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Detection of Inflammatory Circulating Endothelial Cells Using Human Umbilical Vein Endothelial Cells Detached from Culture Dishes by Tumor Necrosis Factor-alpha as Control Cells

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Abstract

Background: Systematic inflammatory response syndrome (SIRS) with disseminated intravascular coagulation (DIC) is a severe disorder in critically ill patients and closely related to progression of vascular endothelial injury. Early diagnosis is required to prevent the progression of organ dysfunction. Circulating endothelial cells (CECs) might increase in vascular endothelial injury and play an important role in DIC diagnosis. However, the CEC detection method has not been standardized. This study aimed to establish a method for CEC detection in a critical care setting. We used human umbilical vein endothelial cells (HUVECs) detached from culture dishes by tumor necrosis factor-alpha (TNF-α) as control cells for CECs.

Methods: Cultured HUVECs were incubated in medium with TNF-α (100 ng/mL), and cells detached from culture dishes after 24 h were used as TNF-HUVECs. Cell surface molecules of normal HUVECs, TNF-HUVECs, and blood cells were analyzed using flow cytometry (FC) to search for appropriate markers for detecting CECs. Normal HUVECs and TNF-HUVECs were added to the blood and detected using FC and the immunobead method (IB) for comparing two methods. CECs were measured in healthy volunteers and intensive care unit (ICU) patients using FC.

Results: CD146 and CD105 were highly expressed in HUVECs and superior for separation of UVECs from whole blood cells. Mean detection rates of normal HUVECs were 75% in FC and 82% in IB. However, mean detection rates of TNF-HUVECs were 64% in FC and 27% in IB ($p < 0.05$). Mean CEC counts from 20 healthy volunteers and 16 ICU patients were 2.8 cells/mL and 4.3 cells/mL, respectively. In one ICU patient with SIRS-induced DIC, CECs were elevated by 49 cells/mL.

Conclusion: CD146 and CD105 are suitable for detecting endothelial cells from blood. FC is superior to IB for detecting endothelial cells in severe inflammatory states.

Keywords: Circulating endothelial cells; Human umbilical vein endothelial cells; Tumor necrosis factor-alpha; Control cells; Flow cytometry; Vascular endothelial injury; Systematic inflammatory response syndrome; Disseminated intravascular coagulation; Intensive care unit patients; Disseminated intravascular coagulation; Intensive care unit patients

Abbreviations: SIRS: Systematic Inflammatory Response Syndrome; DIC: Disseminated Intravascular Coagulation; CECs: Circulating Endothelial Cells; HUVECs: Human Umbilical Vein Endothelial Cells; TNF-α: Tumor Necrosis Factor-alpha; FC: Flow Cytometry; IB: Immunobead Method; ICU: Intensive Care Unit; UEA1: Ulex Europaeus Agglutinin 1; RT: room temperature; 7-AAD: 7-Aminoactinomycin D; PBS: Phosphate Buffered Saline; PE: Phycoerythrin; APC: Allophycocyanin; FITC: Fluorescein Isothiocyanate; FSC: Forward Scatter; SSC: Side Scatter; SOFA: System Organ Failure Assessment

Introduction

Circulating endothelial cells (CECs) are mature vascular endothelial cells released from vascular beds into the blood. The CEC count is increased by vascular endothelial injury in systemic vascular inflammation and many vascular diseases, including myocardial infarction, vasculitis, and cancer [1-6]. CEC count is correlated with survival in coronary artery disease [2]. Further, in antineutrophil cytoplasmic antibody-associated vasculitis, inflammatory activity and CEC levels are correlated [3]. Accurate quantification of CECs would allow evaluation of the pathophysiological progression of vascular endothelial injury.

Critically ill patients tend to have serious inflammation such as systemic inflammatory response syndrome (SIRS) [7]. SIRS leads to SIRS-associated coagulopathy and disseminated intravascular coagulation (DIC), which is closely related to progression of vascular endothelial injury [8,9]. Early and appropriate diagnosis of DIC in critically ill patients with SIRS is required to avoid multiple organ dysfunction. The establishment of a method for CEC detection could play an important role in preventing severe DIC and multiple organ dysfunctions.

Previously reported methods for CEC detection were controversial. Measurements of CECs in healthy subjects show large differences [2,4-6,10-17]. An appropriate positive control needs to be used for accurate measurement of CECs. Tumor necrosis factor-alpha (TNF-α) is one of the major proinflammatory cytokines and contributes to endothelial injury in SIRS. Therefore, endothelial cells detached by TNF-α might be an appropriate positive control for CECs in critically ill patients with SIRS-induced DIC.

This study aimed to establish a method for CEC detection in a critical care setting. We used human umbilical vein endothelial cells (HUVECs) detached from culture dishes by TNF-α as control cells control cells for CECs.

Materials and Methods

Ethical approval

All study protocols were approved by the Institutional Review Board of Nagoya University Hospital (Approval Number 2012-0336). All participants provided written informed consent. The HUVECs were obtained from a commercial source (200-05n; Cell Applications Inc, San Diego, USA). All experiments were performed at the Nagoya University Graduate School of Medicine (Nagoya, Japan).

Preparation of normal-HUVECs and TNF-HUVECs

HUVECs were incubated with vascular endothelial growth medium (EGM-2; Lonza, Basel, Switzerland) in culture dishes at 37°C and 5% CO_2 until they reached approximately 50% confluence. TNF-α (100 ng/mL; Wako, Osaka, Japan) was administered and the detached cells, after 24 h, were used as TNF-HUVECs.

To generate control cells against TNF-HUVECs, HUVECs were incubated in EGM-2 without TNF-α. The adherent cells were trypsinized after 24 h and used as Normal-HUVECs.

Analysis of cell surface molecules of Normal-HUVECs, TNF-HUVECs, and blood cells

One hundred microliters of cell suspensions (1×10^7 cells/mL) of Norma-HUVECs, TNF-HUVECs, and blood cells from a healthy volunteer were incubated with fluorescence-conjugated ulex europaeus agglutinin 1 (UEA1) and antibodies against CD146, CD105, CD31, CD309, CD34, CD45, and CD133 for 30 min at room temperature (RT). The samples were analyzed using a flow cytometer (FACSCanto 2; BD Biosciences, San Jose, USA). FlowJo® software (Tree Star Inc., Ashland, USA) was used for data analysis. 7-Aminoactinomycin D (7-AAD) was used as a marker of dead cells. Normal-HUVECs and TNF-HUVECs labeled with endothelial markers (CD146, CD105, CD31, and UEA1) and Hoechst 33342 were examined on microscope slides with a confocal microscope (A1RMP, Nikon, Tokyo, Japan).

Detection of normal-HUVECs and TNF-HUVECs in blood

Blood was obtained from healthy volunteers with written consent. Normal-HUVECs and TNF-HUVECs were labeled with Hoechst 33342 and the cell concentrations were determined by counting the number of Hoechst-positive cells using a Nageotte chamber (Sunlead Glass Corp, Saitama, Japan) and a fluorescence microscope (BX51; Olympus, Tokyo, Japan). One hundred microliters of a suspension of Normal-HUVECs and TNF-HUVECs (1×10^3 cells/mL) was added to blood and analyzed using flow cytometry (FC) and the immunobead method (IB). Normal-HUVECs and TNF-HUVECs were defined as Hoechst 33342^+/ CD146$^+$/ CD45$^-$ or ^{dim}/ CD105$^+$/ CD133$^-$

in FC and Hoechst 33342^+/ CD146$^+$/ UEA1$^+$ in IB. This procedure was performed eight times for each study.

FC protocol

One milliliter of blood was incubated with lysing solution (BD Biosciences, San Jose, USA) in a tube at RT for 15 min. Ten milliliters of phosphate buffered saline (PBS) with 0.5% BSA and 2 mM EDTA was added, the tube was centrifuged at 400 × g for 5 min, and the supernatant was then discarded. The sample was incubated at RT for 10 min with 200 μL of blocking solution containing 10 μL of FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) and 100 μL of mouse serum (Sigma-Aldrich, St. Louis, USA). Then, it was mixed and incubated at RT for 30 min with 300 μL of antibody solution containing 0.2 μL of phycoerythrin (PE)-labeled anti-CD146, 4 μL of allophycocyanin (APC)-labeled anti-CD105, 4 μL of PE-Vio770-labeled anti-CD45, 1 μL of biotin-labeled anti-CD133, 1 μL of fluorescein isothiocyanate (FITC)-labeled anti-biotin, and 2 μL of Hoechst 33342. The sample was dissolved in 1 mL of 2% paraformaldehyde following washing and analyzed using a FACSCanto® 2 flow cytometer. TNF-HUVECs and blood cells of a healthy volunteer were used as controls in the FC gating strategy.

IB protocol

Four milliliters of blood was incubated with lysing solution in a tube at RT for 15 min. Ten milliliters of buffer (0.5% BSA and 2 mM EDTA in PBS) was added, the tube was centrifuged at 400 × g for 5 min, and the supernatant was discarded. The sample was dissolved in 1 mL of buffer, incubated with 10 μL of FcR blocking reagent at 4 °C for 10 min, and then incubated with 100 μL of anti-CD146-coated beads in a rotating mixer at 4 °C for 30 min. One milliliter of buffer was added, the tube was placed on a magnet (Magna-stand 8; Chisso, Tokyo, Japan), and the supernatant was discarded. The sample was incubated at 4°C for 30 min with 100 μL of solution containing 10 μg of FITC-conjugated UEA1 and 1 μg of Hoechst 33342. The sample was dissolved in 150 μL of buffer following washing and examined using a Nageotte chamber and a fluorescence microscope. Anti-CD146-coated beads were obtained by incubating 1 μg anti CD146 antibody and 25 μL of Dynabeads® (Veritas, Oslo, Norway) in a rotating mixer at 4°C for at least 1 h.

Detection of CECs in healthy volunteers and ICU patients

Blood was obtained from healthy volunteers and intensive care unit (ICU) patients within 24 hours (day 1), 48-72 hours (day 3), 96-120 hours (day 5) and 168-192 hours (day 8) following ICU admission. The first 3 mL of blood was discarded and the next 5 mL was used. CECs were measured using FC and defined as Hoechst 33342^+/ CD146$^+$/ CD45$^-$ or ^{dim}/ CD105$^+$/ CD133$^-$.

Antibodies and materials

Antibodies and materials were obtained from the following suppliers: anti-CD146, PE-labeled anti-CD146, FITC-labeled anti-CD105, and APC-labeled anti-CD45 from Abcam (Cambridge, UK); APC-labeled anti-CD105, PE-Vio770-labeled anti-CD45, biotin-labeled anti-CD133, FITC-labeled anti-biotin, PE-labeled anti-CD309, FITC-labeled anti-CD34, APC-labeled anti-CD133 from Miltenyi Biotec; FITC-labeled anti-CD31 and 7-AAD from Biolegend (San Diego, USA); FITC-conjugated UEA1 from Vector Laboratories (Burlingame, USA); and Hoechst 33342 from Dojindo (Kumamoto, Japan).

Statistical analysis

Statistical comparisons of detection rates of Normal-HUVECs and TNF-HUVECs between FC and IB were done using Bland-Altman analysis. Differences in CEC counts of healthy volunteers and ICU patients were evaluated using the Student's t-test. P-values < 0.05 were considered statistically significant.

Results

Sensitivity of endothelial cell markers in HUVECs

CD146, CD105, CD31, and UEA1 had higher sensitivity than the other markers in HUVECs, as shown in Figure 1 and Supplementary Table 1.

Specificity of endothelial cell markers in HUVECs

CD146 and CD105 were superior to CD31 and UEA1 for separation of HUVECs from whole blood cells, as shown in Figure 2 and Supplementary Table 2.

Characteristics of TNF-HUVECs

Almost TNF-HUVECs were dead cells (7-AAD positive, Figure 3C) and had heterogeneous cell surface molecules and nuclei fragmentation, as shown in Figure 3B. Antigenicities of CD146 and CD105 were decreased in TNF-HUVECs as compared to that in Normal-HUVECs, as shown in Figure 3C and Supplementary Table 3.

FC gating strategy for detection of CECs

Almost all TNF-HUVECs were detected, and blood cells were excluded by the gating strategy, as shown in Figure 4.
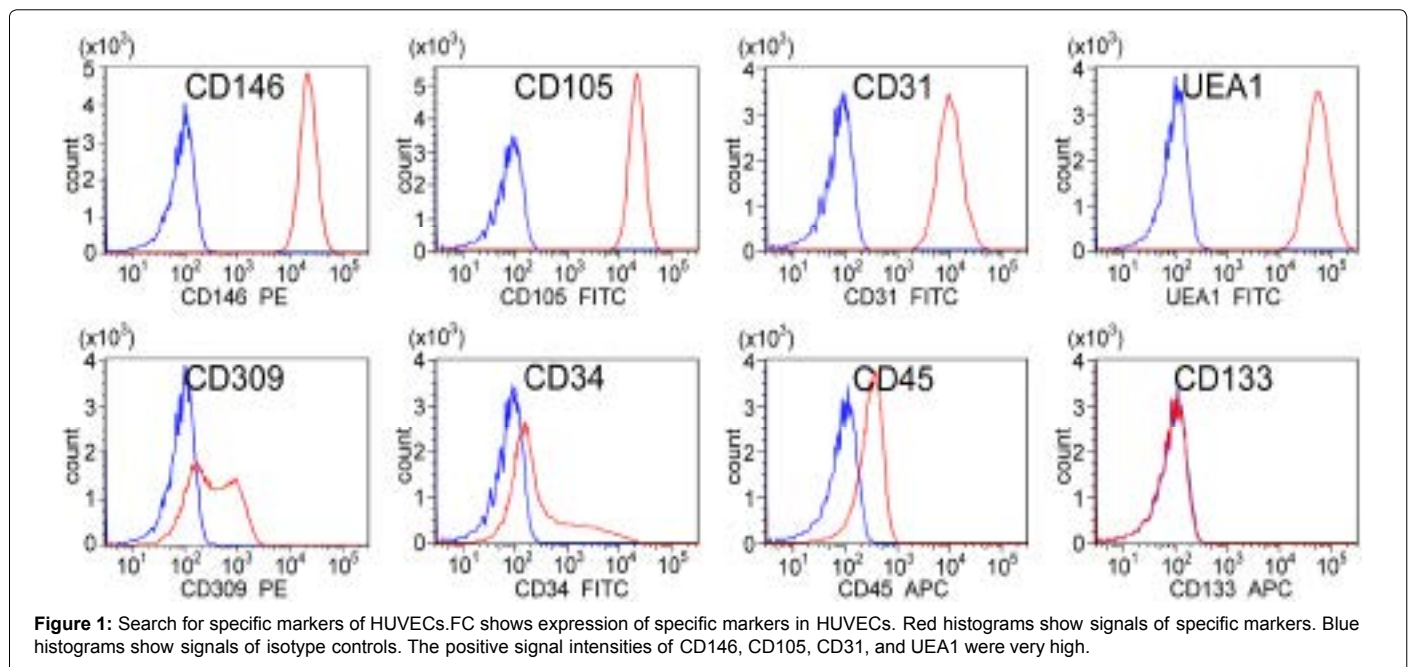
Comparison of FC and IB

The detection rate of Normal-HUVECs was not significantly different between by FC and by IB. However, the detection rate of TNF-HUVECs was significantly lower by IB than by FC, as shown in Table 1.

Detection of CECs in healthy volunteers

Twenty healthy volunteers (12 men and 8 women) participated in this study. The mean age was 38 years (range, 25-51 years). The mean CEC count was 2.8 cells/mL (range, 0–11 cells/mL).

Detection of CECs in ICU patients

Sixteen ICU patients (11 men and 5 women) participated in the study. These patients had no cancer. The mean age was 72 years (range, 57–90 years). The main causes of ICU admission were the following disorders: acute heart failure (5), sepsis (4), acute pancreatitis (2), acute aortic dissection (1), acute myocardial infarction (1), cerebral infraction (1), diabetic ketoacidosis (1), and femur fractures (1). The median Japanese Association for Acute Medicine DIC scores and System Organ Failure Assessment (SOFA) scores [8] of 16 ICU patients were 2.0 (range, 1–8) and 6.0 (range, 2–18), respectively. The mean CEC count was 4.3 cells/mL (range, 0–49 cells/mL). There was no significant difference between ICU patients and healthy volunteers
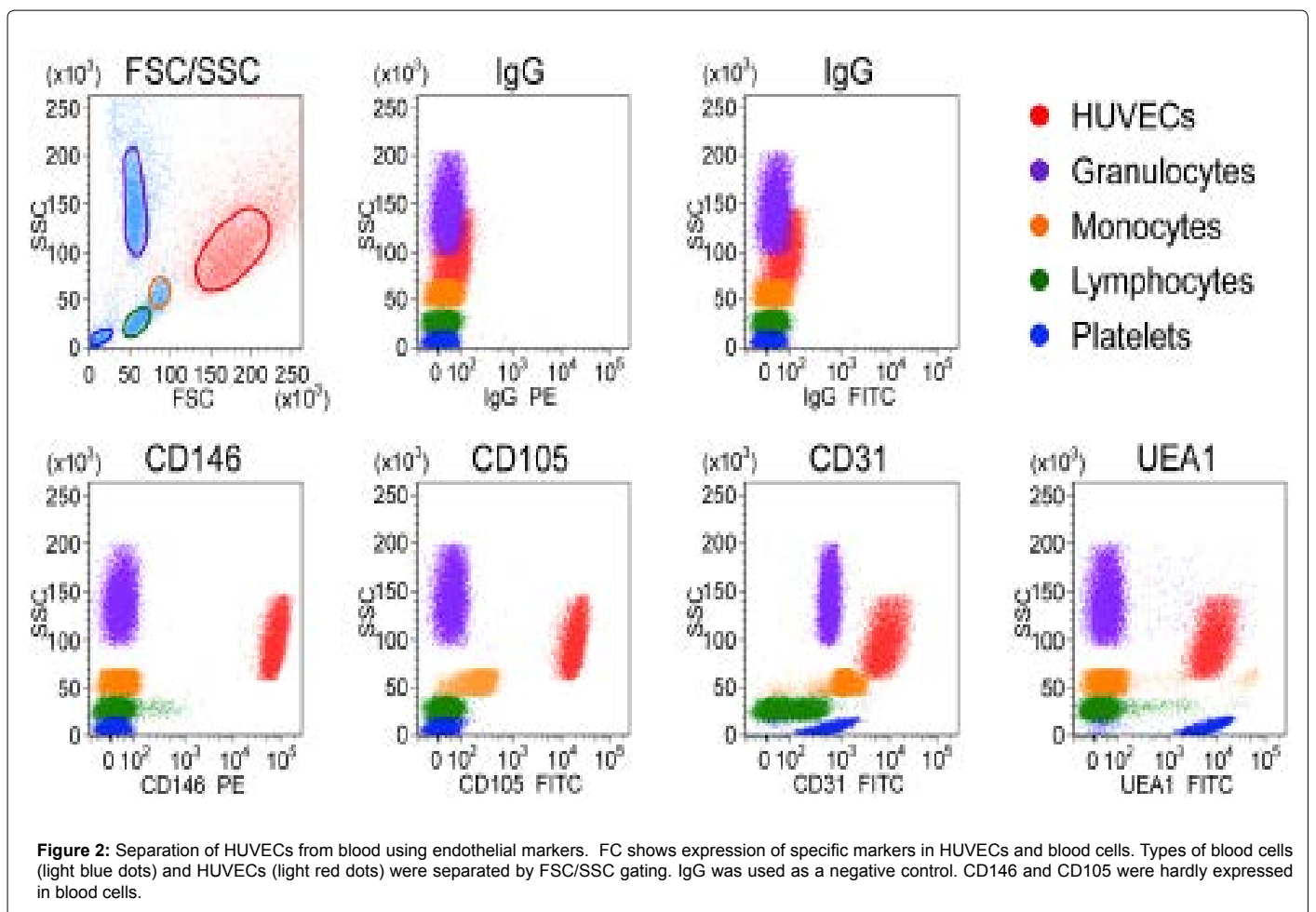

Figure 1: Search for specific markers of HUVECs. FC shows expression of specific markers in HUVECs. Red histograms show signals of specific markers. Blue histograms show signals of isotype controls. The positive signal intensities of CD146, CD105, CD31, and UEA1 were very high.

	FC		IB	
Normal-HUVECs	75%	(62–89%)	82%	(72–106%)
TNF-HUVECs	64%	(44–79%)	27%*	(22–31%)

Data shows the mean (range) of detection rates of Normal-HUVECs and TNF-HUVECs in blood by FC and IB. * P < 0.05.

Table 1. Detection rates of Normal-HUVECs and TNF-HUVECs.

	Day 1	Day 3	Day 5	Day 8
CECs (cells/mL)	49	13	12	3
Japanese Association for Acute Medicine	8	8	7	7
DIC scores				
SIRS criteria	3	3	2	0
PLT (count/µL)	5000	26000	26000	33000
PT-INR	1.55	1.48	1.38	1.30
FDP (µg/mL)	72.1	89.1	34.5	36.0
SOFA scores	15	16	13	9
Respiration	4	3	2	2
Coagulation	4	3	3	3
Liver	1	2	1	0
Cardiovascular	3	3	3	1
Neurogical	1	2	1	1
Renal	2	3	3	2

Table 2. CEC counts and clinical data for an ICU patient.

Figure 2: Separation of HUVECs from blood using endothelial markers. FC shows expression of specific markers in HUVECs and blood cells. Types of blood cells (light blue dots) and HUVECs (light red dots) were separated by FSC/SSC gating. IgG was used as a negative control. CD146 and CD105 were hardly expressed in blood cells.
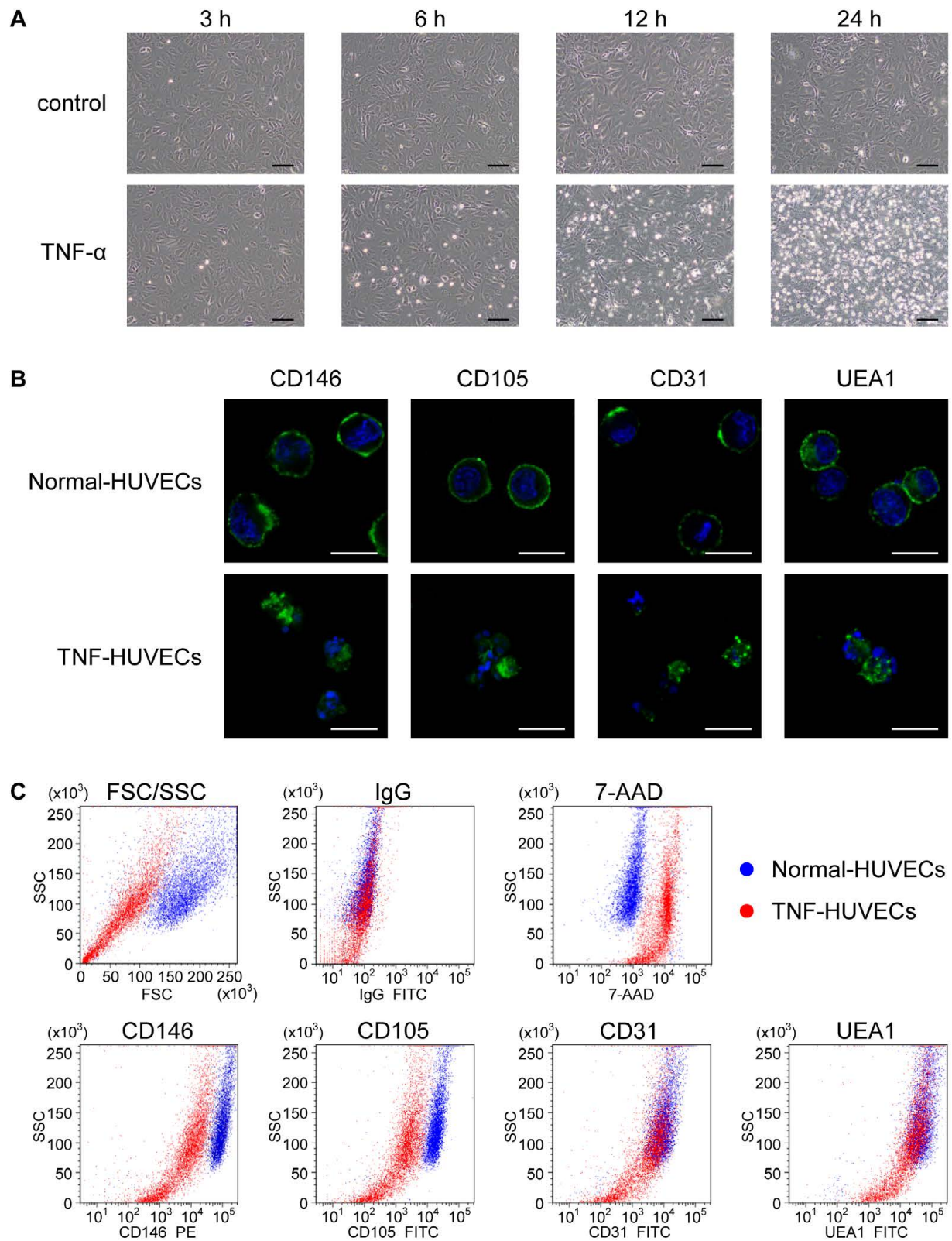
Figure 3: Characteristics of TNF-HUVECs. (A) Phase-contrast micrographs of cultured HUVECs show that detached cells in a culture dish are greatly increased at 12–24 h after administration of 100 ng/mL of TNF-α. Scale bar indicates 100 μm. (B) Confocal micrographs show cell surface molecules and nuclei in Normal-HUVECs and TNF-HUVECs. Blue indicates nuclei stained with Hoechst. Green indicates endothelial markers labeled with FITC. Scale bar indicates 20 μm. (C) FC shows comparison of Normal-HUVECs and TNF-HUVECs. Signal intensity of CD146 and CD105 was much lower in TNF-HUVECs than in Normal-HUVECs. Most TNF-HUVECs were positive for 7-AAD.

in CEC counts. However, in one ICU patient, CECs were elevated. The patient was a 71-year-old man who developed a type A aortic dissection and underwent total aortic replacement. SIRS-induced DIC was caused by surgical site infection on the 18th day following

surgery. He was admitted to the ICU on the 25th day. Antibiotics and recombinant human soluble thrombomodulin were administrated against the infection and DIC. CECs gradually decreased with the improvement of Japanese Association for Acute Medicine DIC scores and SOFA scores in table 2. He was discharged from the ICU on the 10th day following admission.

Discussion

CECs have been defined as mature vascular endothelial cells released into the blood in several types of vascular endothelial injury [1], but it is not known if this definition is reliable for critical illness with SIRS. In a septic mouse, it was observed that vascular endothelial cells proceeded to apoptosis and detached from the vascular beds [18], which indicated that the detached CECs would not be normal in SIRS. Therefore, control cells for CECs should not be normal endothelial cells in patients with SIRS.

TNF-α is known as a major proinflammatory cytokine and a potent inducer of endothelial cell injury. In this study, TNF-α could induce detachment of HUVECs from the culture dish. These detached cells would be suitable for use as a positive control for CECs in critically ill patients with SIRS.

We searched for appropriate markers for detecting CECs. CD34 [11-15], CD31 [6,11,16-17], and UEA1 [2,3,10,13] had been widely used in previous studies for detecting CECs because of the expression in pan-endothelial cells. However, the expression of CD34 was actually low in HUVECs, as shown in Figure 1 and Supplementary Table 1. CD31 was expressed on platelets and leukocytes in addition to HUVECs, as shown in Figure 2 and Supplementary Table 2. Therefore, discriminating CECs from platelets [19] and monocytes [20,21] might be difficult using CD31. UEA1 had strong signal intensity in platelets, which was identical to that in HUVECs, as shown in Figure 2. We concluded that CD146 and CD105 had high sensitivity and the highest specificity to HUVECs.

We demonstrated that the detection rate of TNF-HUVECs was lower by IB than by FC, although that of Normal-HUVECs was similar by IB and by FC, as shown in Table 1. This indicates that IB and FC are equivalent in the detection of normal endothelial cells but FC is superior to IB in the detection of endothelial cells in severe inflammatory states, such as TNF-HUVECs. It is reasonable to use FC for detecting CECs of SIRS patients because CECs are endothelial cells detached from vascular beds by severe inflammation like TNF-HUVECs. The low detection rate of TNF-HUVECs in IB would be because of the low antigenicities of CD146 in TNF-HUVECs, as shown in Figure 3C and Supplementary Table 3. The antigenicities of CD146 in CECs of an ICU patient were as low as TNF-HUVECs, as shown in Figure 5 and Supplementary Table 4. For detecting cells with low antigenicities, it is important to combine fluorescent dyes with high signal-to-noise ratio (PE, APC) with endothelial markers and to adjust the range of the gating in FC, as shown in Figure 4.

We have two additional recommendations for detecting inflammatory CECs. The first is that forward scatter (FSC) and side scatter (SSC) gating should not be used. The range of SSC

of CECs was thought to be identical to that of mononuclear cells [7,11,13,15,17], but it was actually more varied, as shown in Figure 5. Furthermore, it is known that about 25% of CECs in patients with myocardial infarction have multiple nuclei [22], which indicate that the range of FSC and SSC of CECs is very wide. It is not reasonable to determine the ranges of FSC and SSC for the detection of CECs. Our second recommendation is that positive and negative markers for CECs should be combined and a pentagon polygon gate be used in each gating, as shown in Figure 4. False positive CECs might be induced by high intrinsic fluorescent intensity if two positive markers were combined and a square gate was used.

We detected the CECs in human blood under the conditions described above. In our study, a mean value of 2.8 cells/mL of CECs was noted for healthy volunteers. On the other side, previous studies reported values between 140 and 13,400 cells/mL [6,15-17]. They could include false positive blood cells because of the use of CD31 as the marker of CECs, as mentioned above.
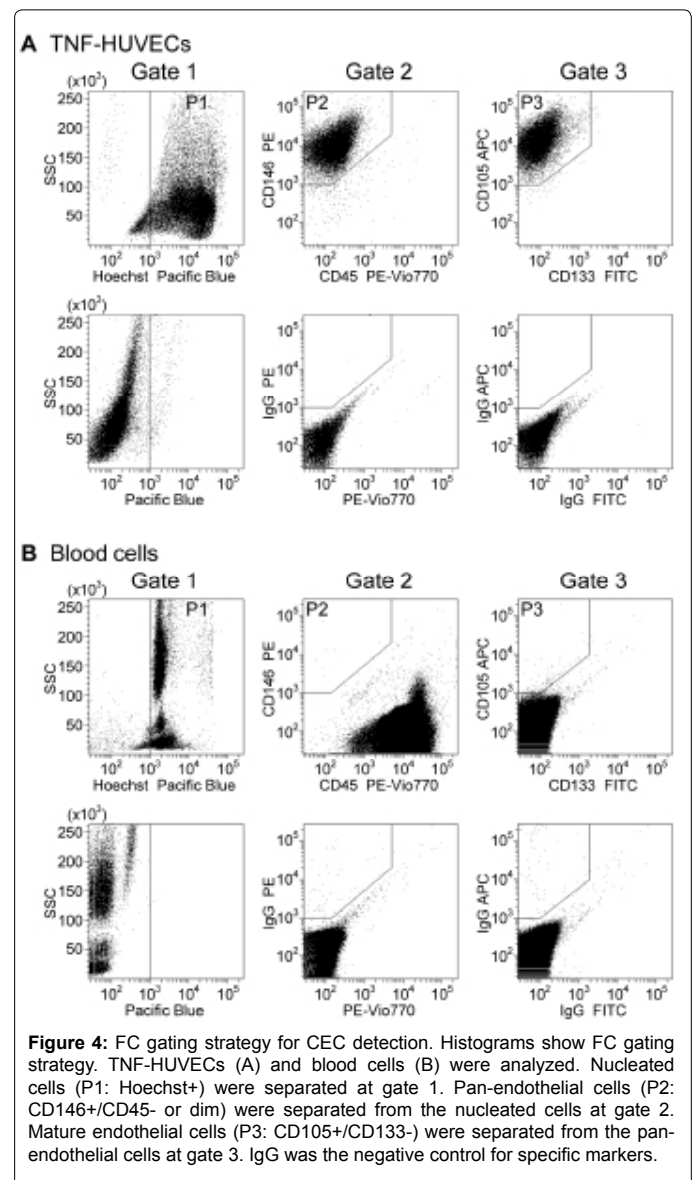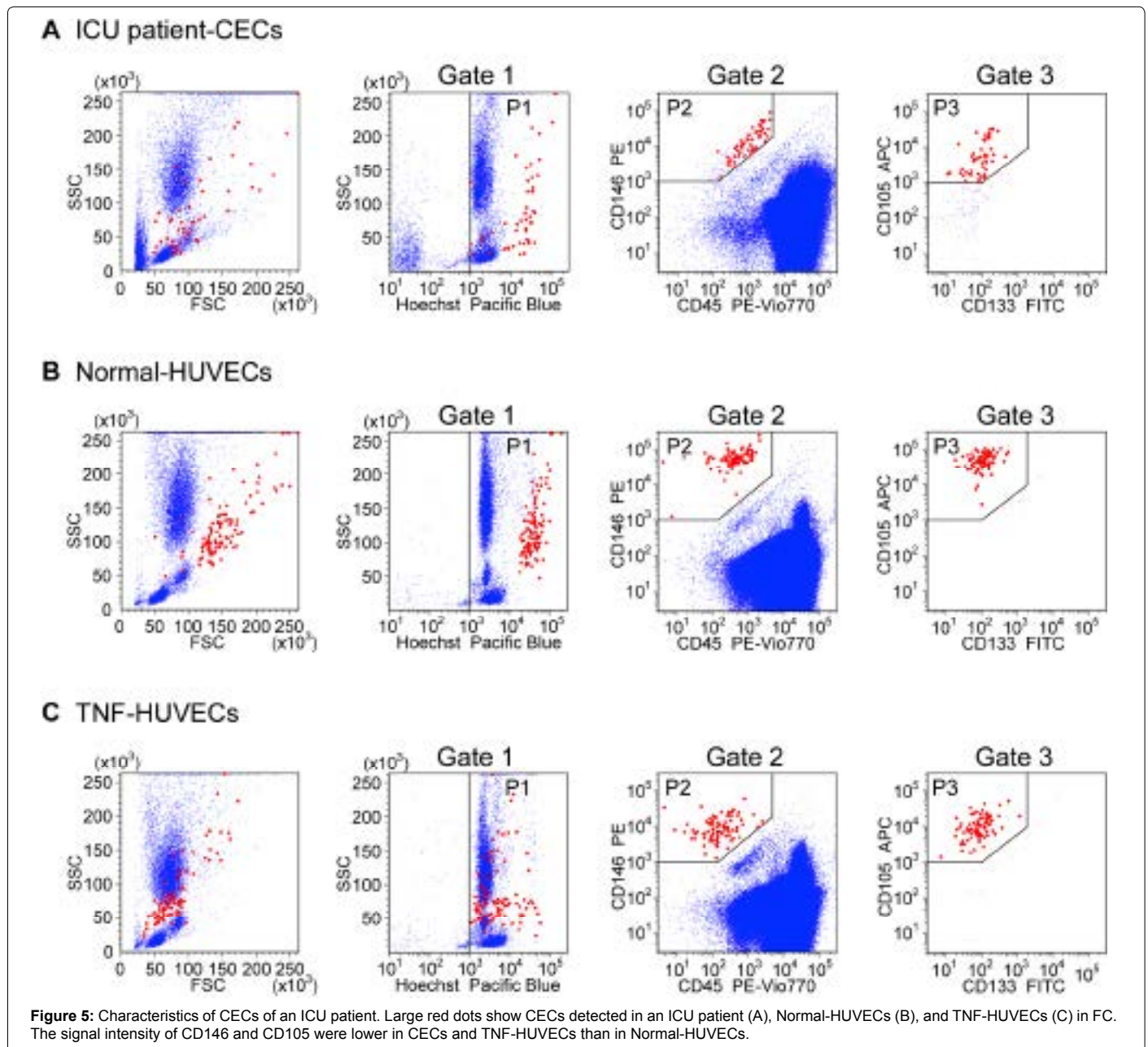

Figure 4: FC gating strategy for CEC detection. Histograms show FC gating strategy. TNF-HUVECs (A) and blood cells (B) were analyzed. Nucleated cells (P1: Hoechst+) were separated at gate 1. Pan-endothelial cells (P2: CD146+/CD45- or dim) were separated from the nucleated cells at gate 2. Mature endothelial cells (P3: CD105+/CD133-) were separated from the pan-endothelial cells at gate 3. IgG was the negative control for specific markers.

Figure 5: Characteristics of CECs of an ICU patient. Large red dots show CECs detected in an ICU patient (A), Normal-HUVECs (B), and TNF-HUVECs (C) in FC. The signal intensity of CD146 and CD105 were lower in CECs and TNF-HUVECs than in Normal-HUVECs.

In one ICU patient, the number of CECs was significantly correlated with clinical severity, as shown in Table 2. CECs might play an important role as a clinical marker for severity of vascular endothelial injury in critically ill patients. In SIRS, endothelial cells are activated by inflammatory cytokines, such as TNF-α, and produce von Willebrand factor and adhesion molecules. These are able to induce the attachment of neutrophils and platelets to endothelial cells [23]. If neutrophils are attached to CECs similarly, neutrophils-attached CECs may not be detected. However, the attachment of neutrophils to CECs was not found in healthy subjects and ICU patients using IB in our study. On the other hand, aggregation of neutrophils and platelets was found in ICU patients. Finally, FC using CD146, CD105, and CD45 Could be effective in excluding aggregated neutrophils and platelets in SIRS patient with DIC.

In conclusion, FC is superior to IB for detecting endothelial cells in severe inflammatory states in our protocol. TNF-HUVECs are effective control cells for detecting inflammatory CECs.

Acknowledgments

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