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Discarded leukoreduction filters: A new source of stem cells for research, cell engineering and therapy?

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Abstract New adult stem cell sources, devoid of the technical/ethical/economical barriers of those presently available, would favor the ongoing development of in vitro cell engineering and transplantation.
Hematopoietic transplantation opened the way to and remains the most successful cell transplantation procedure. CD34+ cells that include hematopoietic stem cells (HSCs) and hematopoietic progenitors (HPs) are presently harvested from bone marrow (BM), cord blood or peripheral blood (after being mobilized from BM). The panel of potential donors, the quantities of collected cells and some other technical/medical problems still represent limiting factors to their transplantation in some patients. Steady state peripheral blood (SSPB) contains very low frequencies of CD34+ cells. They are trapped in leukoreduction filters (LRFs), which are discarded after the preparation of therapeutic red blood cell concentrates from individual blood donations. We recently developed a procedure allowing the easy and rapid elution of CD34+ cells from LRFs and we showed that they are functionally similar to those harvested from other sources.
After providing an overview of the sources, interests and limitations of therapeutic HSCs presently available, we will provide arguments based on our and others' results suggesting that SSPB could become an attractive source of HSCs for hematopoietic transplantation and of other cell types for various research/development procedures.

Contents

Introduction 737
Current therapeutic HSC sources: interests and limitations 737
Bone marrow 737

Abbreviations: BM, bone marrow; CB, umbilical cord blood; G-CSF, granulocyte colony stimulating factor; HPs, hematopoietic progenitors; HSCs, hematopoietic stem cells; iPS cells, induced pluripotent stem cells; LRFs, leukoreduction filters; PB, peripheral blood; RBCs, red blood cells; SSPB, steady state peripheral blood; WBCs, white blood cells.

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Mobilized adult blood HSCs . . . 737
Umbilical cord blood . . . 738
Steady state peripheral blood as a source of HSCs? . . . 738
Functional CD34+ cells can be recovered from LRFs . . . 738
Do discarded LRFs contain HSCs? . . . 738
Interest of LRF CD34+ cells for research and therapy . . . 739
Facing the low amount of CD34+ cells in one blood sample . . . 739
Other biological uses of cells eluted from LRFs () . . . 739
Concluding remarks . . . 740
Acknowledgments . . . 741
References . . . 741

Introduction

Transplantation of autologous or allogeneic hematopoietic stem cells (HSCs) dramatically improved the disease free survival of numerous patients with hematological diseases and presently remains the only widely used cellular transplantation procedure (Deeg and Bartenstein, 2011; Gladstone and Fuchs, 2012). Bone marrow (BM) HSCs are characterized by their life-long capacities to either remain quiescent or proliferate and to either self-renew or differentiate (Lerner and Harrison, 1990; Bradford et al., 1997). These tightly regulated balances allow the life-long homeostatic production of mature functional blood cells, as well as the adaptation/reconstitution of hematopoiesis after infection, hemorrhage or myeloablative stress. During the last 40 years the continuous improvement of our knowledge about hematopoiesis and the development of suitable in vitro techniques for the large-scale isolation, maintenance and expansion of HSCs have increased the numbers and indications of hematopoietic transplantations. They have reduced in parallel the duration of the post-transplantation cytopenia and its related morbidity/mortality consequences.

In 1984, the identification of the CD34 glycoprotein as a marker of HSCs and of hematopoietic progenitors (HPs) (Civin et al., 1984) opened the way to their isolation from BM, umbilical cord blood (CB) and blood mobilized CD34+ cells and to the development of transplantation with in vitro expanded cells. However medical, economical and/or technical constraints still limit their therapeutic use in some patients and/or countries. The main purpose of this brief review is to discuss the potential interest of steady state peripheral blood (SSPB) CD34+ cells trapped in leukoreduction filters (LRFs), which are discarded after the preparation of therapeutic red blood cell (RBC) concentrates, as a new alternative source of HSCs for research and hematopoietic transplantation. We will also mention and discuss briefly some other possible uses for the CD34+ cells and other cell types eluted from these LRFs.

Current therapeutic HSC sources: interests and limitations

Bone marrow

About 50 years ago, successful human hematopoietic transplantations confirmed that transfusion of a limited number of HSCs and HPs collected from human BM was able to give rise to the rapid short-term (HPs) and long-term (HSCs) reconstitution of all hematopoietic lineages in patients (Mathe et al., 1959; Thomas, 1999). However, obtaining BM derived HSCs for therapy faces several technical and/or economical difficulties: i) it is an invasive procedure performed under general anesthesia, thus leading to a very low but real morbidity/mortality risk that cannot be ignored for allogeneic healthy BM donors (Pamphilon et al., 2009); ii) due to the very low percentage of CD34+ cells, an important volume of BM (500 to 1500 mL) must be collected and then processed through a multistep procedure to eliminate maturing granulocytes, platelets and RBCs. Collecting this large volume of BM induces a moderate but real donor RBC depletion that has also to be considered; and iii) the socio-economical impact of a BM donation is costly if one considers that it requires a brief hospitalization and a limited period of restricted activity. The appearance of non-invasive and easy alternative sources of HSCs in numbers sufficient for adults' engraftment reduced the transplantation of BM cells to about 20%.

Mobilized adult blood HSCs

Mobilization of HSCs and HPs from their specific BM niches to the peripheral blood (PB) after chemotherapy was first described in 1976 (Richman et al., 1976). It rapidly led to the development of cell therapy procedures that used the capacity of various chemotherapeutic agents and of some growth factors (granulocyte colony stimulating factor [G-CSF] mostly) to induce the BM to blood egress of numerous CD34+ cells (Siena et al., 1989), containing HSCs and primitive HPs (To et al., 1984; Körbling et al., 1986; Sato et al., 1994). Three major reasons explain that blood mobilized CD34+ cells are now the major source of therapeutic HSCs: i) the good security and tolerance of the G-CSF conditioning processes; ii) the rapid, complete and long term hematopoietic recovery provided by blood mobilized HSCs; and iii) the technical improvements of cytapheresis materials and procedures. However, the drug/growth factor "mobilization process" has also some side effects (bone pain, fatigue, changes in blood cells ratios) that must be taken into consideration, especially for healthy donors (Bosi and Bartolozzi, 2010). In addition, mobilization is inefficient in a low percentage of "poor mobilizers" (Benboubker et al., 2001; To et al., 2011). Despite these limitations, transplantation of blood mobilized CD34+ cells progressively became the standard procedure for auto- and allo-graft (To et al., 2011; Körbling and Freireich, 2011) since it largely improved the benefit/risk ratio for both the

donor and the recipient and the benefit/cost ratio for the public health system.

Umbilical cord blood

CD34+ cells are present at a higher percentage and have a better competitive repopulating capacity than those of adult BM and of blood after mobilization (Wang et al., 1997; Holyoake et al., 1999; Rosler et al., 2000). The interest and the reliability of CB HSCs for allogeneic transplantation are evidenced by the fact that more than 12,000 CB transplantations were performed since 1988 (Delaney et al., 2010). However, the low number of HSCs and HPs per CB sample (mean: 4×10^6 CD34+ cells, range: 1.8 to 12×10^6) restricted its use to pediatric patients below 30 kg for many years since successful transplantation requires more than 2×10^5 CD34+ cells per kg. In adults (50 to 100 kg), who need to receive 1 to 2×10^7 CD34+ cells for successful transplantation, the small number of HSCs obtained from one CB leads to a delayed and insufficient myeloid and lymphoid engraftment, which induces high hemorrhagic and infectious risks (Delaney et al., 2010; Brunstein and Laughlin, 2010). Simultaneous transplantation of two CB units was showed to overcome this problem (Barker et al., 2005). Several clinical trials confirmed that the transplantation of two CB units allows a complete hematological reconstitution in delays comparable to those observed with BM and blood mobilized CD34+ cells (Brunstein et al., 2007; Oran and Shpall, 2012).

Steady state peripheral blood as a source of HSCs?

The limitations described above and some results published years ago (briefly mentioned below and reviewed in Körbling and Freireich, 2011) encouraged us to explore LRFs as an alternative new source of CD34+ cells for basic research, development and future cell therapy/engineering. Indeed, in the 80's, it was shown by three groups that autologous HSCs from SSPB collected by repeated cytapheresis allowed a complete hematological reconstitution in delays comparable to those observed with BM (Kessinger et al., 1986; Reiffers et al., 1986; To et al., 1984). Some years later, it was evidenced that SSPB contains 1 to 4×10^3 CD34+ cells/mL (Bender et al., 1991; Herbein et al., 1994) and that their counting by flow cytometry was a reliable marker of their engrafting capacity (Siena et al., 1991).

Functional CD34+ cells can be recovered from LRFs

In most countries, elimination of white blood cells (WBCs) and platelets from blood donations is mandatory in order to prevent or reduce transfusion incidents/accidents. LRFs are now commonly used for preparing therapeutic RBC concentrates. A 400 to 450 mL blood donation bag contains from 0.4 to 1.6×10^6 CD34+ cells that represent a potential source of HSCs and HPs if they can be eluted from LRFs. In 2006, we brought the "proof of principle" that the elution of functional CD34+ cells and colony-forming-unit-granulocyte-macrophage (CFU-GM) was easily feasible and that their numbers were similar with two types of LRFs, having different technical designs and whatever the age of the blood donor (Ivanovic et al., 2006). We recently developed a miniaturized, rapid and easy 4-step procedure (Fig. 1) allowing to obtain $0.4 \pm 0.05 \times 10^5$ highly enriched CD34+ cells per LRF (yield: 60 ± 5%, purity rate: 84 ± 4%) (Peytour et al., 2010). Their functional properties (cell cycle status, growth rate and myeloid differentiation in liquid culture, or colony-forming ability in semi-solid culture) were rather similar to those found by others and us for SSPB and CB CD34+ cells (Herbein et al., 1994; Ivanovic et al., 2006; Hermitte et al., 2005). Some differences with CB CD34+ cells were however noticed concerning their cell cycle entry and their myeloid differentiation kinetics that are delayed respectively by 24 h and 4 days. These results suggest that the CD34+ cells present in SSPB are quiescent thus requiring more time to proliferate after cytokine stimulation.

Do discarded LRFs contain HSCs?

In 1951, Brecher and Cronkite demonstrated that SSPB cells contain long-term HSCs that are able to reconstitute normal hematopoiesis after parabiosis between irradiated and non-irradiated animals (Brecher and Cronkite, 1951). Later works confirmed the presence of HSCs and HPs in human and animal SSPB (Storb et al., 1977; McCredie et al., 1971; Kessinger et al., 1986; Reiffers et al., 1986). While it was not

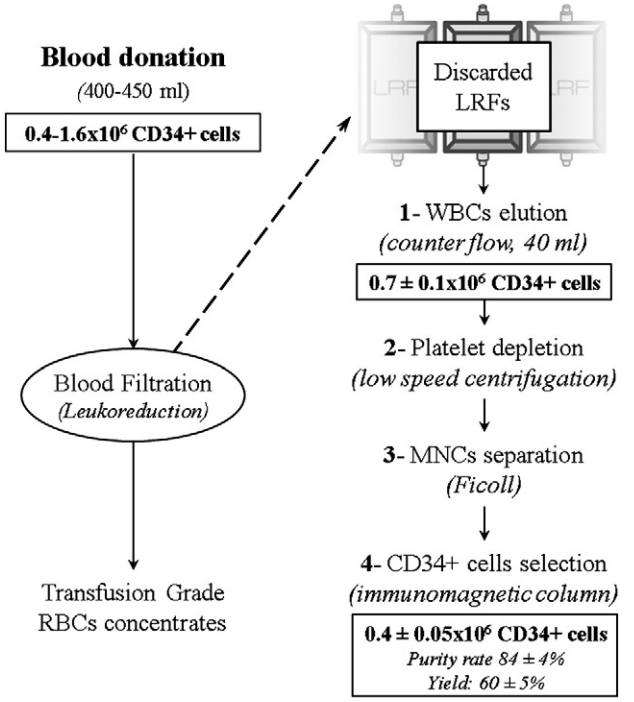

Figure 1 Leukoreduction filters (LRFs): a new source of CD34+ cells for cell engineering and therapy? Discarded LRFs are processed through a rapid (<5 h) and easy 4-step procedure less than 24 h after blood donation. The number of CD34+ cells per blood donation (0.4 to 1.6×10^6 cells) was calculated using published data (Bender et al., 1991; Herbein et al., 1994). The numbers of CD34+ cells after step 1 and step 4 were measured by flow cytometry on our samples. LRF: leukoreduction filter, RBCs: red blood cells, WBCs: white blood cells.

extensively explored, it is often considered that SSPB CD34+ cells do not engraft immunodeficient mice. Recently, by using NOD/SCID mice, we confirmed the data of Hirayama (Hirayama et al., 2003) that SSPB CD34+ cells exhibit a low engraftment capacity and demonstrated that this capacity is dramatically enhanced after ex vivo culture of these cells (Brunet de la Grange et al., Stem Cell Research, 2013). Furthermore, by using an improved animal model (NOD/SCID/IL2r-γ^{null} (NSG) mice), we demonstrated a clearly detectable engraftment ability of steady state SCID repopulating cells (SRC) inside SSPB CD34+ population (unpublished results). These results justify to explore SSPB CD34+ cells as an alternative and innovative approach to the widely used hematopoietic transplantation of CD34+ cells issued from BM, CB or from blood after their mobilization.

Interest of LRF CD34+ cells for research and therapy

This abundant and easy source of CD34+ cells is presently unexploited. For example, the French national transfusion institute (EFS) discards and destroys by incineration more than 3×10^6 LRFs per year. We now commonly use LRF CD34+ cells as a source of HSCs and HPs for research and development purposes because: i) extraction and purification of CD34+ cells from LRFs are rapid and easy; ii) pooling cells eluted from several LRFs allows to easily adapt the number of purified CD34+ cells to the experimental design requirements; and iii) pooling reduces the inter-experiment variations, observed with individual human material, that can be a problem for HSC and HP quantitative tests. Automation and standardization of the process would allow to constitute homogeneous and secured large CD34+ cell pools, then cryopreserved and easily available in public or private cell repositories for the scientific community. These CD34+ cell vials would be helpful for projects requiring homogeneous and standardized cellular material. LRF CD34+ cells could also become an interesting alternative source for HSC transplantation since they are permanently available in most transfusion centers. The HLA diversity of healthy blood donors is at least equivalent to the one of CB samples stored in biobanks. In addition, freshly harvested cells would be easily and rapidly available from a file of healthy volunteers without the technical, ethical and cost constraints related to long-term cryopreservation of human CB cells.

Facing the low amount of CD34+ cells in one blood sample

Injection to recipients of the small quantities of CD34+ cells harvested from one blood donation would lead to a delayed and impaired hematopoiesis reconstitution with severe (probably lethal) infectious and hemorrhagic complications. Transplantation of patients with CD34+ cells from several adult healthy donors is quite unfeasible for immunological reasons. So, despite its potential interest in terms of cost, ethic and availability, SSPB cannot be used today for patients' transplantation. However, it is interesting to notice that transplantation of patients with 8-pooled SSPB apheresis samples allowed a complete hematopoietic transplantation (Kessinger et al., 1986). Apheresis of 2.5 to 6 total blood volumes (as commonly used for harvesting blood CD34+ cells mobilized by G-CSF injections) would provide 1 to 2×10^7 SSPB CD34+ cells from one patient in a single round, thus making this transplantation approach feasible if coupled with an efficient expansion procedure. If one takes into consideration the real possibility to cryopreserve the cells issued from several filters of the same donor over a long period (several donations per year), the potential clinical value of these steady state PB cells becomes obvious. We recently developed innovative clinical grade procedures able to expand CB HSCs and HPs in numbers sufficient to overcome this therapeutic challenge (Ivanovic et al., 2011; Duchez et al., 2012), leading to very short WBCs (2/3 days) and platelets (one week) cytopenias. These excellent clinical results were obtained by culturing purified CD34+ cells in a serum-free medium (HP01-Medium, Macopharma, Tourcoing, France) containing antioxidants and supplemented with a specific combination of cytokines, both of them favoring the self-renewal/differentiation balance of HSCs. This improved culture medium will indeed be tested for SSPB CD34+ cells extracted from LRFs and could allow to develop clinical scale expansion procedures devoted to make these cells transplantable to adults.

Other biological uses of cells eluted from LRFs (Fig. 2)

Most of the potential therapeutic uses of engineered cells and tissues did not yet reach the bedside level due to limitations such as: i) technical/ethical difficulties to obtain some types of stem cells; ii) insufficient knowledge of the mechanisms controlling their self-renewal/differentiation balance and their homing to one specific tissue; and iii) still suboptimal in vitro cellular reprogrammation procedures. Adult stem cells are promising sources for regenerative medicine (Zuba-Surma et al., 2012; Sánchez et al., 2012). Those trapped in LRFs from healthy blood donors are thus an interesting potential material since discarded filters are easily available in large quantities. We suggest below some possible applications for these cells. LRF CD34+ cells are good candidates for cell/tissue engineering such as cardiac cell therapy. Indeed, according to the recent procedure set-up by Losordo et al. (2007)) and latter confirmed by a phase II study (Losordo et al., 2011), an intramyocardial injection of a low dose of G-CSF-mobilized autologous CD34+ cells (1×10^5 cells/kg) led to a significant functional cardiac improvement. Autologous SSPB CD34+ cells collected by apheresis could be thus compatible with this application and could be easily tested. Industrial production of RBCs could also be one important opening for LRF CD34+ cells since the procedure developed by the group of Douay (Douay, 2010) with CB cells will probably reach the therapeutic level in the next few years. We recently demonstrated that LRF CD34+ cells could also give rise to RBC production ex vivo (Vlaski et al., 2009). Using SSPB instead of CB would facilitate the recruitment of numerous samples from a large panel of donors.

Reprogrammation of CD34+ cells from LRFs towards induced pluripotent stem cells (iPS cells) could also be proposed and tested. Chou et al. (2011) and others cited by them succeeded in obtaining iPS cells from adult blood CD34+ cells without viral integration and showed that blood cells have numerous technical, ethical and biological advantages when compared to other cell sources. Homogeneous standardized CD34+ cell

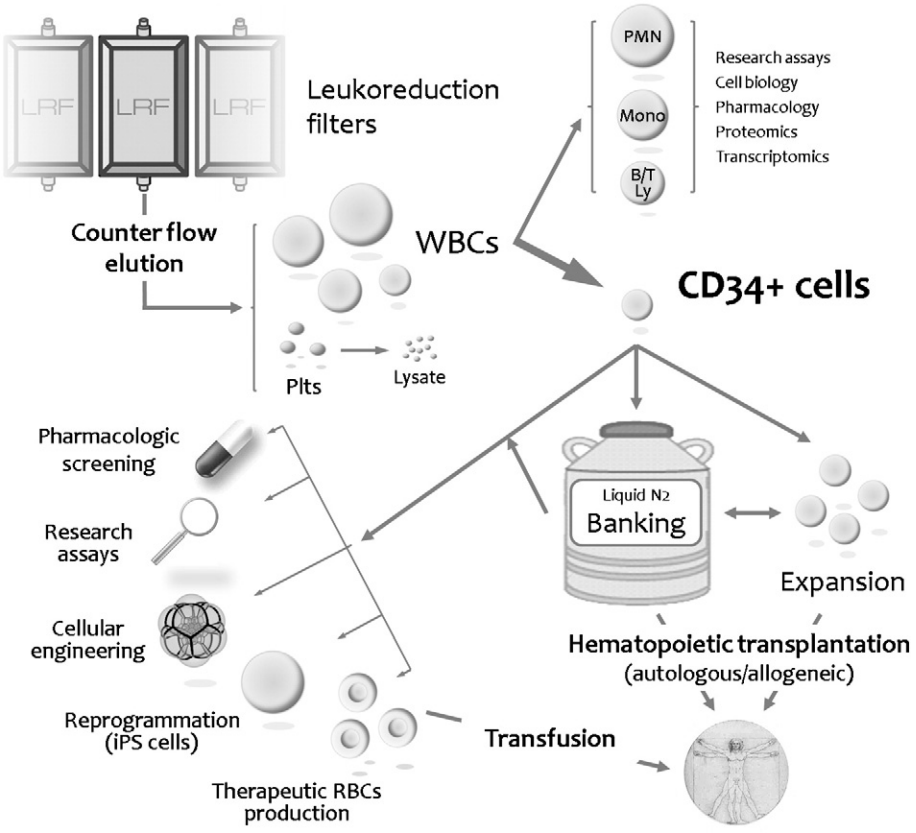

Figure 2 Discarded leukoreduction filters: a source of various cell types for multiple biological applications? Various WBC types and platelets trapped in LRFs are recovered by counter flow elution. The schema shows their potential uses. B/T Ly: lymphocytes B and T, iPS: induced pluripotent stem cells, LRF: leukoreduction filter, Mono: monocytes, Plts: platelets, PMN: polymorphonuclear cells, RBCs: red blood cells, WBCs: white blood cells.

batches available from cell repositories would also be of major interest for pharmacological tests exploring the toxicity/efficiency or the cell signaling pathways of new molecules (Zuba-Surma et al., 2012). Predictive storage of unmodified CD34+ cells from SSPB should also be taken into consideration. Even if the professional risk of massive irradiation or chemical toxicity concerns a very limited number of people, the predictive storage of their autologous SSPB CD34+ cells could be helpful to treat them in case of BM destruction.

Despite the fact that some articles were devoted to lymphocytes and various WBC elution from LRFs and to their experimental uses (Meyer et al., 2005; Dietz et al., 2006; Néron et al., 2006), this cell source was not extensively used by immunologists or other cell biologists. The development of cell repositories delivering specific cell samples ready-to-use, standardized and virally safe could still favor the "industrial" use of LRFs.

Concluding remarks

HSC transplantations represent the best opportunity of definitive cure for patients with poor prognosis hematological malignancies. Availability of good quality grafts, which are HLA compatible (allogeneic transplants), devoid of malignant cells (autologous transplants), virally safe and containing sufficient numbers of HSCs and progenitors, remains a limiting factor for a minority of patients. Collecting CD34+ cells by BM aspiration or by blood apheresis after cytokine mobilization is costly because it requires sophisticated materials/procedures to process graft cell suspensions in cell therapy units and an important medical presence during several days to take care of the patient and/or donor. CB samples represent an interesting source of HSCs for allogeneic transplantations since they offer a large panel of HLA diversity, they are harvested without an invasive procedure and they can be easily cryopreserved for years. But the long-term storage of thousands of individual vials in specialized cell repositories (mandatory for covering the population HLA diversity) is costly and the low number of HSCs in each CB sample still limits their use in adults even if some recent reports show that the simultaneous transplantation of two CB resolved most of these problems. Using SSPB CD34+ cells could represent an alternative. Indeed the large panel of healthy blood donors (if also volunteers for entering in a file of HLA typed CD34+ cell donors) would improve the social and medical efficiency of the selection of HLA matched donors. It would reduce its financial cost by reducing the number and size of bio-banks as compared to CB. The whole process going from LRF elution to transplantation of expanded HSCs still has to be improved, standardized and developed according to GMP rules. This project is worth being realized since LRFs are a source of HSCs technically and ethically easy to obtain that will extend the clinical possibilities of hematopoietic transplantation. For

similar reasons, other cell types trapped in LRFs should also be considered as alternatives to other cell sources for various biological uses.

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