Clinical Evidence That Very Small Embryonic-Like Stem Cells Are Mobilized Into Peripheral Blood in Patients After Stroke

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Background and Purpose—In a murine model of stroke, we identified a population of very small embryonic-like (VSEL) stem cells (SCs) in adult murine bone marrow that could be mobilized into peripheral blood (PB). This raised the question of whether a similar population of cells is mobilized in human stroke patients.

Methods—We evaluated a number of cells that corresponded to VSEL SCs in the PB of 44 stroke patients and 22 age-matched controls. After each patient’s stroke, PB samples were harvested during the first 24 hours, on day +3, and on day +7 and then compared with normal controls. The circulating human cells with the phenotype of VSEL SCs were evaluated in PB by real-time quantitative polymerase chain reaction, fluorescence-activated cell sorting analysis, and direct immunofluorescence staining. In parallel, we also measured the serum concentration of stromal derived factor-1 by ELISA.

Results—In stroke patients, we found an increase in the number of circulating cells expressing SC-associated antigens, such as CD133, CD34, and CXCR4. More important, we found an increase in the number of circulating primitive cells expressing the VSEL phenotype (CXCR4linCD45small cells), mRNA for Octamer-4 and Nanog, and Octamer-4 protein. All changes were accompanied by an increased serum concentration of stromal derived factor-1. Additionally, we found a positive correlation between stroke extensiveness, stromal derived factor-1 concentration in serum, and the number of CXCR4VSEL SCs circulating in the PB.

Conclusions—We conclude that stroke triggers the mobilization of CXCR4VSEL SCs that have potential prognostic value in stroke patients. However, the potential role of these mobilized cells in brain regeneration requires further study. (Stroke. 2009;40:00-00.)

Key Words: ischemic stroke ■ very small embryonic-like stem cells ■ stem cells ■ mobilization

The circulating bone marrow (BM)-derived stem/progenitor cells could have an important role in the turnover of stem cells (SCs) in peripheral tissues. For example, hematopoietic (H)SCs continuously circulate at a very low level in peripheral blood (PB). A pool of HSCs is maintained in marrow tissue that is distributed to bones located in distant parts of the body. These HSCs can be mobilized into PB during pharmacologic mobilization by administering granulocyte colony stimulating factor (G-CSF).1

On the other hand, compelling evidence shows that in addition to HSCs, BM contains a population of non-HSCs that could be mobilized into PB during tissue/organ injuries similarly to HSCs. Accordingly, cells that express markers for mesenchymal (M)SCs,2 fibrocytes,1 skeletal progenitors,4 endothelial progenitors,5 liver oval SCs,6 and kidney7 and bronchial epithelium progenitors8 were identified in PB in various tissue/organ injury models.

Experiments performed in animal models of stroke have revealed that human BM- or umbilical cord blood–derived stem/progenitor cells may contribute to damaged brain tissue regeneration.9 Similarly, in the murine model of stroke, the pharmacologic mobilization of BM-derived cells into the PB accelerated functional recovery of damaged brain.10 Based on these encouraging reports, the first feasibility studies have been proposed that use SCs for brain regeneration in human patients after stroke.11 However, more basic studies are...
needed to clarify the mechanisms by which SCs could improve damaged neural tissues.

We recently reported an increase in the number of CXC chemokine receptor 4 (CXCRI4) mononuclear cells (MNCs) circulating in PB that are enriched for several tissue-specific developmental markers during G-CSF–induced mobilization as well as after heart infarct or stroke. On the basis of this finding, we postulated that a population of CXCR4+ tissue-committed SCs (TSCSCs) resides in the BM. These TSCSCs respond robustly to a chemotactic gradient of stromal derived factor-1 (SDF-1). Because SDF-1 is upregulated in a hypoxia-dependent manner in damaged organs, TCSCs could be mobilized into the PB in response to this chemotactic factor. We also suggested that in addition to SDF-1, mobilization of these cells is also directed by other chemotactic factors, such as hepatocyte growth factor/scatter factor (HGF/SF), leukemia inhibitory factor (LIF), and vascular endothelial growth factor (VEGF). Thus, a concept emerged where chemotactic factors that are upregulated in damaged tissues may orchestrate the release of non-HSCs from BM into PB.

Furthermore, recent work from our group revealed 4 significant attributes of murine BM-derived CXCR4+ TSCSCs. They are small, contain large nuclei with unorganized euchromatin, express stage-specific embryonic antigen-1 on the surface and, in their nuclei, the early embryonic transcription factors such as Octamer-4 (Oct-4) and Nanog show high telomerase activity. On the basis of these observations and on the cells’ ability to differentiate in vitro into cells from all 3 germ layers, we changed their name from the initially described “TSCSCs” to the more appropriate “very small embryonic-like (VSEL) SCs.” The capability of VSEL SCs to express mRNA for several TSCSC markers could be explained by the open status of the chromatin in these cells, which allows transcription of several genes, including those involved in tissue/organ development.

From our observations in the murine model of stroke that a population of primitive cells related to VSEL SCs was mobilized into the PB, we investigated whether cells displaying the VSEL phenotype and morphology could also be released into the PB of human stroke patients. Using multiparameter analysis, we present evidence for the first time that VSEL SCs could be detected in circulating PB as a population of small CXCR4+ /CD45- cells that express Oct-4 protein. Thus, we assert that stress related to the stroke triggers VSEL mobilization from the BM and perhaps other SC niches as well. However, the potential of these cells in brain tissue regeneration requires further study.

Subjects and Methods

Study Population

Subjects were recruited from the patient population of the Clinic of Neurology at Pomeranian Medical University in Szczecin, Poland. We enrolled 44 patients afflicted with ischemic stroke who were admitted within 24 hours of the first symptom onset and 22 healthy control subjects. In each case, the stroke had been precisely documented in clinic by computed tomography scan. According to the definition of Oxfordshire Community Stroke Project, based on clinical examination and cranial computed tomography findings, including volumetric analysis, patients were classified into the following 4 clinical subgroups: total anterior circulation infarcts (TACI), partial anterior circulation infarcts (PACI), posterior circulation infarcts (POCI), and lacunar infarcts (LACI). Additionally, we distinguished 2 subgroups of patients with different extensiveness of stroke, ie, group A (small and medium: patients diagnosed with LACI and PACI) and group B (large: patients diagnosed with TACI).

Patients diagnosed with POCI were included in group A or B on the basis of clinical examination and cranial computed tomography analysis. Approval from the local ethics committee was obtained for enrollment. Moreover, each patient gave written, informed consent for his or her involvement.

Laboratory Measurement

EDTA-anticoagulated PB samples (2×2.7 mL) were drawn within 24 hours after first symptoms onset, on day +3, and on day +7 from stroke patients and once from controls. The absolute numbers of leukocytes and lymphocytes in PB were determined at the same time with an automatic cell counter (Cell-Dyn 3500, Abbott Diagnostics, Santa Clara, Calif). The full population of PBMCs was obtained after lysis of red blood cells with 5% bovine calf serum (HyClone, Logan, Utah). The following monoclonal antibodies directly conjugated with phycoerythrin (PE) or allophycoerycin, (APC) were used in this study: PE–anti-CD34 (BD Biosciences Pharmingen); APC–anti-CXCR4 (BD Biosciences Pharmingen); and APC–anti-CD133 (Miltenyi Biotec, Auburn, Calif). To determine the proportion of CD34+/CD133+, CXCR4+/CXCR4−, and CXCR4+/CD45+ cells, we used dual-color flow cytometric analysis. Samples stained with an appropriate isotype control (BD Biosciences Pharmingen) were examined in parallel. PBMCs were stained in saline (PBS; Ca2+− and Mg2+−free) supplemented with 5% bovine calf serum (Hyclone, Logan, Utah). The full population of PBMCs was obtained after lysis of red blood cells with 5% bovine calf serum (HyClone, Logan, Utah). The following monoclonal antibodies directly conjugated with phycoerythrin (PE) or allophycoerycin, (APC) were used in this study: PE–anti-CD34 (BD Biosciences Pharmingen); APC–anti-CXCR4 (BD Biosciences Pharmingen); and APC–anti-CD133 (Miltenyi Biotec, Auburn, Calif). To determine the proportion of CD34+/CD133+, CXCR4+/CXCR4−, and CXCR4+/CD45+ cells, we used dual-color flow cytometric analysis. Samples stained with an appropriate isotype control (BD Biosciences Pharmingen) were examined in parallel. PBMCs were stained in PBS and analyzed by FACS Aria (BD Biosciences, San Jose, Calif) and Cell Quest software (BD Biosciences). Typically, 50 000 events were acquired to determine the proportion of the examined subpopulation within the MNC population. The number of early TSCSCs was expressed as the absolute number of cells per 1 μL of whole blood.

Additionally, a single-cell suspension was stained for lineage markers (CD356, CD235a, CD3, CD66b, CD24, CD19, CD14, CD16, CD2) conjugated with fluorescein isothiocyanate, CD45 conjugated with APC, and CD44 conjugated with APCFITC (BD Pharm Lyse Buffer). After being washed, cells were analyzed by fluorescence-activated cell sorting (BD Biosciences). At least 105 events were acquired and analyzed by Cell Quest software (BD Biosciences). The population of lin-CD45-CXCR4+ VSEL SCs and lin-CD45+CXCR4+ cells that are highly enriched for HSCs were analyzed (Figure 1). The absolute number of lin-CD45-CXCR4+ cells per 1 μL of PB was calculated on the basis of absolute leukocyte count multiplied by the percentage of specific positive cells/100. Lin-CD45-CXCR4+ cells were expressed as a percentage of total events.

Real-Time Reverse Transcriptase–Polymerase Chain Reaction

To analyze mRNA levels for pluripotent (Oct-4, Nanog) and early neural (glial fibrillary acidic protein [GFAP], nestin, βIII-tubulin, Olig1, Olig2, Sox2, Musashi-1) markers, total mRNA was isolated from PBMCs with the RNeasy Mini Kit (Qiagen GmbH). Subsequently,
mRNA was reverse-transcribed with a first-strand cDNA synthesis kit (Fermantas International Inc, Burlington, Canada). Quantitative assessment of Oct-4, Nanog, GFAP, nestin, βIII-tubulin, Olig1, and Olig2, Sox2, Musashi-1 mRNA levels was performed by real-time reverse transcription polymerase chain reaction (RT-PCR) with an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, Calif). Primers were designed with Primer Express software (Applied Biosystems). A 25-μL reaction mixture contained 12.5 μL SYBR Green PCR Master Mix and 10 ng of cDNA template, 5'-GAT GTG GTC CGA GTG TGG TTC T-3' (forward) and 5'-GTC TGG CTT GAT-3' (reverse) primers for Oct4; 5'-GCA GAA GGC CTC AGC ACC TA-3' (forward) and 5'-AGG TTC CCA GTC GGG TTC A-3' (reverse) primers for Nanog; 5'-GTG GGC AGG TGG GAG CTT GAT TCT-3' (forward) and 5'-CTG GGG CGG CCT GGT ATG ACA-3' (reverse) primers for GFAP; 5'-ACA CCT GTG CCA GCC TTT CTT-3' (forward) and 5'-TGA ACA CTC TAG ACC CAC CCG A-3' (reverse) primers for nestin; 5'-TTC TGG GAA GTG CCT ACC TAC AGT GAT GA-3' (forward) and 5'-CGA GTC GCC GAC GTA GTG G-3' (reverse) primers for βIII-tubulin; 5'-AGG TAA CCA GCC GTC TCA CAG T-3' (forward) and 5'-CGG TAC TCC TGC GTG TTA ATG A-3' (reverse) primers for Olig1; 5'-GGC GGC CAA CTA CAT CCT-3' (forward) and 5'-GCC TCA CCA GTC GCT TCA A-3' (reverse) primers for Olig2; 5'-TCA CGC AAA AAC CGC GAT-3' (forward) and 5'-TAT ACA AGG TCC ATT CCC CCG-3' (reverse) primers for Sox2; and 5'-ATA AAG TGC TGG CGC AAC ATG CG-3' (forward) and 5'-TGG TTC GAG TCA CCA TCT TGG-3' (reverse) primers for Musashi-1. The threshold cycle (Ct), ie, the cycle number at which the amount of amplified gene of interest reached a fixed threshold, was subsequently determined. Relative quantification of Oct-4, Nanog, GFAP, nestin, βIII-tubulin, Olig1, and Olig2, Sox2, Musashi-1 mRNA expression was performed with the comparative Ct method. The relative quantization value of target, normalized to an endogenous control –actin (housekeeping) gene and relative to a calibrator, is expressed as \(2^{-\Delta\Delta Ct}\) (fold difference), where \(\Delta Ct = Ct\) of target genes (Oct-4, Nanog, GFAP, nestin, βIII-tubulin, Olig1, Olig2, Sox2, Musashi-1) – [Ct of endogenous control gene (β-actin)], and \(\Delta\Delta Ct = \Delta Ct\) of gene samples.

To avoid the possibility of amplifying contaminating DNA, several precautions were taken. First, all primers for real-time RT-PCR were designed with an intron sequence inside the cDNA to be amplified. Second, reactions were performed with appropriate negative controls (template-free controls). Third, a uniform amplification of the products was checked by analyzing the melting curves (dissociation graphs). Fourth, the melting temperature was 57°C to 60°C, and the probe melting temperature was at least 10°C higher than the primer melting temperature. Finally, gel electrophoresis was performed to confirm the correct size of the amplification and the absence of nonspecific bands.

**Fluorescence Analysis**

CXCR4+/CD45− cells were sorted from the PB of stroke patients at day +3 after stroke. Cells were fixed in 3.7% paraformaldehyde...
Table 1. Characteristics of Stroke Patients and Control Subjects

<table>
<thead>
<tr>
<th>Characteristics, %*</th>
<th>Stroke Patients</th>
<th>Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>57</td>
<td>56</td>
</tr>
<tr>
<td>LACI</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>PACI</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>POCI</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>TACI</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Stroke extent, small/medium/large</td>
<td>52/5/43</td>
<td>1/4</td>
</tr>
<tr>
<td>Clinical status, good/mild/severe</td>
<td>66/23/11</td>
<td>1/4</td>
</tr>
<tr>
<td>Hypertension</td>
<td>80</td>
<td>30</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>52</td>
<td>13</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>27</td>
<td>11</td>
</tr>
<tr>
<td>Smoking</td>
<td>27</td>
<td>7</td>
</tr>
<tr>
<td>Obesity</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Overweight</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>Previous stroke</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>49</td>
<td>5</td>
</tr>
<tr>
<td>Chronic atrial fibrillation</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Statins</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme inhibitors</td>
<td>63</td>
<td>15</td>
</tr>
<tr>
<td>Aspirin</td>
<td>77</td>
<td>3</td>
</tr>
<tr>
<td>Cloxane</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Age, yr†</td>
<td>68±12</td>
<td>64±11</td>
</tr>
<tr>
<td>High-sensitivity C-reactive protein, mg/L†</td>
<td>7±10</td>
<td>1/4</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL†</td>
<td>375±80</td>
<td>1/4</td>
</tr>
<tr>
<td>White blood cells, cells/μL†</td>
<td>8.7±2.8</td>
<td>7.1±2.8</td>
</tr>
</tbody>
</table>

*Values are percentages.
†Values are mean±SD.

The concentrations of SDF-1 were measured with a commercially available, high-sensitivity ELISA kit SDF-1 Quantikine human immunoassays (R&D Systems, Minneapolis, Minn) according to the manufacturer’s protocol.

Statistical Methods
Nonparametric tests were used because percentage distributions of circulating cells were significantly different from the normal distribution (Shapiro-Wilk test, P<0.05). Significance of changes in the number and SDF-1 serum concentration measured at 24 hours, day +3, and day +7 after stroke was assessed with Friedman’s ANOVA and the Wilcoxon signed-rank test. Spearman rank correlation coefficients (Rs) were calculated to measure associations between the number of cells and SDF-1 plasma concentrations. The Mann-Whitney test was used to compare the number of cells and SDF-1 serum concentrations between 2 subgroups of patients with different extensiveness of stroke (ie, group A vs and group B). Differences between 4 clinical subgroups of stroke patients were tested with the Kruskal-Wallis ANOVA followed by the Mann-Whitney test. P<0.05 without adjustment for multiple comparisons was considered statistically significant.

Results
The characteristics of the patients are summarized in Table 1. The stroke and control groups were matched for age. Of 44 patients, 12 had a history of previous stroke.

Changes in the Numbers of CD34+, CXCR4+, and CD133+ Cells Circulating in PB of Patients After Stroke
The absolute numbers of cells circulating in PB that express CD34, CXCR4, and CD133 markers are shown in Table 2. Compared with control group values, we noticed an increase in the number of CD34+ CXCR4+ CD133+ cells in PB at 24 hours, day +3, and day +7 after stroke (Table 2). This increase was statistically significant for CD34+ and CXCR4+ cells as well as for double-positive CD34+CXCR4+ cells (Table 2). We also observed an increase in the number of circulating CD133+ cells and double-positive CD34+CD133+ cells; however, these changes were not statistically significant. Furthermore, we observed some differences in the number of CD34+, CD34+CD133+, and CXCR4+ cells in the patients with PACI compared with other subtypes of cerebral infarction (LACI, POCI, TACI). In the patients with PACI, the number of circulating CXCR4+ cells was higher in the 24-hour poststroke blood draw (P<0.05).

Table 2. Absolute Numbers of Early Stem/Progenitor Cells Expressing Surface Antigens CD34, CD133, CXCR4 in PB From Patients With Ischemic Stroke at Different Time Points (24 Hours and Poststroke Days +3 and +7) and in the Healthy Control Group

<table>
<thead>
<tr>
<th></th>
<th>Stroke Patients at 24 hours</th>
<th>P vs Control*</th>
<th>Stroke Patients at Day +3</th>
<th>P vs Control*</th>
<th>Stroke Patients at Day +7</th>
<th>P vs Control*</th>
<th>Stroke Patients at Day +7</th>
<th>P vs Control*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+</td>
<td>3.6 [2.0]</td>
<td>&lt;0.001</td>
<td>13.6 [15.4]</td>
<td>&lt;0.01</td>
<td>11.3 [9.9]</td>
<td>&lt;0.01</td>
<td>11.9 [10.7]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CD133+</td>
<td>3.0 [7.1]</td>
<td>NS</td>
<td>9.8 [12.6]</td>
<td>NS</td>
<td>7.9 [39.2]</td>
<td>0.06</td>
<td>6.8 [10.6]</td>
<td>NS</td>
</tr>
<tr>
<td>CD34+/CD133+</td>
<td>0.7 [1.6]</td>
<td>NS</td>
<td>1.1 [2.9]</td>
<td>NS</td>
<td>1.2 [1.7]</td>
<td>NS</td>
<td>1.2 [1.7]</td>
<td>NS</td>
</tr>
<tr>
<td>CXCR4+</td>
<td>104 [409]</td>
<td>&lt;0.0001</td>
<td>506 [927]</td>
<td>&lt;0.01</td>
<td>563 [1007]</td>
<td>&lt;0.01</td>
<td>563 [1007]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CXCR4+/CD34+</td>
<td>0.9 [2.4]</td>
<td>&lt;0.0001</td>
<td>9.0 [10.5]</td>
<td>&lt;0.0001</td>
<td>7.3 [8.1]</td>
<td>&lt;0.001</td>
<td>7.3 [8.1]</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are expressed as median (interquartile ranges) of CD34+, CD133+, and CXCR4+ cells per μL of PB.
*Man–Whitney test.
†Friedman’s ANOVA followed by Wilcoxon signed-rank test.

Plasma Concentrations of SDF-1
for 20 minutes, permeabilized by 0.1% Triton X-100, washed in PBS, and stained with antibodies to Oct-4 (1:300, mouse monoclonal IgG; Chemicon International, Inc, Temecula, Calif) and GFAP (1:200, mouse monoclonal IgG; Chemicon). The nuclei were labeled with 4',6-diamidino-2-phenylindole (Molecular Probes, Eugene, Ore). The fluorescence images were collected with the TE-FM Epi-Fluorescence system attached to a Nikon inverted microscope Eclipse TE300 and captured by a CoolSnap HQ digital B/W CCD camera (Roper Scientific, Tucson, Ariz).
The Number of Small CXCR4⁺CD45-SCs Increases in PB of Stroke Patients

Next, we focused on the presence of cells in the PB that express the VSEL SC (CXCR4⁺CD45-) and HSC (CXCR4⁺CD45-lin) phenotypes. We noticed a statistically significant (at 24 hours, P<0.05; at day +3, P<0.01; at day +7, P<0.05) increase in the percentage of circulating CXCR4⁺CD45- lin- cells in stroke patients compared with normal controls (Table 3 and Figure 1). The percentage of circulating VSEL SCs (CXCR4⁺CD45- lin-) increased 24 hours after the onset of symptoms, reaching a maximum on poststroke day +3 and decreasing after day +7. At the same time, we also observed significant differences in the number of circulating CXCR4⁺CD45- lin- cells that are enriched for population of HSCs between normal controls and stroke patients (Table 3).

Expression of Pluripotent SC Markers in Circulating PBMCs by Real-Time RT-PCR

Real-time RT-PCR was used to detect the expression of mRNA for pluripotent SC markers in PBMCs derived from patients at different time points after stroke as well as from control PBMCs. Figure 2 shows that we found a significant increase in expression of pluripotent SC markers (Oct-4, Nanog) in circulating PBMCs from patients after stroke compared with the control group. The highest level of mRNA for these genes was observed 24 hours after stroke and subsequently decreased with time. However, mRNA levels were elevated compared with control PBMCs.

Expression of Early SC Markers in Circulating PBMCs

In parallel, we evaluated expression of mRNA for VSEL markers by real-time quantitative PCR (Figure 2) and immunofluorescence staining (Figure 3). We noticed an increase in expression of mRNA for Oct-4 and Nanog in PBMCs at 24 hours and day +3 after stroke. At the same time, CXCR4⁺CD45- cells sorted from PB expressed VSEL marker Oct-4 and GFAP, the early neuronal SC marker (Figure 3). Real-time quantitative PCR analysis of PBMCs from stroke patients also revealed an increase in mRNA expression for several early neural markers, such as GFAP, nestin, βIII-tubulin, Olig1, Olig2, Sox2, and Musashi (Figure 4).

Table 3. Circulating lin’CD45’CXCR4+ VSEL SCs (Shown as Percentage and Absolute No. of Cells) and Circulating lin’CD45’CXCR4+ HSCs (Shown as Absolute No. of Cells) in PB From Patients With Ischemic Stroke at 24 Hours, Day +3, and Day +7 Compared With the Healthy Control Group

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Stroke Patients at 24 Hours</th>
<th>Stroke Patients at Day +3</th>
<th>Stroke Patients at Day +7</th>
<th>Stroke Patients at 24 Hours†</th>
<th>Stroke Patients at Day +3†</th>
<th>Stroke Patients at Day +7†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lin-CD45-CXCR4+ [%]</td>
<td>0.024 [0.02]</td>
<td>0.047 [0.062]</td>
<td>&lt;0.05</td>
<td>0.057 [0.067]</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>0.046 [0.062]</td>
</tr>
<tr>
<td>Lin-CD45-CXCR4+ [No. of cells per μL]</td>
<td>1.71 [1.22]</td>
<td>4.72 [9.63]</td>
<td>&lt;0.05</td>
<td>5.77 [4.41]</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>4.18 [6.59]</td>
</tr>
<tr>
<td>Lin-CD45+CXCR4+ [No. of cells per μL]</td>
<td>0.3 [0.99]</td>
<td>2.43 [5.3]</td>
<td>&lt;0.01</td>
<td>1.25 [1.97]</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>2.26 [2.58]</td>
</tr>
</tbody>
</table>

Data are expressed as median (interquartile ranges).
*Friedman’s ANOVA followed by Wilcoxon signed-rank test.
†Mann-Whitney test.

Figure 2. Changes in the expression of markers for pluripotent VSEL SCs in PBMCs in stroke patients and the control group. PBMCs were harvested from patients who experienced ischemic stroke within 24 hours of symptom manifestation and at poststroke days +3 and +7. The expression of mRNA for pluripotent markers (Oct-4, Nanog) in the same number of cells was quantified by real-time RT-PCR and compared between groups. Data are mean±SD.

Figure 3. Expression of Oct-4 on CXCR4⁺CD45- cells sorted by FACS Aria from PBMCs from patients with stroke harvested on poststroke day +3. Nuclei were identified by 4′,6-diamidino-2-phenylindole. All images were photographed under plan Apo60× A/1.40 oil objective lens (Nikon, Tokyo, Japan).
The levels of mRNA for these markers increased up to 8.0 times at poststroke day +3 and remained elevated until day +7.

**SDF-1 Serum Concentration Is Elevated in Stroke Patients**

Because SDF-1 is a strong chemotactic factor for CXCR4+ SCs, we studied changes in SDF-1 serum concentrations in patients after stroke (Figure 5). We found that the levels of SDF-1 were elevated in stroke patients compared with normal levels in the control group. This increase, however, was statistically significant only at day +7. When we compared the elevation of SDF-1 concentration in serum between patients with different localizations of stroke, the patients with POCI displayed lower levels of SDF-1 compared with other subtypes of cerebral infarction ($P=0.09$).

**Correlations Between SDF-1 Serum Concentration and Circulating SCs**

We found that the increase in the number of cells circulating in PB expressing early SC markers CD34+CD133+ and CXCR4+CD34+ 24 hours after stroke was correlated positively with the increase in serum concentration of SDF-1 ($R_S=0.46$, $P<0.05$; $R_S=0.31$, $P=0.094$, respectively).

**Comparison Between SDF-1 Plasma Concentration and Number of Circulating SCs and Selected Patient Parameters**

Finally, we also evaluated the correlation between the level of circulating SCs after stroke and the extensiveness of stroke, arbitrarily dividing the subjects into 2 groups according to extensiveness of the stroke: group A (small and medium) and group B (large). We noticed a positive correlation between the extensiveness of stroke and the number of circulating CXCR4+ cells. Accordingly, significantly more CXCR4+ cells circulated in PB 24 hours after stroke in group B compared with group A ($P<0.001$). At the same time, we noticed an association between serum SDF-1 levels and the recurrence of stroke. We found significantly higher serum concentration of SDF-1 at 24 hours after stroke in patients having their first stroke compared with those with recurrent strokes ($P<0.05$).
Discussion

Occlusion of a cerebral artery leads to focal ischemia that results in damage to neurons and glial cells. Data from mouse and rats have shown that stroke and the subsequent death of neurons lead to increased proliferation of neural precursors that are located in the subventricular zone, hippocampus, and olfactory bulb.12,22,23 Unfortunately, this response is not effective enough to fully restore morphology and function of damaged neural tissue.

It has been postulated that, in response to organ damage, SCs could be mobilized into PB from BM and perhaps other tissue-specific niches.17 These SCs would home to the damaged tissues24 and attempt to contribute to regeneration. We observed that the number of circulating SCs expressing pluripotent and tissue-specific markers increases in PB after G-CSF administration,12 heart infarct in humans13 and mice,25 and in a murine model of stroke.14 Although these cells may act somehow in regeneration from small tissue injuries, their contribution toward repairing more extensive tissue damage requires further study.

Recently, we identified a population of VSEL SCs in adult murine BM that express several pluripotent SC markers and purified them at the single-cell level.18 These SCs are mobilized into PB after G-CSF administration as well as during toxic liver and skeletal muscle damage.13 In the current study, we observed that the number of cells in the PB that express VSEL markers increases in patients after stroke. This increase was observed 24 hours after stroke and remained visible up to day +7.

In addition to VSEL SCs, we also observed an increase in the number of circulating cells that are enriched for HSCs (CXCR4+/CD45lin), as well as CD34+ and CD34+/CXCR4+ cells that contain both HSCs and progenitor cells. This increase in the number of circulating CD34+ cells is in agreement with our previously published data, which show that the numbers of circulating CD34+ cells as well as clonogenic colony-forming units granulocyte-macrophage and burst-forming units-erythroid were significantly higher in the patients’ PB during the first 6 days after stroke than in healthy controls.13

In this study, we observed that PBMCs express both pluripotent (Oct-4, Nanog) and neural (GFAP, nestin, βIII-tubulin, Olig1, Olig2, Sox2, and Musashi-1) SC markers. The increased expression of mRNA for Oct-4 and Nanog in PBMCs in patients after stroke corresponded to those observed by us in an experimental murine model of stroke.14 However, maximal increased expression of neural SC markers in humans was delayed by 2 days (1 day in mice vs 3 days in humans).14 Moreover, the kinetics of changes in expression of mRNA for early SCs correspond to changes that we recently observed after heart infarct in humans4 and mice.25

In this study, we observed differences in SC mobilization in cases of POCI and PACI compared with other subtypes of stroke. This classification is of prognostic significance19 because patients with POCI have the best chance of recovery. Accordingly, PACI is associated with the highest risk of early recurrence of stroke (ie, within 3 months), but it is not associated with high mortality and significant disability.

The changes in the number of CXCR4+ SCs circulating in PB were paralleled by changes in expression of α-chemokine SDF-1. The SDF-1–CXCR4 receptor axis serves an important function in the retention of SCs in BM. Whereas SDF-1 is secreted by BM osteoblasts and fibroblasts, CXCR4 is expressed on HSCs and several types of non-HSCs, including VSEL SCs and neural SCs.18,26–28 Murine embryos with CXCR4 or SDF-1 knockout display several defects in their ability to develop BM, brain, and cerebellum tissues.29 Accordingly, SDF-1 signaling regulates key events during early cortical development in vitro and maintains proliferation of cortical progenitors, possibly through a mechanism involving connexin 43–mediated intercellular coupling. Moreover, SDF-1 signaling upregulates the differentiation of cortical γ-aminobutyric acid outputting neurons, which is independent of the sonic signaling pathway and enables the elongation and branching of axons of cortical glutamatergic neurons.30

It is also widely accepted that the upregulation of SDF-1 expression in damaged organs acts in chemoattracting CXCR4+ SCs necessary for organ regeneration. In the murine model of stroke, we demonstrated that the expression of SDF-1 increased during the first week after stroke and that SDF-1 was 1 of the important chemoattractants for recruiting neural SCs to supernatants from damaged brain tissue.14 In this study, we provide data that the serum concentration of SDF-1 increases in patients after stroke. This may be 1 of the factors that mobilized SCs from the BM into PB. However, we were unable to measure the concentration of SDF-1 in the immediate vicinity of the stroke location in these patients. However, it is most likely that the serum increase of SDF-1 is positively correlated with an increase in expression of SDF-1 in the damaged brain.34 Interestingly, we observed a positive correlation between the number of circulating CD34+/CD133− and CXCR4+/CD34+ cells in PB and the serum concentration of SDF-1. In the available literature, data on the correlation between these 2 variables and organ damage are contradictory. For example, in patients after heart infarct, some authors found a direct correlation4 while others determined the inverse to be true.31

However, although the increase in circulating VSEL SCs in PB (≈3 times) that we observed was relatively small, it is important to remember that mobilization and homing are a dynamic process. Thus, it is very likely that cells released into the circulation may rapidly home to the damaged tissues, enter SC niches in other organs, or even return to SC niches in BM. This means that the number of cells detected in a “frozen time lapse” is only 1 of the parameters of VSEL trafficking and does not reflect the total number of these cells released into PB during stroke. This increase, however, was statistically significant and similar to those we observed for circulating VSEL SCs in murine models of heart infarct32 as well toxic skeletal muscle and liver injuries.33

In conclusion, our study demonstrates for the first time that the mobilization of CXCR4+ CD45lin VSEL SCs and progenitor cells expressing early neural markers into PB occurs in patients with ischemic stroke. We conclude that stress related to the stroke triggers the mobilization of SCs from BM and perhaps other SC niches. We hypothesize that if
these cells will turn out to be human counterparts of VSELs identified in mice,18 they could be potentially purified from the PB, expanded ex vivo, and used.

Disclosures

None.

References

Clinical Evidence That Very Small Embryonic-Like Stem Cells Are Mobilized Into Peripheral Blood in Patients After Stroke

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