Circulating Very Small Embryonic-Like Stem Cells in Cardiovascular Disease

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Abstract Very small embryonic-like cells (VSELs) are a population of stem cells residing in the bone marrow (BM) and several organs, which undergo mobilization into peripheral blood (PB) following acute myocardial infarction and stroke. These cells express markers of pluripotent stem cells (PSCs), such as Oct-4, Nanog, and SSEA-1, as well as early cardiac, endothelial, and neural tissue developmental markers. VSELs can be effectively isolated from the BM, umbilical cord blood, and PB. Peripheral blood and BM-derived VSELs can be expanded in co-culture with C2C12 myoblast feeder layer and undergo differentiation into cells from all three germ layers, including cardiomyocytes and vascular endothelial cells. Isolation of VSELs using fluorescence-activated cell sorting multiparameter live cell sorting system is dependent on gating strategy based on their small size and expression of PSC and absence of hematopoietic lineage markers. VSELs express early cardiac and endothelial lineages markers (GATA-4, Nkx2.5/Csx, VE-cadherin, and von Willebrand factor), SDF-1 chemokine receptor CXCR4, and undergo rapid mobilization in acute MI and ischemic stroke. Experiments in mice showed differentiation of BM-derived VSELs into cardiac myocytes and effectiveness of expanded and pre-differentiated VSELs in improvement of left ventricular ejection fraction after myocardial infarction.

Keywords Very small embryonic-like cells · Bone marrow · Cardiomyocyte

Introduction

Rapid progress in the field of experimental studies on cardiovascular regeneration is being translated to the clinical application of stem cells (SC) isolated from the bone marrow (BM) or the myocardium (cardiac stem cells, CSC). Aim of this approach is to promote the myocardial recovery in patients with acute myocardial infarction (MI) or to improve the cardiac function in the setting of ischemic cardiomyopathy. So far, there is no proof that use of SC can lead to bona fide cardiac regeneration and the mechanism of beneficial effects observed in some studies is probably mediated by paracrine effects leading to neoangiogenesis, reduction of apoptosis, as well as recruitment of CSC to the site of the ischemic injury [1]. At the current state of clinical application of SC, there is no convincing data showing the superiority of any particular-type cells or their source, so heterogenous population of BM-derived mononuclear cells (MNC) is used most often; however, some recent studies assess the efficiency of selected subpopulations of BMC, such as CD133+, CD34+CXCR4+ cells, mesenchymal stromal cells (MSC), or CSC [2].
Despite the encouraging experience from trials using BM-derived MNC novel types of SC carrying higher reparatory potential are clearly needed. Such populations include CSC [3], engineered BM-derived progenitor cells (e.g., C-Cure) [4], allogeneic MSC [5], and pluripotent stem cells (PSC). PSC can be produced using gene transfer (induced pluripotent stem cells) [6, 7] or isolated from the adult tissues (very small embryonic-like stem cells [VSELs] [8]). Isolation of PSC from adult tissues seems to be very promising approach because cells obtained in such way are ethically acceptable, however efficient methods of isolation and expansion in culture of human cells are still not available [9, 10]. This review discusses the recent data on characteristics and potential clinical application of VSELs.

Potential Role of PSC, Including VSELs in Adult Organisms

Adult tissue PSC represent a population of epiblast-derived progeny which survive into adulthood in different locations in BM and solid organs. We hypothesized that these cells, including VSELs, migrate during embryogenesis along with hematopoietic stem cells (HSCs) to the BM, and their migration follows the gradient of chemotactants, including chemokine stromal cell-derived factor-1 (SDF-1) [11]. Their potential role is to be a reserve population of SC and tissue-committed progenitor cells which can be mobilized after tissue injury. VSELs are primitive cells expressing the markers typical for primordial germ cells including Stella, Fragilis, Nobox, Hdac6, and CXCR4. We hypothesize that quiescent VSELs serve as a reserve pool of PSC and are part of the physiological mechanism of tissue repair and renewal of resident SC [11]. Their quiescence is a safety mechanism preventing the formation of teratomas.

Isolation and Sources of VSELs

Initially, rare population of VSELs was isolated from adult murine BM by Kucia et al. by multiparameter fluorescence-activated cell sorting (FACS). Our group established the criteria for sorting of VSELs based on the presence of several surface markers and the diameter of the cells. Figure 1 shows
the gating strategy used for FACS sorting. The detailed description of the protocol was published elsewhere. Briefly, the initial step is the lysis of red blood cells to obtain the fraction of nucleated cells. Erythrocyte lysis buffer is used instead of Ficoll centrifugation because the latter approach might deplete the population of very small cells [12].

Subsequently, cells are stained with antibodies against Sca-1 (murine VSELs) or CD133 (human VSELs), pan-hematopoietic antigen (CD45), hematopoietic lineage markers (lin), and CXCR4 and sorted using a multiparameter, live sterile cell sorting systems (MoFlo, Beckman Coulter; FACSAria, Beckton Dickinson) [8]. We used “extended lymphocyte gate” to include events with diameter 2–10 μm, including approximately 95% of VSELs. The width of the gate was validated by using synthetic beads of predefined size (1–15 μm) [12]. Proper definition of the gate is crucial because this area of the cytogram includes not only cells, but also cellular debris [13, 14].

Several other approaches to define the population of small cells were used, including ImageStream system, which is a combination of FACS and immunofluorescent (IF) microscope and allows to “decode” any particular events on cytogram and visualize it to confirm morphology and presence of markers consistent with VSELs phenotype [13, 14]. VSELs were so far isolated in mice (BM, peripheral blood, fetal liver, and several solid organs in adult animals including brain, heart, retina, kidneys, pancreas, skeletal muscles spleen, and thymus) and humans [15]. In humans, VSELs were successfully isolated from umbilical cord blood (UCB) and peripheral blood (PB). Currently, our group investigates the presence of VSELs in adult BM and myocardium [16, 17].

Structural, Molecular, and Functional Characteristics of Freshly Isolated VSELs

VSELs confer a very small population of cells, and their number is as low as 0.030±0.008% of total BM cells in mice. Both murine BM-derived as well as human PB-derived VSELs have significantly smaller diameter than monocytes and granulocytes, and are larger than platelets [4, 5]. In addition, some differences between murine and human VSELs were observed. Human UCB- and PB-derived VSELs consist of lin CD45-CXCR4+CD133+ and consistently with differences in mean size of leukocytes and RBC between humans and mice are larger than murine (~6–7 μm) [13]. Population of Sca-1+lin CD45+ cells was enriched for PSC markers, such as Oct-4, Nanog, SSEA-1, Rex1, and Dppa3, Rif-1. In addition, VSELs express CXCR4 and migrated to the SDF-1 gradient. Several imaging tools were used to define the morphology of VSELs on the single cell level. Studies using transmission electron microscopy confirmed that VSELs differed in several aspects from HSC. As shown on Fig. 2, VSELs are significantly smaller than HSCs (3–6 vs. 6–8 μm) and have higher nucleus/cytoplasm ratio. Nucleus is large, contains open-type chromatin and is surrounded by the narrow rim of cytoplasm with numerous mitochondria. Therefore, their morphology is consistent with primitive PSC [8, 18, 19]. VSELs display non-hematopoietic immunophenotype (lin CD45+) and do not offer radioprotection in lethally irradiated recipient mice. Freshly isolated and expanded VSELs do not form hematopoietic colonies in vitro [13, 20]. Distinct immunophenotype and size are major criteria for isolation of VSELs, and the presence of PSC markers was confirmed using real-time RT-PCR, at protein level by IF staining and ImageStream system. Importantly, because of the possibility of the detection of pseudogenes raised by some investigators, our group recently demonstrated that the promoters of Oct-4 and Nanog in VSELs contain transcriptionally active chromatin [21]. Importantly, VSELs and HSCs are different not only in terms of their morphology.

Freshly isolated VSELs were expanded in co-culture with C2C12 myoblast feeder layer and after 7–10 days, formed sphere-like clusters consisting of a few hundred cells resembling embryoid bodies (VSEL-derived spheres, VSEL-DSs). VSEL-DSs expressed placenta-like alkaline phosphatase. Co-culture allowed for expansion of VSELs, and after isolation of expanded VSELs, we demonstrated their capacity to differentiate into cell lines from all three germ layers, such as mesodermal cardiomyocytes, ectodermal neural cells, and endodermal pancreatic cells [22]. Importantly, the pluripotent features of VSELs are retained in the population of mobilized to PB murine VSELs. Such circulating cells showed expression of PSC markers on the level similar to ES-D3 murine embryonic cells. Such observations support the hypothesis that mobilized cells might contribute to tissue repair because of their broad differentiation capacity [18].

VSELs display morphological and molecular features of PSCs. Several molecular markers are consistent with PSC phenotype, such as Oct-4 and Nanog, presence of bivalent domains in promoters of developmentally important transcription factors (Sox21, Nkx2.2, Dlx1, Lbx14, Hlx9), and partially reactivate inactivated the X chromosome in female PSCs. VSELs differentiate into cells from all three germ layers. Importantly, the pluripotent features observed in murine cells were not demonstrated in human VSELs. In fact, VSELs neither fulfill the criteria for blastocyst complementation nor show the ability to form teratomas in immunodeficient mice. The quiescence of VSELs isolated from adult tissue can be explained by their quiescence related to epigenetic modification of crucial imprinted genes, such as Igf2 and RasGRF1. Quiescence is probably a mechanism of prevention against the formation of teratomas [21].
Mobilization, Circulation, and Homing of VSELs

Bone marrow-derived SC must undergo rapid mobilization in order to participate in tissue repair. In addition, there must be a variety of chemical signals to which the VSELs must respond to orchestrate their homing and engraftment into the ischemic or otherwise damaged tissue (irradiation, burns) [23]. Data from animal studies showed rapid mobilization of SC from the BM to the peripheral blood, as evidenced by an increase of circulating cells enriched with early cardiac markers (GATA-4 and Nkx2.5/Csx), which migrated along the SDF-1 gradient [24, 25].

Acute Myocardial Infarction

Acute MI leads to a generalized inflammatory response with increased production and release of inflammatory markers, and also of chemoattractant factors such as kinins, chemokines, cytokines, and growth factors, components of the complement cascade. This coexists with subsequent mobilization of SC and endothelial progenitor cells as well as leukocytes [24, 26–28].

In healthy humans, a very small number of VSELs [0.8–1.3 cells/μL] can be detected reflecting continuous efflux of SC from the BM [10, 25, 28]. During acute MI, the number of VSELs is significantly higher and the expression of mRNA of PSC and cardiac markers is up-regulated [25]. Numerous hematopoietic and inflammatory cytokines known to regulate mobilization of SC are up-regulated in acute coronary syndromes. Several factors involved in trafficking of VSELs are SDF-1, leukemia inhibitory factor, hepatocyte growth factor, and stem cell factor-CD117 axes [24, 29, 30]. Figure 3 shows the mechanisms of mobilization and homing of VSELs in acute MI.

The number of mobilized VSELs is dependent on several factors, such as age, presence of diabetes, and the extent of myocardial damage [18]. In our hands, mobilization of VSELs was reduced in elderly and diabetic patients with acute MI [31]. Also, patients with more severely impaired cardiac function (reduced left ventricular ejection fraction, high levels of cardiac troponins) had reduced number of circulating cells [25, 32].

Ischemic Stroke

Similar to acute MI, ischemic stroke was associated with significant increase of the number of circulating VSELs in PB. These cells expressed increased levels of mRNA for neural markers (GFAP, nestin, beta-III-tubulin, Olig1, Olig2, Sox2,
and Musashi) as well as PSC markers (Oct-4, Nanog). This suggests that mobilization of VSELs is an important mechanism in different forms of tissue ischemia [33].

Physical Exercise

Physical exercise, especially a regular one, was shown to increase the number and improve the function of circulating SC and progenitor cells. Our preliminary data showed that intensive treadmill exercise induced the transient mobilization of VSELs into peripheral blood. An increased number of VSELs was detected as early as after the exercise, and circulating cells showed increased expression of mRNA for PSC (Oct-4, Nanog), cardiac (GATA-4, Nkx2.5/Csx, MEF2C), and endothelial markers (VE-cadherin). Mobilization was negatively correlated with an extent of coronary...
Potential Application of VSELs and VSEL-Enriched Populations in Clinical Trials

The measurement of the number of VSELs might, after validation in larger population, serve as a prognostic marker in acute MI because the preliminary data showed inverse correlation with the extent of myocardial injury and presence of co-morbidities, such as diabetes. Another approach is to use the cells for prevention of left ventricular dysfunction after MI. We developed a protocol for expansion and differentiation of BM-derived VSELs into cardiac myocytes. The differentiation of murine VSEL-derived cardiac myocytes (CM) closely resembles the same process in embryonic stem cell-derived CM [8, 34]. Dawn et al. showed that direct intramyocardial injection of freshly isolated VSELs in mice with reperfused MI improved global and regional left ventricular contractility. Moreover, the beneficial effects were observed after 35 days of follow-up. The histopathology study showed rare VSEL-derived cardiac myocytes in the recipients’ heart muscle [35]. Additionally, expansion and pre-differentiation of VSELs in cardiopoiesis-guided media for 5 days before the injection increased their effectiveness leading to increase of left ventricular ejection fraction, myocardial systolic thickening, and attenuated remodeling after 35 days post-MI [36]. Clinical studies using autologous VSELs are needed to validate these promising experimental data, providing that clinically approved protocols for cell expansion and differentiation are available. Several other emerging stem cell technologies such as CSC, genetically engineered bone marrow progenitor cells (e.g., EPC over-expressing endothelial nitric oxide synthase), allogeneic bone marrow-derived MSC and cardiopoiesis-guided bone marrow-derived mesenchymal cardiopoietic cells are under translation into clinical use.

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