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CD34/CXCR4 stem cell dynamics in acute stroke patients

Anna Gójska-Grymajło[1], Walenty M. Nyka[1], Maciej Zieliński[2], Zenon Jakubowski[3]

[1]Department of Neurology, Medical University of Gdańsk, Poland, [2]Department of Clinical Immunology and Transplantology, Medical University of Gdańsk, Poland, [3]University Laboratory Center, University Clinical Centre, Medical University of Gdańsk, Poland

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Abstract

Introduction: *Non-hematopoietic stem cells may be a source of paracrine and structural regeneration for brain damaged by acute ischemic stroke. In this study, we investigated correlations of CD34-, CD34/CXCR4-, and CXCR4-positive peripheral blood CD45-negative stem cells with the neurological and functional status of 34 acute stroke patients.*
Material and methods: *Blood was sampled and assessed by flow cytometry on days 1, 2, 4, and 6 after stroke onset. Parallel to blood sampling and after 3 and 6 months, patients were assessed with the National Institute of Health Stroke Scale, Barthel Index, Scandinavian Stroke Scale, and modified Rankin Scale. Blood was also sampled in 15 control subjects matched for age and sex, without history of previous stroke.*
Results: *We observed very low levels of the observed stem cells resident in peripheral blood. Higher baseline numbers of all 3 stem cell types correlated with better neurological or functional status on admission. Additionally, higher increases in CD34- and CD34/CXCR4-positive stem cell number and lower increase in CXCR4-positive cells correlated with initially worse neurological status. However, increased CD34- and CD34/CXCR4-positive cell induction in patients correlated with better functional/neurological status after the 6-month follow-up.*
Conclusions: *We report that the number and dynamic changes in levels of non-hematopoietic CD34-, CD34/CXCR4-, and CXCR4-positive stem cells tend to correlate with acute stroke patients' neurologic and functional status.*

Key words: *cerebral ischemia, stroke, stem cells, antigens, CD34, CXCR4, prognosis.*

Introduction

Long experience using stem cells in treatment of hematological diseases and knowledge of differentiation of various types of cells into certain tissue progenitors (mesenchymal, muscle, liver, endothelial) [24,29,30] have inspired a number of studies on stem cell treatment in animal models of ischemic cerebral stroke [3,10,14,19,27,28]. However, there have been only a few human studies [2,13,17].

Some studies conducted in the last few years focused on the role of endothelial stem cells in stroke progression and prognosis [4,6,9,18,23], or in diseases linked to stroke pathogenesis [8,16]. Only a few studies have emerged recently that focus on or include non-hematopoietic stem cells [5,22,25], or stem cells void of markers suggesting a more differentiated, specialized character [20]. These cells may become a feasible source for regeneration serving paracrine or structural regeneration of the brain. Some studies suggest an important

Communicating author:
Anna Gójska-Grymajło, Department of Neurology, Medical University of Gdańsk, 7 Dębinki St, 80-212 Gdańsk, Poland, phone +48 583 49 23 00, fax +48 58 349 23 30, e-mail: annagojska@gumed.edu.pl

role of CXCR4 antigen in luring stem cells to ischemic lesions [15,20]. Another important issue affecting stem cells for stroke treatment is their change in number during the first days after stroke occurrence [11,12,20,25]. Apart from the baseline and total number of stem cells, their dynamics may also play a substantial role in the process that occurs in ischemic brain lesions.

In the present study we examined the levels of CD34-, CD34/CXCR4-, and CXCR4-positive stem cells in the peripheral blood of acute ischemic stroke patients and a control group. Additionally, we investigated correlations between the levels and dynamics of these cells with the functional and neurological status of stroke patients.

Material and methods

Study population and blood sampling

The study included 34 patients (17 women and 17 men, mean age 71 years) with acute ischemic stroke hospitalized in the Department of Neurology of the Medical University of Gdańsk, Poland, and 15 control subjects matched for age and sex (8 women and 7 men, mean age 73 years), without a history of previous stroke. Patients were included in the study within the first 24 h after the occurrence of ischemic cerebral stroke (mean 12 h ± 7.36 after symptom onset). Positive qualification for thrombolytic treatment constituted the exclusion criterion for the study. In each patient, ischemic stroke was confirmed by neurological examination and computed tomography. Immediately after inclusion in the study and on days 2, 4, and 6, 3 ml of EDTA-anticoagulated peripheral blood (Becton Dickinson Vacutainer) was collected from each patient. Blood from the control group was sampled once, incidentally. The study was approved by the local ethics committee. All the patients and control subjects provided written, informed consent for involvement in the study.

Flow cytometry

CD34- and CXCR4 (CD184)-positive stem cells were assessed using flow cytometry (Cytomics FC500; Beckman Coulter, CXP System Software). Blood samples (100 µl) were incubated with CD45-FITC (clone J.33), CD184-PE (clone 12G5), and CD34-PC7 (clone 581) (Beckman Coulter) reagents according to the manufacturer's protocol. IOTest 3 Lysing Solution was then used to lyse the erythrocytes in the labeled blood sample. Flow-Count Fluorosphere particles (100 µl) were then added for absolute cell count. Samples were analyzed up to 2 h. CD45-negative cells were analyzed as the population among which CXCR4- and CD34-positive cells were identified. Dead cells, which could nonspecifically bind to reagents, were excluded using 7-ADD Viability Dye (Beckmann Coulter). We evaluated 100,000 events. The number of cells was reported as the percentage of CD45-negative cells. Absolute numbers of evaluated cells were presented as cells/µl.

Neurological and functional assessment

Parallel to blood collection (days 1, 2, 4, 6), on day 9, and 3 and 6 months after the occurrence of stroke, patients were assessed using the National Institute of Health Stroke Scale (NIHSS), Barthel Index (BI), Scandinavian Stroke Scale (SSS), and modified Rankin Scale (mRS). Additionally, patients were classified into 4 subgroups according to the Oxfordshire Community Stroke Project definition: total anterior circulation infarcts (TACI), partial anterior circulation infarcts (PACI), posterior circulation infarcts (POCI), and lacunar infarcts (LACI) [1].

Statistical analysis

The chi-squared test was used to compare cerebrovascular risk factors between ischemic stroke patients and control subjects. The Mann-Whitney U test was used to compare stem cell numbers between the stroke and control groups. The Mann-Whitney U and Spearman rank correlation tests were used to identify correlations between stem cell numbers and neurological/functional scale results and Oxfordshire Community Stroke Project subgroups. Probability values (P) < 0.05 were considered statistically significant. Statistical analyses were performed using STATISTICA data analysis software, version 8.0 (StatSoft, Inc. 2008).

Results

Data concerning stroke patients and control subjects are presented in Table I. On admission, the mean assessment scores among stroke patients were: NIHSS, 10.5 ± 6.3; SSS, 15.5 ± 6; BI, 23.03 ± 26.48; and mRS, 4.42 ± 0.83 points. No significant difference in stroke severity was observed between men and women.

Cell subgroups and baseline levels

We evaluated 3 subgroups of CD45-negative stem cells according to the antigens present on their surface:

Table I. Baseline characteristics of the stroke and control groups

Variables	Stroke	Control	*P*-value
Age, years ± SD	71 ± 12	73 ± 9.9	0.6
Sex, male (%)	17 (50)	7 (46.7)	0.92439
Hypertension (%)	29 (85.3)	8 (53.3)	0.02299
Atrial fibrillation (%)	14 (41.2)	3 (20)	0.13288
paroxysmal	5 (14.7)	0	0.04828
chronic	9 (26.5)	3 (20)	0.46035
Diabetes mellitus (%)			
oral drugs	6 (17.6)	3 (20)	0.56696
insulin-dependent	1 (2.9)	0	0.70833
Coronary artery disease (%)	14 (41.2)	5 (33.3)	0.60145
myocardial infarction (%)	11 (32.4)	1 (6.7)	0.05246
Smoking (%)	13 (38.2)	0	0.00353
Alcohol abuse (%)	8 (23.5)	1 (6.7)	0.15809
Previous stroke (%)	4 (11.7)	0	
6-month mortality (%) [days from stroke, mean ± SD]	11 (33.3) [41.5 ± 39]	–	

Data are presented as mean values. The χ^2 test was used to compare cerebrovascular risk factors between ischemic stroke patients and control subjects.

CD34, CD34/CXCR4, and CXCR4. The number of CD34/CXCR4-positive and CD34-positive cells was very low in both stroke patients and control subjects. The number of CXCR4-positive cells was higher but demonstrated greater variability, with a wide range of observed values.

On days 1, 4 and 6, the percentage of CD34-positive stem cells was significantly lower in the stroke group than the control group (D1: $P = 0.03$, D4: $P = 0.002$, D6: $P = 0.0004$). The number of CD34/CXCR4-positive stem cells was also lower in the stroke group, but the difference was not significant. The percentage of CXCR4-positive cells was similar in both groups, but the absolute number of these cells was higher in stroke patients (significantly higher on day 2, $P = 0.032$) (Table II).

Stem cell numbers, dynamics, and neurological/functional status

CD34-positive cells

On day 1, we observed lower numbers of CD34-positive stem cells in patients with more severe stroke. Patients assigned a NIHSS score < 12 points, denoting "mild stroke", demonstrated a significantly higher percentage of CD34-positive stem cells than patients assigned ≥ 12 points, denoting "severe stroke" (0.27% vs. 0.09%, $P = 0.035$). Similarly, on day 1, patients assigned to the PACI subgroup according to the Oxfordshire Community Stroke Project classification demonstrated higher percentages of CD34-positive stem cells than patients in the TACI subgroup (0.24% vs. 0.09%, $P = 0.04$). The situation reversed over the following days, with more severe stroke correlated with higher numbers of CD34-positive cells. Patients with NIHSS ≥ 12 points on day 4 had significantly higher absolute numbers of CD34-positive stem cells on day 4 than patients with NIHSS < 12 points (1.52 cells/µl vs. 3.0 cells/µl, $P = 0.029$). Patients with < 3 CD34-pos-

Table II. Total cell numbers and percentage of CD34/CXCR4-, CD34-, and CXCR4-positive stem cells on days 1, 2, 4, and 6 in acute stroke patients and control subjects

	Control group	Patients Day 1	*P*-value	Patients Day 2	*P*-value	Patients Day 4	*P*-value	Patients Day 6	*P*-value
CD34/CXCR4	0.73 ± 1.03 [0.28 ± 0.43]	0.69 ± 0.63 [0.08 ± 0.12]	0.65 0.41	0.79 ± 0.72 [0.11 ± 0.15]	0.44 0.65	0.68 ± 0.85 [0.07 ± 0.12]	0.96 0.17	0.96 ± 1.17 [0.069 ± 0.11]	0.64 0.28
CD34	2.8 ± 1.74 [0.57 ± 0.63]	2.36 ± 2.73 [0.19 ± 0.23]	0.07 0.037	2.5 ± 2.38 [0.27 ± 0.3]	0.43 0.063	2.09 ± 1.59 [0.14 ± 0.17]	0.14 0.002	1.71 ± 1.48 0.11 ± 0.15	0.03 0.0004
CXCR4	31.6 ± 30.4 [8.45 ± 10.34]	100 ± 168 [6.57 ± 8.03]	0.15 0.83	104.5 ± 154 [7.21 ± 8.3]	0.032 0.78	118.5 ± 193 [7.94 ± 8.87]	0.07 0.64	110.8 ± 167.9 [8.1 ± 8.97]	0.09 0.819

Upper line: total cell numbers [cells/µl] ± SD.
Lower line, in brackets: percentage [%] of all CD45-negative stem cells ± SD.
P – probability value; comparison between the number of cells in acute stroke patients and control subjects, in whom blood was sampled once, incidentally.

itive cells/μl on day 4 demonstrated a greater increase of BI points (i.e., better improvement) between days 1 and 9 than patients with ≥ 3 stem cells/μl (29.3 vs. 5.0, $P = 0.026$).

Interestingly, we found that more severe stroke correlated with greater increase/dynamics of CD34-positive cells. In patients assigned to the TACI subgroup, we observed a percentage increase in CD34-positive stem cells between days 1 and 6, in contrast to their decrease in PACI-subgroup patients (–0.13% vs. 0.03%, $P = 0.015$). However, the ability to induce higher numbers of cells also appeared to be associated with better neurological outcome over a long-term follow-up. We found that patients who were assessed as having NIHSS scores < 12 points after 6 months demonstrated a greater increase in the total number of CD34-positive cells between days 1 and 6 compared to patients with NIHSS scores ≥ 12 (0.18% vs. –1.5%, $P = 0.046$).

CD34/CXCR4-positive stem cells

Similar but less pronounced correlations with neurological and functional scales were observed in the case of CD34/CXCR4-positive stem cells. We found significant associations between better mRS ($r = –0.38$, $P = 0.024$) and BI results on day 1 ($r = 0.41$, $P = 0.019$) and higher percentages of CD34/CXCR4-positive stem cells on day 2. Dynamic changes in this cell group were also similar to those of CD34-positive cells. The percentage of CD34/CXCR4-positive cells increased over 6 days in patients assigned a NIHSS score ≥ 12 ("severe stroke") on day 1 and decreased in patients initially assigned a NIHSS score < 12 points ("mild stroke") (0.05% vs. –0.05%, $P = 0.022$). Similarly, we observed a tendency for patients in the TACI subgroup to demonstrate a higher increase in percentage of CD34/CXCR4-positive cells between days 1 and 6 compared with PACI patients (0.05% vs. –0.02%, $P = 0.083$).

Again, greater increases in CD34/CXCR4-positive cells correlated with better long-term outcome. Patients assigned BI scores ≥ 95 points on a 6-month follow-up demonstrated a higher percentage increase in total number of CD34/CXCR4-positive cells between days 1 and 4 than patients assigned BI scores < 95 points (0.25% vs. –0.66%, $P = 0.045$). Only this group of stem cells correlated with 6-month mortality: lower total CD34/CXCR4-positive stem cell number on day 1 correlated with higher risk of death during the 6-month follow-up period (alive: 0.86 cells/μl vs. death: 0.36 cells/μl, $P = 0.03$).

CXCR4 stem cells

Similar to CD34- and CD34/CXCR4-positive stem cells, we observed lower levels of CXCR4-positive stem cells in patients with more severe stroke on day 1 based on neurological and functional assessments. However, over the following 6 days the associations of CXCR4-positive cells diverged from those of the other 2 cell groups, in that lower numbers of CXCR4-positive cells continued to be associated with more severe stroke. Patients who demonstrated a lower total CXCR4-positive cell number on day 1 were at higher risk of being categorized with "severe stroke" (NIHSS score ≥ 12 points) on day 2 (42.58 cells/μl vs. 134.09 cells/μl, $P = 0.019$). Patients with > 43 CXCR4-positive cells/μl (median cell number) on day 1 and > 38 cells/μl on day 4 had significantly fewer NIHSS points on day 4 than patients with cell numbers below the abovementioned thresholds (D1: 6.12 vs. 10.5, $P = 0.049$; D4: 6.06 vs. 10.8, $P = 0.017$). Moreover, we found that patients with > 38 CXCR4-positive cells/μl on day 4 demonstrated a significantly greater decrease in points on the mRS scale (i.e., improvement of functionality) from day 1 to day 9 (–1.25 cells/μl vs. –0.33 cells/μl, $P = 0.015$).

With respect to cell dynamics, patients with more mRS points (≥ 3; i.e., poorer global functionality) on day 9 demonstrated a decreased total number of CXCR4-positive stem cells from day 1 to 2 (–4.45 cells/μl vs. 14.5 cells/μl, $P = 0.027$) and from day 1 to 6 (–9.09 cells/μl vs. 31.8 cells/μl, $P = 0.04$) in comparison with patients with mRS < 3 on day 9 (better global functionality). TACI patients demonstrated a greater decrease in total CXCR4-positive stem cell number from days 4 to 6 compared with PACI patients (–0.17% vs. 0.12%, $P = 0.04$). We did not observe any long-term associations for this subgroup of stem cells.

Discussion

The aim of the present study was to observe non-hematopoietic (CD45-negative) stem cells induced in peripheral blood by ischemic processes taking place in the brain (SDF-1 – CXCR4 axis). Accordingly, we observed 3 groups of non-hematopoietic stem cells, CD34-positive, CD34/CXCR4-positive, and CXCR4-positive, in patients with ischemic cerebral stroke. Non-hematopoietic stem cells, in the literature often described as circulating progenitor cells [5,22,25], are a heterogeneous population of cells able to differentiate into various cell types including neurons, astrocytes, and oligo-

dendrocytes as well as endothelial cells [22]. Endothelial cells, in particular, have been the focus of much recent interest [5,16,18,31]. Our chosen group of CXCR4-positive cells also includes very small embryonic-like cells (VSEL), which have the potential to differentiate into 3 germ layers [20]. Thus, this group of stem cells may serve as a source of broad paracrine and structural regeneration for cerebral tissue.

However, we found that the number of CD34/CXCR4- and CD34-positive stem cells was very low in both the stroke and control groups. Results of other studies seem to confirm this finding, although comparison is difficult due to the variability of chosen surface antigens, flow cytometry methods, and definitions of observed stem cell groups. The number of non-hematopoietic stem cells reported in these studies was in the order of a few cells per microliter [20,22,26], with only one study reporting hundreds of cells per microliter [16]. In our study, the CXCR4-positive subgroup was much more numerous than the CD34- and CD34/CXCR4-positive subgroups of cells, in the order of 100 cells/µl. Additionally, the number of CXCR4-positive cells was highly variable, with a wide range of observed values.

Interestingly, we observed non-significantly lower levels of CD34- and CD34/CXCR4-positive stem cells in stroke patients compared to control subjects. A similar correlation for CD34-positive stem cells was reported in the CADASIL study and in a study of chronic stroke patients, in whom atherosclerosis was more advanced [16,22]. This finding may suggest usage of CD34-positive CD45-negative stem cells in the process of atherogenesis and repetitive ischemic brain insults. However, in other studies, levels of CD34-positive stem cells were higher among patients than in the control group [7,20,21]. Notably, however, these studies on CD34-positive stem cells included both CD45-positive and CD45-negative cells, which may suggest a different role of hematopoietic and non-hematopoietic stem cells in the pathogenesis of ischemic cerebral stroke.

Paczkowska *et al.* [20] reported an increased number of CXCR4-positive, CD45-negative, Lin-negative VSEL cells in stroke patients compared to healthy controls. Our study seems to confirm this finding, although we found a statistically significant difference only on day 2. Importantly, however, we observed a much wider range of stem cells; the VSEL cells observed by Paczkowska *et al.* comprise only a small part of our group of cells.

Observations of the dynamic changes in CD34-, CD34/CXCR4-, and CXCR4-positive cells confirmed their differences. CD34-positive cell numbers increased, reached a maximum on day 2, and then decreased. Taguchi *et al.* [25] observed an increase in CD34-positive cells (CD45-negative and -positive) lasting for 7 days, to a level that persisted for 14 days, and then a decrease to baseline levels on day 30.

In the case of percentage values, we observed similar trends in CD34- and CD34/CXCR4-positive stem cells; however, we observed the highest total number of CD34/CXCR4-positive stem cells on day 6. In contrast, Paczkowska *et al.* [20] observed the highest number of CD34/CXCR4-positive cells (CD45-negative and -positive) 24 h after the occurrence of stroke. The highest mean total number of CXCR4-positive stem cells in our study was observed on day 4, similar to the result of Paczkowska *et al.* [20], who reported the highest number of CXCR4-positive, CD45-negative, Lin-negative cells on day 3.

Differences between studies regarding the numbers of stem cells found on individual days may prove that stem cell quantity is the result of very dynamic processes that occur during cerebral ischemia. CD34-positive stem cells released into blood circulation may abruptly localize to ischemic tissues and their bursts can be so short-lived that blood sampling every 24 h may be insufficient to evaluate the global number of released cells [7,20]. We hypothesize that short periods of CD34-positive stem cells present in peripheral blood may underlie their different functions compared to CXCR4-positive cells, which we observed in clearly higher numbers, but with a wide range of values. Our other findings may also serve to confirm this hypothesis. We found that higher numbers of all 3 cell types in the first 24 h after stroke occurrence correlated with patients' better baseline neurological or functional status. This finding is supported by almost all the studies on ischemic stroke patients, irrespective of the type of stem cells observed [5-7,18,20,22,23,26].

Furthermore, we found that the neurological status of ischemic stroke patients correlated not only with initial stem cell numbers but also with subsequent changes in their numbers. High baseline CD34-positive cells, with a small increase (or decrease) in cell number during the observation period (6 days), correlated with better baseline neurological/functional status of stroke patients. Patients with more severe stroke on admission had fewer CD34-positive cells on day 1 and

a greater increase in the following days. A similar pattern of correlations was present in CD34/CXCR4-positive stem cells, although the associations were weaker. However, only the CD34/CXCR4-positive stem cell population demonstrated a negative correlation with mortality, with lower total number of CD34/CXCR4-positive cells on day 1 correlated with increased risk of death during the 6-month follow-up period ($P = 0.03$).

Nevertheless, our results suggest that patients who demonstrated a greater increase in CD34- and CD34/CXCR4-positive stem cells had better neurological/functional status at a long-term follow-up (6 months). Some confirmation of this hypothesis can be found in the study of Dunac *et al.* [7], where points on the NIHSS scale correlated with individual amplitudes of increase in CD34-positive peripheral blood mononuclear cells. Patients classified as "strong inducers" of these cells were characterized by better NIHSS outcome after 1 and 3 months.

In contrast, better baseline neurological and functional status of stroke patients correlated with higher numbers of CXCR4-positive stem cells on day 1 and with their increase or smaller decrease in the following days. Interestingly, however, we did not observe any long-term associations for CXCR4-positive stem cells.

Conclusions

Non-hematopoietic CD34-, CD34/CXCR4-, and CXCR4-positive stem cells are found in the peripheral blood of ischemic stroke patients in very low numbers; however, their levels and dynamics correlate with patients' neurological and functional status. Higher baseline numbers of all 3 stem cell types correlated with better baseline neurological or functional status. Furthermore, greater increases in CD34- and CD34/CXCR4-positive stem cells and smaller increase in CXCR4-positive stem cell numbers following the occurrence of stroke correlated with initially more severe stroke (worse neurological/functional status). However, at long-term follow-up (6 months), patients who showed greater increases in the number of CD34- and CD34/CXCR4-positive cells demonstrated better neurological or functional status.

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