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Expansion of human hematopoietic cells from umbilical cord blood using roller bottles in CO_2 and CO_2 free atmosphere

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SUMMARY

In this work, we evaluated the expansion of human hematopoietic stem cells from umbilical cord blood in roller bottles (RB). The Iscove´s modified Dulbecco's medium, the Stem Pro 34-SFM medium, and the L-15 Leibovitz's medium for cultures in CO_2-free atmosphere were assessed. At day 5 of culture, total colony forming unit expansions of 14.44±3.74, 11.20±6.37 and 17.25±3.65 folds were attained, respectively. The expansion reached using L-15 medium in RB was around 10 times higher than the achieved in the static control cultures. To our knowledge, this is the first report of cultures in CO_2 free atmosphere to expand cord blood human hematopoietic stem cells and it opens a new branch of possibilities for culturing and clinical applications.

HIGHLIGHTS

- Roller Bottle cultures suit hematopoietic stem cell expansion
- CO_2 free atmosphere cultures improve human hematopoietic stem cell expansion

INTRODUCTION

Human hematopoietic stem cells (HHSC) can be obtained from several sources such as bone marrow (BM), mobilized peripheral blood (MPB) and umbilical cord blood (UCB) [1]. BM transplants have been a life saving tool for more than 40 years in the treatment of malignant and non-malignant diseases such as leukemias and aplastic anemia [2], but the collection procedure is invasive and matching donors are not always available. Umbilical cord blood is an approach with high potential for HHSC transplantation. The advantages of UCB are a non-invasive collection procedure, the possibility of establishing cord blood banks, easier finding of compatible donors and a lower risk of host versus graft disease [3,4], but the main drawback is the small amount of HHSC that can be obtained from UCB (0.4- 1.0 $X10^9$ total mononuclear cells).

A wide range of possible therapeutic applications for HHSC is being studied; therefore strategies to exploit the UCB potential, such as *in vitro* expansion are increasingly needed. *In vitro* expansion of HHSC has been proven viable and safe, since expanded cells can be transplanted to patients without risk [5,6]. Therefore, several studies have been focused on the culture and expansion of HHSC. The proposed strategies include from 2-dimensional and three-dimensional static cultures to different types of bioreactors including *airlift*, perfusion chambers, stirred tanks, spinner flasks and rotating wall vessels with promising results [7-9]. Unlike most animal cell cultures where cell-products are harvested and cells are disposable, the major interest of HHSC culture are the cells themselves. HHSC require adequate oxygen and nutrient flow, which may be achieved with agitated bioreactors, but since they grow in suspension they are sensitive to shear stress and the mechanisms to sparge oxygen can cause cell damage. A lower agitation rate could minimize shear stress

[10]. Roller Bottles (RB) are a simple strategy for culturing adherent and suspended cells, they can be operated without specialized training, they are easily scalable for clinical purposes and they involve very low capital investment [11]. RB have been used for a long time to culture animal cells [12], and are now being used to culture various types of cells including hybridoma [11]. RB provide a good choice to culture suspension cells that are sensitive to shear stress because they can be operated at very low agitation rates.

In this work, we evaluated the utility of roller bottles to expand HHSC from UCB comparing the non-static and static cultures in different culture media with recombinant cytokines.

MATERIALS AND METHODS

UCB collection and processing

UCB samples from full-term deliveries were kindly provided by local hospitals, according to their ethic committee's guidelines. The UCB-MNC separation procedure has been described elsewhere [16]. Briefly, 40-80 ml blood 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.

Culture Media

Three culture media were tested: Iscove´s modified Dulbecco's medium (IMDM, Gibco), Leibovitz's L-15 (L-15, Gibco) and Stem-Pro® 34SFM (Stem Pro, Gibco). IMDM and L-15 were supplemented with 10% fetal bovine serum (Gibco). Media were supplemented with human recombinant cytokines (Peprotech): 2 ng/ml interleukin-3 (IL 3), 5 ng/ml interleukin-6 (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).

Roller bottles cell culture

RB cultures were started with 0.5 $x10^6$ MNC/ml or 10 $x10^3$ $CD34^+$ cell/ml into 500 ml glass roller bottles (Wheaton) containing up to 25 ml of culture medium. Cultures were maintained for 14 days at 37°C in an incubator (Shel lab) at 5% CO_2 atmosphere for the IMDM and Stem Prom cultures, whereas experiments in L-15 medium were maintained in a CO_2-free incubator (Shel lab). 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 control.

Colony-forming cell assay and mononuclear cell counting

Number of colony-forming cell (CFC) was determined from methylcellulose-based semisolid cultures (Metho Culture; StemCell Technologies) containing per ml: 50 ng SCF, 10 ng IL-3, 10 ng GM-CSF, and 3 U. Plates were inoculated with 10 000 to 40 000 cell/ml and incubated for 14 days at 37°C and 5% CO2. Hematopoietic colonies were classified as

described previously [20]. MNC concentration and viability were determined by cell counting with the Trypan Blue exclusion method using a hemacytometer.

Data processing

Error bars in all graphs represent the standard error of the mean.

RESULTS

Total cell expansion

The total cell growth kinetics for the cultures of HHSC under static and dynamic conditions is illustrated in fig 1. Panel A shows a typical static-culture using IMDM. For this sample, the maximum cell concentration was $1.22x10^6$ cell/ml at day 13 and $0.063x10^6$ cell/ml at day 10 for the static and RB culture respectively. Fig 1B shows the cell growth of one representative sample cultured in Stem Pro. After the initial death phase of 5 days, the maximum cell concentration was reached at day 10 of culture, with 1.07x $x10^6$ and 2.83x $x10^6$ cell/ml for the static and RB cultures respectively. The cultures in Stem Pro reached higher total cell numbers, which can be confirmed comparing panel B to panel A, which is the same sample cultured in IMDM. Fig 1C shows the total cell growth of a culture in L-15. Nearly all controls showed a death phase of only 3 days, but most RB cultures pass through this phase starting the total cell growth from the beginning of the experiment. In this case, the maximum cell concentration, achieved at day 10 of culture, was $1.23x10^6$ and $1.21x10^6$ cell/ml for the static and RB culture respectively.

Fig. 2 illustrates the total cell fold expansion in our cultures. Panel A shows the IMDM cultures. Some cultures showed a death phase of 5 days; subsequently most control cultures showed continuous growth reaching a maximum cell concentration between days 5 and 13 ranging from 2.44 to 5.30-folds, with a mean of 3.30±0.97 folds at day 13 of culture.

However, in the RB cultures each sample showed a unique behavior indicating even a decrease on total cells during the experiment ranging from 0.47 to 5.40 fold-expansion in different days of culture, with a mean of 1.85±0.51 folds at day 13 of culture. Both static and RB cultures showed the same tendency, at days, 3, 5 and 7 the growth was increasing equivalently in both systems, and by days 10 and 13 we observed the same trend to increase total cell numbers but in the static controls at a major extent.

Fig 2B shows the total cell fold expansion in the Stem Pro cultures. The maximum expansion ranged from 0.5 to 2.14 folds in the controls and from 0.35 to 5.66 in RB on different days for each sample. For this medium, the growth was comparable for both systems from days 0 to 7, with a slightly higher growth for the static cultures. For days 10 and 13 we observed a huge variability on the total cell numbers. The mean maximum fold expansion, achieved at day 13 of culture, was 1.18±0.23 and 1.17±1.06 for the static and RB cultures respectively.

Total cell fold expansion achieved in L-15 cultures is shown in Fig. 2C. The maximum expansion attained ranged from 0.67 to 3.3 and from 0.75 to 2.50 folds for the static and RB cultures respectively, in different days depending on the samples. The growth in both static and RB cultures provided comparable results of total cells for the same samples. For this medium we observed the maximum growth between days and 7 and 10 of culture, but the total increase on cells was lower than for the other two media. The maximum fold expansion in L-15 cultures was 1.35±0.49 and 1.31±0.39 for the static and RB cultures respectively. Comparing panels A and C, the control IMDM cultures showed a higher average total cell expansion than the L-15 controls at days 10 and 13, but for days 3 to 7, both systems showed approximately the same fold expansion in IMDM and L-15 media.

The Stem Pro cultures had a lower total cell expansion from day 3 to day 13 in both static and RB systems compared to the IMDM cultures.

Expansion of total hematopoietic progenitors

The progenitor expansion achieved in static and RB cultures in IMDM is shown in figure 3A. For RB cultures, we found a mean colony forming cell (CFC) fold expansion of 14.44±3.74, 16.87±5.30 and 9.16±4.15 at days 5, 10 and 13 respectively; meanwhile the static controls achieved 10.10±2.58, 14.02±3.21 and 10.96±4.10 respectively at the same days. Progenitor expansion was observed in this work as early as day 3 of culture (data not shown) despite the fact that the cultures showed a total cell decrease. Confirming these, at day 5, even when the total cell numbers was still lower than the initial, the progenitors in RB cultures were already expanded.

The mean CFC fold expansion for the samples cultured in Stem Pro is shown in figure 3B; in these cultures we achieved an 11.20±6.37 CFC-fold expansion on day 5 in RB cultures, whereas for the static control the expansion was 7.74±1.67. However, for days 10 and 13, the progenitor expansion achieved in RB was almost a half of the static controls. Fig. 3C shows the total progenitor fold expansion attained in L-15 cultures of different samples. On day 5, the mean total progenitor fold expansion in RB was 17.25±3.65, on day 10 it was 17.35±8.81 and on day 13 it remained the same with an 18.39±9.49 fold expansion.

Fed-batch cultures using roller bottles

We also performed 15 days fed-batch cultures in IMDM, providing the cultures with fresh media and cytokines in order to support a longer expansion. Fig 4 shows the mean progenitor fold expansion achieved in two fed-batch cultures of the same sample. Feeding

the culture on day 3, resulted in a progenitor fold expansion increase from 17.84 to 27.47 on day 5, and from 0.28 to 19.99 on day 13 but on day 10 it decreased from 24.43 to 18.17. Feeding the culture on day 7 did not increase total progenitor expansion in any of the days tested. The static control cultures showed the same behavior than the RB (data not shown).

DISCUSSION

RB-cultures in all media tested allowed a 15-20 total progenitor fold expansion on day 5 of culture. Progenitor expansion may be affected by the individual variation since samples were not related at all. IMDM is an improved synthetic medium created for rapidly multiplying cell cultures. This medium, supplemented with different amounts of FBS and cytokines has been widely used for the culture and expansion of HHSC [7, 10, 16, 20] showing good results for CFC expansion in most experiments. We used IMDM for our RB system, obtaining expansion results comparable to other cell culture strategies (table 1). Our RB mimic the expansion found in the controls, but they have the advantage of being able to support larger volumes.

We also tested two other media: Stem Pro and L-15. Stem Pro is a serum free medium created to support the growth of $CD34^+$ hematopoietic cells and total cells in static cultures of bone marrow $CD34^+$ hematopoietic cells [21]. The use of Stem Pro may reduce the risk of immune reactions and infection due to serum [13]. However, Stem Pro-RB cultures showed reduced total cell growth and progenitor expansion in most of our experiments. L-15 medium is buffered by phosphates and free-base amino acids instead of the sodium bicarbonate system used by IMDM. It can be used in non-sealed containers as our RB. We found that IMDM and L-15 cultures reached similar progenitor expansion on day 5, but L-

15 permitted the longest total CFC expansion, it remained around 17 times, for all the days tested; therefore it allows a higher and longer CFC expansion than IMDM.

Commercial spinner flasks bioreactors are available to expand HHSC; for example the Dideco Pluricell system with a limit volume of 38 ml achieved a maximum mean expansion of 230 fold in MNC and 21 fold in $CD34^+$ cells. A lower agitation rate would minimize shear stress. For instance, rotating wall vessel bioreactors have been used up to 6 rpm to culture HHSC, achieving 21.7±4.9 fold progenitor expansion [15]. It is important to develop new methodologies to culture HHSC which are easy to perform, safe and do not promote progenitor cells differentiation. This is the first report on expansion of HHSC-UCB in roller bottles. We showed that roller bottles are suitable for the expansion of hematopoietic progenitors since they had a slightly higher total cell expansion than the static cultures, and they allowed progenitor expansion to a greater extent. We used 1 ml control cultures because it has been demonstrated that T-flasks with larger volumes do not show significant cell expansion [15]. We attained total cell expansions comparable to those reported using Spinner flask or stirred bioreactors [16-18], but below the cell expansion attained in Pluricel system or other RB-like devices [10, 19]. RB can be an alternative to the use of culture bags, where up 31-fold CFC expansion has been attained, but the reduced volume and the nutrient rechange and oxygen made difficult the scaling up [14].

In this work, we demonstrated that Roller Bottle short-term UCB-MNC cultures allow progenitor expansion up to 18.39 times in L-15 medium. Roller Bottles are suitable to culture MNC from human umbilical cord blood in all media tested in this work *e.g.* L-15, Stem Pro and IMDM. We demonstrated that L-15 medium is a good choice to culture

HHSC and it does not require CO_2 control. Roller bottles without CO_2 atmosphere are simple to operate, have low requirements of cytokines, and favor HHSC expansion. The L-15 RB cultures would be easily scalable, and therefore they could have a great potential for clinical applications. Nevertheless, expanded progenitors must be evaluated for safety, engraftment and utility in transplants.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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FIGURE AND TABLE LEGENDS

Table 1. Comparison of different protocols for the expansion of HHSC.

Figure 1. Typical growth kinetics of human hematopoietic cells from umbilical cord blood in static and roller bottles. A) Cultures in IMDM B) cultures in Stem Pro medium C) Cultures in L-15 medium.

Figure 2. Maximum total cell expansion in static and roller bottles. A) Cultures in IMDM. B) Cultures in Stem Pro medium C) Cultures in L-15 medium.

Figure 3. Total Progenitor Expansion in static and roller bottles. A) Cultures in IMDM. B) Cultures in Stem Pro medium C) Cultures in L-15 medium.

Figure 4. Progenitor expansion in fed batch culture of mononuclear stem cells.