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SHORT REPORT

Long-term repopulating hematopoietic stem cells and "side population" in human steady state peripheral blood

Philippe Brunet de la Grange [a,b,c], Marija Vlaski [a,b,c], Pascale Duchez [a,b,c], Jean Chevaleyre [a,b,c], Veronique Lapostolle [b,c], Jean-Michel Boiron [a,b,c], Vincent Praloran [b,c], Zoran Ivanovic [a,b,c,*]

[a] Etablissement Français du Sang Aquitaine-Limousin, 33035 Bordeaux, France
[b] CNRS (UMR 5164), 33076, Bordeaux, France
[c] Univ. Bordeaux Segalen (UMR 5164), 33076, Bordeaux, France

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Abstract This report brings the first experimental evidence for the presence of long-term (LT) repopulating hematopoietic stem cells (HSCs) and Side Population (SP) cells within human steady state peripheral blood $CD34^+$ cells. *Ex vivo* culture, which reveals the LT-HSC, also increases short-term (ST) HSC engraftment capacity and SP cell number (as well as the SP subpopulations defined on the basis of CD38, CD90 and CD133 expression) which are very low in freshly isolated cells. Thus, *ex vivo* incubation either allows the expansion of the small fraction of HSCs or reveals "Scid Repopulating Cells – SRC" that are present in the initial $CD34^+$ cell population but unable to engraft. In addition, among these $CD34^+$ cells, we confirm the presence of committed progenitors at frequencies similar to those found in cord blood $CD34^+$ cells. These cells, obtained from leukoreduction filters (LRFs) and rejected in the course of the preparation of red blood cell concentrates, are an abundant and reliable material for obtaining committed progenitors, short- and long-term HSCs of therapeutic interest, especially after the *ex vivo* expansion phase. Our results open a perspective to set up new therapeutic protocols using expanded LRFs-recovered $CD34^+$ cells as a source of HSCs for autologous or allogeneic transplantation.

Introduction

The existence of circulating hematopoietic stem cells (HSCs) in steady state was suggested by the early experiments of parabiosis in rats (Brecher and Cronkite, 1951) and baboons (Storb et al., 1977). These observations have been corroborated by hematopoietic reconstitution of irradiated animals *via* injection of steady state peripheral blood cells (Goodman and Hodgson, 1962; Cavins et al., 1964; Storb et al., 1968;

Abbreviations: PB, peripheral blood; HSC, hematopoietic stem cell; LT, long-term; ST, short-term; SP, Side Population; SRC, Scid-Repopulating Cells; LRF, leukoreduction filters; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte–monocyte-colony stimulating factor; SCF, stem cell factor; MGDF, megakaryocyte-derived growth factor.

* Corresponding author at: Etablissement Français du Sang Aquitaine Limousin, BP 24, 5 Place Amélie Raba Léon, 33035 Bordeaux Cedex, France. Fax: +33 5 56 90 75 51.
E-mail address: zoran.ivanovic@efs.sante.fr (Z. Ivanovic).

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Kőrbling et al., 1979). Although several clinical studies in the 80s demonstrated a beneficial effect of autologous (Kőrbling et al., 1986; Reiffers et al., 1986) and allogeneic (Kessinger et al., 1989) peripheral blood cell transplantation, the presence of HSC in human steady state blood was not confirmed by specific experiments. However, the presence of committed and multi-lineage progenitors in steady state peripheral blood has been established both in the animal models and in humans (McCredie et al., 1971; Chervenick and Boggs, 1971; Barr et al., 1975; Ivanovic et al., 1997, Ivanovic and Milenkovic, 1999), and these cells have been identified as belonging to the $CD34^+$ population, whose frequency is very low in steady state blood (1 to 4 cells/μl) (Bender et al., 1991). Surprisingly, after a phenomenon called "mobilization" *i.e.* increase in the number of committed progenitor cells in blood after chemotherapy (Richman et al., 1976) and cytokine treatment (GM-SCF or G-CSF) (Siena et al., 1990), had been discovered, the studies on steady state peripheral blood cells were almost abandoned. Nevertheless, a couple of studies was published demonstrating the presence of HSC in blood using *ex vivo* LTC-IC (Long Term Culture Initiation Cells) (Udomsakdi et al., 1992) approach and *in vivo* (SRC; Scid Repopulating Cells) (Hirayama et al., 2003) approach. Nowadays, it is considered that the *in vivo* approach, based on the repopulation of hematopoietic tissues of immunodeficient mice, enables estimation of the real HSC capacity. Furthermore, depending on the approach – analysis of recipient mice several weeks after injection of human cells, or their bone marrow cell collection and injection to the secondary recipients which will be analyzed several weeks afterwards – it is possible to get insight into two populations of the heterogeneous HSC compartment: the short- (ST) and long-term (LT) repopulating hematopoietic stem cells, respectively (reviewed in: Ema, 2010). Importantly, the approach using secondary recipients is now regarded as a "gold standard" to reveal the LT-HSC.

The present study used described "gold standard" approach, as well as a complex phenotypic analysis (analysis of the Side Population — "SP" cells were combined with CD34, CD38, CD90 and CD133 expression analyses) to question the presence of primitive HSC in steady state blood of healthy humans.

Material and methods

Recovery of leukocytes and MNC from the LRF

The blood for transfusion purposes is collected only from the voluntary healthy blood donors. The cells were recovered from filters (T2975, Fresenius Kabi, Louviers, France) by counterflow elution as described (Ivanovic et al., 2006a, 2006b). The cells were then centrifuged at low gravity (10 min at 20 °C, 320 *g*; Heraeus Multifuge S3, Yutz, France) to reduce the platelets contamination of the buffy coat. The platelet rich supernatants were discarded and the buffy coats containing residual RBC recovered and loaded on Ficoll (Bicoll separating solution — Biochrom AG, Berlin, Germany). The MNC fraction was harvested after 20 min of centrifugation at 290*g* (Heraeus Multifuge S3, Heraeus Multifuge S3, Yutz, France), then washed and resuspended in buffer for the $CD34^+$ isolation. The cells from myeloma patients were obtained by leukapheresis after the standard mobilization protocol (4 g/m^2 intravenous (iv) cyclophosphamide followed by subcutaneous filgrastim (Amgen, Neuilly sur Seine, France; 5 μg/kg/day) until all leukapheresis procedures were collected) (Boiron et al., 2006). The lowest $CD34^+$ cell incidence before starting leukapheresis procedures was 20/μl for the series of patients whose results were considered in this study.

Isolation of $CD34^+$ cells

$CD34^+$ cells from MNC fraction issued from LDFs were isolated by using Miltenyi's (Miltenyi Biotec, Paris, France) "indirect" immunomagnetic technique (human $CD34^+$ progenitor cell isolation kit "Macs") (Ivanovic et al., 2006a, 2006b): two passages by using the LS columns (Vario Macs Device). This approach enabled, in spite of very low starting concentration of $CD34^+$ cells, relatively good $CD34^+$ purity (80–98%). $CD34^+$ cells from myeloma patients after mobilization – "Mobilized $CD34^+$ cells" – were selected directly from leukapheresis products using Isolex 300i (Miltenyi Biotec, Paris, France) device (Ivanovic et al., 2006b).

Detection of CFU-GM, BFU-E and CFU-Mix committed progenitors

Freshly isolated $CD34^+$ cells (isolated from LRF or cord blood) and their progeny after culture were plated in methylcellulose cytokine-supplemented kits (Stem Alpha ID, Saint Clement les Places, France) and cultured for 14 days in 35 mm Petri dishes (NUNC, Roskilde, Denmark). The colonies (>50 cells) were then counted (Ivanovic et al., 2006a, 2006b).

Evaluation of *ex vivo* expansion capacity of LRF-recovered $CD34^+$ cells

Freshly isolated $CD34^+$ cells were seeded at 20 × 10^3 cells/ml, and cultured in 175 cm^2 flasks (NUNC, Roskilde, Denmark) during 7 days in liquid (serum-free medium Macopharma HP01) cultures supplemented by SCF, G-CSF and MGDF (100 ng/ml each), IL-3 (0.5 ng/ml) (Ivanovic et al., 2004).

Total cell counting

The cells were counted using automatic cell counter (Cell Dyn 3000, Abbott, Rungis, France).

$CD34^+$ cell detection and immunophenotypical analysis

The $CD34^+$ cell concentrations/purities were detected following the recommendations of the International Society of Hematotherapy and Graft Engineering (Sutherland et al., 1996) as previously described (Ivanovic et al., 2006a, 2006b). Briefly, three-color fluorescence was used to detect viable $CD45^+/CD34^+$ cells that were counted directly by a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA) by using trucount microbeads (Beckton Dickinson, San Jose, CA). For phenotypic characterization of cultured cells, the following fluorescein-coupled monoclonal antibodies were used: anti-CD13 (PE), anti-CD33 (PE), anti-CD61 (FITC)