The High Yield Expansion and Megakaryocytic Differentiation of Human Umbilical Cord Blood CD133+ Cells

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Received: 1/Aug/2010, Accepted: 26/Apr/2011

Abstract

Objective: Despite many benefits, umbilical cord blood (UCB) hematopoietic stem cell (HSC) transplantation is associated with low number of stem cells and slow engraftment: in particular of platelets. So, expanded HSCs and co-transfusion of megakaryocyte (MK) progenitor cells can shorten this period. In this study, we evaluated the cytokine conditions for maximum expansion and MK differentiation of CD133+ HSCs.

Materials and Methods: In this experimental study, The CD133+ cells were separated from three cord blood samples by magnetic activated cell sorting (MACS) method, expanded in different cytokine combinations for a week and differentiated in thrombopoietin (TPO) for the second week. Differentiation was followed by the flow cytometry detection of CD41 and CD61 surface markers. Colony forming unit (CFU) assay and DNA analysis were done for colonogenic capacity and ploidy assay.

Results: CD133+ cells showed maximum expansion in the stem span medium with stem cell factor (SCF) + FMS-like tyrosine kinase 3-ligand (Flt3-L) + TPO but the maximum differentiation was seen when CD133+ cells were expanded in stem span medium with SCF + Interleukin 3 (IL-3) + TPO for the first and in TPO for the second week. Colony Forming Unit-MK (CFU-MK) was formed in three sizes of colonies in the mega-cult medium. In the DNA analysis; 25.2 ± 6.7% of the cells had more than 2n DNA mass.

Conclusion: Distinct differences in the MK progenitor cell count were observed when the cells were cultured in stem span medium with TPO, SCF, IL-3 and then the TPO in the second week. Such strategy could be applied for optimization of CD133+ cells expansion followed by MK differentiation.

Keywords: Expansion, Differentiation, CD133+, Megakaryocyte, Cord Blood

Introduction

Transplantation of allogenic and autologous hematopoietic stem cells (HSCs) is used widely for reconstituting the hematopoietic cells after high dose chemotherapy and radiotherapy and some hematologic diseases. In the past, the main source of the HSC, for transplant was the bone marrow (1). Then mobilized peripheral blood by granulocyte-colony stimulation factor (G-CSF) was used as an alternative source of stem cells. It is more convenient and follows by a quicker recovery of neutrophils and platelets (2), but sometimes poor mobilization happens (3). The use of cord blood transplantation in pediatric patients has been established in 2000 (4, 5). However, it has two major limitations: the HSCs may be sufficient for children, but not for adults, and there is a delayed engraftment, especially in the platelets’ (Plt) recovery. Multiple plt transfusion carries the risk of alloantibody formation and plt refractoriness (6). Also clinical trials have shown that recombinant thrombopoietin (TPO) stimulates the megakaryocyte (MK) formation in vivo, but it does not shorten its maturation time (7). So, co-transfusion of HSC and MK progenitor cells can shorten this period. There are several protocols regarding the influence of some cytokines and chemokines to expand and differentiate HSCs including: Interleukin-3 (IL-3), IL-6, IL-9, IL-11, interferon-γ (IFN-γ), FMS-like tyrosine kinase 3liband (Flt3-L), stem cell factor (SCF), TPO, erythropoietin (EPO), stromal derived factor-1 (SDF-1), and macrophage inflammatory protein-1 (MIP-1) (8-11). In some studies, the use of TPO as a key cytokine for megakaryocytic differentiation showed low expansion and early apoptosis in ex vivo cultures (12-14), while addition of other cy-
tokines improved MK expansion and differentiation. In the present study, the maximum potential of in vitro expansion of CD133+ umbilical cord blood (UCB) cells in the presence of Flt3-L, TPO, SCF and IL-3 for a week, and optimal differentiation of expanded CD133+ cells in the presence of TPO as an MK active cytokine was studied.

Materials and Methods

Collection of cord blood

In this experimental study, human UCB samples were collected from consenting women who had normal full-term pregnancy without any complications and signed the testimonial form. This research also was confirmed by Tarbiat Modares University Ethic Group. Cord blood samples were collected in 20 ml CPDA bags and processed within 24 hours of collections.

CD133+ cell separation

Mononuclear cells were separated from the UCB, using Ficoll Hypaque (density 1077 g/cm³, Pharmacia, Sweden) density centrifugation at 2500 rpm for 30 minutes at 25°C, and washed by phosphate buffer saline containing 5% bovin serum albumin (Stem Cell Technology, Canada). The CD133+ fraction was enriched with Magnetic Activated Cell Sorting (MACS) method (Miltenyi Biotec, Canada) according to the manufacturer’s instructions. The procedure was performed twice to obtain higher purity of the selected CD133+ cells. The efficiency of purification was verified by flow cytometry (Partec PAS III, Germany), counterstaining with a Monoclonal Antibody (MoAb) CD133-PE (Miltenyi, Canada) and MoAb CD34-FITC (Miltenyi, Canada) and MoAb CD41-PE and CD61-FITC (DAKO, Denmark) were used to confirm the negativity of the MK series.

Cell culture, expansion and differentiation

The UCB CD133+ cells were cultured in serum free stem span (Stem Cell Technology, Canada) medium in the tissue culture flask T25, and maintained at 37°C in a fully humidified atmosphere with 5% CO2. Ten cytokine combinations were added at 1st and 3th-day: 1- SCF (100 ng/ml) + TPO (100 ng/ml). 2- SCF (100 ng/ml) + TPO (100 ng/ml) + Flt3-L (100 ng/ml). 3- SCF (100 ng/ml) + TPO (100 ng/ml) + IL-3 (10 ng/ml). In order to MK differentiation, after a week, the cells were counted and transferred into 6-well tissue-culture plates in serum free stem span media with TPO (100 ng/ml). The TPO was added twice a week. Differentiation was followed by Flow cytometric analysis of CD41 and CD61 surface marker expression. CFU-MK for colonicogenic capacity and DNA analysis for ploidy detection of MK progenitors were done.

Table 1: Total number of cells, number of CD133+ cells and fold expansion of CD133+ cells during 7 days of culture (mean ± SD)

| Cytokine conditions | Total cell density (×10⁵ cells/flask T25) | Total cell density (×10⁵ cells/flask T25) | Content of CD133+ cells (%) | Expansion fold of CD133+ cells |
|---------------------|------------------------------------------|------------------------------------------|-----------------------------|-------------------------------|
| SCF+TPO             | 8.4 ± 2.8                                | 154.2 ± 84.0                             | 66.8 ± 12.7                 | 14.7 ± 7.5                    |
| SCF+Flt3L+TPO       | 8.4 ± 2.8                                | 570.0 ± 155.8                            | 77.8 ± 5.8                  | 62.1 ± 13.7                   |
| SCF+IL-3+TPO        | 8.4 ± 2.8                                | 442.9 ± 123.0                            | 76.5 ± 4.4                  | 46.9 ± 7.1                    |
Cells differentiation
Cell differentiation was evaluated by flow cytometry after two weeks of culture (Table 2, Fig 2). The most MK differentiated cells were formed in the third condition.

CFU-MK
CFU- colony formingunit-megakaryocyte produced three size colonies in the Mega-cult medium. There were 65.3 ± 13.5 small size colonies with 3-21 cells, 4.6 ± 1.5 medium size colonies with 21-49 cells and 2.3 ± 1.5 large size colonies with more than 49 cells (Fig 3).

DNA analysis
The cells percent (mean ± SD) in the G0G1 phase was 45.9 ± 8.3 and in the 2N was 13.7 ± 3.5; this value for the cells in the 4N was 4.9 ± 1.3 and in total 25.2 ± 6.7% of the cells had more than 2N DNA mass (Fig 4).
Discussion

UCB stem cells transplantation, despite of many benefits is associated with slow engraftment, in particular of platelets because of low numbers of HSC and MK progenitor cells. Possible approaches to expansion have been obtained, and co-transfusion of large number of ex vivo generated human MK cells is a way to shorten thrombocytopenia period.

In this study, we investigated the effects of various cytokine combinations in the high expansion and MK differentiation of CD133+ HSCs. We have used two -step cytokine conditions including: 1. Expansion with different cytokine cocktails and 2. differentiation with TPO. According to the results, though the expansion of CD133+ cells in SCF, TPO and Flt3-L cocktail is more than in other conditions and this is favorable with some studies (15-17), however, when the MK differentiation is the main aim of expansion, TPO, SCF and IL-3 are the best choice. IL-3 made the expanded cells more capable to differentiation (18). So the total amount of MK progenitors after two weeks of treatment was higher when we used TPO, SCF, IL-3 for CD133+ expansion. This finding is in agreement with Kashiwakura et al. (19) report that has provided the role of TPO, SCF, and IL-3 in CFU-Meg formation. Besides Piacibellond et al. (20) and
Ueda et al. (21) reported that the combination of SCF, Flt3-L and TPO can expand more primitive HSCs and has lower effect on the progenitor cells. According to Kuter and Begly report, Flt3-L is dispensable (22), and TPO alone can induce high MK purity, but low MK expansion, which is the same as when TPO is combined with Flt3-L or IL-11(22, 23). In this manner, there are many studies on the other varieties of cytokines (IL-6, IL-9, IL-11, and some hormones) to identify optimum cocktails for expansion and differentiation (24, 25). For instance, Amiphosine can expand and produce MK progenitors as high as 83 folds (26).

All together, we preferred to use one-week expansion prior to MK differentiation and used three group cytokine combinations, because of previous reports on their effect on MK expansion and purity differentiation (27).

For MK differentiation follow up, we used CD41 and CD61 surface expression detection. CD41 expresses earlier than other markers (28), and at the end of the second week, the bright expression of CD41 and CD61, as shown in (Fig 1), was observed.

The degree of MK progenitor maturation was tested by ploidy analysis in day the 14, and in average, about 25% of total cells had more than 2N ploidy, where most of the differentiated cells were megakaryoblast at that time. Further, based on previous studies, more ploidy of MKs could be obtained if the culture was followed more (29).

Colonogenic capacity of MK progenitors is an important criterion. We used Mega-cult media designed for MK colonies growth. After 14 days, from the total of 1000 cells cultured in a 30 mm petri, about 70 colonies were formed. Because of limited Mega-cult culture data in other studies, comparison of our results with those of other achieved studies is not possible.

**Conclusion**

We observed distinct differences in the MK progenitors cells count, when we used TPO, SCF, IL-3 and then TPO in the second week. Such strategy could be applied for optimization of CD133+ cells expansion followed by MK differentiation.

**Acknowledgments**

We would like to thank Iran Blood Transfusion Organization (Tehran,Iran) for financial supporting this project. There is no conflict of interest in this article.

**References**

1. van den Oudendijk S, von dem Borne AE, de Haas M. Differences in megakaryocyte expansion potential between CD34+ stem cells derived from cord blood, peripheral blood, and bone marrow from adults and children. Exp Hematol. 2000; 28(9): 1054-1061.
2. Heike T, Nakahata T. Ex vivo expansion of hematopoietic stem cells by cytokines. Biochim Biophys Acta. 2002; 1592(3): 313-321.
3. Platzbecker U, Prange-Krex G, Bornhauser M, Koch R, Soucek S, Aikele P, et al. Spleen enlargement in healthy donors during G-CSF mobilization of PBPCs. Transfusion. 2001; 41(2): 184-189.
4. Stanevsky A, Goldstein G, Nagler A. Umbilical cord blood transplantation: Pros, cons and beyond. Blood Rev. 2009; 23(5): 199-204.
5. Gluckman E, Locatelli F. Umbilical cord blood transplants. Curr Opin Hematol. 2000; 7(6): 353-357.
6. Bruno S, Gunetti M, Gammatoni L, Dane A, Cavalion I, Sanavio F, et al. In vitro and in vivo megakaryocyte differentiation of fresh and ex vivo expanded cord blood cells: rapid and transient megakaryocyte reconstitution. Haematologica. 2003; 88(4): 379-387.
7. Zheng C, Yang R, Han Z, Zhou B, Liang L, Lu M. TPO-independent megakaryocyte differentiation. Crit Rev Oncol Hematol. 2006; 65(3): 212-222.
8. Cortlin V, Garnier A, Pineault N, Lemieux R, Boyer L, Proulx C. Efficient in vitro megakaryocyte maturation using cytokine cocktails optimized by statistical experimental design. Exp Hematol. 2005; 33(10): 1182-1191.
9. Dimitriou H, Vorgia P, Siliakaki E, Mavroudis D, Markaki EA, Koumantakis E, et al. In vitro proliferative and differentiating characteristics of CD133(+) and CD34(+) cord blood cells in the presence of thrombopoietin (TPO) or erythropoietin (EPO), potential implications for hematopoietic cell transplantation. Leuk Res. 2003; 27(12): 1143-1151.
10. Yang M, Li K, Chui CM, Yuen PM, Chan PK, Chuen CK, et al. Expression of interleukin (IL)1 type I and type II receptors in megakaryocytic cells and enhancing effects of IL-1beta on megakaryocytopenesis and NF-E2 expression. Br J Haematol. 2000; 111(1): 371–380.
11. Metcalf D, Carpinelli MR, Hyland C, Mifsud S, Dirago L, Nicola NA, et al. Anomalous megakaryocytopenesis in mice with mutations in the c-Myb gene. Blood. 2005; 105(9): 3480-3487.
12. Kaushansky K, Drachman JG. The molecular and cellular biology of thrombopoietin: the primary regulator of platelet production. Oncogene. 2002; 21(21): 3359-3367.
13. Baldjuni A, d’Apolito M, Arcelli D, Conti V, Pecci A, Pitera D, et al. Cord blood in vitro expanded CD41 cells: identification of novel components of megakaryocytopenesis. J Thromb Haemost. 2006; 4(4): 848-860.
14. Fox NE, Priestley G, Papayannopoulou T, Kaushansky K. Thrombopoietin expands hematopoietic stem cells after transplantation. J Clin Invest. 2002; 110(3): 389-394.
15. Kie JH, Yang WI, Lee MK, Kwon TJ, Min YH, Kim HO, et al. Decrease in apoptosis and increase in polyploidization of megakaryocytes by stem cell factor during ex vivo expansion of human cord blood CD34+ cells using thrombopoietin. Stem Cells. 2002; 20(1): 73–79.
16. Proulx C, Boyer L, Hurnanen DR, Lemieux R. Preferential ex vivo expansion of megakaryocytes from human cord blood CD34+ enriched cells in the presence of thrombopoietin and limiting amounts of stem cell
factor and Flt-3 ligand. J Hematother Stem Cell Res. 2003; 12(2): 179–188.
17. Ungerer M, Peluso M, Gillitzer A, Massberg S, Heinzmann U, Schulz C, et al. Generation of functional culture-derived platelets from CD34+ progenitor cells to study transgenes in the platelet environment. Circ Res. 2004; 95(5): e36–44.
18. Fock EL, Yan F, Pan S, Chong BH. NF-E2-mediated enhancement of megakaryocytic differentiation and platelet production in vitro and in vivo. Exp Hematol. 2008; 36(1): 78-92.
19. Kashiwakura I, Takahashi K, Monzen S, Nakamura T, Takagaki K. Ex vivo expansions of megakaryocytic progenies from placental and umbilical cord blood CD34+ cells in serum-free culture supplemented with proteoglycans extracted from the nasal cartilage of salmon heads and the nasal septum cartilage of whale. Life Sci. 2008; 82(19-20): 1023-1031.
20. Piacibello W, Gammaitoni L, Bruno S, Gunetti M, Fagioli F, Cavalloni G, et al. Negative influence of IL3 on the expansion of human cord blood in vivo long-term repopulating stem cells. J Hematother Stem Cell Res. 2000; 9(6): 945-956.
21. Ueda T, Tsuji K, Yoshino H, Ebihara Y, Yagasaki H, Hisakawa H, et al. Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. J Clin Invest. 2000; 105(7): 1013-1021.
22. Kuter DJ, Begley CG. Recombinant human thrombopoietin: Basic biology and evaluation of clinical studies. Blood. 2002; 100(10): 3457-3469.
23. Su RJ, Li K, Yang M, Zhang XB, Tsang KS, Fok TF, et al. Platelet-derived growth factor enhances ex vivo expansion of megakaryocytic progenitors from human cord blood. Bone Marrow Transplant. 2001; 27(10): 1075–1080.
24. Bruno S, Gammaitoni L, Gunetti M, Sanavio F, Fagiol F, Aglietta M, et al. Different growth factor requirements for the ex vivo amplification of transplantable human cord blood cells in a NOD/SCID mouse model. J Biol Regul Homeost Agents. 2001; 15(1): 38-48.
25. Goodarzi A, Kheirandish M, Pourfathollah A, Siadat SD, Pourpak Z. Characterization of megakaryocyte progenitor cells differentiated from umbilical cord blood CD133+ and CD133- cells. Yakhteh. 2007; 9(3): 190-195.
26. Kashiwakura I, Murakami M, Inanami O, Hayase Y, Takahashi TA, Kuwabara M, et al. Effects of amifostine on the proliferation and differentiation of megakaryocytic progenitor cells. Eur J Pharmacol. 2002; 437(1-2): 19-25.
27. Guerriero R, Mattia G, Testa U, Chelucci C, Macioce G, Caselia I, et al. Stromal cell–derived factor 1alpha increases polyploidization of megakaryocytes generated by human hematopoietic progenitor cells. Blood. 2001; 97(9): 2587–2595.
28. Schipper LF, Brand A, Reniers N, Melief CJ, Willemze R, Fibbe WE. Differential maturation of megakaryocyte progenitor cells from cord blood and mobilized peripheral blood. Exp Hematol. 2003; 31(4): 324-330.
29. Avecilla ST, Hattori K, Heissig B, Tejada R, Liao F, Shido K, et al. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. Nat Med. 2004; 10(1): 64-71.