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Bone Marrow-Derived Pluripotent Very Small Embryonic-Like Stem Cells (VSELs) Are Mobilized After Acute Myocardial Infarction

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Abstract

The adult bone marrow (BM) harbors Sca-1+/Lin-/CD45- pluripotent very small embryonic-like stem cells (VSELs), which can differentiate *in vitro* into several lineages, including cardiac and vascular lineages. Since mobilization of stem/progenitors from the BM is a prerequisite for their participation in organ repair, we investigated whether VSELs are mobilized into the peripheral blood (PB) after acute myocardial infarction (MI). Wild-type mice (C57/BL6 strain, 6- or 15-wk-old) underwent a 30-min coronary occlusion followed by reperfusion (groups III–V, VIII–X, n=6–12/group) or a 1-h open-chest state (sham controls, groups II and VII, n=8–12/group); mice were sacrificed 24 h, 48 h, or 7 days later and PB samples were harvested. Controls (groups I and VI, n=6/group) were sacrificed without any intervention. By flow cytometry, VSELs were barely detectable in PB under baseline conditions but their levels increased significantly at 48 h after MI, both in younger (6-wk-old) and older (15-wk-old) mice (3.33±0.37 and 7.10±0.89 cells/μl of blood, respectively). At 48 h after MI, qRT-PCR analysis revealed significantly increased levels of mRNA of markers of pluripotency (Oct-4, Nanog, Rex-1, Rif1, and Dppa1) in PB cells of 6-wk-old (but not 15-wk-old) infarcted mice compared with either controls or sham controls. Confocal microscopy and ImageStream analysis confirmed that mobilized VSELs expressed Oct-4 protein, while Sca-1+/Lin-/CD45+ hematopoietic stem cells did not. This is the first demonstration that Oct-4+ pluripotent stem cells (VSELs) are mobilized from the BM into the PB after acute MI. This phenomenon may have pathophysiological and therapeutic implications for repair of infarcted myocardium.

Keywords

Very small embryonic-like stem cells; myocardial infarction; bone marrow; pluripotent; Oct-4; mobilization

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INTRODUCTION

Numerous studies indicate that the adult bone marrow (BM) harbors stem/progenitor cells that replenish not only the hematopoietic system, but also cells in other organs. BM-derived cells (BMCs) have been shown to participate in tissue repair following injury to several organs, including the brain, liver, lung, kidney [1–5] as well as the heart [6–11]. Cardiomyocytes derived from BMCs have been noted in the heart after myocardial infarction (MI) [6,7,10]. The egress of primitive cells from the BM into the blood is an essential first step for effective tissue repair by BMCs [12,13].

Although BMCs have been shown to promote tissue repair, the underlying mechanisms remain unclear. Generation of multilineage cells from BMCs has been proposed as a mechanism for BMC-mediated tissue repair, and it is plausible that pluripotent BMCs, capable of multilineage differentiation, are mobilized from the BM after tissue injury followed by homing and tissue reconstitution. The adult BM harbors several types of primitive cells, including hematopoietic stem cells (HSCs) [14] and a multitude of nonhematopoietic primitive cells, such as mesenchymal stem cells (MSCs) [15], multipotent adult progenitor cells (MAPCs) [16], the marrow isolated adult multilineage inducible (MIAMI) cells [17], tissue-committed stem cells (TCSCs) [18], and BM-derived multipotent stem cells [19]. Recently, we have identified a rare population of nonhematopoietic primitive cells in the BM that are positive for Sca-1 and negative for both lineage markers (Lin) and the panleukocyte marker CD45 (Sca-1⁺/Lin[−]/CD45[−]) [20]. Because these cells express a number of markers associated with a pluripotent state (SSEA-1, Oct-4, Nanog, and Rex-1) and differentiate *in vitro* into components of all three germ-layers, we have named these cells ‘very small embryonic like stem cells’ (VSELs) [20–23]. Besides markers of neural, endothelial, muscle, and pancreatic tissues, VSELs are enriched in mRNA for cardiac-specific antigens (Nkx2.5/Csx, GATA-4, MEF-2C) and can acquire a cardiomyocytic phenotype *in vitro* [20,24]. VSELs represent a distinct population of stem cells and do not express markers commonly expressed in MSCs, including CD90, CD29, and CD105 [25]. We have also reported that murine BM-derived VSELs are mobilized after various forms of tissue injury [12,26]. On the basis of the above observations, we postulated that pluripotent VSELs might be mobilized into the peripheral blood (PB) after acute MI. Mobilization of pluripotent BMCs into the PB has not been previously documented.

Accordingly, using a well-established murine model of MI [27], we investigated (i) whether VSELs are mobilized from the BM into the PB after an acute MI, and (ii) whether mobilization of VSELs is influenced by age. We used a comprehensive approach (flow cytometry, mRNA analysis by qRT-PCR, and immunocytochemistry) to determine both the absolute cell numbers as well as the kinetics of mobilization. The mobilization of VSELs (Sca-1⁺/Lin[−]/CD45[−]) was directly compared with that of Sca-1⁺/Lin[−]/CD45⁺ hematopoietic stem cells (HSCs). Our results show that the levels of Sca-1⁺/Lin[−]/CD45[−] VSELs increase in the PB soon after acute MI both in young and older mice, concomitant with an increase in markers of pluripotency in the PB, although the expression of these genes declines with age.

MATERIALS AND METHODS

All experiments were performed in accordance with the guidelines of the Laboratory Institutional Animal Care and Use Committee (IACUC). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Experimental protocol

Ten groups (n=6–12/group) of wild-type mice (C57BL/6 strain, Jackson Laboratory, Bar Harbor, ME) were used. Groups I–V were 6-wk-old, whereas groups VI–X were 15-wk-old.

Mice in groups III–V and VIII–X underwent coronary occlusion/reperfusion, while groups II and VII (sham controls) underwent a sham procedure (1-hour open-chest state) without coronary occlusion. Infarcted mice were sacrificed at 24 h (groups III and VIII), 48 h (groups IV and IX), or 7 d (groups V and X) after MI, while sham controls (groups II and VI) were sacrificed at 24 h after sham procedure for analysis of cell mobilization. Mice in groups I and VI were sacrificed without any intervention and served as controls.

Myocardial infarction

The experimental preparation has been described in detail [27]. Briefly, mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.), intubated, and ventilated using a small rodent ventilator. Body temperature, heart rate, and arterial pH were carefully maintained within the physiological range throughout the experiments. Using a sterile technique, the chest was opened through a midline sternotomy. An 8-0 nylon suture was passed with a tapered needle under the left anterior descending coronary artery 2 mm from the tip of the left auricle, and a nontraumatic balloon occluder was applied on the artery. Myocardial infarction was induced by inflating the balloon occluder for 30 min. Successful performance of coronary occlusion and reperfusion was verified by visual inspection (i.e., by noting the development of a pale color in the distal myocardium upon inflation of the balloon and the return of a bright red color due to hyperemia after deflation) and by observing S-T segment elevation and widening of the QRS complex on the ECG during ischemia and their resolution after reperfusion [27]. Following reperfusion, the chest was closed in layers and mice were allowed to recover. To replenish perioperative fluid loss, Dextran 40 (10% v/v in 0.9% sodium chloride) was infused after surgery. Mice were euthanized at serial time-points and blood samples were collected for flow cytometry, mRNA analysis, and immunocytochemistry.

Flow cytometric analysis and sorting of VSELs and HSCs from peripheral blood

The scheme for flow cytometric analysis and sorting is illustrated in Figure 1. The full population of PB leukocytes (PBLs) was obtained after lysis of RBCs using 1x BD Pharm Lyse Buffer (BD Pharmingen, San Jose, CA). Cells were stained for CD45, lineage markers, and Sca-1 for 30 min in medium containing 2% fetal bovine serum (FBS). The following fluorochrome-conjugated anti-mouse antibodies were used: rat anti-CD45 (APC-Cy7; clone 30-F11), anti-CD45R/B220 (PE; clone RA3-6B2), anti-Gr-1 (PE; clone RB6-8C5), anti-TCR $\alpha\beta$ (PE; clone H57-597), anti-TCR $\gamma\delta$ (PE; clone GL3), anti-CD11b (PE; clone M1/70), anti-Ter119 (PE; clone TER-119) and anti-Ly-6A/E (Sca-1, biotin; clone E13-161.7, followed by staining with PE-Cy5-conjugated streptavidin) (all from BD Pharmingen). Cells were washed and re-suspended in RPMI 1640 medium with 10% FBS. The percentage of VSELs and HSCs among PBLs was analyzed by flow cytometry using MoFlo (Dako, Carpinteria, CA). The total leukocyte count (per unit volume of PB) was determined using the Hemavet 950, WBC hematology system (Drew Scientific, Oxford, CT). The absolute number of VSELs and HSCs in 1 μ l of blood was computed from the respective percentage contents and the total leukocyte count. For immunocytochemistry and confocal microscopy, Sca-1⁺/Lin[−]/CD45[−] VSELs and Sca-1⁺/Lin[−]/CD45⁺ HSCs were isolated accordingly to a previously described sorting strategy (Fig. 1) [21].

Immunocytochemistry and confocal microscopy

Freshly isolated, PB-derived Sca-1⁺/Lin[−]/CD45[−] VSELs and Sca-1⁺/Lin[−]/CD45⁺ HSCs were plated for 24 h on 22-mm-diameter plates coated with poly-L-lysine (Sigma). Cells were fixed with 4% paraformaldehyde solution for 20 min and permeabilized with 0.1% Triton X-100 for 5 min at room temperature (RT). Blocking with 10% donkey serum (Jackson ImmunoResearch, West Grove, PA) was performed for 30 min at RT to avoid non-specific binding of antibodies. Cells were incubated with primary antibodies against Oct-4 (mouse

monoclonal IgG, Chemicon, 1:200) and CD45 (FITC-conjugated rat monoclonal IgG1, clone 30-F11, BD Pharmingen, 1:100) for 2 h at 37°C. Following washing, cells were incubated with TRITC-conjugated donkey anti-mouse IgG secondary antibody (Jackson ImmunoResearch, 1:200) for 2 h at 37°C. Nuclei were stained with DAPI (Invitrogen) for 10 min at 37°C. Immunofluorescent photomicrographs were acquired using a LSM 510 confocal microscope (Carl Zeiss, Thornwood, NY).

Analysis of surface antigen and Oct-4 expression by ImageStream

The full population of PB leukocytes (PBLs) was obtained after lysis of RBCs using 1x BD Pharm Lyse Buffer (BD Pharmingen). Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 solution for 5 min and stained for Oct-4 using an anti-mouse/human Oct-4 antibody (purified, monoclonal IgG1, Chemicon) followed by incubation with an anti-mouse IgG secondary antibody (BioLegend). Cells were then washed and stained for Sca-1, CD45, and lineage markers. Based on the detection channels available in the ImageStream system, we employed a “cocktail” of rat anti-mouse antibodies against various hematopoietic lineage markers conjugated with PE (clones as described above for flow cytometric analysis), as well as anti-CD45 antibodies (PE; clone 30-F11) and anti-Ly-6A/E antibodies (Sca-1, biotin; clone E13-161.7, followed by staining with streptavidin conjugated with PE-Cy5) (all from BD Pharmingen). In addition, the following antibodies were included in the “lineage cocktail” to exclude circulating MSCs: anti-mouse/rat CD90 (PE, clone: HIS51, eBioscience), anti mouse/human CD29 (PE, clone: HMβ1-1, BioLegend) and anti-mouse CD105 (PE, clone: MJ7/18, eBioscience). Nucleated objects were visualized with the addition of 7-aminoactinomycin D (7-AAD) for 10 min before analysis (40μM; BD Pharmingen). Samples were analyzed with the ImageStream System 100 (Amnis Corporation, Seattle, WA). Signals from FITC, PE, 7-AAD and PE-Cy5 were detected by channels 3, 4, 5, and 6, respectively, while side scatter and brightfield images were collected in channels 1 and 2, respectively.

Assessment of expression of pluripotent genes by quantitative real-time RT-PCR (qRT-PCR)

Total mRNA was isolated from the PBL fraction with the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) and reverse-transcribed with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Quantitative assessment of mRNA expression of markers characterizing pluripotent stem cells (Oct-4, Nanog, Rex-1, Rif1, and Dppa1), hematopoietic cells (Scl), and β2-microglobulin was performed by qRT-PCR using an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The primer sequences (designed with the Primer Express software) have been previously described [20]. A 25-μl reaction mixture containing 12.5 μl of SYBR Green PCR Master Mix and 10 ng of forward and reverse primers was used. The threshold cycle (Ct), i.e., the cycle number at which the amount of amplified gene of interest reached a fixed threshold, was subsequently determined. Relative quantitation of mRNA expression was performed with the comparative Ct method. The relative quantitative value of target, normalized to an endogenous control (β2-microglobulin gene) and relative to a calibrator, was expressed as $2^{-\Delta\Delta Ct}$ (-fold difference), where $\Delta Ct = (Ct \text{ of target genes [Oct-4, Nanog, Rex-1, Rif1, Dppa1, Scl]}) - (Ct \text{ of endogenous control gene } [\beta 2\text{-microglobulin}])$, and $\Delta\Delta Ct = (\Delta Ct \text{ of samples for target gene}) - (\Delta Ct \text{ of calibrator for the target gene})$. To avoid the possibility of amplifying contaminating DNA (i) all of the primers for real-time RT-PCR were designed to contain an intron sequence for specific cDNA amplification; (ii) reactions were performed with appropriate negative controls (template-free controls); (iii) a uniform amplification of the products was rechecked by analyzing the melting curves of the amplified products (dissociation graphs); and (iv) the melting temperature (Tm) was 57–60°C, and the probe Tm was at least 10°C higher than primer Tm. Three independent experiments were performed for each set of genes.

Statistical analysis

Data are mean $\pm$ SEM. The concentration of cells and the quantitative mRNA data (-fold changes in mRNA levels) for cardiac-specific transcriptions factors and those associated with a pluripotent state were analyzed with a one-way ANOVA. If the ANOVA showed an overall difference, *post hoc* contrasts were performed with Student's *t*-tests for unpaired data, and the resulting probability values were adjusted according to the Bonferroni correction. A $P<0.0025$ was considered statistically significant. All statistical analyses were performed using the SPSS (version 8.0) statistical software (SPSS Inc., Chicago, IL).

RESULTS

VSELs are mobilized into the peripheral blood after MI

The numbers of circulating VSELs (Sca-1+/Lin-/CD45-) and HSCs (Sca-1+/Lin-/CD45+) were examined at 24 h, 48 h, and 7 days after MI. At each time point, the percent content of VSELs and HSCs in PB was estimated as exemplified in Figure 1. The absolute numbers of both cell populations per microliter of blood were computed from the respective total leukocyte counts. Combining the percentage of circulating primitive cells with the number of PB cells avoided possible confounding effects of dilution.

In control mice, the number of circulating VSELs was very low (0.98 ± 0.20 and 1.44 ± 0.37 VSELs/ μ l of blood in 6- and 15-wk-old mice, respectively) (Figure 2). The number of circulating VSELs in sham-operated animals at 24 h after the open-chest procedure was similar to that in respective untreated controls (1.20 ± 0.15 and 1.65 ± 0.20 VSELs/ μ l of blood in 6- and 15-wk-old mice, respectively) (Figure 2), indicating that opening the chest, in itself, is not sufficient to mobilize VSELs. Circulating VSELs increased significantly at 24 h after MI in 6-wk-old mice (2.28 ± 0.30 VSELs/ μ l of blood [group III]; $P<0.0025$ vs. both untreated controls and sham controls) (Figure 2). In both age groups, the number of mobilized VSELs peaked at 48 h after MI (3.33 ± 0.37 [group IV] and 7.10 ± 0.89 [group IX] VSELs/ μ l of blood in 6- and 15-wk-old mice, respectively; $P<0.0025$ vs. respective untreated and sham controls) (Figure 2). At 7 days after MI, circulating VSELs were similar to those observed in the respective untreated controls (1.62 ± 0.37 [group V] and 1.63 ± 0.27 [group X] VSELs/ μ l of blood in 6- and 15-wk-old groups, respectively) (Figure 2).

Compared with the number of VSELs, the number of circulating Sca-1+/Lin-/CD45+ HSCs in PB of control mice was greater (5.47 ± 0.81 [group I] and 6.48 ± 0.77 [group VI] HSCs/ μ l of blood in 6- and 15-wk-old mice, respectively) (Figure 3). The number of HSCs did not change significantly at 24 h after sham surgery in either age group (7.69 ± 0.66 [group I] and 5.46 ± 0.74 [group VI] HSCs/ μ l of blood in 6- and 15-wk-old mice, respectively). However, circulating HSCs increased at 24 h after MI (9.20 ± 0.82 [group III] and 8.82 ± 0.53 [group VIII], in 6- and 15-wk-old mice, respectively). HSC mobilization was even greater at 48 h after MI (15.19 ± 1.31 [group IV] and 12.96 ± 1.12 [group IX] HSCs/ μ l of blood in 6- and 15-wk-old mice, respectively; $P<0.0025$ vs. respective untreated and sham controls) followed by a decline at 7 days after MI (11.69 ± 0.92 [group V] and 8.99 ± 0.82 [group X] HSCs/ μ l of blood in 6- and 15-wk-old mice, respectively, $P<0.0025$ vs. sham controls) (Figure 3).

The peripheral blood is enriched in pluripotent primitive cells after acute MI

To confirm the enrichment of PB with VSELs after MI, we evaluated the expression of markers of pluripotency in PB-derived cells harvested at different time-points after coronary occlusion/reperfusion. For this purpose, we employed qRT-PCR to detect mRNA for markers of pluripotency including Oct-4, Nanog, Rex-1, Rif1, and Dppa1. In 6-wk-old mice sacrificed at 48 h after acute MI, we found that the PB was indeed enriched in cells containing mRNA for these markers (10.01 ± 1.98 , 6.02 ± 1.66 , 5.28 ± 1.68 , 2.07 ± 0.99 and 3.18 ± 0.49 -fold increase,

respectively, in mRNA levels of Oct-4, Nanog, Rex-1, Rif1, and Dppa1 compared with untreated controls, $P < 0.05$ for all comparisons, Figure 4, **panel A**). In contrast, in PB cells from 15-wk-old mice the respective mRNA levels of these markers were only 2.24 ± 0.95 , 1.41 ± 0.36 , 2.61 ± 0.84 , 3.43 ± 0.98 and 2.03 ± 1.01 -fold higher compared with untreated controls (Figure 4, **panel B**), indicating that in older mice mobilized cells express considerably lower levels of genes associated with a pluripotent state. At 48 h after MI, we also observed increased mRNA levels of the marker of hematopoietic cells (Scl) in 6-wk-old mice (4.32 ± 1.54 -fold higher compared with respective untreated controls) but not in 15-wk-old mice (0.82 ± 0.32 -fold difference) (Figure 4, **panels A and B**).

Mobilized VSELs isolated from the peripheral blood express Oct-4

The expression of Oct-4 (a marker of pluripotency) at the protein level in mobilized VSELs was examined by immunocytochemistry. For this purpose, both VSELs and control HSCs were isolated from the full population of PB cells by FACS. Sorted cells were stained for CD45 and Oct-4, markers for hematopoietic cells and pluripotency, respectively. Confocal microscopic analysis following immunostaining confirmed that the mobilized and sorted Sca-1+/Lin-/CD45- VSELs were very small ($< 5 \mu\text{m}$ in diameter), were negative for CD45, and expressed Oct-4 (Figure 5, **lower panels**). In contrast, sorted Sca-1+/Lin-/CD45+ HSCs were considerably larger than VSELs, were positive for CD45, and did not express Oct-4 (Figure 5, **upper panels**). These observations were further confirmed by ImageStream analysis, which enables concomitant examination of cellular morphology as well as antigen expression. During ImageStream analysis of mobilized cells, expression of Oct-4 was noted exclusively in very small cells, which were Sca-1+/Lin-/CD45- and exhibited a morphology consistent with the VSEL phenotype (Figure 6). Moreover, Oct-4+ very small cells that were Sca-1+/Lin- were uniformly negative for CD45, indicating that mobilized VSELs with a characteristic cellular morphology and surface marker expression profile did not express even low levels of CD45. Conversely, Oct-4 expression was absent in CD45+ HSCs.

DISCUSSION

Mounting evidence indicates that the adult BM harbors various primitive cells that possess the ability to repair nonhematopoietic organs. In this regard, exogenous cytokine-induced mobilization of BMCs has been shown to be beneficial after stroke as well as MI [6,28]. Moreover, the identification of BMC-derived cells in various injured tissues, including brain, liver, kidney, lung, and heart, indicates that tissue injury can induce mobilization of BMCs from the marrow into the PB [1–5,7,8]. However, the mobilization of pluripotent stem cells after acute MI has never been reported.

Using complementary methods (flow cytometry, qRT-PCR, confocal microscopy, and ImageStream analysis), we report, for the first time, that pluripotent VSELs expressing Oct-4 are mobilized early after acute MI. We did not observe any significant difference in the levels of VSELs in the PB of untreated healthy animals vs. those subjected to an open-chest sham procedure, indicating that surgery in itself does not mobilize pluripotent cells from the BM stem cell pool. In mice subjected to MI, the levels of circulating VSELs were elevated at 24 and 48 h followed by a return to the levels observed in untreated control mice at 7 days. The observations made by flow cytometry were confirmed by qRT-PCR analysis.

Previous studies have shown that various types of BMCs are mobilized after MI. These include hematopoietic stem cells [29,30], mesenchymal stem cells [31], endothelial progenitor cells [29,32], and other distinct subpopulations characterized by surface markers. Circulating CD34+ progenitors [30,33] and CD34+/CXCR4+, CD34+/c-kit+, c-met+ subpopulations [34,35] have been observed in patients after an acute MI. Studies in animals have shown the presence of BM-derived c-kit+, CD31+ cells in the infarcted myocardium after MI [36]. The progenitor

cells detected in PB of patients with acute MI express increased levels of mRNA of early cardiac (GATA-4, Nkx2.5/Csx, and MEF2C) and endothelial (VE-cadherin and von Willebrand factor) markers [34]. Similar results have been obtained in mice [18]. However, the content of pluripotent cells (as reflected by expression of markers of pluripotency) in these mobilized subpopulations was not investigated in the above studies [18,34]. In this study we documented the presence of pluripotent VSELs in blood after MI via a comprehensive approach. First, using flow cytometry, VSELs were identified in the PB by their typical phenotype (Sca-1⁺/Lin⁻/CD45⁻). Second, greater mRNA levels of markers of pluripotency (Oct-4, Nanog, Rex1, Rif-1, and Dppa1) were detected by qRT-PCR. Third, we verified by confocal microscopy the expression of Oct-4, a marker of pluripotency, at the protein level in VSELs, but not in the control population (Sca-1⁺/Lin⁻/CD45⁺ HSCs). Finally, using ImageStream analysis, we confirmed that Oct-4-expressing cells in the peripheral blood exhibit the VSEL phenotype characterized by a very small size and a distinctive surface antigen expression profile. Importantly, by ImageStream analysis mobilized Oct-4⁺ VSELs were negative for surface markers that are commonly expressed in MSCs, including CD90, CD29, and CD105, indicating that the mobilized MSCs were unlikely to be the source of the primitive markers detected by qRT-PCR.

In addition to examining the time-course of VSEL mobilization after MI, we sought to determine whether the release of these cells differs between young (6-wk-old) and older (15-wk-old) mice. Using flow cytometric analysis of surface markers, we found that the kinetics of mobilization of VSELs was similar in 6- and 15-wk-old mice. However, at 48 h after MI (when mobilization peaked), the levels of mRNA for markers of pluripotency, such as Oct-4, Nanog, Rex-1, Rif1, and Dppa1, were lower in 15-wk-old mice compared with 6-wk-old mice. These data indicate that although cells with phenotypic attributes of VSELs (Sca-1⁺/Lin⁻/CD45⁻) were released into the PB of older animals, these cells lost markers of pluripotency with age, an observation that is consistent with previous reports regarding attrition of pluripotency and functionality of stem cells with aging [37–39].

The observation that VSELs are mobilized after MI has important implications for the repair of cardiac and other tissues. We have previously shown that VSELs express markers of pluripotency such as Oct-4, Nanog, and Rex-1 at the mRNA and protein levels [20–22]. We have also documented that under appropriate culture conditions, VSELs give rise to cellular spheres akin to embryoid bodies, expand efficiently resembling cultured embryonic stem cells, and differentiate into the components of all three germ layers *in vitro* [20–22]. Mobilization of these primitive cells from the BM to the PB after MI would be the first step in their involvement in cardiac repair. Therefore, the present findings of markedly increased trafficking of VSELs in the PB early after MI raises the possibility that these pluripotent cells may contribute to myocardial repair in this setting. Our observations also imply that enhancing the mobilization of endogenous VSELs via cytokine or growth factor administration may be utilized therapeutically to promote repair after MI. The development of methods for *in vitro* expansion of VSELs is currently being investigated in our laboratory. Such methods might enable exogenous administration of expanded VSELs to effect myocardial regeneration after MI.

In conclusion, our results demonstrate, for the first time, that pluripotent Sca-1⁺/lin⁻/CD45⁻ VSELs are mobilized from BM after acute MI. The circulating levels of pluripotent VSELs peak early (48 h) after MI, followed by a decrease at 7 days. Consistent with these observations, the PB of infarcted animals is enriched in cells expressing markers of pluripotency (Oct-4, Nanog, Rex1, Rif-1, and Dppa1), although the expression of these genes in VSELs declines with age. The present observations warrant future studies to identify the potential contribution of VSELs to cardiac repair after MI.

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Figure 1.

Flow cytometric analysis of VSELs circulating in the peripheral blood (PB). PB samples were collected at 24 h, 48 h and 7 days after acute MI; at 24 h after sham surgery (sham control); and from untreated mice (control). The full population of PB leukocytes (PBLs) was stained for Sca-1, lineage markers, and CD45. PBLs were visualized in the dot-plot representing their forward (FSC) vs. side scatter characteristics (SSC), which are related to the size and granularity/complexity of cellular contents, respectively. Agranular, small (between 2–10 μ m in size) events, which contain the VSEL population, were included in region R1 (**Panel A**). Cells from region R1 were further analyzed for the expression of Sca-1 and lineage markers (Lin), and only Sca-1⁺/Lin⁻ events were included in region R2 (**Panel B**). Cells from region R2 were subsequently analyzed based on CD45 expression, and CD45⁻ and CD45⁺ subpopulations were visualized on histograms (**Panel C**, regions R3 and R4, respectively). This panel shows representative dot-plots from different groups and time-points. These analyses were repeated a minimum of three times in each mouse, and the percentages reflect the average content of each subpopulation in total PBLs from repeated analyses from each representative mouse. According to the FSC, **Panel D** shows the size of Sca-1⁺/Lin⁻/CD45⁻ cells (VSELs) and Sca-1⁺/Lin⁻/CD45⁺ cells (HSCs) in regions R5 and R6, respectively. Red circles indicate the predominant localization of cells in each subpopulation.

Figure 2.

Time-course of VSEL mobilization after acute MI. Shown is the absolute number of circulating Sca-1⁺/Lin⁻/CD45⁻ VSELs per microliter of blood in untreated (control), sham-operated (sham control), and infarcted mice at 24 h, 48 h, and 7 days after MI. **Panels A and B** represent data obtained from 6- and 15-wk-old mice, respectively. The absolute numbers were calculated based on the percent content of VSELs among PBLs and the total leukocyte count in the peripheral blood. Data are mean $\pm$ SEM. ●, mean; ○, individual mice. * P <0.0025 vs. controls as well as sham controls.

Figure 3.

Time-course of HSC mobilization after acute MI. The figure shows the absolute numbers of circulating Sca-1+/Lin-/CD45+ HSCs per microliter of blood in untreated (control), sham-operated (sham control), and infarcted mice at 24 h, 48 h, and 7 days after MI. **Panels A and B** represent data obtained from 6- and 15-wk-old mice, respectively. The absolute numbers were calculated based on the percent content of HSCs among PBLs and the total leukocyte count. Data mean $\pm$ SEM. $\circ$, mean; $\circ$, individual mice. * $P<0.0025$ vs. controls as well as sham controls in respective age groups.

Figure 4.

mRNA levels of markers of pluripotency (Oct-4, Nanog, Rex1, Rif1, Dppa1) and of hematopoietic stem cells (Scl) in peripheral blood-derived cells from 6- and 15-wk-old mice after acute MI (**Panels A and B**, respectively). Cells isolated from the blood of animals in each experimental group were pooled together to obtain the average content of mRNA at each time point. qRT-PCR was performed in triplicate for all samples. The -fold increase in mRNA content was compared with controls. The average values were calculated based on three reactions. Data are presented as mean $\pm$ SEM. PSC, pluripotent stem cell.

Figure 5.

Expression of Oct-4 in peripheral blood (PB)-derived VSELs. Representative confocal microscopic images of a mobilized VSEL (**lower panels**) and HSC (**upper panels**) isolated from the PB at 24 h after MI. Sca-1+/Lin-/CD45- VSELs and Sca-1+/Lin-/CD45+ HSCs were isolated by FACS followed by immunostaining. The **upper panels** show a Sca-1+/Lin-/CD45+ cell (HSC), which is positive for CD45 (FITC, green fluorescence), a marker of hematopoietic cells, and negative for Oct-4 (TRITC, red fluorescence). The **lower panels** show a Sca-1+/Lin-/CD45- cell (VSEL), which is negative for CD45 and positive for Oct-4, a marker of pluripotent cells. Nuclei were stained with DAPI (blue fluorescence). Tr, transmission image.

Figure 6.

Expression of Oct-4 in peripheral blood (PB)-derived VSELs by ImageStream analysis. Representative ImageStream images of a mobilized VSEL isolated from the PB at 24 h after MI. The Oct-4⁺ (FITC, green fluorescence) VSEL is very small in size (brightfield images), positive for Sca-1 (magenta fluorescence), and negative for Lin and CD45 (**upper panels**). The **lower panels** demonstrate localization of Oct-4 and Sca-1 relative to the nuclear marker 7-AAD in the same VSEL.