Mobilization of human hematopoietic stem/progenitor-enriched CD34+ cells into peripheral blood during stress related to ischemic stroke

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Abstract: The bone marrow-derived stem/progenitor cells were demonstrated to play an important role in a regeneration of damaged tissue. Based on these observations we asked whether the stroke-related stress triggers mobilization of stem/progenitor cells from the bone marrow into the peripheral blood, which subsequently could contribute to regeneration of damaged organs. To address this issue, the peripheral blood samples were harvested from patients with ischemic stroke during the first 24 hrs as well as after the 48 (2nd day) and 144 hrs (6th day) since the manifestation of symptoms. In these patients we evaluated the percentage of hematopoietic stem/progenitor-enriched CD34+ cells by employing flow cytometry and the number of hematopoietic progenitor cells for the granulocyto-monocytic (CFU-GM) and erythroid (BFU-E)-lineages circulating in peripheral blood. We concluded that stress related to ischemic stroke triggers the mobilization of hematopoietic stem/progenitor cells from the bone marrow into peripheral blood. These circulating stem/progenitor cells may play an important role in the process of regeneration of the ischemic tissue. (www.cm-uj.krakow.pl/FHC)

Key words: Stem/progenitor cells - CD34+ cells - Bone marrow - Stroke

Introduction

Ischemic stroke leads to the degeneration of brain tissue supplied by the occluded vessel. This produces a lesion cavity and results in neurological deficits. The disease is the leading cause of death and disability worldwide [16]. Organ and tissue repair is a constant phenomenon which occurs during normal life [12]. This process may be driven by stem cells that reside in bone marrow and in other tissues [15, 17]. Bone marrow (BM)- or mobilized peripheral blood (mPB)-derived stem cell implants have been reported to regenerate damaged organs, including brain [2, 14, 20, 25]. Bone marrow-derived stem cells have been shown to be able to differentiate in *in vitro* cultures into neurons, cardiac myocytes, smooth muscle- and endothelial cells [4, 5, 11, 19]. Similarly, they were reported to regenerate ischemic brain tissue *in vivo*. Human bone marrow mesenchymal stem cells engrafted into the cortex surrounding the area of infarction significantly improved the functional performance in limb placement test in rats [25]. In the same study, histological examination revealed that transplanted human mesenchymal stem cells expressed markers for astrocytes, oligodenroglia and neurons. Subcutaneous administration of granulocyte colony-stimulating factor (G-CSF) into rats resulted in diminished cerebral infarction and improved motor performance after right middle cerebral artery ligation [20]. These observations awoke many hopes for the development of new stem cell based therapeutic strategies to ameliorate neurological deficits in patients after stroke.

However, whether the bone marrow-derived progenitor/stem cells play a role in the regeneration of damaged tissue is not clear at this point. Generally, the beneficial effect of these cells in regeneration of damaged organs could be explained by (1) trans-dedifferentiation/plasticity of hematopoietic stem cells [1], (2) paracrine secretion of angiopoietic factors from BM-derived stem/progenitor cells which leads to an improved vascularization of the damaged organ, or (3) what we have recently postulated - by the presence of a heterogeneous population of tissue-committed stem

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cells (*e.g.*, for myocardium and endothelium) in the BM [15, 17].

The circulating pool of stem cells can be increased by pharmacological mobilization from the BM. The progenitor/stem-enriched CD34+ cells could be mobilized into peripheral blood by administration of growth factors (*e.g.* granulocyte colony-stimulating factor G-CSF), or chemotherapeutics (*e.g.* cyclophosphamide), or a combination of both [22]. Mobilized peripheral blood hematopoietic stem cells are increasingly used for allogeneic hematopoietic transplantations. The expression of the CD34 surface antigen characterizes a heterogeneous population of cells including hematopoietic stem/progenitor cells (HSPC), endothelial progenitor cells (EPC), mature endothelial cells and, as we recently reported, tissue-committed stem cells (TCSC) [6]. Although the true role of the CD34 molecule continues to be debated, CD34+ HSPC have been functionally defined as capable of generating progenitor-derived clones *in vitro* and, by their potential, of reconstituting the lymphomyelopoietic system in myelocompromised hosts [13, 21, 24]. CD34+ cells were also reported to be mobilized after heart infarct [23]. However, there is no data available on mobilization of CD34+ cells after ischemic stroke.

Thus, the aim of this study was to evaluate the stroke-related stress mobilization of progenitor/stem-enriched CD34+ cells from the bone marrow into peripheral blood. We evaluated the total number of circulating CD34+ cells by flow cytometry and the number of circulating hematopoietic clonogeneic progenitors: CFU-GM (colony forming unit of granulocytes and macrophages) and BFU-E (burst forming unit of erythrocytes) by employing *ex vivo* cell culture assays in patients with ischemic stroke. The evaluation of these cells in peripheral blood was performed during the first 24 hrs of manifestation of symptoms as well as on the 2nd and 6th day afterwards.

Materials and methods

Patients. Peripheral blood samples (4 mL) were harvested from 25 patients with ischemic stroke. The samples were collected during the first 24 hrs of manifestation of symptoms as well as on the 2nd and 6th day of the stroke. In each case the stroke had been precisely documented clinically by computer tomography (CT). Patients were recruited from the inpatient population of the Clinic of Neurology, Pomeranian Medical University, Szczecin, Poland. Peripheral blood samples were also harvested from 12 healthy donors, which were treated as a control group. An approval from the Local Ethical Committee was obtained. Moreover, the donors gave written informed consent in each case. The patients' characteristics is summarized in Table 1.

Clonogeneic assays. Light-density mononuclear cells (MNC) were obtained after centrifugation over Gradisol L (Polfa, Poland) as described previously [9]. Cells isolated from peripheral blood were subsequently depleted of adherent cells (A-MNC), plated in methylcellulose cultures and stimulated to form granulocyte-monocytic

Table 1. Characteristics of patients with ischemic stroke and healthy control group

	Control	Stroke
Age [yrs]	58±26	67±29
Men/Women	7/3	16/10
Hypertension [%]	0	79
Hypercholesterolemia [%]	0	"
Diabetes [%]	0	21
Smoking [%]	0	25

(CFU-GM) and erythroid (BFU-E) colonies. CFU-GM colonies were stimulated with GM-CSF (granulocyte-macrophage colony stimulating factor) + IL3 (interleukin-3). BFU-E colonies were stimulated with specific human recombinant growth factors: EPO (erythropoietin) + KL (kit ligand) + IL3, as described elsewhere [18]. The *ex vivo* colonies were subsequently identified and counted using an inverted microscope, as described previously [18]. Cultures were performed in quadruplicate.

Flow cytometry. At the same time the percentage of CD34+ cells in peripheral blood was evaluated by employing immunostaining with anti-CD34 monoclonal antibodies (Becton-Dickinson, CA, USA) and flow cytometry (FACScan, Becton-Dickinson, CA, USA), as described previously [8] (Fig. 1).

Automatic cell count. The absolute number of leukocytes and lymphocytes in peripheral blood were determined at the same time with an automatic cell counter (Cell-Dyn 3500, Abbott, USA).

Statistical analysis. The arithmetic means and standard deviations were calculated on an IBM computer using Statistica. The cells were cultured in quadruplicate at each point. The data were analyzed using Kruskal-Wallis test. The values showing significant differences in the Kruskal-Wallis test were next analyzed using the Mann-Whitney U-test. Statistical significance was defined as $P < 0.05$.

Results

We found that the number of CD34+ cells was significantly higher ($P < 0.05$) in peripheral blood of patients after ischemic stroke as compared to the healthy control group (Fig. 2). Furthermore, we noticed differences in the number of myeloid (CFU-GM) and erythroid (BFU-E) progenitors circulating in the peripheral blood of patients with ischemic stroke as compared to healthy controls (Fig. 3). The number of circulating clonogeneic CFU-GM, was significantly higher after 24 hrs, on day 2 and 6 after stroke ($P < 0.05$). In the case of BFU-E, the number of these cells in peripheral blood was also slightly higher, however, this increase was not statistically significant.

The absolute number of leukocytes and lymphocytes in peripheral blood of patients with ischemic stroke during the first 24 hours of manifestation of symptoms, on the 2nd day and on the 6th day afterwards, and of healthy control group is shown in Table 2.

CONTROL
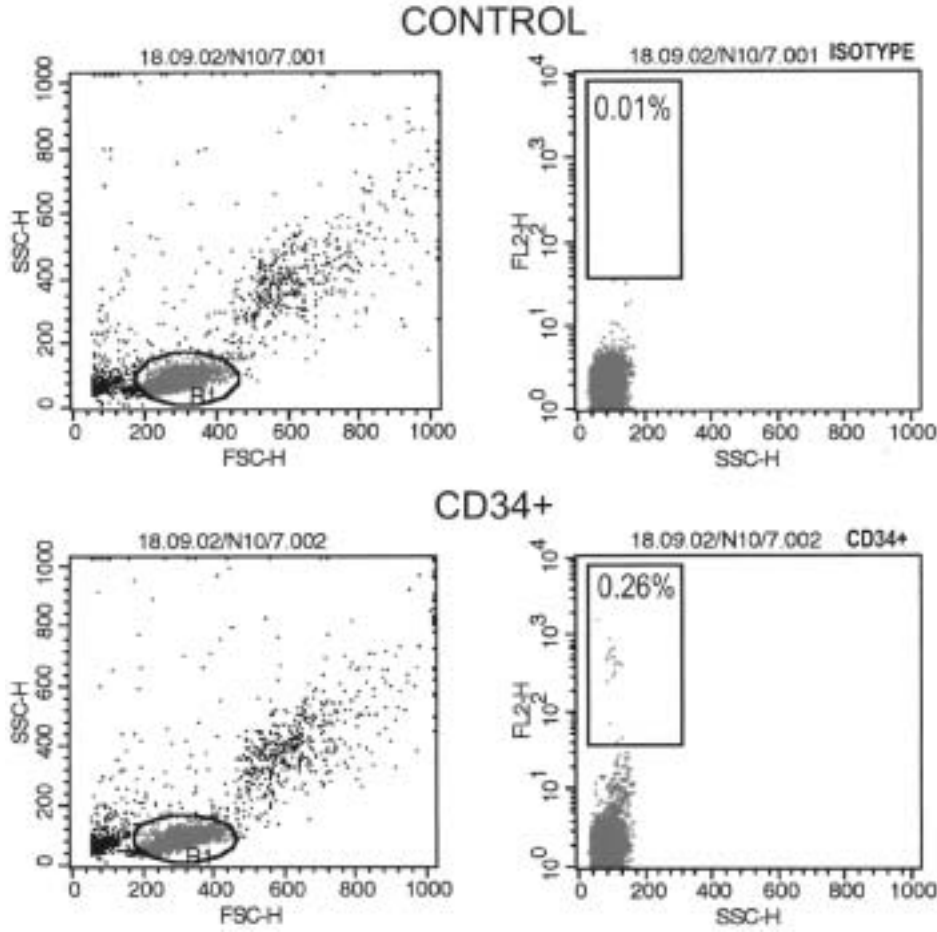
Fig. 1. Representative flow-cytometry measurements of the number of CD34+ cells in peripheral blood of patient with ischemic stroke. CD34+ cells were detected by employing phycoerythrin-conjugated anti-CD34 monoclonal antibodies. Isotype-matched antibodies were used as a control.
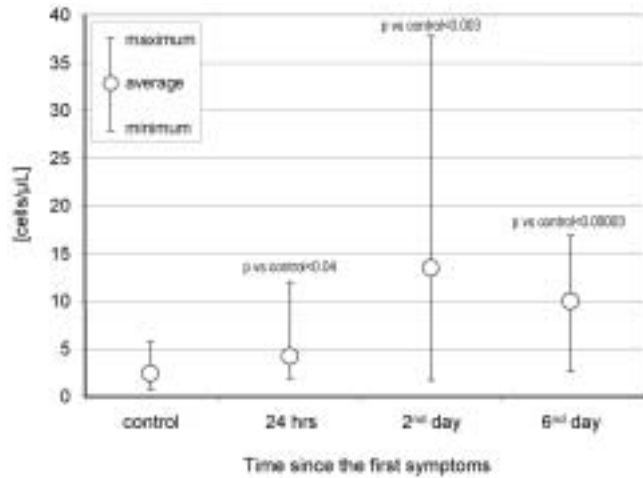
Fig. 2. Number of circulating CD34+ cells in patients with ischemic stroke 24 hours (24 hrs), 48 hrs (2nd day) and 144 hrs (6th day) after manifestation of symptoms and in healthy control group (control). The number of CD34+ cells is expressed per μL of peripheral blood.
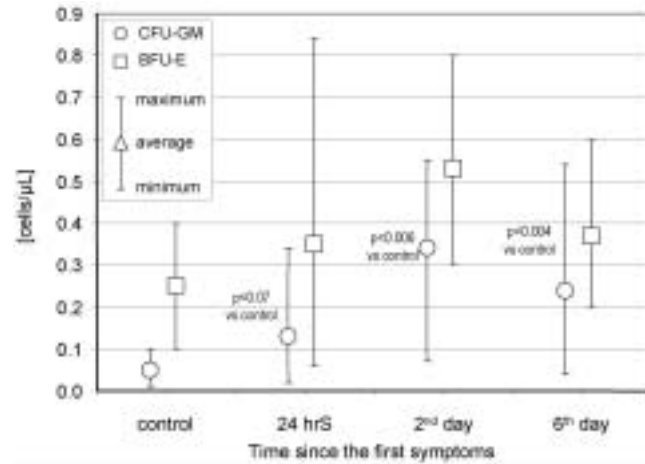
Fig. 3. Number of circulating early hematopoietic stem/progenitor cells (clonogenic CFU-GM and BFU-E) in patients with ischemic stroke 24 hours (24 hrs), 48 hrs (2nd day) and 144 hrs (6th day) after manifestation of symptoms and in healthy control group (control). The number of clonogeneic progenitors are expressed per μL of peripheral blood.

Of note, no significant correlation was found in any patient between the number of circulating hematopoietic progenitor cells (CFU-GM and BFU-E) and circulating CD34+ cells on one hand and evaluated parameters such as patient age, sex, presence of diabetes mellitus, hypertension and smoking habits on the other.

Table 2. The absolute number of leukocytes and lymphocytes in peripheral blood of patients with ischemic stroke during the first 24 hrs of manifestation of symptoms, on the 2nd and 6th day afterwards, and of healthy control group. Standard deviations are shown in brackets.

	Control	24 hrs	2nd day	6th day
Number of leucocytes [G/L]	6.2 (3.6)	8.7 (2.3)	11.0 (2.2)	10.6 (3.9)
Number of lymphocytes [G/L]	2.3 (1.7)	2.66 (1.2)	3.37 (2.0)	2.37 (0.5)

Discussion

Human hematopoietic stem and progenitor cells (HSPC) reside in a complex bone marrow microenvironment consisting of a diverse population of stromal cells and extracellular matrix produced by these cells [7]. However, under physiological conditions relatively small amounts of primitive stem cells continuously leave BM and circulate in the peripheral blood in order to keep in balance the stem cell pool distributed in remote tissue niches that are located in different organs [3]. The number of these cells may be increased in peripheral blood after so-called pharmacological mobilization [22]. The multiple mechanisms and factors that regulate HSPC mobilization from bone marrow into peripheral blood are still not fully understood. We attempted to answer the question whether stroke-related stress may also trigger mobilization of early stem/progenitor-enriched CD34+ cells from the bone marrow into the peripheral blood. The results obtained in our study support this notion.

Accordingly, we noticed by employing flow cytometry a higher number of circulating CD34+ cells in stroke-affected patients as compared to the healthy controls. We also demonstrate here for the first time that the number of circulating CD34+ cells as well as clonogeneic CFU-GM and BFU-E is significantly higher during the first 6 days in patients affected by stroke.

In the available literature there are no data published on the number of circulating CD34+ cells in stroke patients. We are aware that at this point it is still premature to speculate on the biological function of these mobilized early hematopoietic stem/progenitor cells. First, these cells may accompany mobilization of other more primitive hematopoietic and non-hematopoietic stem cells (*e.g*., endothelial-, neural- or cardiac-stem cells) that are mobilized into peripheral blood in an attempt to regenerate damaged tissues (*e.g*., brain). Thus, they could serve as an indicator of stroke-related stress and reflect the general response of an organism to tissue/organ damage. Second, as we demonstrated in the past, that hematopoietic CD34+ progenitor cells are a rich source of several pro-angiopoietic factors [10]. Thus, it is possible that they may home to the damaged tissues/organs and by secreting angiopoietic factors contribute to neovascularization. These possibilities will be tested in our further investigations.

In conclusion, our study has demonstrated for the first time that CD34+ clonogeneic hematopoietic stem/progenitor cells are mobilized into peripheral blood in patients after stroke. Thus, stress related to stroke may increase the number of early CD34+ HSPC in peripheral blood. Whether along with these stroke-mobilized hematopoietic stem/progenitors other tissue-committed stem cells, *e.g.* neural TCSC or endothelial TCSC are also mobilized, will be the subject of our further investigations. We postulate that these circulating stem/progenitor cells may play an important role in regeneration of ischemic brain, however, further studies are needed to elucidate the mechanisms involved in this phenomenon.

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