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EXPANSION OF CD34$^+$ HUMAN HEMATOPOIETIC CELLS FROM UMBILICAL CORD BLOOD USING ROLLER BOTTLES

EXPANSIÓN DE CÉLULAS CD34$^+$ DE SANGRE DE CORDÓN UMBILICAL EN FRASCOS GIRATORIOS

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Abstract

Hematopoietic stem cell (HSC) transplantation is limited by the initial CD34$^+$ cell content in cord blood units; the aim of this work was the *in vitro* expansion of HSC to overcome this issue. Supplemented IMDM roller bottle cultures of CD34$^+$ cells from human umbilical cord blood were established for hematopoietic progenitor expansion. The maximum total colony forming cells (CFC) expansion was achieved after 5 days of culture, being 16.94±2.82 folds in roller bottles and 17.28±4.47 in static cultures. However, the maximum total cell fold expansion was attained after 10 days of culture. It was of 20.31±6.18 for the roller bottles and 26.45±9.89 for the static cultures. The efficacy of the roller bottles system for short-term cultures of CD34$^+$ cells and expanding hematopoietic progenitors in IMDM was demonstrated; encouraging the use of this culture system for other experiments and may be used for future clinical applications.

Keywords: *CD34$^+$, expansion, hematopoietic stem cell, bone marrow transplantation, cell culture, roller bottles.*

Resumen

El transplante de células madre hematopoyéticas está limitado por la cantidad de células CD34$^+$ presentes en las unidades de sangre de cordón umbilical; el objetivo de este trabajo fue obtener la expansión *in vitro* de células madre hematopoyéticas. Se establecieron cultivos de células CD34$^+$ de sangre de cordón umbilical en medio IMDM suplementado con citocinas para promover la expansión de progenitores hematopoyéticos. En 5 días de cultivo en frascos giratorios se obtuvo la máxima expansión de unidades formadoras de colonias (UFC) totales de 16.94±2.82 veces, mientras que en cultivo estático fue de 17.28±4.47. La máxima expansión de células totales fue de 20.31±6.18 veces en frasco giratorio y de 26.45±9.89 veces en cultivo estático a los 10 días de cultivo. Se demostró la eficacia del sistema de frascos giratorios con medio IMDM para el cultivo a corto plazo de células enriquecidas CD34+ provenientes de sangre de cordón umbilical y para la expansión de progenitores hematopoyéticos, potencializando el uso de este sistema para otros experimentos y aplicaciones clínicas a futuro.

Palabras clave: CD34$^+$, expansión, células madre hematopoyéticas, trasplantes de médula ósea, cultivo celular, frascos giratorios.

1 Introduction

Mature blood cells are short-lived cells that need to be replaced continuously. The circulating numbers are maintained by a high throughput production system that delivers 10^{11}-10^{12} hematopoietic cells daily (Gordon *et al*., 2002). The bone marrow (BM) "niche" hosts the hematopoietic stem cells (HSC), which are responsible for all the newly produced blood cells and for their own renewal. Damage of bone marrow by radiation or

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malfunctioning of HSC may result in an impaired blood cells production. Bone marrow transplants have been performed since half a century ago with the aim of repopulating a patient´s damaged bone marrow with healthy donated HSC (Bortin, 1970). HSC self-renewal, differentiation and engraftment mechanisms are still the object of study of many investigations and there is still an evident lack of knowledge about them (Broxmeyer, 2010). The surface markers of early hematopoietic progenitors are still undefined, but $CD34^+$ cells transplants have demonstrated total recovery after mieloablative processes (Nimgaonkar *et al*., 1995). Bone marrow transplants are effective but the collection of BM is an invasive and painful procedure and a compatible available donor must be found.

In 1988, the first umbilical cord blood (UCB) successful transplant was reported (Gluckman *et al*., 1989). UCB is a HSC source that can be obtained painlessly from full term delivery cords with the consent from the parents. It can be cryopreserved for future use and presents low graft-versus-host disease risk (Broxmeyer, 2010). In the past two decades UCB successful transplants have been rapidly increasing (Rubenstein *et al*., 1998; McKenna and Sheth, 2011) for hematological and non-hematological disorders. Despite the proved value of UCB transplants, there are some limitations mainly due to the low content of stem cells in the UCB samples. The success of transplants is related to the cell dose and the minimum effective number of transplanted cells is 5x105 CD34+cells/kg (Koller and Palsson 1993; McAdams *et al*., 1996; Van Haute *et al*., 2004), reducing the possibility of transplants to low weight patients. Alternative strategies have been evaluated to overcome this problem. Sophisticated *ex vivo* culture approaches have been targeted by several groups to increase hematopoietic stem cells before transplant (Takizawa *et al*., 2011; Andrade-Zaldívar *et al*., 2008). Previously, our research group designed an artless roller bottle (RB) system that was used to culture total mononuclear cells (MNC) from UCB allowing a 17.25±3.65 fold expansion of HSC (Andrade-Zaldívar *et al*., 2011). This culture system allowed culturing larger volumes than the traditional static cultures without affecting the UCF expansion, which could be an important advantage for clinical applications. The present work aimed to research whether the roller bottle system can sustain HSC expansion in cultures of a $CD34^+$ selected population from UCB.

2 Materials and methods

For this investigation a total of 5 UCB units were used. General methods for UCB collection and processing, colony assays and cell counting have been already published (Andrade-Zaldívar *et al*., 2011). Recalling, UCB samples from full-term deliveries were collected in 50 mL sterile Falcon tubes containing 1 mL of 3% EDTA and kindly provided by local hospitals, according to their ethic committee's guidelines. Blood samples were processed 1 to 10 hours after collection. Briefly, samples were centrifuged 30 min at 850 g, then 5-7 ml of white inter-phase cells and plasma were transferred into a 15 ml falcon tube and diluted 1:2 with Phosphate Buffered Saline pH 7.2 (PBS). Cells were transferred to 15 ml Falcon tubes containing 7 ml of Ficoll-Paque Plus (Pharmacia) at room temperature, and centrifuged 30 min at 1250 g. MNC ring was aspirated and transferred to a clean tube, washed twice with PBS and resuspended in 1 ml of IMDM (Iscove's modified Dulbecco's medium).

2.1 $CD34^+$ cells enrichment

The EasySep® Kit (StemCell Technologies) was used for positive selection according to manufacturer directions. Briefly, 4.38×10^5-1.13×10^6 cells were centrifuged for 5 min at 2100 g, supernatant was discarded and cells were washed with PBS, then centrifuged at 2100 g for 5 min and re-suspended in 200 μl of PBS containing 2% fetal bovine serum (FBS) in a round bottom tube. Ten μl of *Cocktail Positive Selection Easy Sep*^{TM} for $CD34^+$ cells, were added and the mixture was incubated 15 min at room temperature (RT), then 5 μl of nanoparticules *Easy Sep*^{TM} were added and the mixture was incubated 10 min at RT. Two ml de 2% FBS PBS were added and the tube was incubated inside a magnet for 5 min, supernatant was discarded without separating the tube from the magnet and the procedure was repeated from the addition of 2 ml of PBS containing 2% FBS for 4 more times. The cells remaining inside the tube after the washes are $CD34^+$ and were used for our experiments.

2.2 Culture media

Culture media was chosen aiming to obtain results comparable to other published work with $CD34^+$ cell cultures. The experiments were performed using IMDM (Gibco) supplemented with 10% fetal bovine serum (Gibco) and human recombinant cytokines (Peprotech): 2 ng/ml interleukin (IL) 3, 5 ng/ml IL 6, 5 ng/ml stem cell factor (SCF), 5 ng/ml granulocyte colony stimulating factor (G-CSF), 5 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF), 5 ng/ml flt3 ligand (Flt 3), and 3 U/ml erythropoietin (EPO).

2.3 Roller bottles cell culture

Each RB batch culture was started by seeding.0.5x105 CD34+ MNC/ml from a single UCB unit in 500 ml glass roller bottles (Wheaton) containing up to 20 ml of culture medium. Cultures were maintained for 14 days -starting at day 0- at 37°C in an incubator (Shel lab) with 5% CO_2 atmosphere. Roller bottles were set in a bottle bench top roller (Wheaton) at 1 rpm. 24-well plates with 1 ml of the respective culture medium were used as static control cultures. The RB caps were kept slightly opened for the cultures.

Sampling was performed at each indicated time by taking out the RB or the plates from the incubator; removing the caps and washing out the interior walls with the same culture media to recover attached cells, then 0.3 ml of media were removed for total cell counting and/or colony forming cell (CFC) assays. Medium was not added, removed or altered in any other way during these cultures.

Total cell counting was performed excluding non-viable cells. Samples were separated in two eppendorf tubes before counting when the CFC assays were done. Then 1000 viable cells were seed in 1 mL of Methocult media (Stem cell Technologies) in 35 mm Petri dishes and incubated at 37°C with 5% CO_2 atmosphere by 14 days. Colonies were identified by direct microscopic analysis.

3 Results and discussion

3.1 CD34$^+$ enrichment

The collected UCB samples had an average volume of 45±3.2 ml. The mononuclear cells content in the samples was $1.07\times10^8\pm0.14\times10^8$. From the MNC, an average of $8.80\times10^5\pm2.75\times10^5$ CD34$^+$ cells were recovered, which represents the 0.82±0.26% of the cells.

3.2 Total cell expansion

Five cultures of CD34$^+$ cells from UCB were performed in supplemented IMDM. Fig. 1a shows the typical growth kinetics observed. The culture started with 0.5×10^5 cells and after 13 days it ended up with 16 to 19 times the initial cell number. The maximum total cell number was 8.0×10^5 and 9.5×10^5 for the static and Roller bottle cultures, respectively. The average total cell fold expansion is shown in Fig. 1b. Cells started to multiply from the beginning of the culture and continued the expansion until day 10 of the experiment in both the static and the roller bottle conditions. At day 3, total cells in the static culture had a 4.18±3.42 fold increase and in the RBs a 2.97±2.05 fold increase. After 5 days the number of total cells increased 10.58±4.46 and 7.38±2.13 folds for the static and RB conditions, respectively. At day 10, the total cells increased 26.45±9.89 folds in the static and 20.31±6.18 folds in the RB. By the end of the experiment, the fold expansion decreased to 11.28±3.48 in the static and to 12.17±4.47 in the RB cultures. Total cell expansion was bigger in the static cultures compared to the RB maybe because the shear forces present in the agitated system did not favor total cell expansion (Adamo *et al*., 2009) .

3.3 Total hematopoietic progenitor expansion

The average total progenitor expansion achieved in 5 cultures of UCB HSC in IMDM media is shown in Fig. 2. On day 5, total CFC increased 17.28±4.47 and 16.94±2.82 folds for the static and the roller bottle cultures, respectively. However, for day 10 the CFC fold expansion decreased to 3.00±2.76 for the static and 2.43±1.89 folds for the roller bottle cultures. On day 13 the progenitor fold expansion was very difficult to evaluate since we were unable to count the progenitor colonies in three of the five samples cultured in the clonogenic assay.

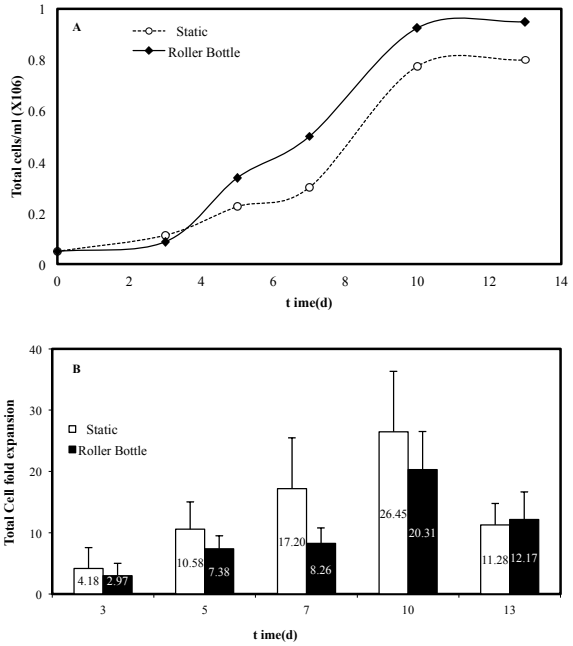

Fig. 1. A) Typical total cell growth kinetics in static and roller bottle cultures of CD34+ cells from human umbilical cord blood. B) Maximum total cell expansion in static and roller bottle cultures of CD34+ cells from human umbilical cord blood. Data represents the mean of five unrelated samples and the bars show the standard error.

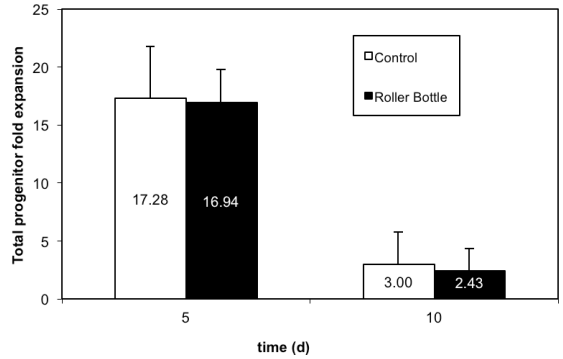

Fig. 2. Total Progenitor Expansion in static and roller bottle cultures of umbilical cord blood CD34$^+$ cells. Data represents the mean of five unrelated samples and the bars show the standard error.

3.4 Total cell expansion vs total hematopoietic progenitor expansion

From the start of the cultures of the $CD34^+$ enriched fraction a rapid total cell increase was observed, avoiding the initial cell death phase previously reported for total MNC cultures (Andrade-Zaldívar *et al*., 2011). For the enriched cultures, the total progenitor expansion was evaluated since day 5 of culture. At that moment, both the total cell number and the progenitor expansion were increased. However, the increase in progenitors was greater than the increase in total cells. It is likely that at this time of culture the added cytokines were in the proper concentration to drive the expansion of progenitors over the expansion of total cells. At day 10, a proliferation of total cells at a major extent was observed but the progenitor expansion decreased possibly due to the nutrients consumption.

Table 1. Expansion of hematopoietic progenitors starting with UCB CD34+ cells cultures.

System	Volume (ml)	Media	Maximum total cell proliferation		Maximum progenitor expansion		Reference
			Folds	Day	Folds	Day	
Teflon bags / 14 days 0.25×10^5 cells/ml	50	CellGRO, 20ng/ml IL3, 100ng/ml SCF and TPO, 300ng/ml Flt3	17±7	6	14±9	6	(Querol, 2003)
24 well plates/ 30 days 0.5×10^5 cells/ml	1	Myeloculth, 12.5%FBS, 12.5% horse serum, 10ng/ml SF, IL3, IL6, GM-CSF, G-CSF and 3U/ml EPO	500	25	10.45	30	(Flores-Guzman *et al*., 2002)
24 well plates/ 14 days 2×10^5 cells/ml	1	IMDM, 130ng/ml SCF, (ng/ml IL3, 20ng/ml IL6, 13ng/ml G-CSF, 50 ng/ml Flt3, 0.4U/ml EPO	12.2±4	14	7.6±4.1	7	(Fietz *et al*. 1999)
4 well plates/ 6 days 0.5×10^5 cells/ml	1	IMDM, 25% FCS, 50ng/ml SCF, IL3 and IL6, 100ng/ml Flt3	18.19±10.05	6	12.50±14.51	6	(Capmany *et al*. 1999)
		Stem Pro, 2% albumin, 50ng/ml SCF, IL3 and IL6, 100ng/ml Flt3	16.71±7.23	6	13.60±8.77	6	
Static culture/ 7 days 0.25×10^5 cells/ml	NR	IMDM, 10% FBS, 500 IU/ml IL1, 10ng/ml SCF, IL3 and GM-CSF	18.23±4.73	7	12.81±9.40	7	(Case *et al*. 1997)
24 well plates/ 14 days 0.25 to 0.5×10^5 cells/ml	1	IMDM, 10% FBS, 2 ng/ml IL 3, 5 ng/ml IL 6, 5 ng/ml SCF, 5 ng/ml G-CSF, 5 ng/ml GM-CSF, 5 ng/ml Flt-3, 3 U/ml EPO.	26.45±9.89	10	17.28±4.47	5	This work
Roller Bottles/ 14 days 0.25 to 0.5×10^5 cells/ml	9-20	IMDM, 10% FBS, 2 ng/ml IL 3, 5 ng/ml IL 6, 5 ng/ml SCF, 5 ng/ml G-CSF, 5 ng/ml GM-CSF, 5 ng/ml Flt-3, 3 U/ml EPO.	20.31±6.18	10	16.94±2.82	5	This work

Also, the rapidly increasing cell number may be affecting the "stemness" of the progenitor cells by the accumulation of other growth factors (Piacibello *et al*., 1998). For mammalian cells it has been shown that variations on media composition reduce up to 20% the viability of cells (Espinosa-Ayala *et al*., 2007). By the end of the experiment the total cells decreased considerably in both static and RBs. Since adding more media to the cultures implies more manipulation, it would be worth to shorten the culture periods to avoid cell death and progenitor death or differentiation.

These results contrast with the reported for total MNC cultured in the same RB system, in which the total cell increase remained low (up to 2 fold) and steady throughout the culture whereas the progenitor expansion was high (around 15 fold) and steady on days 5 and 10 of culture and only decreased to 9 folds by day 13 (Andrade-Zaldívar *et al*., 2011). But for both, total or enriched MNC cultures, the higher progenitor expansion was achieved with relative low total cell proliferation. This data, together with the lack of the death phase in $CD34^+$ cultures, may be suggesting that a higher progenitor expansion could be found even earlier in the cultures of the enriched fractions.

The RB cultures of human UCB $CD34^+$ cells achieved a maximum progenitor expansion comparable to other published work, but in most cases the other cultures were smaller in volume, including our own static control culture (Table 1). It has been demonstrated that larger static cultures in T-flasks or other culture plates do not show significant expansion (Liu *et al*., 2006) and that primitive cells cultured in static conditions may lose their stem features (Unsworth and Lelkes, 1998). RBs are easily scalable to large volumes and they are suitable for the culture of suspended cells such as MNC (Berson and Friederichs, 2008). In RB experiments, it was possible to perform cultures 20 times larger than the statics and therefore 20 times more total expanded progenitors

We previously reported the use of the RBs for the culture of total MNC from UCB and the present work aimed to look for the usefulness of the system using a sub-population of these cells. The results brought out interesting differences in the behavior of the hematopoietic progenitors under the same conditions of culture, but surrounded by total MNC or by the $CD34^+$ fraction only.

It is also relevant that in enriched cultures total cell proliferation was constantly rising throughout the cultures, but total progenitors did not increased simultaneously. The selected cytokine cocktail was previously optimized and evaluated by our group (De León A., *et al*., 1988, Andrade Zaldívar *et al*., 2011) and showed promising results in CFC expansion, we wanted to contrast our previous results in total MNC cultures with the $CD34^+$ cultures aimed in the present work. In the later case may be an increase in the cytokines doses will be useful for maintaining CFC expansion for longer periods.

All these findings may be pointing to a meaningful difference that would help to decide whether to use total MNC or selected fractions for the expansion of hematopoietic progenitors depending on the purpose and length of the cultures. To our knowledge there is not available related work and therefore further research is required.

Future investigations may also include gathering more data by monitoring the $CD34^+$ and other fractions during the cultures by fluorescence-activated cell sorting or immunocytochemistry and verifying the phenotype of the cultured cells at different stages.

Conclusion

A major restriction for transplantation of UCB hematopoietic progenitors is the low quantity of cells. The expansion of such progenitors to clinical useful dosages is still a challenge. Our results are comparable in fold expansion to other published work, however we explored a newly reported system and shorter periods of culture for human UCB $CD34^+$ cells.

Roller bottles are an appropriate system for short-term cultures of UCB $CD34^+$ enriched cells. In this work they allowed a mean 16.94±2.82 fold expansion. We need to research further the differences that the RB system may be producing in all the cells involved in the culture. It is demanding to keep optimizing this simple technological approach to improve progenitor cell expansion and to demonstrate the feasibility of the expanded population for clinical studies.

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Abbreviations

BM, bone marrow; CD, cluster of differentiation; CFC, colony forming cell; EDTA, Ethylenediaminetetraacetic acid; EPO, erythropoietin; FBS, fetal bovine serum; Flt 3, flt3 ligand; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; HSC, hematopoietic stem cell; IMDM, Iscove's modified Dulbecco's medium; IL , interleukin; MNC, mononuclear cells; PBS, phosphate buffered saline; RB, roller bottle; RT, room temperature; SCF, stem cell factor; UCB, umbilical cord blood.

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