Research article

Evidence for proangiogenic cellular and humoral systemic response in patients with acute onset of spinal cord injury

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Context/objective: Traumatic spinal cord injury (SCI) leads to disruption of local vasculature inducing secondary damage of neural tissue. Circulating endothelial progenitor cells (EPCs) play an important role in post-injury regeneration of vasculature, whereas endothelial cells (ECs) reflect endothelial damage.

Methods: Twenty patients with SCI were assessed during the first 24 hours, at day 3, and day 7 post-injury and compared to 25 healthy subjects. We herein investigated EPC and EC counts by flow cytometry as well as the levels of soluble factors (SDF-1, HGF, VEGF, Ang2, EGF, endoglin, PLGF, FGF-2, ET-1, BDNF, IGF-1) regulating their migration and proangiogenic function. To better characterize peripheral blood (PB) cells, global gene expression profiles of PB-derived cells were determined using genome-wide RNA microarray technology.

Results: We found significantly higher EPC (CD34⁺/CD133⁺/VEGFR2⁺) as well as EC (VEGFR2⁺) count in PB of patients with SCI within 7 days post-injury and the increased HGF, ET-1, Ang2, EGF, and PLGF plasma levels. Global gene expression analysis revealed considerably lower expression of genes associated with both innate and adaptive immune response in PB cells in patients.

Conclusion: Collectively, our findings demonstrate that SCI triggers bone marrow-derived EPC mobilization accompanied by increased circulating EC numbers. Significant changes in both chemoattractive and proangiogenic cytokines plasma levels occurring rapidly after SCI suggest their role in SCI-related regenerative responses to injury. Broadened knowledge concerning the mechanisms governing of human organism response to the SCI might be helpful in developing effective therapeutic strategies.

Keywords: Endothelial cells, Angiogenic factors, Endothelial progenitor cells, Immune response, Spinal cord injury

Spinal cord injury (SCI) is an important cause of permanent loss of neurological function and disability in young people throughout the world. In patients undergoing acute SCI, an initial mechanical tissue damage is followed by secondary degeneration with blood vessel loss resulting in decreased blood flow, hypoxia, and edema of the neural tissue.¹ Blood–spinal cord barrier (BSCB) breakdown and altered permeability facilitate inflammatory cell infiltration and augment neural disruption and demyelination processes.²

Restoration of vascular compartment and prevention of BSCB damage are critical for patients and result in prevention of secondary neural damage to the spinal cord. It has become evident from a vast body of data that rescue of blood vessels results in improved outcome following SCI.^{3,4} Both endothelial progenitor cells (EPCs) and angiogenic support are critical to the endogenous regenerative response to trauma after vasculature damage.⁵ Endothelial progenitors mobilized from bone marrow (BM) are recruited to the site of injury where the regenerative process is coordinated by a number of growth factors.⁶ A plethora of factors influencing this process is wide and includes the specific site

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of injury, infiltration of blood cells, changes in gene expression on intact cells, consequences of trauma.

EPCs play a critical role in angiogenesis and vasculogenesis, thus they may represent a population of cells critical for repair of spinal cord following SCI. Identification of EPCs is typically based on the cell surface expression of the protein or an *ex vivo* cultivation of mononuclear cells or CD34⁺ cells under specific conditions. It is well established that early EPCs are positive for CD34, CD133, and VEGFR2 surface antigens. While differentiation has been shown to be associated with changes in the marker expression profile, including a loss of the CD133 surface marker.^{7,8} In this study, we employ immunological method of EPC identification and characterize both early EPCs (CD34⁺/CD133⁺/VEGFR2⁺) and late EPCs (CD34⁺/VEGFR2⁺). In animal models of acute SCI, it has been found that a number of EPC colonies from circulating peripheral blood (PB) mononuclear cells peaked on day 3 post-injury.⁴ Moreover, BM-derived EPCs recruited into the site of injury markedly increased on day 7 and correlated with neovascularization and astrogliosis.⁴ However, nothing is known about the behavior of EPCs and ECs in patients with SCI. There are two proposed mechanisms of action underlying the beneficial effects of BM-derived EPCs. The first one is associated with idea that stem/progenitor cells (SPCs) exert therapeutic benefits through cell replacement. The second mechanism involves the secretion of trophic factors. The beneficial effect through trophic factor secretion is the growing concept in the field of cell therapy.

The organism's systemic response to stress triggered by acute injury involves secretion of chemoattractive factors to recruit BM-derived SPCs, including EPCs, to the site of injury and proangiogenic factors for vascular protection and restoration. In this study, the effect of SCI on plasma levels of several chemoattractive factors as well as proangiogenic peptides and vascular regulatory factors (vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), stromal cell-derived factor 1 (SDF-1), Angiopoietin 2 (Ang2), epidermal growth factor (EGF), endoglin, placental growth factor (PLGF), basic fibroblast growth factor (FGF-2), endothelin-1 (ET-1), brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1)) has been examined in patients with SCI compared to healthy control subjects. Because of this functional nature of these peptides on a number of cells, and tissues, they may play a significant role in organism regenerative responses to vascular damage. We have therefore examined the plasma concentrations of these factors in the acute phase of SCI in patients. We

wondered if these peptides might change in their levels because of an increased release or perhaps as a direct result of tissue damage.

In this study, we showed the kinetics of BM-derived EPC numbers in PB, kinetics of circulating endothelial cell (EC) counts, and systemic response to trauma after SCI involving angiogenic factors in patients with acute SCI. The aim of this study was also to determine the expression patterns of PB-derived nucleated cells (NCs) in patients with acute SCI. Our data obtained from microarray gene expression profiling of NCs isolated from PB of patients with SCI at different time points, revealed different expression patterns characterizing PB-derived NCs from patients with SCI compared to healthy subjects.

Materials and methods

Subjects

Twenty patients with acute SCI from the Department of Orthopaedics, Traumatology, and Musculoskeletal Oncology, Pomeranian Medical University in Szczecin, and 25 healthy controls were included in this study (Table 1). We enrolled patients with SCI who were admitted within 24 hours of the injury. The

Table 1 Demographic data of the case and control group

Parameters	Study group (N = 20)	Control group (N = 25)
Clinical features		
Mean age	39.1 ± 19.2	35.6 ± 8.4
Range	18–76	20–59
Male sex	95%	100%
Dislocation	55%	–
Compression fracture	65%	–
Flexion	15%	–
Other	35%	–
Fracture level	20%	–
C1–TH1	60%	–
TH2–TH12	35%	–
L1–L3	5%	–
Number of injured segments		
One	30%	–
Two	35%	–
Three	30%	–
High energy mechanism	35%	–
Fracture of other bones	25%	–
Internal organ injuries	15%	–
Intensive care unit (ICU) stay	50%	–
Prednisolone therapy	90%	–
Decompression	80%	–
Hypertension	5%	20%
Smoking	30%	20%
Obesity	0%	0%
Overweight	15%	25%
Alcohol	55%	0%
Laboratory parameter		
Plasma hsCRP, mg/l	14.1 ± 20.3	–
Plasma fibrinogen, mg/dl	315.4 ± 106	–
WBC, g/l	11.25 ± 3.08	6.64 ± 1.64

causes of injury were traffic accidents in eight patients, falls in eight, and others causes in four. The exclusion criteria for the patients with SCI, as well as the control subjects, included current history of acute inflammatory disease, chronic inflammatory disease, neoplastic disease, and renal failure. The study adhered to the tenets of the Declaration of Helsinki, and approval was obtained from the Local Research Ethics Committee. Moreover, written informed consent for the patient's involvement was obtained.

Laboratory measurement

Ethylenediaminetetraacetic acid (EDTA)-anticoagulated PB samples (2×2.7 ml) were drawn within 24 hours after injury, at day 3, and day 7 from patients with SCI and once from healthy controls. The absolute numbers of leukocytes and lymphocytes in PB were determined with an automatic cell counter (Cell-Dyn 3500, Abbott Diagnostics, Santa Clara, CA, USA). The full population of PB NCs was obtained after lysis of red blood cells using $1 \times$ BD Pharm Lyse

Buffer (BD Biosciences Pharmingen, San Diego, CA, USA). Total mRNA was isolated from 2×10^6 of NCs using a commercially available Rneasy Mini Kit (Qiagen GmbH, Hilden, Germany). Thereafter, mRNA was frozen and stored at -80°C . Plasma samples for measuring SDF-1, HGF, VEGF, Ang2, EGF, endoglin, PLGF, bFGF, ET-1, BDNF, and IGF-1 levels were frozen and stored at -80°C . Samples of PB-derived NCs for assessing EPCs and ECs were processed within 12 hours after drawing.

Flow cytometry

Fig. 1A shows the analysis of EPCs and Fig. 2A presents the analysis of ECs. EDTA-anticoagulated PB samples (2×2.7 ml) were drawn within 24 hours after injury, at day 3, and day 7 from patients with SCI and once from healthy controls and processed within 12 hours. In samples of whole blood, erythrocytes were lysed using BD lysing buffer (BD Biosciences, San Jose, CA, USA) at room temperature for 15 minutes and subsequently washed twice in phosphate-buffered saline

Figure 1 (A) The EPC analysis strategy. Analysis was based on the immunophenotype (CD34⁺/CD133⁺/VEGFR2⁺) of the early EPC and (CD34⁺/VEGFR2⁺) of late EPC. Representative dot plots showing lymphocyte and monocyte gating on the side scatter (SSC) and forward scatter (FSC) (gate P1). Events contained by gate P2 were characterized by the presence of CD34 marker. Cells positive for the CD34 marker were further divided based on the expression of CD133 and VEGFR2 markers. Therefore, cells enclosed in quadrant 2 (Q2) are characterized by the concomitant presence of CD34, CD133, and VEGFR2 antigens and represent early EPCs (CD34⁺/CD133⁺/VEGFR2⁺ cells). While cells enclosed in quadrant 4 (Q4) are positive for VEGFR2 marker and negative for CD133 marker and represent late EPCs (CD34⁺/VEGFR2⁺ cells). (B) Bar graphs showing the number of circulating early EPCs (CD34⁺/CD133⁺/VEGFR2⁺ cells) in the PB of patients with SCI at 24 hours, at day 3, and day 7 post-injury and the control group. (C) Bar graphs showing the number of circulating late EPCs (CD34⁺/VEGFR2⁺) in the PB of patients with SCI at 24 hours, at day 3, and day 7 post-injury and the control group. Data are presented as median, quartiles, interquartile range, minimum, and maximum. *P < 0.05 vs. the control group.

Figure 2 (A) The EC analysis strategy. Representative dot-plots showing lymphocyte and monocyte gating on the side scatter (SSC) and forward scatter (FSC) (gate P1). Events contained by gate P2 were characterized by the presence of VEGFR2 marker. VEGFR2⁺ ECs are enclosed in P2 region. (B) Bar graphs showing the number of circulating ECs (VEGFR2⁺) in the PB of patients with SCI at 24 hours, at day 3, and day 7 post-injury and the control group. Data are presented as median, quartiles, interquartile range, minimum, and maximum. *P < 0.05 vs. the control group. Dot P < 0.05 vs. SCI 24 h. (C) Representative immunofluorescent microscopic images documenting the expression of ET-1 in circulating ECs. PB cells were stained with anti-VEGFR2-PE antibody and subsequently VEGFR2⁺ cells were isolated by Anti-PE MicroBeads. Isolated VEGFR2⁺ cells were stained anti-ET-1. Nuclei were visualized by DAPI staining. Pseudo-coloring was assigned to each stain as follows: anti-VEGFR2 is red, anti-ET-1 is green, and nuclei are blue. All images were captured using the Pathway Bioimager System (BD Biosciences). Representative data are shown. (D) Bar graphs showing the ET-1 plasma levels of patients with SCI at 24 hours, at day 3, and day 7 post-injury and the control group. (E) Bar graphs showing ET-1 mRNA expression levels in PB NCs of patients with SCI at 24 hours, day 3, and day 7 post-injury and the control group. Relative of mRNA expression values are normalized against BMG expression values. Data are presented as median, quartiles, interquartile range, minimum, and maximum. *P < 0.05 vs. the control group. Dot P < 0.05 vs. SCI 24 h.

(PBS), and single-cell suspension was obtained. 1×10^6 cells were stained with fluorescein isothiocyanate-conjugated anti-CD34 (BD Biosciences, Pharmingen, San Jose, CA, USA; clone 581), allophycocyanin-conjugated anti-CD133 (Miltenyi Biotec, Auburn, CA, USA, clone AC133), and phycoerythrin (PE)-conjugated anti-VEGFR2 (R&D Systems Europe, Ltd, Abingdon, UK) mouse monoclonal antibodies. Appropriate isotype antibodies were used as controls (BD Biosciences, Pharmingen, San Jose, CA, USA; Myltenyi Biotec, Auburn, CA, USA). Staining was carried out on ice for 30 minutes. Cells were washed in PBS and analyzed by LSRII (BD Biosciences) using BD FACSDiva software. At least 2×10^5 events were acquired to determine a percentage of specific positive cells within PB NCs. The populations of early $CD34^+/CD133^+/VEGFR2^+$ EPCs, late $CD34^+/VEGFR2^+$ EPCs (Fig. 1A), and circulating ECs ($VEGFR2^+$) (Fig. 2A) were analyzed. The number of EPCs and ECs per 1 μ l of PB was calculated on the basis of the percentage content of these cells detected by flow cytometry multiplied by the absolute number of white blood cells (WBCs) per 1 μ l of PB/ 100^9 .

Quantitative real-time polymerase chain reaction analysis of gene expression

Quantitative analysis of mRNA expression of ET-1 and BDNF was performed in a two-step reverse-transcription polymerase chain reaction (PCR). Total mRNA was isolated from PB-derived NCs using the Rneasy Mini Kit (Qiagen GmbH). Subsequently, mRNA was reverse-transcribed using the First Strand cDNA Synthesis Kit (Fermentas International Inc., Burlington, ON, Canada).

Quantitative real-time PCR (qRT-PCR) was performed using a CFX96 Real-Time PCR Detection System (Bio-rad, Philadelphia, PA, USA). Beta-2 microglobulin (BMG) was selected as a reference gene. A 25 μ l reaction mixture contained 12.5 μ l of iQ Sybr Green Supermix reagent (Bio-rad), 10 ng of cDNA template, and one pair of the primers: 5'-GTCCCTGATGGATAAAGAGTGTG-3' (forward) and 5'-TCACGGTCTGTTGCCTTTGT-3' (reverse) for ET-1, 5'-GATGCTCAGTAGTCAAGTGCC-3' (forward) and 5'-GCCGTTACCCACTACTAATAC-3' (reverse) for BDNF, 5'-AATGCGGCATCTTCAAACCT-3' (forward) and 5'-TGACTTTGTCACAGCCCAAGATA-3' (reverse) for BMG. The real-time cycling conditions were: 1 cycle at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, 60°C for 1 minutes, and 72°C for 15 seconds. All gene expression analyses were done in duplicate.

Calculations were performed by $\Delta\Delta C_t$ relative quantification method. The thresholds were set manually to compare data between runs and C_t values were extracted. All C_t values for each sample were normalized to the value obtained for BMG, the endogenous control gene. Fold change between groups was calculated from the means of the logarithmic expression values.

Enzyme-linked immunosorbent assay

The plasma concentrations of ET-1, VEGF, SDF-1, HGF, BDNF, and IGF-1 were measured by use of the commercially available, high-sensitivity enzyme-linked immunosorbent assay Quantikine kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Absorbance was read at 450 nm with an ELx 808IU automated Microplate Reader (Biotek Instruments Inc., Bad Friedrichshall, Germany). The results were analyzed using log log quadratic or 4PL algorithm curve fit.

Multiplex immunoassay/quantitative assay

Ang2, EGF, endoglin, PLGF, and FGF-2 were quantified simultaneously using the commercial Human Angiogenesis/Growth Factor kit (Merck Millipore, Billerica, MA, USA) on the Luminex analyzer (Luminex Corporation, Austin, TX, USA). The serum samples were diluted 3 $\times$ in sample diluent provided with the kit. Hundred microliters of standard, control, and diluted samples were added to the plate together with 50 μ l of the antibody capture bead mixture, and the plate was incubated for 18 hours (2–8°C). Next day, washing was carried out three times using assay buffer and vacuum filtration. Fifty microliters of diluted biotin-coupled antibody cocktail were added to each well and the plate was incubated for 1 hour followed by washing. Fifty microliters of diluted streptavidin conjugated with PE were added to the plate and incubated for 30 minutes in dark. Finally, after washing, 100 μ l Sheath Fluid were added and the plate was incubated for 5 minutes, after which the analysis was carried out on the Luminex analyzer. Ang2, EGF, Endoglin, FGF-2, and PLGF concentrations were determined from five different standard curves showing median fluorescence intensity vs. protein concentrations.

Immunofluorescent analysis

$VEGFR2^+$ cells were isolated from PB using an Anti-PE MicroBeads Kit (Miltenyi Biotec) according to the manufacturer's instructions. Cells were stained with mouse monoclonal antibody anti-VEGFR2 conjugated with PE and subsequently subjected to

immunomagnetic isolation. Isolated VEGFR2⁺ cells were then immunofluorescence (IF) stained for ET-1. Briefly, cells were fixed in 3.7% paraformaldehyde for 20 minutes, permeabilized using 0.1% Triton X-100 for 10 minutes, washed in PBS, and subsequently stained with primary antibody rabbit polyclonal anti-ET-1 (GeneTex, Inc., Irvine, CA, USA) for 1 hour at room temperature. Next, the cells were incubated with the secondary antibody chicken anti-rabbit AlexaFluor 488 (Life Technologies, Paisley, UK) for 1 hour at room temperature in the dark. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) (BD Pharmingen, Franklin Lakes, NJ, USA) for 5 minutes in the dark. Fluorescent images were captured using the Pathway Bioimager System (BD Biosciences, Rockville, MD, USA).

RNA isolation and Affymetrix GeneChip Microarray and data analysis

Total RNA was isolated from PB NCs derived from patients with SCI and healthy control using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA isolates from three patients with SCI at three time points (24 hours, 3 days, and 7 days after injury) and from three healthy controls were pooled to generate four samples (control, 24 hours, day 3, and day 7) for subsequent experimental procedures. Sense-strand cDNA generated from total RNA using an Ambion WT Expression Kit (Life Technologies) was fragmented and labeled using the GeneChip[®] WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA) and hybridized onto an Affymetrix WT Array Strip. Hybridization as well as subsequent fluidics and scanning steps were performed using an Affymetrix GeneAtlas[™] system (Affymetrix). Microarray data are available in the ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress>) under accession number E-MTAB-2155. The differences in expression of the chosen genes and Gene Ontology (GO) terms were analyzed in the R programming environment using Bioconductor packages.

Statistics

Non-parametric tests were used because most distributions of circulating cells, plasma concentrations, and gene expressions values were significantly different from normal distribution (Shapiro–Wilk test, $P < 0.05$). Significance of changes in the cell counts and SDF-1, HGF, VEGF, Ang2, EGF, endoglin, PLGF, BDNF, and ET-1 plasma concentration as well as gene expression of ET-1 and BDNF measured at 24 hours, day 3, and day 7 post-injury was assessed with

Wilcoxon signed-rank test. The Mann–Whitney test was used to compare the cell counts and cytokine plasma concentrations between the study and control groups and between two subgroups of patients with different clinical characteristics. $P < 0.05$ was considered statistically significant.

Results

The demographic and clinical characteristics of the enrolled patients with SCI including the mechanism of injury and the healthy control group have been summarized in Table 1.

Both early CD34⁺/CD133⁺/VEGFR2⁺ and late CD34⁺/VEGFR2⁺ EPCs are mobilized from BM in response to acute SCI

To investigate whether SCI triggers an increase in EPCs circulating in the PB, we used a flow cytometry to assess the numbers of circulating early CD34⁺/CD133⁺/VEGFR2⁺ and late CD34⁺/VEGFR2⁺ EPCs in patients at first 24 hours of SCI, at day 3, and day 7 post-injury. We found that the numbers of circulating early EPCs were higher at days 3 and 7 after injury in patients with SCI than in healthy subjects (Fig. 1B). While circulating late EPC counts were higher at day 3 post-injury compared to the control group (Fig. 1C). Together, these findings demonstrate that SCI triggers a spontaneous mobilization of both early and late EPCs, which suggests an intrinsic mechanism of ameliorating tissue damage that involves circulating SPCs.

The number of VEGF-R2⁺ ECs is increased in the PB of patients with SCI

Next, we focused on evaluating the number of circulating VEGFR2⁺ ECs in PB of patients with SCI. Abundant evidence indicates that a number of circulating ECs reflects endothelial damage.¹⁰ Accordingly, we evaluated the circulating EC counts and the relationship between ECs and circulating EPCs during vasculature and BSSB injury in the course of acute SCI. Thus, we simultaneously evaluated the number of VEGFR2⁺ ECs in the PB of patients with SCI 24 hours after injury as well as at days 3 and 7 post-injury (Fig. 2B). We found a significantly higher percentage of circulating ECs in patients with SCI at days 3 and 7 post-injury compared with the healthy subjects. These levels peaked at day 3 with the moderate decrease at day 7 post-injury. The pattern of circulating EC kinetics after SCI largely mimics the pattern observed in EPCs.

Table 2 Plasma concentrations of SDF-1, HGF, and IGF-1 in patients with SCI at different time points (24 hours, day 3, and day 7) and in healthy subjects

Cytokine	Control group	SCI 24 hours	SCI day 3	SCI day 7
SDF-1 (pg/ml)	2228 (416)	1519* (663)	1539* (419)	2039 [^] (631)
HGF (pg/ml)	593 (139)	1426* (1028)	1635* (1151)	1348* (987)
IGF-1 (ng/ml)	93.6 (21.0)	74.5 (56.2)	72.3* [^] (59.8)	104.9 [^] (76.8)

Data are expressed as median (interquartile range).

*P < 0.001 vs. control.

[^]P < 0.05 vs. 24 hours.

The plasma levels of chemoattractive factors are changed in patients with SCI

We sought to determine the plasma concentrations of SDF-1 and HGF, which represent the main chemoattractive factors contributing to trafficking, migration, and homing of BM-derived SPCs, including EPCs, and subsequent vascular repair or angiogenesis at the vascular injury site¹¹ (Table 2). HGF plasma level was found to be markedly higher in patients with SCI at each of time points (24 hours, day 3, and day 7) compared to healthy subjects with peak concentration at day 3 post-injury (median: 1635 vs. 593 pg/ml; P < 0.0001). This may suggest an indirect interaction between HGF and circulating EPCs. Surprisingly, we found a significantly lower SDF-1 concentration in patients with SCI during the first 3 days after acute SCI compared to healthy subjects (median: 1519 and 1539 vs. 2228 pg/ml; P < 0.0001; for 24 hours and day 3, respectively). The SDF-1 levels returned to those found in healthy subjects at day 7 post-SCI. We observed a significant increase in SDF-1 levels at day 7 compared to 24 hours and day 3 post-injury. This may suggest that SDF-1 does not play a supportive role in organism response to SCI. While IGF-1 largely mediates the actions of growth hormone, it has many complex functions. HGF-1 has been found as a chemoattractive factor for mesenchymal stem cells.¹² We observed significantly lower plasma concentration of IGF-1 only at day 3 after SCI. However, the plasma

levels of this factor differ significantly within 24 hours, at day 3, and day 7 in patients with SCI.

Together, these findings indicate that increased levels of HGF could contribute to the triggering of pathophysiological mobilization of EPCs from BM into circulation during SCI as well as circulating EPC recruitment to a site of injury.

The plasma levels of proangiogenic cytokines are changed in patients with SCI

Next, we concentrated on evaluating the plasma level changes of VEGF, Ang2, EGF, Endoglin, PLGF, and FGF-2 in PB, which represent the proangiogenic cytokines (Table 3). VEGF is a potent mediator of angiogenesis and vascular permeability. Following experimental SCI in rats, VEGF mRNA expression at the site of injury has been found to be constantly down-regulated within 4 weeks.^{2,13} In our study, the VEGF plasma levels were not significantly higher in patients with SCI compared to the healthy subjects. The result of the present study show only the tendency to elevation of VEGF plasma levels following acute SCI in patients at day 7 post-injury compared to healthy subjects (median: 104 vs. 48 pg/ml, P = 0.86). The next angiogenic cytokine Ang2 was found to be markedly elevated in patients with SCI within the first 24 hours since the injury onset (median: 559 vs. 427 pg/ml; P < 0.01) and subsequently gradually decreased in patients with SCI compared to healthy subjects. Moreover, we found

Table 3 Plasma concentrations of VEGF, Ang2, EGF, Endoglin in patients with SCI at different time points (24 hours, day 3, and day 7) and in healthy subjects

Cytokine	Control group	SCI 24 hours	SCI day 3	SCI day 7
VEGF (pg/ml)	48 (48)	82 (144)	89 (84)	104 (192)
Ang2 (pg/ml)	427 (166)	559* (416)	570 (388)	370 (773)
EGF (pg/ml)	6.3 (8.0)	16.8* (34.7)	2.4 [^] (4.3)	1.8 [^] (6.4)
Endoglin (pg/ml)	241 (335)	206 (326)	233 (132)	127* (126)
PLGF (pg/ml)	0.7 (0.4)	1.4* (2.4)	0.9 (0.8)	1.9* (1.7)

Data are expressed as median (interquartile range).

*P < 0.05 vs. control.

[^]P < 0.05 vs. 24 hours.

that EGF concentration was also significantly higher at 24 hours post-injury in patients with SCI compared to the control group (median: 16.8 vs. 6.3 pg/ml; $P < 0.05$). Endoglin is an accessory TGF- β type III receptor that is expressed on ECs. We found no significant difference in endoglin concentrations measured during the first 3 days of SCI compared to healthy subjects. However, the endoglin levels were significantly lower in patients with SCI at day 7 post-injury compared to the healthy control group (median: 127 vs. 241 pg/ml; $P < 0.05$). PLGF plasma levels were found to be elevated at 24 hours and at day 7 post-injury (median: 1.4 and 1.9 vs. 0.7 pg/ml; $P < 0.05$; for 24 hours and day 7, respectively).

FGF-2 factor was detectable in $<50\%$ of the samples. Analysis for this peptide has not been calculated because of the very small percentage of samples above the detection limit and is not included in the table.

ET-1 plasma levels are considerably elevated without increased intracellular ET-1 expression in PB cells in patients with SCI

ET-1 is a potent vasoconstrictor peptide secreted by ECs in response to catecholamines, and other agonists. It has been shown to induce a direct angiogenic effect on ECs.^{14,15} Thus, we investigated whether ET-1 levels may play a role in the pathophysiology of acute SCI. We found that ET-1 plasma levels were significantly higher in patients with SCI at each of the time points than in controls (Fig. 2D). Moreover, we observed dynamic changes in ET-1 plasma levels in patients with SCI. A peak level was detected at 24 hours with the significant decrease at days 3 and 7 post-injury. Next, we investigated whether ET-1 is produced by circulating ECs in PB of patients. To visualize the circulating ECs with high ET-1 content in PB-derived NCs, an IF analysis was performed. We found that immunomagnetically isolated PB-derived ECs expressed ET-1, as shown by IF staining (Fig. 2C). Since we observed increased EPC and EC counts in blood stream at days 3 and 7 post-injury in patients with SCI, to make our analysis more insightful, we extracted mRNA from circulating PB-derived NCs and examined ET-1 gene

expression levels by qRT-PCR. As shown in Fig. 2E, the intracellular mRNA expression for ET-1 in NCs was not elevated in patients with SCI compared to healthy subjects. Taken together, we observed the elevated ET-1 plasma levels with peak at 24 hours of SCI without the significant increase in ET-1 gene expression in PB-derived NCs. However, we visualized ET-1 expression on protein level in circulating ECs. These findings strongly suggest that ET-1 is involved in the systemic response mechanism of SCI.

BDNF plasma levels and intracellular expression in PB-derived NCs in patients with acute SCI

BDNF plays important role in developing and functioning of central nervous system (CNS). Furthermore, it exerts proangiogenic actions.¹⁶ BDNF is also present in PB and can derive from different sources including platelets, monocytes, and the brain.¹⁷ According to our recent study, various human SPCs are also the source of neurotrophins, including BDNF.¹⁸ Here, we observed that BDNF levels did not differ from those observed in the healthy control group within 3 days post-injury (Table 3). Moreover, BDNF plasma levels were significantly decreased on day 7 after SCI compared to healthy subjects (median: 3285 vs. 7020 pg/ml; $P < 0.05$). We observed the dynamic decrease in BDNF plasma concentrations on day 7 compared to 24 hours post-injury in patients. We investigated also the mRNA expression of BDNF in NCs of patients with SCI. We did not observe any differences of intracellular mRNA expression of BDNF between patients with SCI and healthy subjects (Table 4).

Comparisons of circulating cells and plasma cytokine levels between subgroups of patients with different clinical characteristics

We compared the circulating populations of EPCs and cytokine plasma levels between the two subgroups of patients according to the occurrence of concomitant other bones fractures or internal organ injury. We did not find any significant differences between the subgroups of patients of different clinical characteristics.

Table 4 BDNF plasma concentration and expression in PB-derived NCs in patients with SCI at different time points (24 hours, day 3, and day 7) and in healthy subjects

Neurotrophin	Control group	SCI 24 hours	SCI day 3	SCI day 7
BDNF plasma level (pg/ml)	7020 (6366)	4919 (4823)	4674 (4553)	3285* [^] (4002)
BDNF intracellular (relative expression)	0.574 (1.394)	0.901 (0.866)	0.655 (1.08)	0.708 (0.958)

Data are expressed as median (interquartile range).

* $P < 0.05$ vs. control.

[^] $P < 0.05$ vs. 24 hours.

Table 5 The 10 down-regulated genes associated with the immune response with the largest change in expression for the NCs from patients with SCI at 24 hours post-injury compared to the NCs from healthy subjects

Gene symbol	log ₂ (FC)	Gene name	Entrez GeneID
CD36	-3.801	CD36 molecule (thrombospondin receptor)	948
CD86	-3.76	CD86 molecule	942
HLA-DPA1	-3.65	Major histocompatibility complex, class II, DP alpha 1	3113
IFI44L	-3.514	Interferon-induced protein 44-like	10964
TRAT1	-3.505	T-cell receptor associated transmembrane adaptor 1	50852
CD4	-3.502	CD4 molecule	920
HSP90AB1	-3.412	Heat shock protein 90 kDa alpha (cytosolic), class B member 1	3326
HLA-DRB5	-3.399	Major histocompatibility complex, class II, DR beta 5	3127
MS4A1	-3.339	Membrane-spanning 4-domains, subfamily A, member 1	931
HLA-DMA	-3.242	Major histocompatibility complex, class II, DM alpha	3108

Whole genome microarray analysis of NCs reveals large-scale alterations in gene expression between patients with SCI and healthy subjects

In our continuing efforts to characterize the constitutional response involving PB EPCs to SCI, we analyzed the global gene expression pattern in the NCs at the 24 hours, day 3, and day 7 post-injury. Microarray comparisons of control vs. NCs at the 24 hours post-injury revealed that 369 genes were at least 2-fold down-regulated. In contrast, for patients with SCI at day 3 post-injury, the respective number was 49 and for patients at day 7 post-injury was 34.

Next, all of the differentially expressed genes were classified according to the GO classification of biological processes. Given the systemic response to SCI involving angiogenic factors and circulating EPCs and ECs, we were interested in biological processes up- or down-regulated associated with angiogenesis, vasculogenesis, and endothelium. Unfortunately, we found no specifically represented pathways involved in these processes. However, functional analysis using GO revealed that a number of pathways were specifically represented in the analyzed PB-derived NC populations. Comparing the bioinformatics analysis of the complex gene dataset in PB-derived NCs of patients with SCI to that

of NCs from healthy subjects identified that genes involved in the regulation of immune system process, adaptive immune response, activation of immune response, leukocyte-mediated immunity, lymphocyte-mediated immunity, defense response, humoral immune response, cellular defense response, T-cell activation, B-cell activation, innate immune response, leukocyte activation, leukocyte migration, defense response to virus, cellular response to interferon-gamma, cellular response to type I interferon, and regulation of immune effector process were among the most down-regulated. The top 10 down-regulated genes within these GO pathways with the largest change in expression are presented in Table 5 for the 24 hours, in Table 6 for day 3, and in Table 7 for day 7 post-injury in patients with SCI. A summary of the selected distribution of genes of interest according to the GO classification of biological processes is shown in Fig. 3.

Taken together, our analysis of the global gene expression changes revealed that a large number of genes are expressed in different patterns within the NCs in patients with SCI compared to the healthy control group. First, the decreased expression of genes associated with regulation of immune system and immune response was observed in NCs of patients

Table 6 The 10 down-regulated genes associated with the immune response with the largest change in expression for the NCs from patients with SCI on day 3 post-injury compared to the NCs from healthy subjects

Gene symbol	log ₂ (FC)	Gene name	Entrez GeneID
RSAD2	-4.213	Radical S-adenosyl methionine domain containing 2	91543
IFI44L	-4.149	Interferon-induced protein 44-like	10964
IFIT1	-3.877	Interferon-induced protein with tetratricopeptide repeats 1	3434
SH2D1B	-2.993	SH2 domain containing 1B	117157
CD160	-2.863	CD160 molecule	11126
MX1	-2.78	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	4599
IFI6	-2.659	Interferon, alpha-inducible protein 6	2537
FCER1A	-2.464	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	2205
OAS3	-2.455	2'-5'-oligoadenylate synthetase 3, 100 kDa	4940
HERC5	-2.335	HECT and RLD domain containing E3 ubiquitin protein ligase 5	51191

Table 7 The 10 down-regulated genes associated with the immune response with the largest change in expression for the NCs from patients with SCI on day 7 post-injury compared to the NCs from healthy subjects

Gene symbol	log ₂ (FC)	Gene name	Entrez GeneID
RSAD2	-4.122	Radical S-adenosyl methionine domain containing 2	91543
IFI44L	-3.967	Interferon-induced protein 44-like	10964
IFIT1	-3.605	Interferon-induced protein with tetratricopeptide repeats 1	3434
SH2D1B	-2.986	SH2 domain containing 1B	117157
MX1	-2.536	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	4599
IFI6	-2.535	Interferon, alpha-inducible protein 6	2537
HERC5	-2.522	HECT and RLD domain containing E3 ubiquitin protein ligase 5	51191
CD160	-2.457	CD160 molecule	11126
OAS3	-2.219	2'-5'-oligoadenylate synthetase 3, 100 kDa	4940
THBS1	-2.195	thrombospondin 1	7057

with SCI compared to healthy subjects. Second, expression of genes associated with angiogenesis, endothelium, and vasculature system do not differ in NCs between patients with SCI and healthy control.

Figure 3 Global gene expression changes in human PB-derived NCs from patients with SCI at 24 hours, day 3, and day 7 post-injury and the control group. The heat map represents the expression levels of overexpressed genes (fold change >2). Individual genes are assigned according to the GO classification of specific biological processes listed on the left side of the graph. Each column comprises a set of horizontal lines, each representing a single gene. Levels of gene expression are indicated on a color scale, with yellow corresponding to the highest level of expression and blue corresponding to the lowest level. The range of expression rate of the analyzed genes is shown below the graph. The diagram shows large-scale alterations in gene expression between PB-derived NCs from patients with acute SCI and the control group.

Discussion

Primary injury of spinal cord is considered as initial mechanical injury to the spinal cord that results in the disruption of local neural and vascular structures, while secondary injury is progressive cell damage that spreads on intact, neighboring tissue. It has been postulated that one of the most important factors influencing subsequent post-injury neuronal degeneration is local vascular damage of microcirculation at the site of injury. It has been documented that ECs and blood vessels showed degenerative changes within 30 minutes after SCI and were lost during the first 3 days, causing significant hemorrhage and disruption of vascular autoregulation being an indirect contributor to neuronal damage.¹⁹ Targeting the restoration of vascular compartment post-SCI is important component of therapeutic approaches, especially as vascular signals regulate endogenous neural stem cells. This phenomenon is well known and extends beyond the CNS.

Circulating EPCs play a critical role in angiogenesis and vasculogenesis, thus they may represent a population of cells critical for repair of spinal cord following SCI. In our study, we demonstrated for the first time the elevated number of EPCs in PB triggered by acute SCI in patients. This observation is in line with recent animal studies that report experimental spinal injury to result in the increased number of EPC colonies from circulating mononuclear cells that peaked at day 3 post-injury.⁴ Moreover, in this model, BM-derived EPCs recruited into the site of injury markedly increased at day 7 and positively correlated with neovascularization and astriogliosis.⁴ However, a comparison of the available data regarding the concentration of circulating EPCs is complicated by the heterogeneity of the applied methods. Mobilization of BM-derived EPCs seems to be an important component of organism response to the injury of vasculature in CNS. Accordingly, our recent study has shown the increased percentage of circulating EPCs in patients undergoing ischemic or hemorrhagic

stroke.²⁰ A novel approach to target the regeneration of vascular compartment post-SCI is cellular therapy. Sasaki *et al.*²¹ has administered G-CSF (granulocyte colony-stimulating factor) mobilized CD133⁺ cells to the spinally injured rat and observed improvement in locomotor recovery.

It has become evident from a vast body of literature in rodent models of SCI that the regulation and mediation of repairing processes after SCI are extremely complex. They involve various progenitor cells and the participation of a plethora of neurogenic, angiogenic, anti-apoptotic, and other signals on the cellular as well as systemic level. A number of chemoattractive and growth factors is considered to play a major role in egress of BM-derived EPCs into circulation and recruitment to the site of injury. In a number of tissue damage, the level of SDF-1 has been found elevated and its role in spontaneous mobilization of SPCs has been appreciated. It has been hypothesized that secretion of SDF-1 by ischemic tissue following injury may induce an egress of EPCs from the BM and recruitment through the reversal of the BM SDF-1 gradient. CXCR4, one of two unique receptors of SDF-1, is highly expressed on EPCs. Our previous study also demonstrated that SDF-1 concentration gradually increases in acute phase of ischemic as well as hemorrhagic stroke in patients.²⁰ In the current study, we demonstrate a low concentration of plasma SDF-1 during the first days post-injury with a return to the level that was observed in healthy subjects on day 7 after SCI. Hence, SDF-1 did not seem to contribute in EPCs egress from the BM in patients with SCI. In contrast, at the lesion site in SCI a peak in SDF-1 expression has been found at 7 days post-injury and it correlated positively with the recruitment of stem cells, which were injected into the blood stream.²² Together, these observations indicate an important role of SDF-1 in SPC homing to the site of tissue spinal cord damage but not in the egress of these cells from the BM in SCI. Differences in CNS injury characteristics in patients with stroke and SCI probably result in diverse organism response. Similarly, we observed the lower IGF-1 plasma concentration only at day 3 in patients with SCI. Although, in the analysis of IGF-1 plasma level changes we found significant differences between certain time points. This suggests that the trauma associated with particular injury influences a multiple specific mechanisms important to restore homeostasis and activates reparative response.

Another important angiogenic and chemoattractive factor that we hypothesized could play a role in mobilization of EPCs from the BM to the circulation was

VEGF. VEGF is a potent mediator of angiogenesis and vascular permeability. VEGF binding to its receptor VEGFR2 mediates cell proliferation, migration, and survival via the PI3K-AKT kinase-nuclear factor κ B or mitogen-activated protein kinase p42/44 (MAPK p42/44) signal transduction pathways. VEGF can also stimulate the migration and proliferation of neurons.³ These effects of VEGF suggest a role in neural protection and potential therapeutic applications. Following experimental SCI in rats VEGF mRNA expression at the site of injury has been found to decrease.^{2,13} To address the issue of beneficial effect of VEGF treatment, Herrera *et al.*³ have shown that combined treatment with Angiopoietin-1 via intraparenchymal infection with adenovirus vectors results in increased expression of both proteins in the chronic phase of injury, contributing to reduced lesion volume and improved BSCB integrity. The result of our study showed no significant elevation of VEGF plasma levels following acute SCI in patients. These data indicate that VEGF did not contribute in systemic response to acute spinal cord damage in patients with SCI.

HGF acting via its c-met receptor is a known angiogenic pathway. HGF regulates proliferation and migration of human umbilical vein endothelial cells by VEGF induction.²³ HGF is also involved in BM-derived EPC mobilization process.²⁴ In spinal injury model, constantly elevated HGF expression starting at day 3 post-SCI was observed at the site of injury in rats.¹³ It has been documented that spinal cord intraparenchymal pre-treatment with HGF enhances locomotor recovery and decreased lesion volume with significantly diminished apoptosis of oligodendrocytes and neurons and increased new microvessel formation.²⁵ Moreover, it has been shown that recombinant HGF reduced the disruption of the blood-brain barrier, which is involved in protection against the apoptotic death of cerebral ECs at an early stage after cerebral ischemia.²⁶ In this study, we observed that HGF plasma levels were markedly elevated at each of the time points following SCI compared to healthy volunteers. We speculated that the increased HGF plasma levels immediately after acute SCI were related to degradation of neural tissue of the spinal cord as well as adjacent anatomical structures and reflect the extent of tissue damage in patients with SCI. Taking into consideration a lack of elevation of VEGF and SDF-1 plasma levels in patients with SCI, we do not rule out the possibility that an increased secretion of HGF may promote egress of BM-derived EPCs into circulation. Thus, sustained increased HGF concentration could play a role in vascular reparative processes through regeneration of ECs, promoting

angiogenesis, and inhibition of apoptosis in the injured spinal cord. However, further studies estimating the HGF concentration in cerebrospinal fluid in humans are needed to assess precisely an influence of HGF on local regenerative processes at the place of injury.

Interestingly, in this study, we observed an increase of other proangiogenic cytokines that could play an important role in vasculature protection and regeneration. Proangiogenic cytokine Ang2 is secreted by ECs and interferes with the activation of the Tie2 receptor.²⁷ The first description of the endogenous expression of angiogenic proteins such as Angiopoietin-1, Ang2, and angiogenin in an acute SCI setting have demonstrated up-regulated mRNA and protein levels of Ang2 up to 10 weeks after SCI.^{5,28} Ang2 was localized rather in astrocytes and NG2⁺ oligodendrocyte precursor cells after SCI than in ECs and high levels of Ang2 correlated with enhanced locomotor recovery, suggesting a beneficial role of Ang2 in SCI.^{13,28} Ang2 is expressed by ECs and perivascular smooth muscles at sites of active angiogenesis during the vessel destabilization process.^{5,29} In our study, we observed an elevated plasma Ang2 levels immediately after injury, at 24 hours, and day 3 post-injury in patients with SCI indicating its contribution in organism response to vasculature damage. Our findings are in line with the results previously reported by Ng *et al.* Albeit, they have evaluated Ang2 concentration in human CSF and serum in patients with SCI. They have observed that Ang2 levels in CSF patients with acute SCI are increased from 36 hours post-injury to 5 days.⁵ The maximal difference in serum levels between patients and the control group has been observed at 60 hours post-injury. In addition, in abovementioned study, no significant correlations have been found between Ang2 values and the neurological outcome at 6 or 12 months post-injury.⁵

EGF is a potent angiogenic peptide. Kang and co-workers³⁰ have reported increased levels of EGF in the region of the injury in animal model of the SCI. It has been also demonstrated that EGF stimulated a proliferation of spinal cord progenitor cells via MAPK signaling pathways.³¹ In our study, we observed significantly elevated plasma levels of EGF during the first 24 hours after injury in patients with SCI compared to healthy subjects. PLGF is a member of VEGF subfamily and it plays an important role in angiogenesis and vasculogenesis. Gaál *et al.*³² have tested growth factors for angiogenesis in the CNS using recombinant adeno-associated virus vectors encoding the growth factors that have been injected transcranially to the frontoparietal cerebrum of mice. In that study, PLGF have emerged as the most efficient and safe angiogenic factor for

therapeutic CNS revascularization. In our study, PLGF plasma levels were significantly elevated in patients with acute SCI at 24 hours and day 7 post-injury and it seems to play a role in organism response to SCI. Whereas, soluble endoglin, an antiapoptotic factor, is decreased on day 7 in patients with SCI. Endoglin is an important factor for EC proliferation and migration. These processes can be stimulated in ECs by TGF- β signaling via an Endoglin/T β RII/ALK1 complex.³³ However, a soluble form of endoglin comes from proteolytic cleavage of an extracellular domain of endoglin by matrix metalloproteinase 14 and serves as antagonist for TGF- β signaling and anti-angiogenic factor.³⁴ Our study demonstrates that soluble form of endoglin decreased at day 7 post-SCI. Therefore, the decreased level of soluble endoglin in patients with SCI may contribute to the augmentation of the TGF- β -induced angiogenesis and neuroprotective effects of TGF- β , especially that accumulating data suggest a beneficial role of TGF- β after SCI.³⁵ Thus, taking together our findings, we hypothesize that the immediate increment of concentration of angiogenic cytokines, such as EGF, PLGF, and Ang2 as well as HGF consolidates early after injury onset and might lead to the egress of EPCs from the BM niches to the circulation and subsequently to tissue niches at the local sites of vascular damage, where the expression of these factors is up-regulated. The increase in EPC concentration appears with delay. The possible explanation of the delayed cellular response might be a lack of an increase in concentration of the potent chemoattractive factors such as SDF-1 and VEGF.

ET-1 is a potent vasoconstrictor. Under physiological conditions, it is secreted in small amounts mainly by ECs in response to various factors. Under pathophysiological conditions, however, the ET-1 production is induced in a large number of different cell types, including vascular smooth muscle cells, cardiac myocytes, and inflammatory cells such as macrophages and leukocytes.³⁶ Besides its vasoconstrictive action on arterial wall, ET-1 shows a wide variety of biological effects including neuromodulatory role and mitogenic action. ET-1 presence in the brain was demonstrated in glial cells, invading macrophages and neurons.³⁷ Its action is mediated through two types of receptors, ETR-A and ETR-B, the B type being mainly synthesized in the brain.³⁸ ET-1 exerts a role in glial proliferation under pathological conditions.^{39,40} The role of ET-1 in the pathophysiology of secondary neural damage after SCI has been thoroughly investigated.^{40,41} ET-1 has been implicated in the pathogenesis of post-traumatic ischemia and BSCB breakdown after SCI. Westmark

*et al.*⁴² have found an enhanced ET-1 expression in the spinal cord after SCI and its increased level has correlated with anatomical pattern of BSCB breakdown and altered permeability. Similarly, in the rat injury model, an increase in tissue content of ET-1 has been demonstrated in the lesion site. It seemed to be attributed to an increased expression level of ET-1 in brain capillary ECs and also the exudation of plasma-derived ET-1 through injured brain capillaries due to damage of the BSCB.⁴³

Elevated level of ET-1 has been observed in patients with chronic SCI and seems to be involved in the development of cardiovascular complications in these patients.⁴⁴ However, it has not been demonstrated in patients with acute SCI so far. In our study, we documented for the first time the elevated plasma levels of ET-1 in patients with acute SCI. ET-1 plasma levels remained elevated from 24 hours to day 7 after SCI with peak concentration at 24 hours post-injury. Specific stimuli can induce transcription of ET-1 mRNA and synthesis and secretion of ET-1 in ECs within minutes. The half-life of the mRNA is ~15–20 minutes, and the plasma half-life of ET-1 is ~4–7 minutes.⁴⁵ We found the expression of ET-1 in PB NCs on the same level as in the control group as estimated by qRT-PCR. However, an increase in EC number per microliter reflects activation and damage of endothelium and may be responsible for increased ET-1 plasma levels observed in patients with SCI compared to the control group. In addition, EPCs are also the source of ET-1 and an increment in EPC number in the PB may contribute to the increased total plasma level of ET-1 in patients with SCI. However, the peak concentration of ET-1 was at 24 hours while the increment of circulating ECs reflecting endothelial damage occurs on days 3 and 7 post-injury. The possible explanation is fact that the most severe trauma takes place on the injury onset and at this time the response on endothelium damage consolidates.

ET-1 is an important peptide for critical illness. Accordingly, its elevated levels have been documented in patients with hypoperfusion and sepsis.⁴⁶ SCI is associated with the loss of supraspinal control of the sympathetic nervous system leading to several severe cardiovascular complications including both supine and orthostatic hypotension, autonomic dysreflexia, and cardiac arrhythmias.⁴⁷ The possible explanation of increased ET-1 secretion is compensatory reaction to cardiovascular instability, hypotension, and hypoxia. It could also be the result of local endothelial injury or systemic endothelial activation. Taken complexity of disturbances observed in patients with SCI, it is rather

difficult to indicate one mechanism underlying the increased ET-1 levels in patients. Our previous findings have indicated a potential role of ET-1 in the pathophysiology of EPC mobilization and recruitment into the damaged neural tissue in ischemic stroke.²⁰ As ET-1 is one of these factors that are able to stimulate cell proliferation, it might be involved in both the regeneration of vasculature and neuroregenerative processes.

BDNF, a potent neurotrophin in CNS, plays a neuroprotective role and enhances some regenerative activity after SCI.^{48,49} It has been demonstrated that exogenous delivery of BDNF to animal models of SCI can promote robust axonal growth⁵⁰ and local BDNF application decreases loss of function in the partially transected spinal cord starting 1 day after SCI. BDNF is also present in the circulation and derives from different sources including platelets, monocytes, and the brain.¹⁷ The positive correlations between BDNF levels in the brain and the PB plasma have been documented in rodents.⁵¹ From this time, the plasma levels of BDNF are widely estimated and used in humans as a determinant of the same parameter in the brain. The BDNF plasma level has been studied in a number of psychiatric disorders.⁵² Our very recent study has demonstrated that human SPCs exert humoral activity and produce BDNF.¹⁸ That finding may suggest that circulating mobilized BM-derived progenitor cells could also be the source of BDNF. However, the intracellular BDNF mRNA expression levels in PB-derived NCs were similar to those observed in healthy subjects. Moreover, in this study, we showed that BDNF plasma levels were decreased in patients with SCI at 7 day post-injury compared to healthy subjects and compared to 24 hours post-injury. In fact, it has been shown that BDNF presents a very short half-life in plasma ($t_{1/2} = 0.92$ minutes)⁵³ that could be shortened owing to the severe organism's response and activation of the immune response. The decreased BDNF plasma levels might be related to the inhibition of BDNF synthesis by the activation of mineralocorticoid and glucocorticoid receptors⁵⁴ associated with stress in the course of trauma and circulatory disturbances in patients with SCI. Despite the fact that injury of spinal cord involves acute neural tissue damage and BSPB disruption, it belongs to diseases that do not induce the increased BDNF plasma level and intracellular mRNA expression levels in PB cells.

The observed cellular and humoral response in patients with SCI could be attributed to SCI as well as with concomitant injuries in patients. A number of patients from the study group experienced additional fractures of other bones in addition to vertebral fractures. Similarly, a number of patients simultaneously

suffered from internal organ injuries. Therefore, each injury could contribute to some degree to the observed changes in circulating cell counts as well as cytokine plasma levels. We attempted to analyze the influence of these additional factors on cellular and humoral response of patients with SCI. When we compared subgroups of patients with accompanying injuries and without them, we did not notice significant differences. However, our study group was relatively small to obtain appropriate number of subjects to perform reliable statistical analysis of subgroups. This analysis would require a greater number of patients.

Immune and inflammatory responses are involved in SCI that induces widespread glial activation and recruitment to the CNS of innate (e.g. neutrophils, monocytes) and adaptive (e.g. T- and B-lymphocytes) immune cells. It has been observed in the experimental model that SCI triggers transient increase in leukocyte populations in blood early after SCI with subsequent invasion of neutrophils and monocytes into the injured spinal cord, but it also induces lymphopenia that may contribute negatively to the overall outcome after spinal cord trauma.⁵⁵ In a very recent study, microarray expression profiling has been used by Chamakhah *et al.*⁵⁶ to investigate the changes in the transcriptome of the injured spinal cord in rats. Using GO enrichment analysis, they have revealed among others that both induced innate and adaptive immune responses are strongly and significantly up-regulated at the site of injury.⁵⁶ Moreover, they have found biphasic expression pattern identified in many genes related to immune response suggesting that both resident spinal cord cell types as well as infiltrating blood cells may participate in general inflammatory response within the spinal cord. On the other hand, it is well known that traumatic SCI causes immune deficiencies leading to life-threatening infections in patients. In our study, we performed the expression patterns of PB-derived NCs in patients with acute SCI. Our data obtained from microarray gene expression profiling of NCs isolated from PB of patients with SCI at different time points, revealed different expression patterns characterizing PB-derived NCs from patients with SCI compared to healthy subjects. Global gene expression analysis revealed significantly lower expression of genes associated with both innate and adaptive immune response in PB-derived cells during acute phase of SCI.

Noteworthy, using global gene expression analysis, we found no significant changes in genes expression associated with angiogenesis, endothelium, and vasculature system in PB-derived NCs between patients with SCI and healthy controls. A possible explanation of our

finding is the relatively low percentage of circulating ECs and EPCs among the entire PB cell population.

Conclusions

Acute SCI triggers an organism's systemic response involving an increased number of circulating EPCs and ECs in PB and release of various chemoattractive and growth stimulating factors as well as immunological suppression. The emerging role of vasculature regeneration in recovery in SCI warrants continued investigation into the factors controlling this process. Thus, a better understanding of the organism's response to SCI involving circulating mobilized progenitor cells and various chemotactic, angiogenic plasma factors has considerable relevance from a therapeutic standpoint. As scientific researches and advances become more insightful, there is the raising possibility that SCI will be repairable in the future and that strategies to restore function will be developed.

Disclaimer statements

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Conflicts of interest None.

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