Circulating CD34-Positive Cells Provide an Index of Cerebrovascular Function

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Background—Increasing evidence points to a role for circulating endothelial progenitor cells, including populations of CD34- and CD133-positive cells present in peripheral blood, in maintenance of the vasculature and neovascularization. Immature populations, including CD34-positive cells, have been shown to contribute to vascular homeostasis, not only as a pool of endothelial progenitor cells but also as a source of growth/angiogenesis factors at ischemic loci. We hypothesized that diminished numbers of circulating immature cells might impair such physiological and reparative processes, potentially contributing to cerebrovascular dysfunction.

Methods and Results—The level of circulating immature cells, CD34-, CD133-, CD117-, and CD135-positive cells, in patients with a history of atherothrombotic cerebral ischemic events was analyzed to assess possible correlations with the degree of carotid atherosclerosis and number of cerebral infarctions. There was a strong inverse correlation between numbers of circulating CD34- and CD133-positive cells and cerebral infarction. In contrast, there was no correlation between the degree of atherosclerosis and populations of circulating immature cells. Analysis of patients with cerebral artery occlusion revealed a significant positive correlation between circulating CD34- and CD133-positive cells and regional blood flow in areas of chronic hypoperfusion.

Conclusions—These results suggest a possible contribution of circulating CD34- and CD133-positive cells in maintenance of the cerebral circulation in settings of ischemic stress. Our data demonstrate the utility of a simple and precise method to quantify circulating CD34-positive cells, the latter providing a marker of cerebrovascular function. (Circulation. 2004;109:2972-2975.)

Key Words: cerebral infarction ■ cerebral ischemia ■ antigens, CD34 ■ stem cells

Although it had traditionally been assumed that replacement of damaged endothelium resulted only from outgrowth of preexisting vasculature, recent studies have identified endothelial progenitor cells (EPCs) that appear to contribute to vascular homeostasis and repair. Clinical trials to assess the therapeutic potential of bone marrow–derived mononuclear cells, a rich source of immature cells including EPCs, in hind-limb ischemia and cardiac ischemia have been initiated and have, thus far, provided promising results. Furthermore, immature cells, including CD34-positive (CD34+) cells, have been shown to contribute to maintenance of the vasculature, not only as a pool of EPCs but also as the source of growth/angiogenesis factors. Bone marrow–derived immature cells have also been shown to participate in neovascularization of ischemic brain after experimental stroke. On the basis of these results, we hypothesized that levels of circulating immature cells might be proportional to the resilience of the cerebral circulation to ischemic stress; ie, lower numbers of circulating immature cells might be associated with cerebral ischemia and infarction.

Methods

The institutional review board of the National Cardiovascular Center approved this study. All subjects provided informed consent. Circulating CD34+ cells in 50 μL of peripheral blood were quantified according to the manufacturer’s protocol (ProCOUNT, Becton Dickinson Biosciences). To minimize intersample variation for measurements of CD34+ cells, several methods were used: A nucleic acid dye was added as a threshold reagent; a no-wash technique was performed to eliminate cell loss, and reverse pipetting was used; an internal reference particle was added for determination of absolute cell numbers; and an isotype control, matched for the concentration of anti-CD34 antibody and fluorochrome-to-protein ratio, was included. All measurements were performed in triplicate (Figure 1A, control; Figure 1B, CD34). To quantify other stem cell populations (besides CD34+ cells), immature mononuclear cells were enriched.
from 2 mL of peripheral blood by antibody-mediated depletion of mature cells according to the manufacturer’s protocol (StemCell Technologies) using depletion cocktail, including antibodies to CD2, CD3, CD14, CD16, CD19, CD24, CD56, and CD66b. Enriched immature mononuclear cells were double-stained with peridinin chlorophyll protein (PerCP)-conjugated CD34 antibody (Figure 1D) and phycoerythrin (PE)-conjugated CD133 (Figure 1F), CD117 (Figure 1G), or CD135 (Figure 1H) antibody. The number of cells in a region including brightly stained cells was counted, and immature cells were quantified using CD34:\({}^+\) cells as an internal control. The cumulative intra-assay coefficient of variation was 14\%, 13\%, 14\%, and 15\%, with CD34:\({}^+\), CD133:\({}^+\), CD117:\({}^+\), and CD135:\({}^+\) cell measurements, respectively, from 5 stroke patients.

Atherosclerosis in the common and internal carotid arteries was analyzed by ultrasonography to determine plaque score as described previously.\(^7\) Cerebral infarcts (diameter >5 mm) were counted independently by a neurologist blinded to other parameters under study (number of circulating CD34:\({}^+\), etc) using T1-weighted, T2-weighted, and fluid-attenuated inversion-recovery MRI obtained with a 1.5-Tesla MRI scanner. The diagnosis of hypoperfusion was made angiographically. Regional cerebral blood flow (CBF), cerebral blood volume, oxygen extraction fraction (OEF), and cerebral metabolic rate of oxygen (CMRO\(_2\)) were quantified by conventional steady-state \(^{15}\)O PET using a PET scanner (Shimadzu) as described.\(^8\)

Cerebrovascular function was evaluated in patients with chronic hypoperfusion caused by major cerebral artery (carotid artery or M1 portion of the middle cerebral artery) occlusion or severe stenoses (\(>90\%)\) without a major stroke. Twelve patients with 15 major arterial occlusions or stenoses had PET examinations.

To investigate the mobilization of immature cells after acute cerebral infarction, peripheral blood was quantified at 6 hours and 3, 7, 14, and 30 days after the onset of stroke. The episodes of acute cerebral infarction were confirmed by the diffusion image of brain MRI. Age-matched volunteers who had no history of cerebrovascular diseases and no neuronal deficiency were enrolled as controls (mean age, 67\(\pm\)4 years). Test-retest intraclass correlations were 0.88, 0.75, 0.86, and 0.86 for CD34, CD133, CD117, and CD135, respectively, obtained from 5 volunteers tested twice with an interval of at least 10 days between samples.

Univariate correlations were performed using Pearson’s correlation coefficient and Spearman’s correlation coefficient. Statistical comparisons among groups were determined using analysis of variance. Individual comparisons were performed using Students’ \(t\) test. In all experiments, mean\(\pm\)SE is reported.

**Results**

First, we investigated mobilization of immature cells after acute cerebral infarction (\(n=5\)), focusing on CD34:\({}^+\) cells. The level of CD34:\({}^+\) cells gradually increased to day 7 and remained significantly above the prestroke baseline on days 7 and 14, returning to baseline levels by day 30 (Figure 2A). On the basis of these data, we enrolled 25 patients with a history of atherothrombotic cerebral ischemic events, excluding those who had suffered cerebrovascular or cardiovascular acute ischemic episodes in the 30 days before study, as well as premenopausal females. In this group (>30 days after stroke), no correlation was observed between the interval after stroke and the level of circulating CD34:\({}^+\) cells (\(r=0.009, P=0.97\)). Characteristics of this group included mean age of 68\(\pm\)2 years, 20 men and 5 women, 23 patients receiving antiplatelet therapy, 11 patients receiving antihypertensive therapy, 6 patients receiving therapy for hyperlipidemia, 5 patients receiving therapy for diabetes mellitus (DM), and 16 patients with a current or past history of smoking.

Several factors were found to influence the number of circulating CD34:\({}^+\) cells. Statistical analysis revealed a significant decrease in circulating CD34:\({}^+\) cells in patients with DM (0.5\(\pm\)0.1; non-DM, 1.2\(\pm\)0.1 cells/\(\mu\)L; \(P=0.01\)). In contrast,
no change was observed in patients with hypertension (P=0.61), with hyperlipidemia (P=0.81), with smoking (P=0.64), or based on gender (P=0.36). In addition, treatment with HMG-CoA reductase inhibitors (P=0.81), compared with patients without hyperlipidemia, did not impact the number of CD34+ cells. In the control patient group, a decrease of circulating CD34+ cells was observed with aging (Figure 2B), although this was not observed in the patient group (Figure 2C). Comparing baseline levels of circulating CD34+ cells, there was a significant decrease in the patient group compared with age-matched controls (stroke, 1.1±0.1; control, 1.6±0.2 cells/μL; P=0.02).

We sought a possible correlation between circulating immature cells and the degree of arteriosclerosis of the common and internal carotid arteries in the patients with atherothrombotic cerebral ischemic events. However, there was no significant correlation between arteriosclerosis and circulating CD34+ (Figure 2D). This result was not surprising, because multiple risk factors and cell types contribute to progression of vascular lesions in major arteries. In contrast, because disruption of vascular homeostasis and repair are associated with cerebral infarction, we reasoned that a history of cerebral infarction might correlate with circulating immature cells. A strong correlation was observed between the number of infarcts and the absolute number of circulating CD34+ cells (Figure 2E) and CD133+ cells (Figure 2F). However, no significant correlation with regard to cerebral infarcts was observed with circulating CD117+ cells (Figure 2G) and CD135+ cells (Figure 2H).

**Figure 2.** Levels of circulating CD34+ cells and stroke. Circulating CD34+ cells increased after the onset of stroke and peaked on day 7. A significant increase in circulating CD34+ cells was observed on days 7 and 14 (A). A decrease of circulating CD34+ cell was observed with aging in the control group (B), but no such correlation was observed in the stroke patient group (C). No correlation was observed between the number of circulating CD34+ cells and the degree of arteriosclerosis in major cerebral arteries (D). However, there was a correlation between cerebral infarctions and circulating CD34+ (E) and CD133+ cells (F). In contrast, there was no correlation between cerebral infarction and CD117+ (G) or CD135+ cells (H). Correlation between circulating CD34+ (I) and CD133+ (J) cells and CBF in areas of chronic hypoperfusion was observed. Lower levels of circulating CD34+ cells were correlated with a decrease in CMRO2 (K) but not with a change in OEF (L). *P<0.05 compared with day 0 (based on 2-way ANOVA).
In view of the critical role of endothelium in maintaining CBF, we evaluated cerebrovascular function in patients with chronic hypoperfusion. Direct correlations were observed between CBF (in the chronically hypoperfused area) and circulating CD34+ cells (Figure 2I) and CD133+ cells (Figure 2J). In addition, lower numbers of circulating CD34+ cells (Figure 2K) correlated with diminished CMRO2, although there was no significant increase in the OEF (Figure 2L). These observations suggest a contribution of CD34+ cells in homeostasis and repair of the cerebral circulation and maintenance of brain metabolism. No correlation was observed with the above parameters of vascular function and circulating CD117+ and CD135+ cells. Measurement of angiogenic growth factors in patient plasma, vascular endothelial growth factor, basic fibroblast growth factor, hemopoietic growth factor, and insulin-like growth factor-1 also demonstrated no correlation with indices of cerebrovascular function or the number of CD34+ cells (not shown).

Discussion
We have found that circulating immature cell populations, especially CD34+ and CD133+ cells, are associated with maintenance and repair of the cerebral vasculature. In our study, we used a simple and precise method to count the absolute number of circulating CD34+ cells in a small sample of peripheral blood. Our results indicate that the level of CD34+ cells serves as an index/marker for cerebrovascular function. Analysis of CD133+, CD117+, and CD135+ cells, which identify other populations of immature cells, demonstrated that only CD133+ cells correlated with cerebrovascular function in a manner paralleling CD34+ cells.

Patients with diabetes displayed a significant reduction in the number of circulating CD34+ cells. In view of the microvascular dysfunction that is characteristic of diabetes, this may not be surprising. Similarly, decreased circulating CD34+ cells with increasing age in healthy individuals may be associated with limited vascular renewal in older individuals. It was also of interest to note no change between levels of CD34+ cells in patients taking HMG-CoA reductase inhibitors. The latter results might reflect the positive effect of such drugs countering the negative effect of hyperlipidemia on circulating CD34+ cells. Such conclusions, of course, are at best tentative, because in this first report we have identified associations rather than proved a cause-effect relationship.

These observations suggest that diminished numbers of CD34+ and CD133+ cells impact maintenance and repair of cerebral vasculature. Precise measurement of circulating CD34+ cells provides a marker for cerebrovascular function in the setting of ischemic stress.

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