ cells stained negative for anti-CD146, anti-CD105, and anti-CD144 antibodies but positive for vWF, a common marker of endothelial cells and platelets (Fig. 5B, top) as assessed by immunocytochemical staining and confocal microscopy. These results together with the morphologic phenotypes of
these cells suggest that anucleated CD45<sup>+</sup>/CD31<sup>+</sup> cells are mainly platelets.

**Characterization of nucleated CD45<sup>+</sup>/CD31<sup>+</sup> cells**

To determine whether endothelial cells are included in the nucleated CD45<sup>+</sup>/CD31<sup>+</sup> cell population, CD45<sup>+</sup>/CD31<sup>+</sup> cells were isolated as described previously and stained with antibodies against CD146 (34), CD105 (35), VWF (36), and CD144 (37). Consistent with the flow-cytometric results, most nucleated CD45<sup>+</sup>/CD31<sup>+</sup> cells expressed CD105 but not CD146 (Fig. 5A, top). These CD45<sup>+</sup>/CD31<sup>+</sup>/CD105<sup>+</sup> cells also expressed CD144 (Fig. 5A, bottom).

An increasing body of evidence indicates that monocytes share several functional and immunophenotypic characteristics with endothelial cells (38, 39). Moreover, endothelial progenitor cells or circulating angiogenic cells derived from monocyte/macrophage lineage were reported to promote angiogenesis by secreting angiogenic growth factors (40). To determine whether CD45<sup>+</sup>/CD31<sup>+</sup>/CD105<sup>+</sup> cells possess monocyte/macrophage characteristics in addition to those of endothelial cells, cells were stained with an anti-C68 antibody (macrophage marker) and the anti-VWF antibody. Interestingly, CD45<sup>+</sup>/CD31<sup>+</sup>/CD105<sup>+</sup> cells stained positive for CD68 (Fig. 5B, bottom) but negative for VWF (Fig. 5B, top). Furthermore, these cells expressed the monocyte-specific antigen CD14 (Fig. 5C). CD45<sup>+</sup>/CD31<sup>+</sup> cells (mostly lymphocytes) were used as a negative control for CD68, VWF, and CD14 expression, whereas CD45<sup>+</sup>/CD31<sup>+</sup> cells (mostly monocytes) were used as a positive control for CD68 and CD14 expression (Fig. 5C for CD14 expression). Human umbilical vein endothelial cells and human dermal microvascular endothelial cells were used as positive controls for VWF, CD146, CD105, and CD144 (data not shown).

The specificity of sorted CD45<sup>+</sup>/CD31<sup>+</sup> cell population could be confirmed and the possibility of contamination of sorted cell population with CD45<sup>+</sup>/CD31<sup>+</sup> cells could be excluded because CD45<sup>+</sup>/CD31<sup>+</sup> cells were not detected when the sorted cells by CD45<sup>+</sup>/CD31<sup>+</sup> gate were analyzed by the CD45/CD31 expression (Supplementary Fig. S1).

Collectively, these data indicate that anucleated CD45<sup>+</sup>/CD31<sup>+</sup> cells were mainly platelets, and CD45<sup>+</sup>/CD31<sup>+</sup>/CD105<sup>+</sup> cells were derived from monocytes/macrophages rather than endothelial cells.

**Discussion**

CECs in the peripheral blood have been widely recognized as a marker of angiogenesis. Most studies have relied on multiparametric flow cytometry to identify endothelial cells because of the limited specificity of the markers used for CEC detection, which can also be expressed by other hematopoietic cells (16, 39). CECs are currently defined as cells that express CD31 and other markers such as CD146, CD34, or CD105, but not CD45, a pan-leukocyte marker (7, 11). However, their rareness and phenotypic overlap with other hematopoietic cells have led to controversies about the identification and determination of CECs in peripheral blood. Therefore, the present study focused on assessing the accuracy of current flow-cytometric techniques for the identification of CECs and the immunofluorescence phenotyping of CECs using different markers. Our results suggest that those cells that meet the conventional criteria to be defined as CECs are derived from a monocyte lineage rather than having an endothelial origin.
Autofluorescence is an important consideration when conducting polychromatic flow-cytometric analyses. Because monocytes have higher levels of autofluorescence than lymphocytes, these 2 types of cells should be analyzed separately (29). The frequency of CECs has mostly been analyzed by gating the lymphocyte and monocyte populations as a whole without separation, which can lead to false-negative or false-positive results. To overcome this potential defect, we first gated FSC<sub>low</sub>/SSC<sub>low</sub> fraction (mostly lymphocytes) and then subgated CD45<sup>+</sup>/CD31<sup>+</sup> cells for a more detailed and accurate characterization. In addition, we also showed that FMO is a more accurate control to set the boundaries for the analysis of rare cells such as CECs as described previously (31).

Several studies have shown evidences that CECs, defined as DNA<sup>+</sup> cells with CD34<sup>+</sup>/CD45<sup>+</sup>/CD146<sup>+</sup> or CD31<sup>+</sup>/CD45<sup>+</sup>/CD146<sup>+</sup> immunophenotypes, are significantly elevated in the peripheral blood of patients with cancer than healthy subjects (41, 42). The origin of the cells was confirmed as endothelial cells by morphology, immunohistochemistry, gene expression, and the presence of Weibel–Palade bodies. However, there have been scientific issues to be improved that the effects of various fluorescent compensation methods on the detection of CECs were not considered. Moreover, expression of several lineage markers other than endothelial cell origin should be fully investigated to identify the genuine origin of those cells, because the immunophenotypes can be overlapped among cells.
derived from endothelial cells, hematopoietic progenitors, or monocytes, etc.

On the basis of the cell sorting conditions used, our results did not agree with previous studies (11) in which few CD45+/CD31+/CD146+ cells, which are known to define CECs, were present in the peripheral blood, even in that of patients with cancer. Although the frequency of CD45+/CD31+/CD146+ cells seemed to be significant if isotype controls were used, they remained at background levels in the presence of FMO controls. In addition, our immunocytochemical data showed that none of the isolated CD45+/CD31+ cells expressed CD146. CD146 expression has been reported on activated T-cell subsets in healthy individuals (33). Previously, Duda and colleagues reported that CD146 marks endothelial cells in normal and neoplastic tissues, as well as a subset of T cells (21). These authors reported that CD146 expression was frequently detected on pericytes, and CD45+/CD146+ cells were occasionally contained in the massive hematopoietic cell infiltration in tumor tissues. Our results were in agreement with those of Duda and colleagues in that CD146+ cells were detected exclusively in the CD3− cell population.

We could not detect the CD45+/CD31+/CD146+ cells in the peripheral blood of patients with gynecologic cancer; however, it remains to be further tested and compared among patients with different types or sites of malignancies. For instance, endothelial cells in hemangioma tissues showed negative immunoreactivity for CD146 (43), whereas the expression of CD146 was highly increased in the blood vessels of breast carcinoma (44).

Because flow cytometry is not sensitive enough to obtain reproducible results when analyzing rare cells such as CECs, the results of these analyses should be interpreted with caution (45). The frequency of CECs in the peripheral blood of the healthy population is between 1 × 10−7 and 1 × 10−5 per leukocyte (0–20 cells/mL of venous blood; refs. 20, 46). This level is below the detection threshold for conventional flow cytometry, which is approximately 1 × 10−4 (45). We therefore assumed that the frequency of CECs may be too low for detection by flow cytometry, despite their presence among PBMCs.

On the other hand, the frequency of CD45+/CD31+/CD105+ cells (another potential CEC candidate) was significantly higher in patients with cancer than in the healthy population. Furthermore, the frequency of CD45+/CD31+/CD105+ cells was significantly increased by up to approximately 8-fold in patients with gynecologic cancer compared with healthy volunteers. CD105 (endoglin), a 180-kDa homodimeric integral membrane glycoprotein and a commonly used marker for the detection of CECs next to CD45, was mainly expressed on endothelial cells of capillaries, veins, and arteries (35) but was also detectable on activated monocytes, macrophages, erythroid precursors, fibroblasts, mesangial cells, follicular dendritic cells, and syncytiotrophoblasts (47). Interestingly, our immunocytochemistry data showed that these CD45+/CD31+/CD105+ cells did not express VWF (36), whereas they were positive for CD144 (VE-cadherin; ref. 37). Actually, monocytes express not only CD31 constitutively (48) but also other markers including CD144, KDR, Tie-2, and CD105 when they are activated (49). Therefore, we further analyzed CD45+/CD31+/CD105+ cells with antibodies against monocyte-specific markers such as CD14 and CD68, and showed that CD45+/CD31+/CD105+ cells uniformly expressed both CD14 and CD68. This result indicates that CD45+/CD31+/CD105+ cells have the characteristics of monocytes rather than those of endothelial cells (Supplementary Fig. S2). Prokopi and colleagues showed that the endothelial phenotype may arise in mononuclear cells through the uptake of platelet microparticles (derived from the disintegration of platelets during mononuclear cell preparation) that abundantly contain marker proteins such as CD31 and VWF (50). More importantly, monocytes in the peripheral blood are known to exhibit versatile and flexible differentiation potentials and functions, as they can differentiate into macrophages, dendritic cells, osteoclasts, microglia, or even endothelial-like cells (51). Myeloid lineage cells including monocytes were reported to express markers that were expressed in both endothelial cells and monocytes (22, 49, 52–54). Furthermore, circulating CD31+ cells, which can contribute to the development of new blood vessels, were shown to be monocytes in an elaborate animal model (24).

In summary, our results showed that CD45+/CD31+/CD105+ circulating cells detected by flow cytometry under gating conditions we established, which were significantly increased in the peripheral blood of patients with gynecologic cancer, were not CECs but rather monocytes, suggesting that the conventional flow-cytometric techniques used for the identification of cell subpopulations could be improved by adjusting gating conditions. Further study is needed to identify the biologic significance of these cells and their function in relation to angiogenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H.-K. Yu, J.-S. Kim, S.J. Kim, T.J. Kim
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