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RESEARCH ARTICLE

OVERVIEW OF STEM CELLS AND THEIR RESEARCH IN BURN PATIENTS

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ABSTRACT

An overview of stem cells, their populations, properties, functions and various antigen markers used in stem cell differentiation. A demonstrated analysis of circulating hemopoietic stem cells (HSCs) in humans, specifically in burn patients along with data showing significant rise in absolute and relative quantities of HSCs in burn patients as compared to healthy individuals, an event triggered presumably by the tissue hemopoietic regeneration needs.

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INTRODUCTION

A highly precise orchestration of complex signaling cascades is required in order to coordinate growth of spatially proximate, but physiologically different distinct tissue structures. While this process is facilitated in many cases by proliferation, migration and differentiation of local progenitor cells, selective recruitment of bone marrow-derived stem cells and progenitor cells mobilized into a circulatory pathway, is also thought to play a role.

Stem Cells

Stem cells are a class of a wide variety of undifferentiated cells that share properties of self-renewal, differentiation and self-sustenance, but differ in their differentiation potential and potency (Nimer, 2009; Belousov *et al*., 2005; Lishuk, Mostkova, 2003). The bone marrow acts as a reservoir for multiple stem cell populations. The main types, which, presumably, play a key part in tissue regenerative processes, are: hemopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), very small and embryonic like cells (VSELs), all of which mobilize at varying degrees into the peripheral circulation following injury (including experimental injury). Subsets of these cells have also demonstrated distinct ability to home to a variety of damaged tissues (muscles, heart, kidneys, skin, bone, liver, brain), where presumably, they variably contribute to tissue repair and regeneration via paracrine effects and direct differentiation. Despite this mechanism of endogenous stem cell recruitment, the inability of most adult tissue to regenerate following injury suggests that these mechanisms are not all-powerful and are easily overwhelmed. Therapies, attempting to augment bone marrow stem cell involvement following insult, have been developed and have demonstrated the ability to mitigate injury and enhance regenerative capacity of adult tissue in a variety of preclinical models. Effective clinical translation of these methods, however, still lags behind. Endothelial progenitor cells (EPCs) are rare circulating cells that have the ability to integrate into foci of neovascularization and participate in vasculogenesis, a process of forming new blood vessels, bypassing the use of local angiogenic cell mechanism (Hristov *et al.,* 2003; Urbich, Dimmeter, 2004). Various subpopulations of hemopoietic and non-hemopoietic cells (EPCs) possessing a distinct ability for paracrine effects or self-differentiation have been identified. Mesenchymal stem cells (MSCs) are multipotent, non-hemopoietic stromal cells, which can be isolated from various adult organs and tissues, including bone marrow, adipose tissue and others (Bernardo *et al.,* 2009; Dicker *et al.*, 2005; Pyko, 2007). Mobilized bone marrow MSCs home to injury sites, where they are thought to

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contribute to tissue repair and regeneration through paracrine support of injured cells (HGF, EGF, VEGF, SFRP-4), regulation of extracellular matrix remodeling, immune response (IL-1 antagonist, IL-10) and local progenitor cell proliferation and differentiation. Very small and embryonic like stem cells (VSELs) are a population of primitive pluripotent stem cells found in bone marrow and other adult organs (O'Shea, 1999). They share several features typical for embryonic stem cells: including a small size, a large nucleus surrounded by narrow cytoplasmic rim, open-type chromatin and ability to differentiate into all three germ layers. VSELs have not been studied thoroughly, yet they demonstrate examples of their ability to migrate and integrate into injury sites.

Hemopoietic stem cells (HSCs)

HSCs are self-renewing, multipotent bone marrow cells, responsible for replenishing all cellular components of blood, including leukocytes, erythrocytes and thrombocytes. HSCs are relatively rare, comprising approximately 0.01 - 0.15% of nucleated bone marrow cells, and can be characterized based on their capacity for sustained bone marrow reconstitution (long- vs. short-term HSCs regenerative activity). HSCs are typically isolated based on certain surface antigen expression, and although these profiles are constantly evolving, common definitions include lack of lineage-specific markers and positivity for CD45, C-complex, CD34, CD38 and CD133.

All cells of hemopoietic origin are formed of primitive (uncommitted) blood forming stem cells, localized in bone marrow, which give rise to four main blood cell lineages:

- Erythroid (erythrocytes);
- Megakaryocyte (thrombocyte);
- Myeloid (granulocytes and mononuclear phagocytes);
- Lymphoid (lymphocytes)

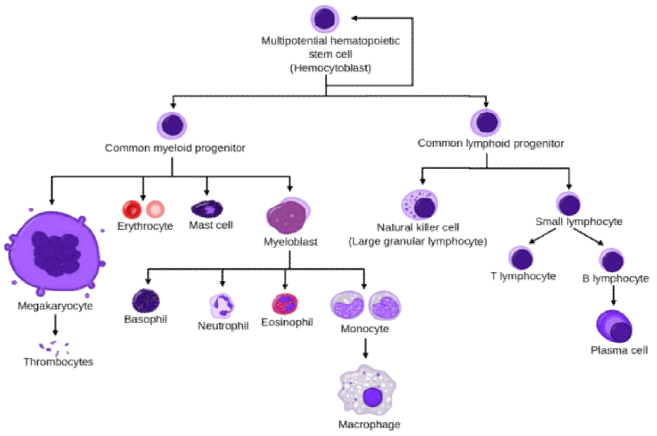

Figure 1. Overview of hemopoiesis

Following various injuries, such as heart failure, stroke, liver damage and skin burns, HSCs have demonstrated ability to mobilize from bone marrow to circulation, although their contribution to tissue repair and regeneration remains uncertain (Baker *et al.,* 2011; Armenian *et al.,* 2012; Kida, McDonald, 2012; Baker *et al.,* 2012; Kramer *et al.,* 2000). They are the central component of after-burn anemia repopulation. Initially, based on early preclinical studies, it was presumed that HSCs can aid in repairing damaged tissue through direct differentiation. However, evidence suggests that HSC and other early progenitor cells can exert a paracrine influence on damaged tissues, along with anti-inflammatory and other effects.

Stem cell "diagnostic" markers

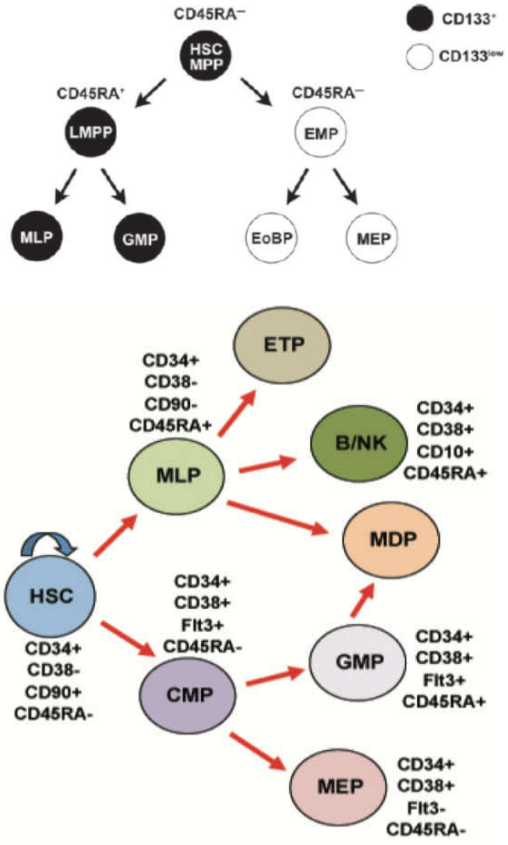

Figure 2. Overview of changes in marker expression during differentiation

Based on existing data about surface markers of cell types listed above, it was decided to use the following combinations of antibodies for identifying the circulating stem cells by method of flow cytofluorimetry: CD34+/CD45+/CD133+, CD38+/CD45+/CD133+. These combinations allow a complex assessment of the widest range of circulating stem cells, hemopoietic stem cells - CD34±/CD38±/ CD45+/ CD133+, EPCs – CD31+/CD34+/CD133+, VSELs – CD34+/ CD133+/CXCR4+.

CD34

CD34 is a membrane protein, an important adhesion molecule that plays a role in early hematopoiesis. CD34 mediates the attachment of stem cells to the bone marrow extracellular matrix or directly to stromal cells. It also serves as a protein scaffold for the attachment of lineage specific glycans, allowing stem cells to bind to lectins expressed by stromal cells or other marrow components. CD34 is a member of CD34 protein family, which also includes Podocalyxin and Endocalyxin on the basis of conserved domains and genomic organization. Although these proteins exhibit similar structure, CD34 is the only member of the three routinely used clinically for the identification of stem cells (Sutherland *et al.,* 1993; Greaves *et al.,* 1995; Kuci*et al.*, 2003). Structurally, CD34 is a single transmembrane spiral whose extracellular N-terminal domain is much larger than its intracellular C-terminus. Of the

3 proteins in the family, CD34 happens to be the smallest. The N-terminal domain contains many serine, threonine, and proline amino acids, which are heavily O-glycosylated and sialylated; in addition, it contains possible N-glycosylation sites. The extracellular portion of the protein has a cysteine-containing globular domain, while the C-terminus of CD34 contains many consensus phosphorylation sites and a PDZ-binding domain. The extensive post-translational modifications described above account for a much higher observed molecular mass (~90 kDa) than that predicted for the protein (~35kDa).

Despite the well-defined structure of the protein and its usefulness in identifying hematopoietic stem cells, the actual cellular functions of the CD34 antigen have remained relatively unknown. Of the many presumed roles of the CD34 protein, evidence most strongly points to the following:

- Promoting the proliferation of hematopoietic progenitor cells;
- Promoting lymphocyte adhesion to vascular endothelium via binding to L-selectin, in addition to improving cellular adhesion in general;
- Preventing the activation of integrins;
- Involvement in hematopoiesis
- Involvement in mobilization of hematopoietic cells

CD38

CD38 is a multifunctional membrane surface glycoprotein, expressed on a wide variety of cell types, including T- and B-lymphocytes in specific developmental stages. CD38 also functions as a transmembrane receptor in signal transduction, as well as an extracellular fermentor (Dërken *et al.,* 1989; Ghia *et al.,* 2003; Tenca *et al.,* 2003). It plays a distinct role in adhesion of lymphocytes to endothelium of vessels. As a transmembrane receptor, CD38 can transmit both positive and negative signals, which regulate T- and B-lymphocyte proliferation and differentiation. There is evidence that the monoclonal antibodies (mAb) of CD38 and CD38-ligand can under certain conditions stimulate or prevent lymphocyte apoptosis in humans. Among other CD38-ligand effects, kinase activation and protein phosphorylation are noted. The extracellular part of CD38 molecule functions as a fermentor, catalyzing synthesis and hydrolysis of cyclical ADP-ribose, which recently has been recognized as a powerful Ca^{2+} mobilizing agent. Fermenting functions of CD38, likely carry immunoregulatory connotation. Given that the cyclical ADP-ribose synthesizes in extracellular sites, it remains unclear how it can function as a typical intracellular signal transduction molecule at the same time. Along with the membrane-bound CD38 antigen, cells produce soluble CD38 (sCD38), possessing a molecular mass of 39 kDa. It has the ability to attach with mAb against CD38 and soluble CD31 antigen (120-130 kDa), which functions as CD38-ligand. It is thought that sCD38 has a similar functional designation as membrane-bound CD38 antigen. Moreover, there is evidence suggesting that sCD38 is able to perform regulatory functions in intracellular interactions in normal and pathological conditions.

CD45

CD45, a leukocyte common antigen, is present on the surface of all hematopoietic cells, except adult erythrocytes. CD45 can be alternatively spliced, which produces isoforms expressed on various lymphocytes. The human memory T cells contain a CD45 isoform RO-epitope, while common T-lymphocytes express the CD45 RA-epitope. CD45 expression is crucial for signal transduction via the T-cell receptor. CD45 cytoplasmic domain has an intrinsic tyrosine phosphatase activity and regulates work in TcR – CD3 complex receptor tyrosine kinase. CD45 is a member of tyrosine protein phosphatase family (PTP). Tyrosine protein phosphatase enzymes are components of signal transduction pathways, and regulate cell growth, differentiation, mitotic cycle and suppress malignant cells. The molecule has an extracellular domain, singular transmembrane-segment and two cytoplasmic catalytic domains. As a tyrosine protein phosphatase receptor, PTPRC binds the ligand in the extracellular domain and sends a signal inside the cell via target protein dephosphorylation in the cytoplasm. This gene is specifically expressed on hemopoietic cells and is a key regulator of signal transduction of T- and B-cell antigen receptors. It is capable of directly interacting with all the antigen receptor components, as well as activateSrc-family kinase, necessary for signal transduction from these receptors. CD45 also suppresses JAK kinases, thus, negatively regulating cytokine receptor signal transduction. PTPRC mRNA undergoes alternative splicing and gives rise to several isoforms.

CD133

Antigen CD133, also known as prominine-1, is a glycoprotein, a member of pentaspan transmembrane glycoprotein family (5 transmembrane proteins, 5-TM), which specifically localizes to cellular protrusions. While the precise function of CD133 remains unknown, it has been proposed as an organizer of cell membrane topology. CD133 is expressed on hemopoietic stem cells, endothelial progenitor cells, glioblastoma, neuronal and glial stem cells, various pediatric brain tumors, as well as kidney, mammary glands, trachea, salivary glands, placenta, digestive tract, testes, and some other adult cell types (Handgretinger *et al.,* 2003; Bonanno *et al.,* 2004; Pfenninger *et al.,* 2007; Pavon *et al.,* 2012).

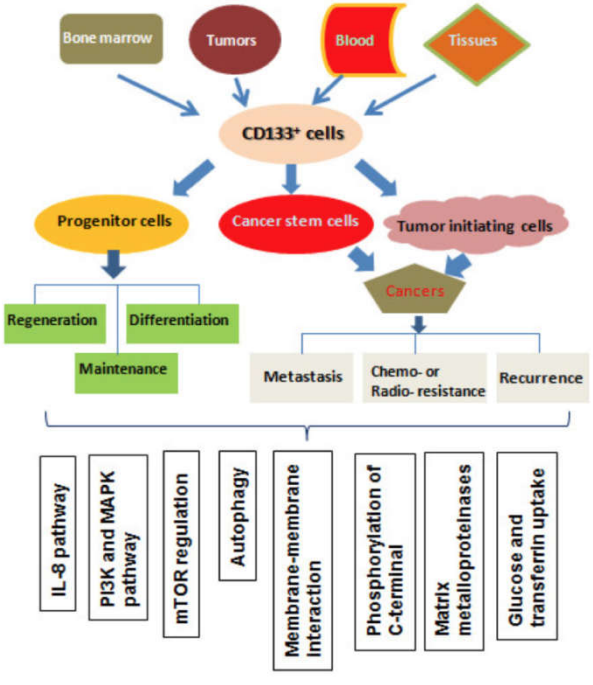

Figure 3. CD133 marker expression on hematopoetic and cancer cells

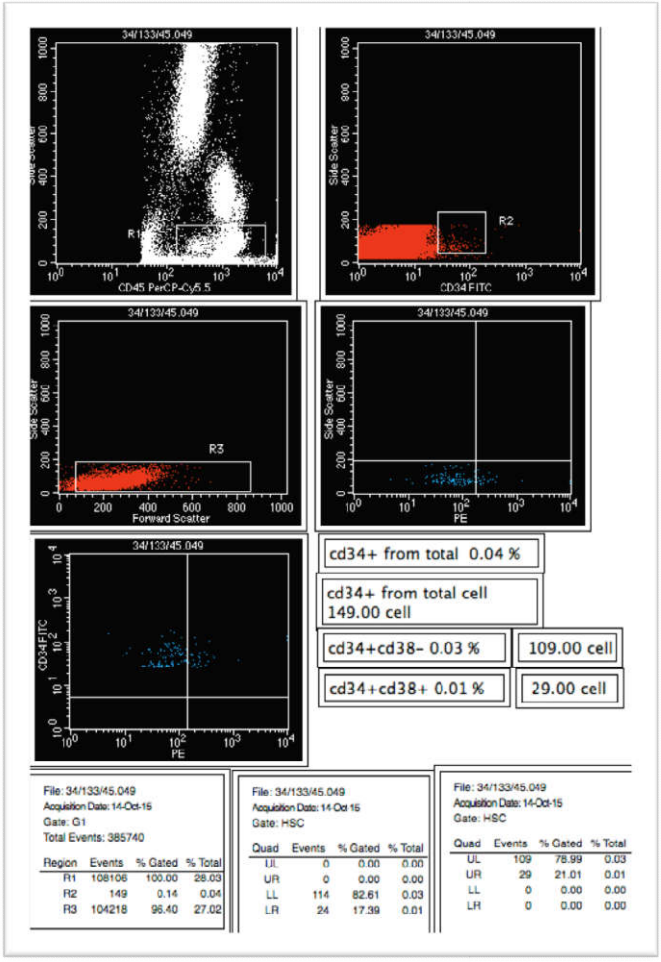

Figure 4. Stages of HSC selection from peripheral bloodstream (flowcytofluorimetry)

In addition to cell markers, we are especially curious about extracellular markers in activation of regenerative processes. This area of interest includes signal proteins and colony stimulating factors. Vascular Endothelial growth factor (VEGF), is a signal protein produced by cells that stimulate the formation of blood vessels. At the present moment, several different factors of this protein family has been identified. VEGF proteins serve as a part of a system responsible for restoring oxygen supply to tissues in situations when blood circulation is not sufficient. Concentrations of VEGF in blood serum rises with bronchial asthma and, possibly, after heavy injuries. The main functions of VEGF include: creating of new blood vessels in embryonic development or after injury, increasing muscle growth after physical exercise, and providing collateral blood circulation support (creating of new vessels to bypass the existing blocked ones). Granulocyte (G-CSF) is a CSF 3 colony stimulating factor, which stimulates the granulocyte colony formation in cell culture. G-CSF stimulates proliferation and differentiation of late progenitor cells into neutrophils, as well as stimulates the brain marrow to release stem cells into bloodstream. The G-CSF and GM-CSF cytokines promote production of neutrophils and monocytes.

MATERIALS AND METHODS

To study the activation of regenerative processes in burn patients (as well as patients with other types acute and chronic pathological conditions), flow cytometry method on a flow cytometer FACSCalibur (Becton Dickinson, USA) was utilized. To determine the relative and absolute quantity of circulating stem cells, a set of monoclonal antibodies marked with fluorochromes FITC and PE was used. To perform an analysis of circulating hemopoietic stem cells, a cell marker analysis protocol was established as follows: CD45(PC5)/ CD34(FITC)/CD38(PE) and CD45(PC5)/CD34 (FITC)/CD133 (PE). This sequence reflects also the stage, where the lineage-negative cells were being isolated. The count yielded at least 500 thousand cells. A polygonal region of lymphocyte cells represented 100% value.

RESULTS

A baseline study was conducted with a group of 10 healthy individuals to compare their results with those of the patients. All volunteers were absolutely healthy without any acute inflammatory or chronic diseases. Mean (average) donor age was 34 years (20 – 60). Cells expressing CD45+/CD34+/ CD38+ and CD45+ /CD34+/CD133+ markers, were categorized as HSCs. Despite additionally used CD38+ or CD133+ additional markers, the HSC count remained steady with validity. HSCs comprised 0,041±0, 0035 and 0,05±0,0037% (P=0,11), accordingly quantitatively 268±25, 22 and 285,6 ± 22,96 cells (P=0,16). From total HSCs, the norm for CD38-negative and CD133-positive adult blood forming stem cells (PBSCs) was at 0,031 ± 0,00277 and 0,0263 ± 0,00263% (P=0,24), quantitatively - 155±13,35 and 187,8±17,8 cells (P=0,16). Accordingly, the norm for CD38-positive and CD133-negative adult PBSCs comprised 0,017±0,0034 and 0,0167± 0,0029% (P>0.05), quantitatively - 71±11,94 and 110,1±19,52 cells (P=0,1). In this instance, once again, no differences were found between the two early and two adult subpopulations of PBSCs. In this way, the peripheral bloodstream total HSC content has shown to comprise 0,041-0,05% (268 - 285,6 cells). Further, total circulating HSCs and their subpopulations in burn patients has been analyzed. The patients consisted of 30 burn patients (16 men and 14 women) with average age of 42,9 years old, at different stages of burn complications with thermal injury covering an average of 45% of body surface. Note, thatHSC and HSC subpopulation content varied greatly in cases with burn complications and could fluctuate from completely absent to high, very high contents. Due to this circumstance, it was impossible to determine average cell content per group and determine true differences among the patient groups. The large data variation ratio among the patients frequently comprised ordinal differences (tenfold). For this reason the burn patient group was divided into three clusters: patients with 1) high, 2) very high or 3) completely absent PBSC content. This approach allowed to determine colossal differences in PBSCs and their subpopulation content in burn patients.

However, like in the case with donors, burn patients within-group overall data did not yield statistical differences in contents of two HSC types (0,07246±0,01043 and 0,07274±0,009781% [P>0,05]; 328,5484±43 and 340,4±40,73 cells [P>0,05]), their early HSC subpopulations (0,04788± 0,008249 and 0,037±0,005813% [P>0,05]; 205,9±29,81 and 160,8±20,27 cells [P>0,05]) and adult HSCs (0,02355± 0,003930 and 0,035±0,005775% [P>0,05]; 111,7±19,55 and 164,3±28,79 cells [P>0,05]). Here, we have a valid result showing no change in differences between corresponding phenotypic groups of the same cell type, despite the use of additional markers CD38+ or CD133+. When the donor and burn patient groups were compared, interesting data surfaced.

50% of the total burn patient group had high 0,1344±0,01962, donors - 0,04125±0,003504% [P<0,0123]; 600,3±82,25, donors - 268±25,22 cells [P≤0,0152]), and 30% with very high (CD34+38+45+)PBSCs (0,1836±0,02895 and 0,04125±0,003 504% [P=0,0015]; 873,1±141,5 and 268±25,22 cells [P=0,0007]), which significantly exceeded the data from the donor group. Similarly significant increase was noted in relative PBSCs (CD34+133+45+) as compared with the donor group. An increase of such magnitude in relative and absolute stem cell count was found in 43,3% patients with high (0,096 ±0,01833 and 0,031±0,002769% [P=0,028]; 360±52,65 and 155±13,35 cells [P=0,028]) and 16,7% with very high increase or early (CD45+34+38-) PBSCs (0,21±0,04487 and 0,031±0, 002769% [P=0,0001] and 593,5±99,31 and 155±13,35 cells [P=0,0005]). Accordingly, in 46,7% of equivalent patient groups, early PBSC subpopulations with (CD45+34+133+) phenotype exhibited a sharp increase in comparison with the donor group. A noteworthy fact, 20% of this patient group had zero PBSC content, while donor group contained no such individuals (P<0,0001).

Finally, similar adult PBSC (CD45+34+38+ и CD45+34+ 133-) subpopulations changes were seen in burn patients, divided again into clusters. First subpopulation showed a sharp increase of PBSC as compared with the donor group, in 53,3% with high increase of cells (0,057±0,009634, donors-0,017±0,00335% [P=0,006]; 207,8±35,69 and 71±11,94 cells [P=0,006]), although 33.3% of patients showed complete absence of this phenotype, while donor group contained no such individuals. Close results were collected while analyzing another adult PBSC subpopulation (CD45+34+133-), but only a third of patients showed no evidence of containing this PBSC subpopulation. In this way, a number of burn patients exhibited sharp increase in two PBSC population content, as well as subpopulation of early and adult PBSC, with valid differences.

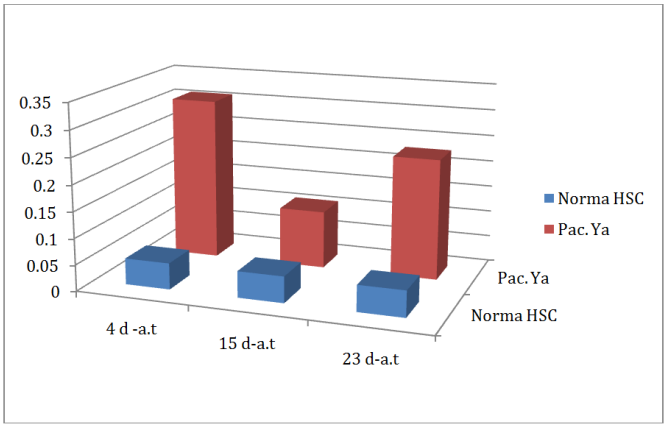

Figure 5. HSC content (% along the ordinate) in patient Ya.at various stages of burninjury. d - days after thermal trauma (a.t)

DISCUSSION

In reviewing possible causes of expressed quantitative modulation of PBSCs and their early and adult populations content, we can infer that expansive and severe thermal trauma with thermal inhalation injury, tubular bone fracture plays a big part in reliably triggering the release of PBSCs from their reservoir into the circulation. Indeed, we have observed a sharp increase in circulating HSC count in patients with autofibrolast transplantation, severe thermal inhalation injury, thermal-chemical or chemical burns, and combined injuries. A proof of this possibility was found in clinical case of patient Y., of 27 years old with combined burn injuries covering 50% of body surface and multiple tubular bone fractures (see Figure 5), whose HSC content % exceeded the norm multiple times and remained in dynamic state for a prolonged period of time. It can be assumed that significant increase of PBSC content in burn patients is brought about by the necessity of these cells in tissue regeneration processes and repair of disrupted hemopoiesis. Increased production of cytokines, nucleic acids and toxic substances can stimulate PBSC proliferation or their intensified migration from corresponding reservoirs. Of special significance is the disappearance of certain PBSC subpopulations in some patients, which can be caused by pool exhaustion of these cells or by their toxic damage. In any case, the PBSC content modulation findings are being discovered for the first time and have a substantial fundamental meaning. They require further pursue of clinical material and discovery of the true causes for such modulation. The HSC kinetics at normal HSC levels is difficult to assess due to lack of research studies, especially ones of early stages. It seems that in burn patients without complications, the normal HSC count either rises or remains within physiological fluctuation parameters, whereas, early HSC deficit is difficult to normalize in dynamic state, which requires a deeper analysis of clinical laboratory data and comparison with clinical burn injury progression. In most cases, patients with HSC deficit exhibited zero values (%) in relative $CD45^{dim}34+38+$ and $CD45^{dim}34+133+$ subpopulation levels, due to deep deficit of absolute blood count of this cell type. In this way, the rising of circulating hemopoietic stem cells in burn patients can be viewed as a natural defense reaction against burn and other types of injury factors. It seems that the rising of circulating HSCs can serve as a beneficial factor, conducive to regeneration and fast healing of burn injuries after autodermoplastics. At the same time, in some cases with patients with weak granulations, slow healing injuries and lysis of autodermotransplants, we observed deep deficit of HSCs and their subpopulations, which may indirectly validate predictive use of markers for forecasting development of chronic complications, recovery process and healing of the injury foci.

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