

Stem Cells, Including a Population of Very Small Embryonic-Like Stem Cells, are Mobilized Into Peripheral Blood in Patients After Skin Burn Injury

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Abstract

Background Developmentally early cells, including hematopoietic stem progenitor cells (HSPCs), as well as very small embryonic-like stem cells (VSELs), are mobilized into peripheral blood (PB) in response to tissue and organ injury (e.g., heart infarct or stroke).

Objective We seek to determine whether these cells are also mobilized into PB in patients with skin burn injuries.

Methods Forty-four (44) patients (33–57 years of age) with total body surface burn area of 30–60%, as well as 23 healthy control subjects, were recruited and PB samples were harvested during the first 24 hours, day +2, and day +5 after burn injury and compared to normal controls. The circulating human CD34⁺CD133⁺ cells

enriched for HSPCs, as well as small CXCR4⁺CD34⁺CD133⁺ subsets of Lin⁻CD45⁻ cells that correspond to the population of VSELs, were counted by FACS and evaluated by direct immunofluorescence staining for pluripotency markers (Oct-4, Nanog, and SSEA-4). In parallel, we also measured by ELISA the serum concentration of factors that regulate stem cell trafficking, such as SDF-1, VEGF, and HGF.

Results Our data indicate that skin burn injury mobilizes cells expressing stem cell-associated markers, such as CD133, CD34, and CXCR4, into PB. More importantly, we found an increase in the number of circulating primitive, small Oct-4⁺Nanog⁺SSEA-4⁺CXCR4⁺Lin⁻CD45⁻ VSELs. All these changes were accompanied by increased serum concentrations of SDF-1 and VEGF.

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Limitations Further studies are needed to fully assess the role of mobilized stem cells in the healing process to see if they can contribute to skin regeneration.

Conclusion Skin burn injury triggers the mobilization of HSPCs and CXCR4⁺ VSELs, while the significance and precise role of mobilized VSELs in skin repair requires further study.

Keywords VSELs · Skin burns · Stem cell mobilization · SDF-1 · CXCR4

Introduction

We have demonstrated in the past that hematopoietic stem/progenitor cells (HSPCs), as well as pluripotent very small embryonic-like stem cells (VSELs), are mobilized into peripheral blood (PB) in patients and experimental animals in response to tissue/organ injury as seen, for example, in heart infarct [1] or stroke [2, 3]. This phenomenon indicates that an intrinsic mechanism, involving circulating stem cells, exists to ameliorate tissue damage. Skin burn injuries are an important clinical problem and we became interested in whether stem cells are also mobilized and circulate in PB in patients that have experienced skin damage due to this type of injury.

It is well known that skin is the organ containing the highest number of stem cells and thus is capable of extensive regeneration following wounding [4]. Epidermal stem cells, which form the basis of this system, reside in several locations, including the interfollicular epidermis, sebaceous glands, and hair follicles, especially in the bulge region [5–11]. However if a large area of skin is burned and local stem cells residing in epidermis are irreversible damaged, the regeneration process is severely impaired or impossible without additional support. Several approaches have been proposed, including coverage of damaged skin by artificial skin equivalents [12–14], topical application of expanded keratinocytes [15–18], or engraftment of bone marrow-derived cells [19–22].

While extensive and deep skin burns lead, in the process of healing, to the formation of scars, smaller burns have a chance to be covered by regenerating epidermis. An important role in the regeneration of burned skin is played by stem cells that line damaged areas or that have survived, scattered throughout less-damaged skin fragments. Another incompletely explored potential source of stem cells that could ameliorate skin damage is the populations of stem cells circulating in PB. In steady-state conditions, a majority of these circulating cells are hematopoietic stem progenitor cells (HSPCs). However, as we have observed in several types of organ tissue injuries (e.g., heart infarct and stroke), there are, in addition to HSPCs, other types of stem cells mobilized, including VSELs. These cells

command particular attention because, being pluripotent, they may differentiate into different types of cells. Therefore, in the current work we became interested in whether VSELs are also mobilized and circulate in patients suffering from skin burn injuries.

We report here that skin injury caused by extensive and deep burns mobilizes stem cells into PB. Moreover, we observed that, in addition to HSPCs, a population of pluripotent VSELs becomes mobilized and circulates in PB. The existence of this putative, intrinsic pro-regenerative mechanism suggests that this phenomenon could be exploited therapeutically by, for example, increasing the number of circulating stem cells by administration of pharmacological mobilizing agents. This however, will require further study.

Materials and Methods

Patients and Peripheral Blood Collection

We enrolled 44 burned patients from the West Pomeranian Heavy Degree Burns Centre in Gryfice, Poland and 23 healthy control subjects in this study (Table 1). The study protocol was approved by the institutional Ethics Committee and written informed consent for peripheral blood (PB) collection was obtained from each patient. EDTA-anticoagulated PB samples (5 mL) were drawn within 24 h after burn, on day +2, and on day +5 from burned patients and once from controls. The absolute numbers of white blood cells (WBC) in PB were determined at the same time with an automatic cell counter SYSMEX XT - 2000i. The full population of PB nucleated cells (PBNCs) was obtained after lysis of red blood cells with BD PharM Lyse (ammonium chloride lysing) reagent (BD Biosciencics, San Jose, CA, USA). Total mRNA was isolated from 10 [6] PBNCs with the commercially available RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Thereafter, mRNAs were frozen and stored at -80°C . Serum samples for measuring SDF-1, VEGF, and HGF levels were frozen

Table 1 Patient characteristic – demographic and clinical data

Patient characteristic	n=44
Age (years)	
Median [range]	47 [33–57]
Sex	
Female	7
Male	37
% TBSA burn	40 [30–60]
Burn degree	
II/III	9
III	30
III/IV	5

and also stored at -80°C . Samples of PBNCs for assessing SCs were processed within 12 h after blood draw. Of note, no one of our patients was receiving immunosuppressive drugs.

Flow Cytometry

All primary and secondary antibodies were obtained from BD Biosciences (San Jose, CA, USA), except the secondary antibody APC, which was obtained from Miltenyi Biotec (Auburn, CA, USA). A single-cell suspension was stained with murine anti-human monoclonal antibodies for lineage markers (CD2, clone RPA-2.10; CD3, clone UCHT1; CD14, clone M5E2; CD66b, clone G10F5; CD24, clone ML5; CD56 clone NCAM16.2; CD16, clone 3 G8; CD19, clone HIB19; and CD235a, clone GA-R2) conjugated with fluorescein isothiocyanate (FITC) and CD45 (clone HI30) conjugated with phycoerythrin (PE). Cells were also stained for the following antigens: CXCR4 (clone 12 G5) for analysis of $\text{CD45}^{\text{lin}^-}\text{CXCR4}^+$ and $\text{CD45}^{\text{lin}^-}\text{CXCR4}^+$ cells, CD34 (clone 581) for analysis of $\text{CD45}^{\text{lin}^-}\text{CD34}^+$ and $\text{CD45}^{\text{lin}^-}\text{CD34}^+$ cells, and CD133 (CD133/1) conjugated with APC for analysis of $\text{CD45}^{\text{lin}^-}\text{CD133}^+$ and $\text{CD45}^{\text{lin}^-}\text{CD133}^+$ cells. At the same time, samples were analyzed for the presence of circulating $\text{CD34}^+\text{CD45}^+$ and $\text{CD133}^+\text{CD45}^+$ hematopoietic stem/progenitor cells. Samples with the appropriate isotype controls were examined in parallel. Staining was carried out on ice for 30 min. Cells were washed and analyzed by LSR II (BD Biosciences, San Jose, CA, USA). At least 2×10^6 [6] events were acquired and analyzed using FACS Diva 6.2 software (BD Biosciences, San Jose, CA, USA) (Fig. 1). The absolute numbers of stem cells per $1 \mu\text{L}$ of PB were calculated on the basis of their percentage content and the absolute number of WBCs.

Isolation and Immunofluorescence Analysis and of Circulating Stem Cells

PB was lysed in BD PharM Lyse (ammonium chloride lysing) solution (BD Biosciences, San Jose, CA, USA). The single-cell suspension of PBNCs obtained was subsequently stained with murine anti-human monoclonal antibodies for lineage markers (CD2, clone RPA-2.10; CD3, clone UCHT1; CD14, clone M5E2; CD66b, clone G10F5; CD24, clone ML5; CD56 clone NCAM16.2; CD16, clone 3 G8; CD19, clone HIB19; CD235a, clone GA-R2) and conjugated with fluorescein isothiocyanate (FITC). CD45 (clone HI30) was conjugated with phycoerythrin (PE) and CXCR4 (clone 12 G5) conjugated with APC (all from BD Biosciences, San Jose, CA, USA). The sample was incubated with antibodies for 30 min on ice, washed in PBS, and then resuspended in PBS. The population of $\text{CD45}^{\text{lin}^-}\text{CXCR4}^+$ cells was sorted by multiparameter, live sterile cell sorting (BD FACSAria II Cell-Sorting System, BD Biosciences, San Jose, CA, USA).

The expression of each antigen was examined in cells from four independent experiments. $\text{CXCR4}^{\text{lin}^-}\text{CD45}^+$ cells were fixed in 3.5% paraformaldehyde for 20 min, permeabilized by 0.1% Triton X100, washed in PBS, pre-blocked with 2% BSA, and subsequently stained with antibodies to SSEA-4 (clone MC-813-70, 1:100, mouse monoclonal IgG, Chemicon Int., Temecula, CA), Oct-4 (clone 9E3, 1:100, mouse monoclonal IgG, Chemicon Int.) and Nanog (1:200, goat polyclonal IgG, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Appropriate secondary Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 594 rabbit anti-goat were used (1:400, Molecular Probes, Eugene, Oregon). The nuclei were identified with DAPI (Molecular Probes, Eugene, Ore). The fluorescence images were collected with the TE-FM Epi-Fluorescence system attached to an Olympus Inverted Microscope IX81 (Olympus, Center Valley, PA).

Plasma Concentrations of SDF-1, VEGF, and HGF

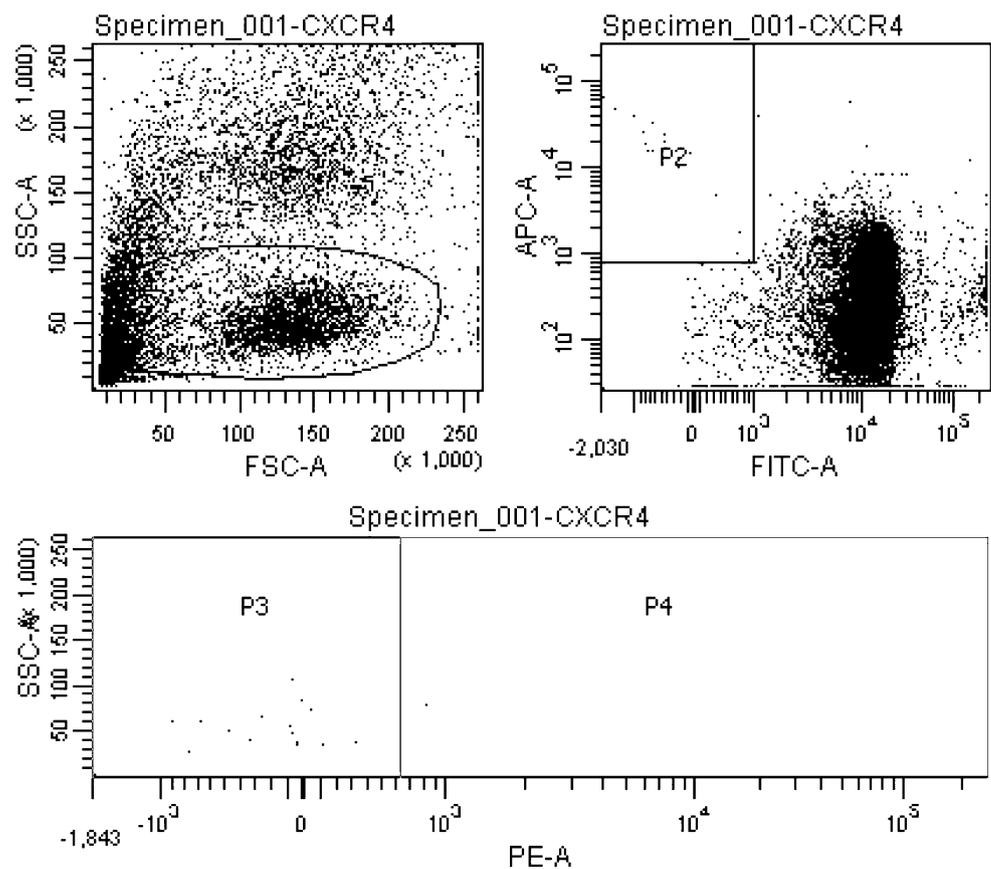
The concentrations of stromal derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) were measured with commercially available, high-sensitivity Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol.

Real-Time Reverse Transcriptase–Polymerase Chain Reaction

To analyze mRNA levels for pluripotent (Oct-4 and Nanog), early epidermal (Trp63, Krt14, and Krt15), and endothelial (VE-cadherin) markers, total mRNA was isolated from PBMCs with the RNeasy Mini Kit (Qiagen GmbH). Subsequently, mRNA was reverse-transcribed with a first-strand cDNA synthesis kit (Fermentas International Inc, Burlington, Canada). Quantitative assessment of mRNA levels was performed by real-time RT-PCR on an ABI 7500 instrument with Power SYBR Green PCR Master Mix reagent. Real-time conditions were as follows: 95°C (15 s), 40 cycles at 95°C (15 s), and 60°C (1 min). According to melting point analysis, only one PCR product was amplified under these conditions. The relative quantity of a target, normalized to the endogenous control β -2 microglobulin gene and relative to a calibrator, is expressed as $2^{-\Delta\Delta\text{Ct}}$ (–fold difference), where Ct is the threshold cycle, $\Delta\text{Ct} = (\text{Ct of target genes}) - (\text{Ct of the endogenous control gene } \beta\text{-2 microglobulin})$, and $\Delta\Delta\text{Ct} = (\Delta\text{Ct of samples for target gene}) - (\Delta\text{Ct of calibrator for the target gene})$. The following primer pairs were used:

Oct-4 F: 5' – GAGCCCTGCACCGTCACC – 3'
 Oct-4 R: 5' – TTGATGTCCTGGGACTCCTCC – 3'
 Nanog F: 5' – GCAGAAGGCCTCAGCACCTA – 3'

Fig. 1 Representative flow cytometric analysis of the expression of the CD45 lineage marker and CXCR4 antigens on circulating PBMNCs in patients with skin burns at 24 h after injury. Upper left: cytogram of PBMNCs with gating area extended to the left. Upper right: P2 shows rare CXCR4⁺ Lin⁻ event. Lower panel: cells from P2 shown for expression of CD45; P3 contains CXCR4⁺ Lin⁻ CD45⁻ VSELs



Nanog R: 5' -AGGTTCCCAGTCGGGTTC- 3'
 Trp63 F: 5' -CCTCGTCCACCAGTCCCTAT- 3'
 Trp63 R: 5' -GGAAGGACACGTGAAACTG- 3'
 Krt14 F: 5' -TTCTGAACGAGATGCGTGA- 3'
 Krt14 R: 5' -GCAGCTCAATCTCCAGGTTC- 3'
 Krt15 F: 5' -GGCTTTGCATGCGCTCTATT- 3'
 Krt15 R: 5' -GCTGCATCTCCTTGCTCCA- 3'
 VE-cadherin F: 5' -TTTTCCAGCAGCCTTTCTA
 CCA- 3'
 VE-cadherin R: 5' -GCGGATGGAGTATCCAATG
 CTA- 3'

Statistical Analysis

Both parametric and nonparametric tests were used to test for differences in absolute numbers of circulating small early stem cells in PB from burn patients at different time points. Many common statistical procedures, such as analysis of variance (ANOVA) assume (1) multivariate normality and (2) homogeneity of variance. Multivariate normality was tested using the Shapiro-Wilk test. Homogeneity of variance was tested using Levene's test. If the assumptions above were fulfilled (both Shapiro-Wilk and Levene tests with p values >0.05), parametric one-way ANOVA was used. In other cases, data were log-

transformed and then again tested for normality and homogeneity of variance. If the variance of log-transformed data was not homogenous, or distributions of log-transformed data were significantly different from a normal distribution, the non-parametric Mann-Whitney test was used.

The significance of changes in SDF-1, VEGF, and HGF cytokine concentrations between the healthy control group and burn patients at day +2 and day +5 was assessed with the Mann-Whitney test. The significance of changes in SDF-1, VEGF, and HGF cytokines between burn patients groups (24 h vs day +2 vs day +5) was assessed with Kruskal-Wallis ANOVA test, followed by the multiple comparisons *post hoc* test.

Results

Cells That Express the Phenotype of Hematopoietic Stem Progenitor Cells (HSPCs) and Very Small Embryonic-Like Stem Cells (VSELs) are Mobilized into PB in Patients with Burn Injuries

To test our hypothesis that deep skin burns, like other types of tissue/organ injury [23, 24], mobilize stem cells into PB, we evaluated the number of circulating human

cells enriched for HSPCs, as well as small VSELs by FACS. A total of 44 patients (33–57 years of age) with total body surface burn area from 30–60% as well as 23 healthy control subjects were recruited for this study (Table 1). PB samples were harvested during the first 24 h, day +2, and day +5 after burn injury and compared to normal controls. The numbers of cells enriched for HSPCs are summarized in Table 2. Interestingly, we observed that the number of CD34⁺ CD45⁺, CD133⁺ CD45⁺, and CXCR4⁺ CD45⁺ cells, which are enriched for HSPCs, as well as CD34⁺CD45⁺lin⁻, CD133⁺CD45⁺lin⁻, and CXCR4⁺CD45⁺lin⁻ cells, which are enriched for developmentally more primitive HSPCs, significantly decreases during the first 48 h after the burn episode. We also observed that after an initial decline, the number of these cells is increased at day 5 after the burn episode. The most visible changes were observed for CD133⁺CD45⁺ and CD133⁺CD45⁺lin⁻ cells.

Next, we analyzed the numbers of small CD45⁻Lin⁻ cells, which are enriched for VSELs. As shown in Table 3, the number of CD34⁺, CD133⁺, and CXCR4⁺ small CD45⁻Lin⁻ cells increased significantly 24 h after the burn episode. In the case of the CXCR4⁺Lin⁻CD45⁺ population of VSELs, this increase also remained highly statistically significant at day 2 and day 5 after the burn event. However, there was no correlation between the extent of the burn injury, the age of the patient, and the number of mobilized cells (data not shown). This could be explained by the fact that our study included mainly patients with extensive burns.

Cells Circulating in PB of Patients after Burns are Enriched for Pluripotency Markers

To confirm that burn injury may induce mobilization of primitive pluripotent stem cells (VSELs) we employed real-time PCR (RQ-PCR) to compare the expression of mRNA for two embryonic transcription factors, Oct-4 and Nanog,

in mononuclear cells isolated from PB of patients suffering from burn injuries and normal controls. Figure 2 shows a significant increase in expression of mRNA for Oct-4 and Nanog 24 h after burn injury ($p < 0.05$). In the case of Oct-4, we also observed that this increase was significant at day 2 after injury.

The expression of pluripotent stem cell markers, Oct-4 and Nanog, was subsequently confirmed by immunocytochemical staining of cells sorted from PB VSELs. We selected for sorting a population of CXCR4⁺CD45⁻Lin⁻ VSELs, that, as shown in Table 3, was significantly enriched in PB of patients 24 h after the burn injury.

As shown in Fig. 3, CXCR4⁺CD45⁻Lin⁻ VSELs express the stage-specific embryonic antigen-4 (SSEA-4) on the surface and the embryonic transcription factors, Oct-4 and Nanog, in the nuclei.

Mobilized PBMNCs Express Markers for Early Epidermal and Endothelial Cells

Next, we became interested in whether cells mobilized into peripheral blood are enriched for mRNA-encoding genes involved in development of early epidermal and endothelial cells. Thus, we again extracted mRNA from mobilized PB mononuclear cells and evaluated the expression of mRNA for early epidermal (Trp63, Krt14, and Krt15) and endothelial (VE cadherin) markers by RQ-PCR. As shown in Fig. 4, the mRNA for these markers increased up to 5 times in the 24 h after the burn event; only in the case of Trp63 did they remain high until day 5 after injury.

Are Circulating CXCR4⁺ Stem Cells Mobilized into PB in an SDF-1-Dependent manner?

Next, we became interested in whether α -chemokine stromal derived factor-1 (SDF-1), which binds to the CXCR4 receptor and is an important chemottractant for CXCR4⁺ stem cells, is involved in mobilization of cells into PB. To

Table 2 Absolute numbers of circulating cells enriched for HSPCs in PB of burn patients at different time points (24 h, and post burn days +2 and +5) and in the healthy control group

	Control group	Burn patients at 24 h	<i>P</i> vs control	Burn patients at day +2	<i>P</i> vs control	<i>P</i> vs 24 h	Burn patients at day +5	<i>P</i> vs control	<i>P</i> vs 24 h	<i>P</i> vs day +2
CD34 ⁺ CD45 ⁺	2.65 [2.27]	1.90 [3.18]	0.480**	1.42 [3.46]	0.231**	0.839*	3.54 [6.96]	0.02**	0.0698*	0.218*
CD133 ⁺ CD45 ⁺	2.63 [1.51]	1.24 [1.51]	0.002**	1.01 [1.78]	0.09***	0.856*	4.37 [3.96]	0.03**	0.001*	0.0003*
CXCR4 ⁺ CD45 ⁺	0.44 [0.52]	0.29 [0.38]	0.159**	0.50 [0.64]	0.321**	0.04*	0.36 [0.43]	0.350**	0.223*	0.158*
CD34 ⁺ CD45 ⁺ lin ⁻	0.54 [1.08]	0.47 [0.45]	0.244**	0.17 [0.38]	0.021**	0.676*	1.25 [2.78]	0.0496**	0.029*	0.004*
CD133 ⁺ CD45 ⁺ lin ⁻	0.23 [0.29]	0.27 [0.34]	0.562**	0.37 [0.40]	0.214**	0.86*	1.13 [2.80]	0.0003***	0.899*	0.626*
CXCR4 ⁺ CD45 ⁺ lin ⁻	0.19 [0.35]	0.25 [0.29]	0.125**	0.20 [0.25]	0.453**	0.119*	0.31 [0.50]	0.189**	0.533*	0.596*

Data are expressed as the number of cells per μ L of blood (median [interquartile range]); Significance of changes assessed with: * HSD Tukey test, ** one way ANOVA, *** U Mann–Whitney test

Table 3 Absolute numbers of circulating small early stem cells in PB from burn patients at different time points (24 h, and post-burn days +2 and +5) and in the healthy control group

	Control group	Burn patients at 24 h	<i>P</i> vs control	Burn patients at day +2	<i>P</i> vs control	<i>P</i> vs 24 h	Burn patients at day +5	<i>P</i> vs control	<i>P</i> vs 24 h	<i>P</i> vs day +2
CD34 ⁺ CD45 ⁻ lin ⁻	0.16 [0.67]	0.61 [1.76]	0.01**	0.37 [0.85]	0.091**	0.276*	0.40 [0.43]	0.403**	0.0002*	0.007*
CD133 ⁺ CD45 ⁻ lin ⁻	0.33 [0.47]	0.40 [0.90]	0.046**	0.43 [0.64]	0.12**	0.816*	0.31 [0.53]	0.133**	0.023*	0.091*
CXCR4 ⁺ CD45 ⁻ lin ⁻	0.31 [0.34]	1.73 [2.40]	<0.0001**	1.52 [1.57]	<0.0001**	0.554*	1.16 [0.54]	<0.0001**	0.087*	0.497*

Data are expressed as the number of cells per μL of blood (median [interquartile range]); Significance of changes assessed with: * HSD Tukey test, ** one way ANOVA, *** U Mann–Whitney test

address this important question, we employed ELISA to measure serum levels of SDF-1 in control patients and patients suffering from burn injuries. At the same time, we also measured serum levels of other chemoattractants involved in stem cell migration, such as hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) (Table 4 and Fig. 5).

Our ELISA measurements show that maximal levels of SDF-1 and HGF were observed 5 days after the burn event (Table 4 and Fig. 5), which correlates with the maximal level of circulating HSPCs and CXCR4⁺ VSELs in PB (Table 2). In contrast, the serum level of VEGF was already

increased 24 h after the burn event and reached a further statistically significant increase in the serum of burned patients 5 days after injury (Table 4).

Discussion

It is well known that HSPCs continuously circulate in PB and lymph throughout the body, both during development and later on in adult life. During development they move between major anatomical sites where hematopoiesis is initiated and/or temporarily active [25]. The stromal derived factor-1 alpha chemokine (SDF-1), which binds to the G-protein coupled, seven-transmembrane-spanning CXCR4 receptor expressed on HSPCs, has been hypothesized to guide HSPCs during early development to colonize BM, and later to play a major role in retention of these cells in the BM microenvironment [26, 27]. The same chemokine-receptor axis is also involved in trafficking of other types of stem cells (e.g., neural stem cells and primordial germ cells) [2, 28–31]. Later in adult life, a small percentage of HSPCs is continuously released from BM niches into the PB, which may be envisioned as a highway by which HSPCs relocate between distant BM stem cell niches in order to keep the total pool of BM stem cells in balance. The number of circulating HSPCs increases in response to i) systemic or local inflammation, ii) strenuous exercise, iii) tissue/organ injury, and iv) pharmacological agents [24, 32]. As recently reported by us, in addition to HSPCs, a population of pluripotent VSELs is also mobilized that circulates at detectable levels in PB in similar situations [1, 2, 23, 33–35]. This phenomenon occurs in patients and experimental animals after heart infarct [1, 23], stroke [2, 3], and administration of stem cell mobilizing agents, such as granulocyte colony stimulating factor (G-CSF) [36] and after injection of a small molecular antagonist of the CXCR4 receptor, AMD3100 [34].

However, the potential role that circulating stem cells are playing in these clinical situations requires further study. We envision that it is an intrinsic propensity of organisms to repair damaged tissues by harnessing circulating stem cells [37]. If this is the case, this

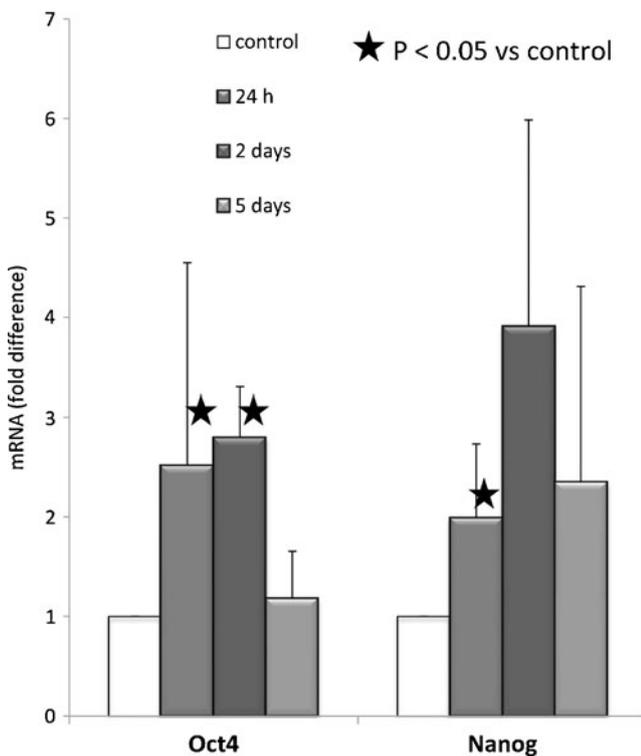
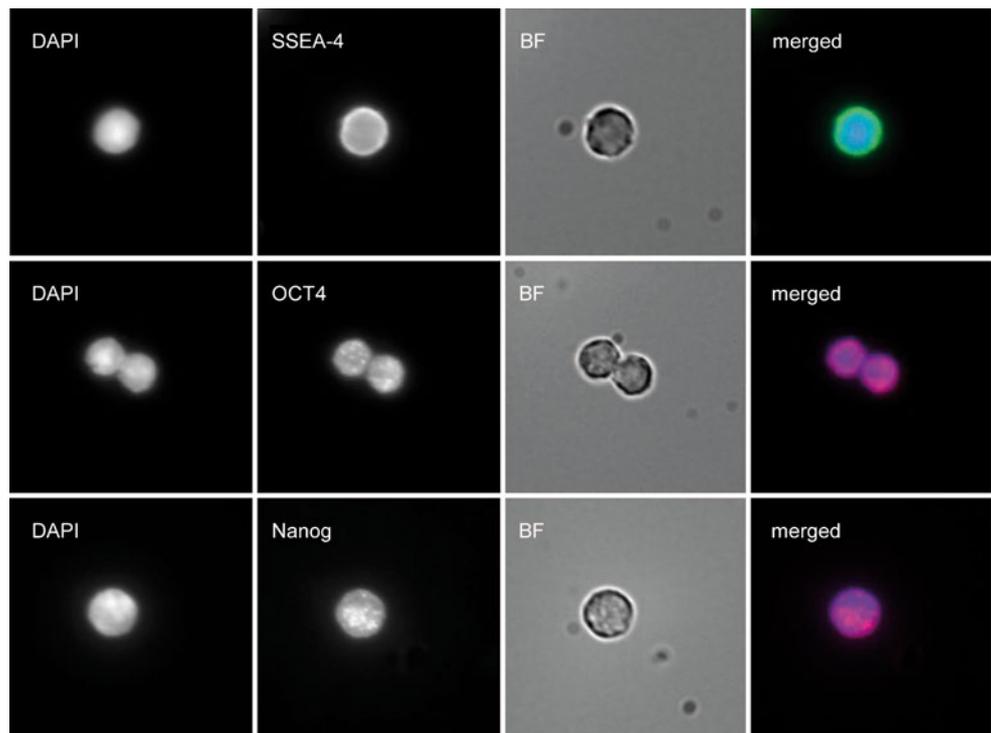


Fig. 2 Combined changes in the expression of markers for pluripotent VSEL stem cells (SCs) in PBMNCs for patients with skin burn injuries and the healthy control group. Data are expressed as fold difference compared to healthy subjects. Data are mean \pm SD. * $p < 0.05$

Fig. 3 Immunocytochemical analysis of VSEL SCs mobilized into peripheral blood. Expression of Oct-4, SSEA-4, and Nanog in CXCR4⁺CD45⁻Lin⁻ cells sorted by FACSARIA from patient PBMNCs 2 days after burn injury. Nuclei are visualized after DAPI staining. The fluorescence images were collected with the TE-FM Epi-Fluorescence system attached to an Olympus Inverted Microscope IX81 (Olympus, Center Valley, PA). Representative data are shown



mechanism could be exploited therapeutically by intravenous or local administration of stem cells or by increasing their mobilization into PB by employing pharmacological agents, such as G-CSF or AMD3100. This treatment

option has already been proposed for patients following heart infarct [38, 39] or stroke [40] and we are currently investigating its potential application in patients following skin burn injury.

Fig. 4 Combined changes in the expression of markers for pluripotent early epidermal and endothelial cells in circulating PBMNCs for burn patients and the control group. Data are expressed as the fold difference compared to control. * $p < 0.05$

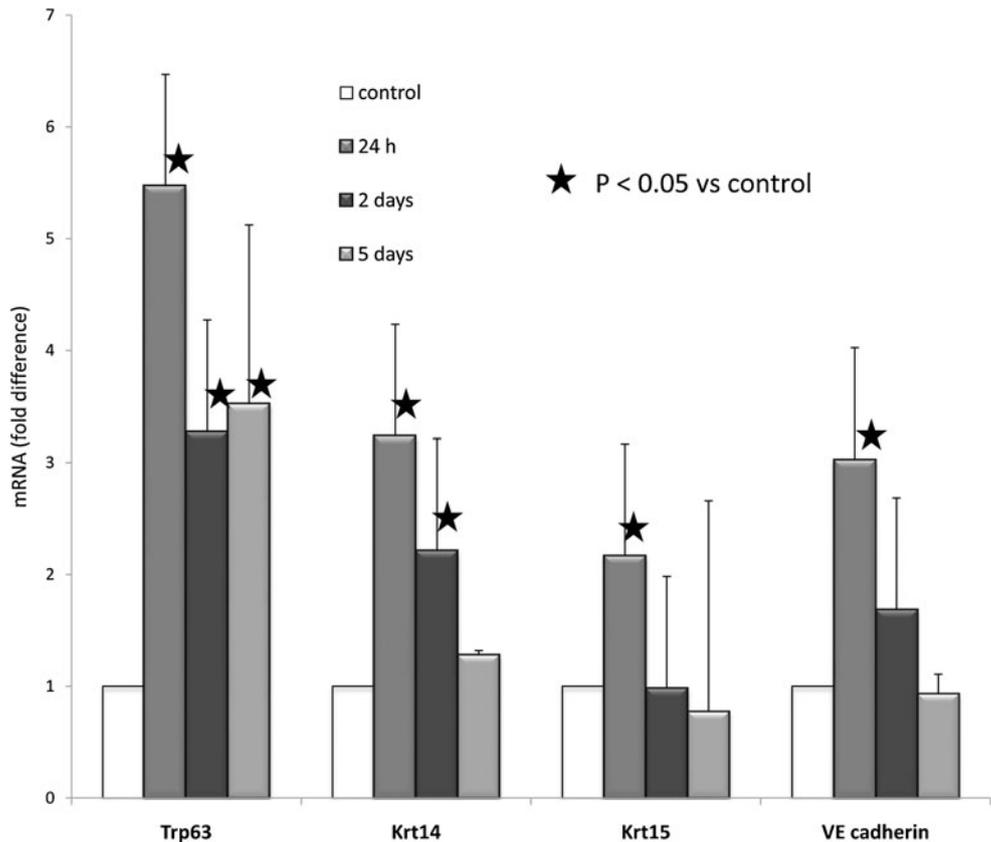


Table 4 Plasma concentration [pg/mL] of cytokines in healthy control and in burn patients at different time points (24 h, and post burn days +2 and +5)

	Control group	Burn patients at 24 h	P vs control	Burn patients at day +2	P vs control	P vs 24 h	Burn patients at day +5	P vs control	P vs 24 h	P vs day +2
SDF-1	2585 [670]	2314 [1915]	0.084*	2208 [1792]	0.006*	1.0**	4004 [3265]	<0.0001*	<0.0001**	<0.0001**
VEGF	670 [131]	4474 [2618]	0.67*	3102 [1690]	0.44*	1.0**	4526 [2702]	0.007*	0.0016**	<0.0001**
HGF	129 [252]	133 [248]	<0.0001*	126 [138]	<0.0001*	0.23**	464 [527]	<0.0001*	1.0**	0.048**

Data shown as a median [interquartile ranges]. Significance of changes assessed with: *U Mann–Whitney test, **multiple comparisons test (Kruskal–Wallis post hoc test)

Because regeneration of skin after extensive burns is one of the therapeutic goals of regenerative medicine, we became interested in whether stem cells are also mobilized into PB in patients suffering from extensive skin burns. In this report, we demonstrate for the first time that HSPCs, as well as VSELs, are mobilized into PB in these patients as well. Interestingly, mobilization of HSPCs increased significantly 5 days after the burn injury, which may reflect the time needed to amplify a pool of cells ready for mobilization in BM and/or the time needed for a PB chemoattractant to reach an effective chemotactic level. In fact, we observed that at this time point SDF-1 reaches its maximal concentration in PB. In contrast, the number of VSELs was already increased significantly in PB 24 h after injury. This may also reflect different biological roles for these cells in responding to skin damage. While VSELs are involved in physiological regeneration of damaged tissues from the beginning, HSPCs are more gradually mobilized to increase the pool of white blood cells in damaged tissues [25, 41], and thus prevent burn-related infections.

There is no doubt that a proper understanding of the mechanisms that govern stem cell mobilization in regener-

ation of damaged tissues will help to develop more efficient strategies to increase their mobilization into PB for clinical purposes. Several mechanisms have been proposed to orchestrate mobilization, but more work is needed to better understand this process. Overall, the mobilization process has been proposed to be directed by i) a decrease in SDF-1–CXCR4 and VLA-4–VCAM-1 interactions in BM (e.g., due to release of proteolytic enzymes or after molecular blockage due to administration of small molecular antagonists), ii) release of neurotransmitters from the synapses of the nerves that innervate the BM microenvironment, iii) activation of the coagulation cascade (e.g., release of uPAR), and finally, as recently proposed, iv) activation of the complement cascade [42–45]. In support of this last possibility, it is known that the complement cascade becomes activated in all mechanisms leading to stem cell mobilization of HSPCs (e.g., systemic inflammation, organ injury, as well as administration of all types of mobilizing drugs) [35, 36, 41, 46, 47].

In our studies we demonstrate that mobilization of CXCR4⁺ stem cells correlates with an increase in SDF-1, HGF, and VEGF plasma levels. We have already reported a similar phenomenon for mobilization of stem cells in experimental animals and patients after acute myocardial infarction [1, 23]. Thus, the SDF-1–CXCR4 signaling axis could play an important role in trafficking of stem cells in tissue/organ injuries. However, our recent research also indicates the involvement of other factors, in particular, small bioactive lipids that may direct mobilization and trafficking of stem cells to injured organs [34]. One of these factors—sphingosine-1-phosphate (S1P)—as we have already demonstrated, is released from circulating erythrocytes and plays a crucial role in egress of stem cells into PB [48, 49]. Of note, release of S1P correlated with activation of complement cascade and formation of the C5b-C9 membrane attack complex (MAC) [34]. It is well known that both complement cascade activation and a reduced level of red blood cell lysis is observed in patients suffering from skin burn injuries. Based on these observations, the roles of complement cascade activation and the release of S1P from circulating erythrocytes

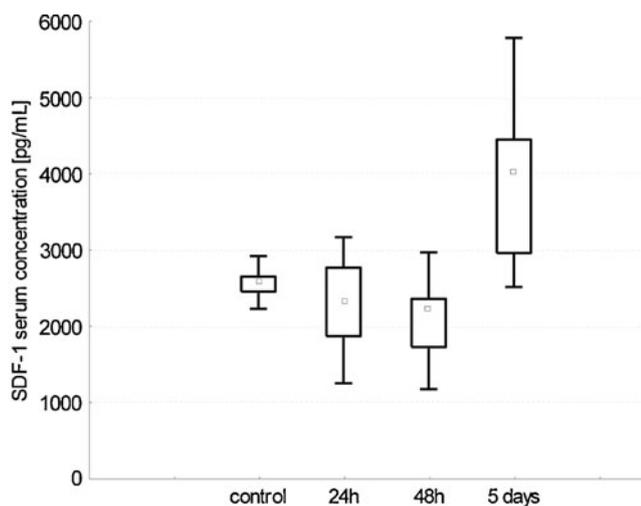


Fig. 5 SDF-1 serum concentration is elevated in burn patients. Box-and-whiskers plot of differences in SDF-1 serum concentration between the healthy control group and burn patients at 24 h, day +2, and day +5

as triggers for stem cell mobilization in patients after burn injuries requires further study.

In this report we have focused on the mobilization of HSPCs and VSELs. We are, however, aware that in addition to HSPCs and VSELs, some other rare stem cells, for example, mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs), may also appear in the PB during various stress situations. Thus, we can envision that in patients following skin burn injury, stem cells circulating in PB are like “paramedics” involved in immune surveillance (HSPCs) or tissue/organ rejuvenation (VSELs, MSCs, and EPCs). For example, during infections related to skin damage, circulating HSPCs (e.g., CFU-GM [colony forming units of granulocytes and macrophages]) may proliferate in damaged tissues and supply granulocytes, monocytes, and dendritic cells to fight the infection, while VSELs, which are at the top of the hierarchy for other types of stem cells (e.g., MSCs and EPCs) [31], are directly involved in regeneration of damaged skin tissues.

Several approaches to employing cells for regenerating skin damaged by extensive burns have been proposed. For example, ex vivo-expanded early epidermal cells [15–18], MSCs [19] and even BM-derived nucleated cells have been successfully employed to accelerate skin regeneration. These approaches, however, are based on using already differentiated cells (e.g., epithelial cells and keratinocytes) or cells whose mechanisms of action are primarily related to paracrine effects (e.g., BM-derived cells). It is known that MSCs, for example, may secrete several growth factors, cytokines [50], or even membrane-derived microvesicles [51] that accelerate the regeneration process. These cells, however, do not form new epidermis or vessels in damaged skin and are eliminated over time. Thus, it is important to identify pluripotent stem cells that will be able to differentiate into all types of cells present in damaged skin (e.g., epidermis, blood vessels, and connective tissues) [52]. Thus, we propose that VSELs [53, 54] could be employed as a population of stem cells for skin regeneration, and currently we are investigating this possibility in patients after burn injury.

In conclusion, the phenomenon of stem cell circulation in patients after burn injury, identified by us, opens up a new area of investigation in dermatology and skin surgery. However, further studies are needed to fully assess the role of mobilized stem cells in the healing process to see if they can contribute to skin regeneration. Furthermore, several questions remain to be addressed on a larger sample of patients. The most important is to address whether the number of stem cells circulating in PB correlates with the extent of skin damage and whether they have any prognostic value. Furthermore, if this phenomenon reflects an intrinsic mechanism to regenerate skin burns with populations of circulating stem cells, could it be exploited

in the future to treat patients? The number of circulating stem cells (including VSELs) could be enhanced by systemic and local delivery of these cells purified from the patient’s BM or by increasing their level in PB after administration of G-CSF or AMD3100. We wish to propose that, while mobilized hematopoietic progenitors play an important role in immune surveillance of damaged tissues and protect them from infections [25], VSELs play a crucial role in regeneration of skin structure.

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