

# A New Protocol for Quantifying CD34<sup>+</sup> Cells

in Peripheral Blood of Patients  
with Cardiovascular Disease

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Increasing evidence points to a role for circulating CD34-positive (CD34<sup>+</sup>) cells in vascular maintenance and neovascularization. Although there are established methods for evaluating absolute numbers of CD34<sup>+</sup> cells in bone marrow or mobilized peripheral blood, there is no convenient and highly reproducible method for quantifying low numbers of CD34<sup>+</sup> cells in blood samples, such as those from the peripheral blood of patients with cardiovascular disease. With current commonly used methods, the mean percentage of CD34<sup>+</sup> cells in leukocyte fractions from such patients was only 0.02%, and the cumulative intra-assay coefficient of variation was ~30%. With use of the protocol described herein, actual counts of CD34<sup>+</sup> cells increased ~5-fold and cumulative intra-assay coefficients of variation were reduced to ~7%. The new method is useful to precisely measure low numbers of CD34<sup>+</sup> cells in samples, and it has potential as a screening tool to evaluate cardiovascular risk in large patient populations. (**Tex Heart Inst J 2006;33:427-9**)

In vasculature maintenance and neovascularization, there is increasing evidence of a role for circulating endothelial progenitor cells (EPCs)—including the populations of CD34-positive (CD34<sup>+</sup>) cells that are present in peripheral blood.<sup>1</sup> As a source of numerous growth and angiogenesis factors at ischemic loci, CD34<sup>+</sup> cells also contribute to vascular homeostasis.<sup>2</sup> Furthermore, initial clinical trials of cell transplantation in treating ischemia of the hind limb<sup>3</sup> and myocardium<sup>4</sup> have shown promising results. On the basis of these observations, circulating EPCs<sup>5</sup> and CD34<sup>+</sup> cells<sup>6</sup> have been evaluated in patients with cardiovascular disease, and strong correlations of their levels with vascular function have been reported. However, procedures to evaluate EPCs and CD34<sup>+</sup> cells are not simple<sup>5</sup>; because of low numbers of circulating CD34<sup>+</sup> cells, routine FACS (fluorescence-activated cell sorter) analysis<sup>7</sup> of CD34<sup>+</sup> cell counts in patients with cardiovascular disease is not feasible. In this report, we demonstrate a new method that facilitates determination of the absolute number of circulating CD34<sup>+</sup> cells in patients with low levels of CD34<sup>+</sup> cells.

## Patients and Methods

This study was approved by the Human Assurance Committee of the National Cardiovascular Center and Osaka Minami Medical Center, and all subjects provided written informed consent. Results of experiments are reported as mean ± standard error.

### Analysis of Peripheral Blood

Three milliliters of heparinized peripheral blood were obtained from 20 patients who had histories of cardiovascular disease: 14 had sustained myocardial infarction, and 9 had sustained cerebral infarction (3 had histories of both). Patients who had experienced vascular events within 30 days of measurement were excluded. The study group included 12 men and 8 women, with a mean age of 74 ± 1.7 years (range, 59–87 yr). Medicines taken by study subjects included anticoagulants (aspirin, 17); anti-hypertensive agents, including calcium-channel antagonists, angiotensin-converting enzyme (ACE) inhibitors, or both (14); and sulfonylureas for glycemic control (5). Patients who were taking HMG-CoA reductase inhibitors (statins) were excluded from the study.

First, we counted circulating CD34<sup>+</sup> cells with ProCount™ (BD Bioscience; San Jose, Calif) and Stem-Kit™ (Beckman Coulter; Marseilles, France), according to the

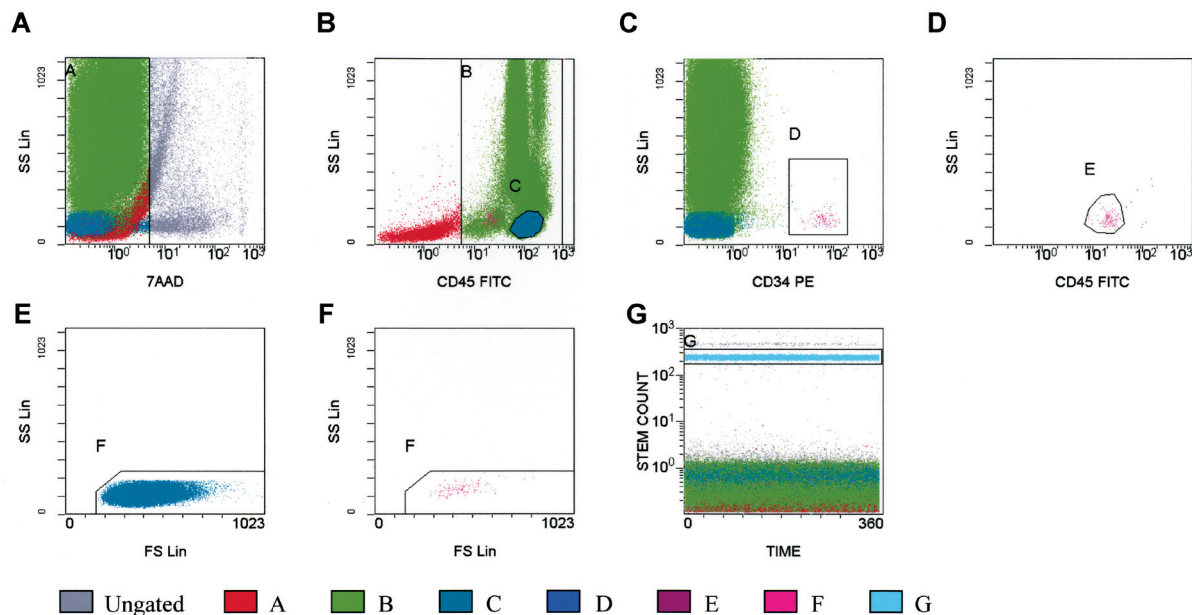
manufacturers' protocols. (These protocols are based on International Society of Hematology and Graft Engineering (ISHAGE) Guidelines<sup>7</sup> and are frequently used for quantification of CD34<sup>+</sup> cells that have mobilized into peripheral blood.) Next, to increase the reproducibility of CD34<sup>+</sup> cell counts, the Stem-Kit protocol was modified as follows: the blood sample volume, antibodies, and lysing solution were doubled. After adding 30  $\mu$ L of internal control particles (stem count: Beckman Coulter), samples were centrifuged for 5 min at 450 G, and 3,860  $\mu$ L of supernatant was removed carefully with a pipette. Samples were analyzed by Coulter CYTOMICS™ FC500 & XL-system II software (Beckman Coulter) for 6 min each (Fig. 1).

## Results

### Increase of CD34<sup>+</sup> Cell Counts

The mean percentage of CD34<sup>+</sup> cells in the leukocyte fraction obtained from mobilized peripheral blood has been reported to be about 0.2% to 0.5%.<sup>7</sup> First, we used the ProCount and Stem-Kit protocols to count circulating CD34<sup>+</sup> cells that had been obtained from

patients with cardiovascular disease (Table I). The mean percentages of CD34<sup>+</sup> cells in the leukocyte fraction were 0.024%  $\pm$  0.003% (range, 0.012%–0.046%) for ProCount and 0.021%  $\pm$  0.001% (range, 0.011%–0.032%) for Stem-Kit. The actual CD34<sup>+</sup> cell counts per analysis were only 16  $\pm$  1 (range, 10–31) for ProCount and 34  $\pm$  4 (range, 16–58) for Stem-Kit. Because the absolute cell count is a major factor in the reproducibility of the measurement,<sup>8</sup> our approach was to modify the original protocol in order to obtain higher numbers of actual CD34<sup>+</sup> cells per count. However, simply increasing sample volume or measurement time does not improve reproducibility, because of these factors: adhesion of internal control particles to cells in the patient sample, and precipitation and aggregation of cells in the sample. Our approach, as outlined under Methods, was to seek a method that produces higher cell counts while maintaining short measurement times. Through the use of our method, the mean CD34<sup>+</sup> cell count increased to a level of 174  $\pm$  18 (range, 88–404) per analysis, and the mean percentage of CD34<sup>+</sup> cells in the leukocyte fraction was 0.019  $\pm$  0.002 (range, 0.014%–0.038%), which was consistent with measure-



**Fig. 1** Quantification of circulating CD34<sup>+</sup> cells by fluorescence-activated cell sorter analysis using our modified, improved protocol. **A)** All events: 7-aminoactinomycin-D viability dye-positive cells (dead cells) were excluded from region A. **B)** Events from region A: All CD45<sup>+</sup> cells (leukocytes) were included in region B. Region C was adjusted to include only lymphocytes (bright CD45, low side-scatter). **C)** Events from regions A and B: Region D was adjusted to include CD34<sup>+</sup> hematopoietic progenitor cells (HPC). **D)** Events from regions A, B, and D: Region E was adjusted to include cells forming a cluster with characteristic CD34<sup>+</sup> HPC (low side-scatter and low-to-intermediate CD45 staining). Brightly stained events were excluded from region E. **E)** Events from regions A and C: Region F was adjusted to include lymph/blast cells, excluding platelet aggregates if present. **F)** Events from A, B, D, and E: Lymph/blast region F identified a cluster of events that met all the fluorescence and light-scattering criteria of ISHAGE Guidelines for CD34<sup>+</sup> HPC. **G)** All events: Region G was adjusted to enclose the internal control.

7AAD = 7-aminoactinomycin-D; CD34 PE = cluster of differentiation 34 phycoerythrin; CD45 FITC = cluster of differentiation 45 fluorescein isothiocyanate; FS Lin = forward-scatter linear scale; ISHAGE = International Society of Hematology and Graft Engineering; SS Lin = side-scatter linear scale

**TABLE I.** Reduction in the Coefficient of Variation Using Our Modified, Improved Protocol for Determining CD34+ Cell Levels in Peripheral Blood

	ProCount	Stem-Kit	Improved Protocol
Actual CD34+ cell counts	16 ± 1	34 ± 4	174 ± 18
Range	10–31	16–58	88–404
CD34+ cells in leukocytes (%)	0.024 ± 0.003	0.021 ± 0.001	0.019 ± 0.002
Range	0.012–0.046	0.011–0.032	0.014–0.038
Circulating CD34+ cells (cells/μL)	0.82 ± 0.05	0.81 ± 0.06	0.88 ± 0.06
Cumulative intra-assay coefficient of variation (%)	30.3	25.7	7.4

ments using the standard method (Table I). The supernatant that was removed during the procedure was also analyzed and was found not to contain either cells or internal control particles.

### Improvement in the Cumulative Intra-Assay Coefficient of Variation

In mobilized peripheral blood, the coefficients of variation have been reported to be about 8% and 4% using ProCount and Stem-Kit, respectively, on the basis of the manufacturers' published information. However, in non-mobilized peripheral blood of patients with cardiovascular disease, the mean percentage of CD34+ cells in the leukocyte fraction was less than 10%, compared with mobilized blood, and the calculated cumulative intra-assay coefficients of variation were 30.3% and 25.7%, as evaluated by ProCount and Stem-Kit, respectively. Our method increased the absolute number of CD34+ cells by about 5-fold during the same measurement period, and it resulted in a reduced (7.4%) cumulative intra-assay coefficient of variation (Table I).

### Discussion

Although our modified method for quantifying CD34+ cells in blood was similar to established methods for the calculation of mean circulating CD34+ cell counts, our method substantially improved reproducibility of the measurement.

The coefficient of variation of CD34+ cell counts is inversely proportional to the square root of the number of CD34+ cells detected in the sample. A minimum of 100 CD34+ cells is required to ensure a coefficient of variation in the range of 10%.<sup>8</sup> Our modified protocol yielded more than 100 CD34+ cells in each count (confirmed by duplicate counting), and the coefficient of variation was reduced to 7%. Simply increasing the sample volume or lengthening the time for measurement of cell numbers does not necessarily improve reproducibility of the counts.<sup>8</sup> Our results indicate that absolute numbers of circulating CD34+ cells in peripheral blood of patients who have low levels of such cells

can now be quantified precisely using a modification of the ISHAGE protocol. This easy method enables precise measurement of the CD34+ cell population of stem cells in peripheral blood and can be broadly applied to screening patients for cardiovascular risk.

### References

1. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;275:964-7.
2. Majka M, Janowska-Wieczorek A, Ratajczak J, Ehrenman K, Pietrkowski Z, Kowalska MA, et al. Numerous growth factors, cytokines, and chemokines are secreted by human CD34(+) cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. *Blood* 2001;97:3075-85.
3. Taguchi A, Ohtani M, Soma T, Watanabe M, Kinoshita N. Therapeutic angiogenesis by autologous bone-marrow transplantation in a general hospital setting. *Eur J Vasc Endovasc Surg* 2003;25:276-8.
4. Hamano K, Nishida M, Hirata K, Mikamo A, Li TS, Harada M, et al. Local implantation of autologous bone marrow cells for therapeutic angiogenesis in patients with ischemic heart disease: clinical trial and preliminary results. *Jpn Circ J* 2001;65:845-7.
5. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003;348:593-600.
6. Taguchi A, Matsuyama T, Moriwaki H, Hayashi T, Hayashida K, Nagatsuka K, et al. Circulating CD34-positive cells provide an index of cerebrovascular function. *Circulation* 2004;109:2972-5.
7. Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. *International Society of Hematotherapy and Graft Engineering, J Hematother* 1996;5:213-26.
8. Sutherland DR, Keeney M, Gratama JW. Enumeration of CD34+ hematopoietic stem and progenitor cells. In: Robinson JP, Darzynkiewicz Z, Dobrucki J, Hyun WC, Nolan JP, Orfao A, Rabinovitch PS, editors. *Current protocols in cytometry*. New York: John Wiley and Sons, Inc.; 2003. Unit 6.4, p. 1-23.