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Hematopoietic stem cells from peripheral blood the perspective of non-mobilized peripheral blood

Vassilios Katsares*, Zissis Paparidis, Eleni Nikolaidou, Anastasia Petsa, Iliana Karvounidou, Karina-Alina Ardelean, Nikolaos Peroulis, Nikolaos Grigoriadis, John Grigoriadis

Biogenea-Cellgenea Ltd, Trade Centre "Plateia", Thessaloniki, Greece; *Corresponding Author: vkatsare@gmail.com

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ABSTRACT

The peripheral blood is a major source of hematopoietic stem cells. Almost for two decades the peripheral blood has been mobilized, in order to enhance the $CD34^+$ concentration. The isolated stem cells from the mobilized peripheral blood are used as an alternative, or in addition to bone marrow derived stem cells. In this paper, a new perspective is being discussed; the use of non-mobilized peripheral blood as an alternative source for hematopoietic progenitor cells. The number of isolated hematopoietic stem cells is evaluated using flow cytometry. The viability can be evaluated using the trypan blue exclusion test, the flow cytometry or automated assays. The isolated hematopoietic stem cells could be used for ex vivo expansion either in static systems or in proper bioreactor systems, prior to cryopreservation and/or transplantation.

Keywords: Non-Mobilized Peripheral Blood; Hematopoietic Stem Cells; Ex Vivo Expansion

1. INTRODUCTION

Since the early 1990s, peripheral blood progenitor cells collected by apheresis have largely replaced bone marrow as a source of hematopoietic stem cells for autologous transplantation [1]. Peripheral blood cells produce more rapid hematopoietic recovery, thereby leading to reduced costs [2-5]. Furthermore, although follow-up is more limited in the PBSC group than in the BM group, no evidence was found that the use of PBSC was associated with an increased risk of chronic GVHD compared to results with BM [6].

2. CHARACTERIZATION OF HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells are normally found in very limited numbers in the peripheral circulation (less than 0.1% of all nucleated cells). It is logical that progenitor cells circulate in the periphery, as this ensures an even distribution of hematopoiesis within the BM [7]. CD34 antigen expression is used as a surrogate marker for hematopoietic stem cells and enumeration of $CD34^+$ cells has been used to quantify progenitor and stem cell content [8].

PBSCs represent a subpopulation of all $CD34^+$ cells ($CD34^+/CD38^{-}$) found in the circulation [9]. $CD34^+$ cell viability was measured by an established flow cytometric method [10]. The method is based on triple staining with anti-CD34-PE, anti-CD45-FITC, and the viability marker 7-actinomycin D, and it allows the calculation of the absolute numbers of viable $CD34^+$ cells. Recently, a new rapid and accurate method has been developed for the viability evaluation based on luminometry [11].

3. CRYOPRESERVATION AND THAWING OF PBPCS

PBPCs are usually harvested and stored in liquid N_2 until reinfusion. Storage at this low temperature will block all enzymatic pathways and metabolism in the cell [12]. Cryopreservative(s) must be added to the PBSC before freezing in order to protect the cells. The concentration of the cryoprotectant and the rate at which the cells are frozen are the main factors governing the survival of the cells. Thereafter the cells are stored in liquid N_2 [13]. Due to their different cell membrane composition and higher osmotic inactive volume, $CD34^+$ cells are better protected from hypertonic shock and ice crystal formation and should be more resistant to cryopreservation damage than the remaining nucleated cell population [14].

A computer-controlled freezer is used for the cryopreservation. In order to ensure rate-controlled freezing an optimized program is developed and adjusted accordingly.

In controlled rate freezing, the concentrated stem cells are frozen down at a rate of $1\text{-}2^0$C/min up to a tempera-

ture point of about –40˚C. Then, the freezing process down to a target of –120˚C is performed at a faster pace, about 3-5˚C/min. For PBSCs the controlled rate freezing process is considered standard [15,16], and was in different reports found to be superior to uncontrolled freezing approaches. This procedure is time consuming and requires staff with a specific expertise. Hence, the use of uncontrolled rate freezing in which the specimen is first cooled down to –4˚C and then directly deposited into a freezer at –80˚C or put into liquid phase nitrogen has been evaluated. Several reports [17-19] established that the uncontrolled method is safe and reveals comparable results to the controlled rate process for PBSCs. A controlled study performed by Perez-Oteyza *et al*. [20] showed that the controlled and uncontrolled rate freezing approach are comparable in terms of viability testing and that only a statistically significant decrease in the CFU-GM clonality assay could be detected in the uncontrolled freezing situation. Recent studies suggested that uncontrolled freezing is also a viable approach for UCB stem cells [21,22].

As a cryoprotectant, a solution containing 50% DMSO in HAES-steril® 10% is used. Prior to freezing, a part of pre-cooled cryosolution (with 50% DMSO) is mixed with three parts of the buffy coat (pre-cooled), to achieve DMSO concentration of 5 or 10% in the final solution. Cryopreservation is then carried out in aliquots in cryogenic vials.

Current protocols for cryopreservation of PBPCs are usually based on the use of 10 percent DMSO in the freezing medium [23]. HPCs can be preserved with 5% DMSO, and such autografts can be safely used for stem cell rescue even after long-term nitrogen storage [24].

Several techniques for the thawing procedure have been proposed. The standard method is warming in a water bath at 37˚C until all ice crystals disappear [19]. A German study compared the thawing of cryopreserved units in a warm water bath with dry heat applied by gel pads at 37˚C. The viability and clonogenic potential were comparable, with a trend towards less infectious contamination in the dry method [25]. Different studies examined the preservation of function when thawed units were incubated at 0-37˚C [19,26].

Akkök *et al.* [25] suggest that even simple single-wash DMSO depletion causes significant $CD34^+$ cell loss. Despite a beneficial impact on the frequency of adverse effects during and after stem cell infusion, this time-consuming procedure caused a delayed PLT recovery and increased requirement for PLT transfusions. The $CD34^+$ cell loss, however, was never critically low, that is, never lower than 2×10^6 per kg. They concluded that single manual washing of autografts is a simple and safe procedure that decreases the frequency of adverse events during and after stem cell infusion. The procedure should be recommended especially for patients with an increased risk of serious toxicity, for example, patients with cardiac amyloidosis.

4. THERAPEUTIC DOSES OF PBSC

Typical doses of $CD34^+$ stem cells used for PBSCs are 2×10^6 cells/kg of recipient body weight or greater. Doses lower than this threshold is associated with prolonged cytopenias and increased early mortality [27]. The use of higher doses of $CD34^+$ cells may lead to quicker engraftment, particularly when doses are greatly increased [28,29]. Platelet recovery appears to be more sensitive to $CD34^+$ doses than neutrophil recovery [29]. Efforts to enrich PBSCT by ex-vivo $CD34^+$ cell selection (positive selection) have resulted in increased rates of GVHD, possibly by altering the cytokine expression patterns of transplanted cells or changing lymphocyte subsets delivered with the graft [30].

Autologous stem cell grafting has been used with varying degrees of success in chronic myelogenous leukemia (CML) [31,32], acute leukemia [33], myelodysplasia [34], and multiple myeloma [35].

Niwa *et al*. [36] reported successful autologous peripheral blood stem cell transplantation with a double-conditioning regimen for recurrent hepatoblastoma after liver transplantation.

Nevskaya *et al*. [37] with preliminary results suggested the feasibility of therapeutic angiogenesis by local implantation of $CD34^+$ and MNC from PB for Systemic Sclerosis ischemic ulcers. Improved endothelial function, stimulatory effects on circulating endothelial precursors kinetics and augmentation of microcirculatory blood flow may contribute to therapeutic potential of the implanted cells.

5. COST ANALYSIS

The cost method involved two sets of data: a data set including patient-related or direct costs, and a data set including nonpatient-related or indirect costs [38]. The patient-related costs comprise the followings: 1) hospitalization and basic medical service, including medical and nursing staff; 2) pharmacy and blood products; 3) procedures such as operating theatre, leukapheresis and cryopreservation. On the other hand, the indirect costs comprise the clinical service department costs, for instance, radiology, clinical chemistry, pharmacy and the nonclinical service departments such as transportation, housekeeping and kitchen services.

A study by Mishra *et al*. [38] reported a cost analysis using mobilized peripheral blood at 2001 prices and the

costs had been recalculated into US$ by using the exchange rates of 1st January 2001. The mean cost for the mobilization/cryopreservation phase per patient was US$ 6544 (range 5114-7273). The mean cost of high dose chemotherapy followed by hospitalization was US$ 25616 (13978-43277). This amounts to total running costs of US$ 32160 (19092-50550). Taken together, staffing, medication and blood products contributed to 74% of total costs. On average, 53% of total costs comprised staff costs, ranging from 39 to 76%. Personnel resources varied from one center to another, from US$ 12608 to US$ 26038 per patient. Pharmacy and blood products contributed 16 and 5%, respectively, of the total costs.

A study by van Agthoven [39] documented total costs of PBSC transplantation at Euro 33742. The author applied a unit cost method where staff costs accounted for 42% of the transplant cost. This relatively large difference in staff costs between Van Agthoven and Mishra *et al*. is notable, and may indicate there are cost variations between different countries, for example, related to wages. Van Agthoven [39] reported a remarkably low cost per patient for the stem cell harvesting and cryopreservation procedures, an average of €4982, and a blood component cost (during the induction chemotherapy regimen, harvesting and transplantation phase), respectively, of €904, €376 and €1680, a total of €2960.

In another study, Ghosh *et al*. [40] reported a PBSCT cost for patients with plasma cell leukemia that ranged from US$ 20000 to US$ 25000. The major part of the costs related to hospitalization, growth factors, blood products, collection and cryopreservation of PBSC.
Hopefully, the use of non mobilized peripheral blood could eliminate the cost, since there are no mobilization drugs and no any special equipment required.

6. EX VIVO EXPANSION OF HSC

The $CD34^+$ surface antigen, which is a glycoprotein expressed on early progenitor cells is present on less than 0.1% of the mononuclear cells in peripheral blood [41]. Many studies have shown that the minimum acceptable dose of HPCs for successful transplantation ranges between $2 – 5 \times 10^6$ $CD34^+$ cells per kg of recipient weight [42]. Furthermore, transplantation of higher doses of $CD34^+$ cells seems to improve haematopoietic recovery and overall survival [43,44]. To try and overcome the problem of low progenitor cell dose, ex vivo expansion of CB-derived cells has been attempted. The true test of this method is whether an expansion technology will be able to provide a reliable, reproducible increase in the number of progenitor cells available from a single unit of CB, resulting in superior rates of engraftment and overall survival in adult patients. A significant hurdle of presently available methods for graft production is the ability to generate an expanded population of committed hematopoietic progenitor cells without compromising the numbers of less differentiated progenitor cells ($CD34^+$ $CD38^-$ or $CD34^+$ Lin^- cells), which are important functional hematopoietic repopulating cells [45].

In order to consistently achieve an adequate cell dose, the processing methods must minimize cell losses. Each additional manipulation of a cellular product potentially leads to further loss of cells. In most studies, $CD34^+$ cell selection is done before initiating cell culture [46], but the $CD34^+$ cell selection itself is associated with a substantial loss of progenitor cells. This cell loss, which may not be significant for smaller children, may become critical in reaching a suitable dose for transplant in older children and adults [47].

To achieve adequate cell doses, many researcher used different ex vivo expansion protocols, either the traditional way, or using a bioreactor system. Beshlawy *et al*. [48] used three cytokine combinations, *i.e.* cell factor alone, IL-3 alone, and both stem cell factor and IL-3. Interleukin-3 enhances the amplification of early and committed progenitor cells without impairing the long-term engraftment of stem cells [49].

Several investigators reported significantly decreased cell viability after cryopreservation [50-51] and attributed this to the effect of thawing and washing to remove the cryoprotectant. Laroche *et al*. [47] stated that thawing and washing result in loss of cells approaching 20% when compared with pre-freeze counts, with the wash step responsible for nearly half of this cell loss. However, Beshlawy *et al*. [48], using umbilical cord blood derived hematopoietic stem cells, detected mean fold expansion of 6.64 ± 3.34 with stem cell factor alone, 7.38 ± 2.86 with both stem cell factor and IL-3, and 8.11 ± 4.49 with IL-3 alone after 2 days culture of the samples frozen for 2 weeks. There were no statistically significant differences in fold expansion between the 3 cytokine combinations before freezing and after 1 week and 2 weeks of freezing. They concluded that although preservation procedures could decrease the count and viability of cord blood HSCs, freezing does not impair their ex vivo expansion potential; however, it results in a significant loss of cell viability.

In a similar study, Moezzi *et al*. [51] used stem cell factor, IL-3, and thrombopoietin and reported levels of expansion of (4.2-4.7 fold) after 7 days of culture of samples cryopreserved for 1 month.

It was shown that a combination of early- and late-acting cytokines, including SCF, thrombopoietin (TPO), G-CSF and IL-3, resulted in only a marginal-fold expansion of late ($CD34^+$) and early ($CD34^+CD38^-$) progenitor

cells, probably due the fact that the late-acting cytokines drive the cultures mainly toward accelerated differentiation [52,53].

On the other hand, cultures with only early-acting cytokines (SCF, TPO, IL-6 and FLT-3 ligand) resulted in better and prolonged expansion of both late and early progenitors [54], which are important for short-term early trilineage engraftment [55-57].

Peled *et al*. [58] suggested that TEPA supports the self renewal division cycle without compromising differentiation capacity of hematopoietic stem cells.

A number of serum-free media have been used over the last few years with different results [59-62]. For obtaining sufficient numbers of progenitor cells for transplant, FCS [63] and autologous plasma [60,61] have been used in clinical expansion protocols. However, Lam *et al*. [61] suggested that, with the appropriate serum-free media and cytokines, FCS may be excluded in clinical expansions. On the other hand, human plasma, which may contain factors that promote cell maturation [64,65] is thus unlikely to add significant value to the expansion.

It has been reported that MSC constitutively secrete various hematopoietic cytokines, among them stem cell factor (SCF), Flt-3 ligand (FL), thrombopoietin (TPO), leukemia-inhibiting factor (LIF), interleukin (IL)-6, IL-7, IL-8, IL-11, IL-12, IL-14, and IL-15 [66-68]. Addition of MSC as a feeder layer has been shown to improve expansion of cord blood HSC, inhibit their differentiation, and decrease their rate of apoptosis [66,69-71]. Li *et al*. [66] demonstrated that bone marrow MSC can increase human adult PBSC expansion as compared with culture in the presence of cytokine alone.

Conventional culture systems such as T-flasks and gas permeable blood bags are the most widely used devices for expanding hematopoietic cells. However, such static culture systems have several inherent limitations. Firstly, lack of mixing results in concentration gradients for dissolved oxygen (DO), pH, cytokines and metabolites. Secondly, the environmental conditions in well-plate and T-flask are not readily monitored or controlled online. Thirdly, static systems require repeated changes of culture medium, which significantly increases the risk of contamination. Hence there is an urgent need for developing bioreactors for HSCs expansion, which overcomes the limitation of mass transport, keeps culture parameters constant and controls differentiation [72].

Several kinds of bioreactors have been applied in the field of HSCs expansion, including stirred tank bioreactor, fixed bed bioreactor and perfusion chamber [73-75]. It is known that hematopoietic cells are extremely sensitive to shear force, hence cells may suffer some physical damage under shear environment like in a stirred tank bioreactor and perfusion chambers [73]. In stirred tank bioreactor, agitation may affect cell surface marker expression, including cytokine receptors, which can have a profound effect on which cells expand and to what extent expansion occurs [73]. A condition with low shear but low concentration gradients is highly desirable for hematopoietic cell expansion [72].

Rotating wall vessel (RWV) bioreactor may provide a technical solution. The RWV bioreactor has several key characteristic features as follows [76]: firstly, fluid flow is near solid body and is laminar at most operating conditions, which avoids the large shear stresses associated with turbulent flow and allows introduction of controlled and nearly homogenous shear fields; secondly, the culture medium is gently mixed by rotation, avoiding the necessity for stirring vanes, which may damage cells by both local turbulence at their surface and the high flow rates created between the vessel walls and the vanes; thirdly, there is no headspace in the RWV bioreactor while in roller bottles, due to incomplete filling of the vessel, the air in the headspace creates turbulence and secondary bubble formation in the culture medium, which are both potent sources of extra shear and turbulence; finally, the RWV bioreactor supports coculture efficiently by bringing different cell types of different size and density together simply and efficiently. So by optimizing the geometry of the bioreactor and operational condition, it is possible to provide a uniform and low shear condition within the bioreactor. At the same time concentration gradient can be minimized.

RWV bioreactors have been used to simulate microgravity in space flight to study how microgravity affects the hematopoiesis of astronauts [77,78], to proliferate BM cells [79]. The RWV bioreactor can provide a 3D suspension culture environment and all hematopoietic cells are suspended in the culture medium effectively, which overcomes the concentration gradients in T-flasks and makes the utilization of cytokines more effective.

The National Aeronautics and Space Administration (NASA) developed two RWV bioreactors for tissue mass culture [80]. The slow turn lateral vessel (STLV) bioreactor has been used to culture several kinds of cells both on Earth and in space. The STLV was operated at 15-30 rpm on Earth and slower in space allowing a free-fall state, reducing the shear stress. The high aspect ratio vessel (HARV) bioreactor has a similar design, but the rotating speed can be slower than STLV. The NASA RWV systems have been used to study the effects of microgravity on murine HSC and evaluating the hematopoietic homeostasis during long space expeditions [81].

The elucidation of mechanisms governing self-renewal and differentiation of HSC is needed to control the in

vitro expansion. Results from pilot clinical trials of transplants using expanded UCB-HSC have shown no adverse effects in the patients. However, more clinical trials must be conducted using expanded HSC for guarantying the safety [82]. Very recently, Delaney *et al*. (2010) claimed that when cord blood progenitors expanded ex vivo in the presence of Notch ligand we infused in a clinical setting after a myeloablative preparative regimen for stem cell transplantation, the time to neutrophil recovery was substantially shortened. This is the first instance of rapid engraftment derived form ex vivo expanded stem/progenitor cells in humans [83].

7. AUTHORS' CONTRIBUTION

All authors contributed substantially to this research. V.K., J.G., and N.G. designed research and collected the data; V.K., Z.P., A.P., E.N., I.K., and K.A.A. performed literature revision; V.K. analysed and interpreted data, and wrote the manuscript. All authors drafted the manuscript, revised it critically and approved it.

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GLOSSARY OF ABBREVIATIONS AND INITIALISMS

BM: Bone Marrow
DMSO: Dimethyl sulfoxide
GVHD: Graft versus Host Disease
HARV: High Aspect Ratio Vessel
hESC: human Ebryonic Stem Cell
HLA: Human Leukocyte Antigen
HPC: Hematopoietic Progenitor Cell
HSC: Hematopoietic Stem Cell
LTC-IC: Long-term culture initiating colony
NASA: National Aeronautics and Space Administration
PBSC: Peripheral Blood Stem Cell
PBSCT: Peripheral Blood Stem Cell Transplantation
PPC: Primitive progenitor cell
RWV: Rotating Wall Vessel
STLV: Slow Turn Lateral Vessel

