Biotechnology Letters (2006) 28: 335–340
DOI 10.1007/s10529-005-5932-2

Kinetic analysis of the *ex vivo* expansion of human hematopoietic stem/progenitor cells

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Received 28 October 2005; Revisions requested 3 November 2005; Revisions received 5 December 2005; Accepted 6 December 2005

Key words: cell expansion, expansion kinetics model, human hematopoietic cells, human stem cells, stroma

Abstract

The total cell expansion of human umbilical cord blood (CB) and adult bone marrow (BM) $CD34^+$-enriched cells cultured in supplemented serum-free media, either over irradiated human feeder layers or in stroma-free systems, were characterized by a simple kinetic model using only two parameters: the specific cell expansion rate, μ, and the death rate constant, k_k. Both CB and BM cells can expand at approximately the same rate (0.21 day^{-1}) in this culture system however, cell death depends on the presence of stroma and the environment in which the cells are cultured.

Introduction

The human body consumes an average of 400 billion mature blood cells per day and this number dramatically increases under conditions of stress (McAdams *et al.* 1996). Hematopoiesis consists in a complex scheme of cell proliferation and differentiation to fulfil this demand in which, the more primitive cells – hematopoietic stem/progenitor cells (HSC) – mature into erythrocytes and the other blood cells. The potential clinical applications of HSC include bone marrow (BM) transplantation and somatic cell gene therapy (Lewis *et al.* 2001), whereas more mature blood cells are required in specific clinical settings, e.g. blood transfusion. Therefore, HSC *ex vivo* expansion and/or differentiation is required in order to obtain a therapeutically significant amount of cells from the available donor samples.

The human hematopoietic stem cell pool is characterized by the expression of the CD34 surface antigen (Berenson *et al.* 1988); however, since the expression of this marker by this population is not exclusive, a combination of CD34 and other antigens is currently used to select HSC. One of the most used is CD38, a marker for hematopoietic commitment (Shih *et al.* 1999); thus, the more immature hematopoietic progenitors are known by the $CD34^+CD38^-$ phenotype.

The HSC microenvironment in the BM consists in mesenchymal tissue composed of a mixed population of endothelial cells, fibroblasts, reticular cells, macrophages and adipocytes, known as stromal cells. These cells support and regulate self-renewal and differentiation of HSC, through cell-to-cell interactions and expression of specific cytokines and growth factors (Majumdar *et al.* 1998). Therefore, to mimic hematopoietic environment *in vitro*, stromal cells have been used as supportive cells of HSC cultures in a two-dimensional reconstitution of the BM microenvironment

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in vivo (Emerson 1996, Majumdar *et al*. 1998, Lobato da Silva *et al*. 2005). In this study, human stroma was used as feeder layer for expansion of HSC collected from CB and BM (Lobato da Silva *et al*. 2005).

Rational approaches to the *ex vivo* expansion of HSC require a better understanding of self-renewal/differentiation mechanisms. Detailed kinetic models can provide a significant insight into the limiting steps involved in cell expansion and differentiation (Lobato da Silva *et al*. 2003). This information is useful not only for the *in vitro* expansion itself, but also for the design and operation of stem cell bioreactors (Cabrita *et al*. 2003).

In the present work we have characterized the total cell expansion by means of a Monod-type kinetic model involving only two parameters: the specific cell expansion rate, μ, and the death constant, k_k. The parameters obtained were used to evaluate the influence of the presence/absence of a human feeder layer on the overall hematopoietic cell (HC) expansion. This kind of approach is limited by the fact that, in the present case, the cells expand but also differentiate and, thus, the expansion rate is likely to change as the cells evolve. However, an average value of the expansion rate can be obtained from this type of models, providing a global measure of culture system performance in terms of total cell expansion *ex vivo*.

Materials and methods

Cell preparation

Umbilical cord blood samples were obtained after mother's informed consent from the Pediatric Stem Cell Transplant Program, at Duke University Medical Center, Durham, USA, and from the Serviço de Ginecologia e Obstetrícia, Hospital D. Estefânia, Lisboa, Portugal. Heparinized human bone marrow was obtained from healthy donors after informed consent. Low density cord blood mononuclear cells (CB MNC) or bone marrow mononuclear cells (BM MNC) were separated by a Ficoll density gradient (1.077 g ml^{-1}) (Sigma) and washed twice in Iscove's Modified Dulbecco's Medium (IMDM), (Gibco Laboratories). CB MNC and BM MNC from each donor were enriched for $CD34^+$ cells using magnetic cell sorting (Miltenyi Biotec Inc.).

Cell expansion

A human stroma based cell culture system established for the *ex vivo* expansion of HSC was used (Lobato da Silva *et al*. 2005). CB and BM $CD34^+$ enriched cells were resuspended in a serum-free medium (5 ml), QBSF-60 (Quality Biological, Gaithersburgh, MD, USA), supplemented with stem cell factor (SCF, 100 ng ml^{-1}), leukaemia inhibitory factor (LIF, 10 U ml^{-1}), fibromyalgia syndrome-related tyrosine kinase 3 ligand (Flt-3 ligand, 100 ng ml^{-1}) and basic fibroblast growth factor (bFGF, 5 ng ml^{-1}) (Peprotech, Rocky Hill, NJ, USA), and cultured over a γ-irradiated feeder layer (15 Gy) or in stroma-free conditions. The cultures were inoculated in T_{25} culture flasks (25 cm^2) in a humidified incubator at 37 °C, with an atmosphere of 5% CO_2 during 2–4 weeks, maintaining periodic half-feed procedure. The *ex vivo* expansion of the cell populations was determined at each time point by microscopic cell counting and viability checked by Trypan Blue Dye exclusion. In order to better visualize the expansion of cells, the data were corrected to account for the half-feed procedure and expressed as total cell expansion (Lobato da Silva *et al*. 2003).

Cell phenotype was determined using monoclonal antibodies against CD34 and CD38 antigens (Becton Dickinson Immunochemistry Systems, San Jose, CA, USA) and analysis was performed in a flow cytometer (FACScan, Beckton Dickinson).

Expansion kinetic model

A first order Monod-type kinetic model for cell expansion and death was used to compute the concentration of viable (X_v) and dead (X_k) cells over time (t). The balance for both viable and dead cells can be written as

$$\frac{dX_v}{dt} = \mu \cdot X_v - k_k \cdot X_v = (\mu - k_k)X_v \quad (1)$$

$$\frac{dX_k}{dt} = k_k \cdot X_v \quad (2)$$

where t is the culture time. Assuming that both μ and k_k are time invariant, integrating Equation (1) between $t = 0$, $X_v = X_0$ and $t = t$, $X_v = X_v$ leads to:

$$X_v = X_0 e^{(\mu - k_k)\cdot t} \tag{3}$$

By substituting Equation (3) in Equation (2), the following relation is obtained:

$$\frac{dX_k}{dt} = k_k \cdot X_0 . e^{(\mu - k_k)\cdot t} \tag{4}$$

This equation can be integrated between $t = 0$, $X_k = 0$ (100% viability upon culture inoculation) and $t = t$, $X_k = X_k$ leading to:

$$X_k = k_k \left(\frac{X_0}{\mu - k_k}\right) \cdot \left[e^{(\mu - k_k)\cdot t} - 1\right] = k_k \cdot K \tag{5}$$

from which the value of k_k can be estimated; Equations (3) and (5) allow the estimation of both μ and k_k.

Parameter estimation was carried out by non-linear least-squares regression, using an Excel spreadsheet. Confidence intervals for the parameters thus obtained were computed using the bootstrap resampling technique (Press *et al.* 1992).

Results and discussion

We have established a stroma-based culture system that allows the *ex vivo* expansion of HSC, from both CB and BM (Lobato da Silva *et al.* 2005). Significant CB cell expansion was only observed in the presence of a stromal layer; with respect to BM cells, although cell expansion was observed either in the presence or in the absence of stroma, higher total expansion values were obtained when cells were cultured over a feeder cell layer.

Since CB cells could not be expanded under stroma-free conditions, only the expansion parameters for CB $CD34^+$-enriched cells expanded over human feeder cells were estimated in this work (Table 1). Figure 1 illustrates the model fitting to the experimental values for a representative experiment of CB $CD34^+$-enriched cells. The μ values ranged from 0.14 to 0.24 day^{-1}, whereas k_k ranged from 0.004 to 0.035 day^{-1}. These results suggest that the initial cell number is not determining for total cell expansion, since within the range of starting cell concentration, 0.18–2.54 $\times 10^5$ cells ml^{-1}, μ variations fall within the experimental error.

The same procedure was used with the results of BM cultures with and without a stromal layer.

Table 1. Cord Blood (CB) and Bone Marrow (BM) expansion parameters estimated when culturing different initial cell numbers in the presence (+)/absence (–) of human stroma layers (n.d., not determined).

Starting population				Stroma	Expansion parameters	
	No.	X_0 (cells)	$\%CD34^+CD38^-$		μ (day^{-1})	k_k (day^{-1})
CB	1	1.2×10^6	n.d.	+	0.21 ± 0.13	0.013 ± 0.003
CB	2	9.0×10^4	n.d.	+	0.24 ± 0.04	0.035 ± 0.011
CB	3	7.5×10^5	n.d.	+	0.19 ± 0.05	0.031 ± 0.006
CB	4	1.27×10^6	n.d.	+	0.15 ± 0.03	0.011 ± 0.003
CB	5	8.5×10^5	0.23	+	0.14 ± 0.05	0.004 ± 0.001
BM	1	3.25×10^6	23	–	0.36 ± 0.03	0.057 ± 0.005
BM	1	3.25×10^6	23	+	0.30 ± 0.16	0.020 ± 0.003
BM	2	1.76×10^6	8.2	–	0.24 ± 0.03	0.057 ± 0.006
BM	2	1.76×10^6	8.2	+	0.21 ± 0.02	0.024 ± 0.008
BM	3	6.2×10^6	7.4	+	0.13 ± 0.02	0.009 ± 0.003
BM	4	2.5×10^6	1.7	+	0.14 ± 0.02	0.005 ± 0.001
BM	5	9.3×10^5	0	+	0.17 ± 0.03	0.008 ± 0.001
BM	6	4.3×10^6	0.65	+	0.11 ± 0.03	0.014 ± 0.003
BM	7	1.7×10^6	0.63	+	0.30 ± 0.06	0.015 ± 0.002

The estimated parameters are given together with their 95% confidence interval.

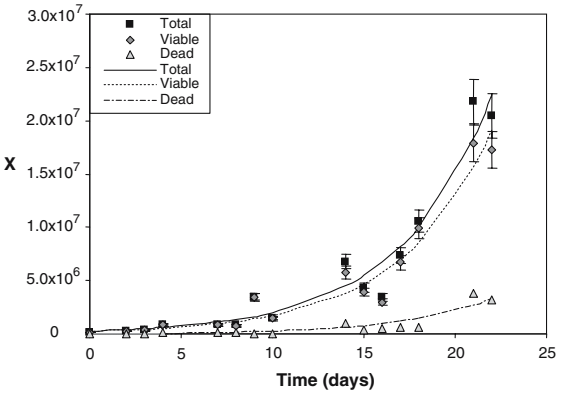

Fig. 1. Example of model fitting (lines) to the experimental time course values of total (■), viable (◆) and dead (△) cell numbers (X) for the CB no. 2 experiment expanded over a stromal layer.

Figure 2 depicts an example of the model fitting. Table 1 shows that μ values range from 0.11 to 0.36 day^{-1} and, although the two data points for higher initial cell number (BM no.3 and BM no.6) are among the ones that have a lower μ value, there does not seem to be a systematic variation.

Within the range of starting cell concentration that was used, the specific expansion rates of BM $CD34^+$-enriched cells are similar to the CB-$CD34^+$-enriched cells, and an average μ value of 0.21 day^{-1} can be estimated for both CB and BM $CD34^+$-enriched cells. Two BM cultures were performed without a stromal layer: both from the values in Table 1 and from Figures 3

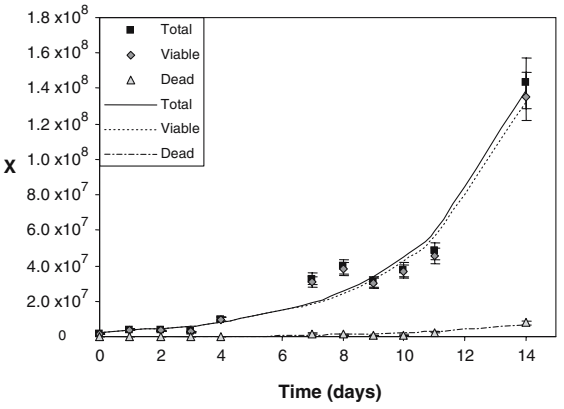

Fig. 2. Example of model fitting (lines) to the experimental time course values of total (■), viable (◆) and dead (△) cell numbers (X) for BM no. 7 experiment expanded over a stromal layer.

and 4, the μ values seem to be slightly higher than the corresponding experiments using a stromal layer, but still fall within the same range. Since it was observed that a higher fold-increase was observed for cultures with a stromal layer, this suggests that expansion rate, μ, might not be the only determining factor for cell expansion performance in terms of fold increase values. Other parameters, such as the death rate (k_k), also affect the outcome of hematopoietic cell expansion.

The values of k_k for BM experiments with a stromal layer varied between 0.005–0.024 day^{-1} and, again, no significant influence of the initial cell number was observed. However, cell death in the absence of a stromal layer seems to be significantly higher (0.057 day^{-1}), which explains why total expansion is lower when no stromal layer is present. Also in the case of k_k, the values obtained for BM stroma containing cultures seem to fall within the same range as those for CB, and an average of 0.016 day^{-1} was estimated.

The specific constants for both cell growth (μ) and cell death (k_k), determined by the Monod-type kinetic model are in accordance with the kinetic constants calculated by a predictive kinetic model developed to interpret HSC expansion and differentiation (Lobato da Silva *et al.* 2003) and correspond to the time average of the respective rate constants for the expansion and death of the different types of cells generated in culture. The conclusion that death rates are determining in the total fold-increase is also in accordance with the observations in the predictive model.

Stromal cells can induce positive or negative effects on hematopoiesis, but the mechanism of these interactions are not fully established (Stein *et al.* 2004, Moore *et al.* 2005). Yoo *et al.* (2003) reported apoptosis suppressing effects of stromal cells which are, globally, in accordance with the reduced k_k values obtained in our studies for cultures over a stromal layer.

Our results do not allow the identification of any clear relation between initial cell number and level of stem/progenitor enrichment and the expansion rate, suggesting that, globally, HSC from two different sources, BM and CB, can be expanded at the same rate in this culture system. However, our data quantitatively indicates the

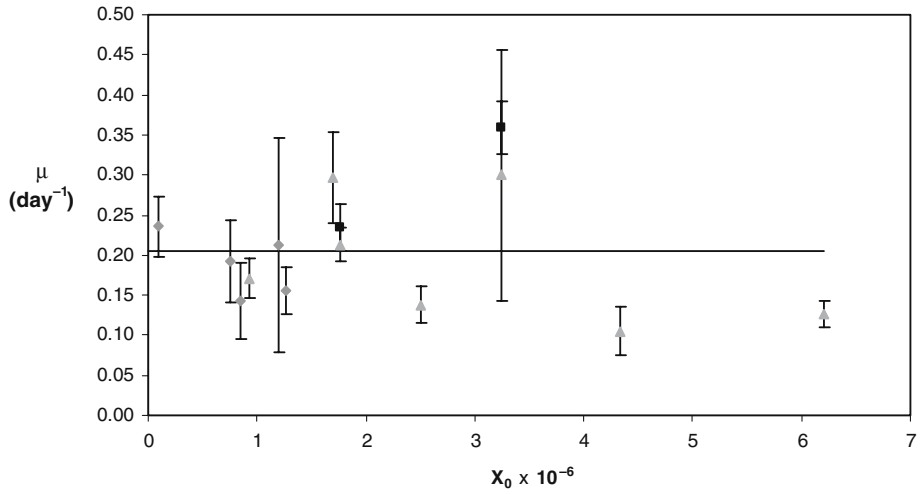

Fig. 3. Total cell expansion growth rates as a function of the initial cell number (X_0) for the cultures over irradiated feeder cells. (◇) CB with stromal layer, (■) BM with no stromal layer and (△) BM with stromal layer. Horizontal line corresponds to the average of all values. Vertical bars correspond to estimated errors in the parameters as obtained by the bootstrap technique.

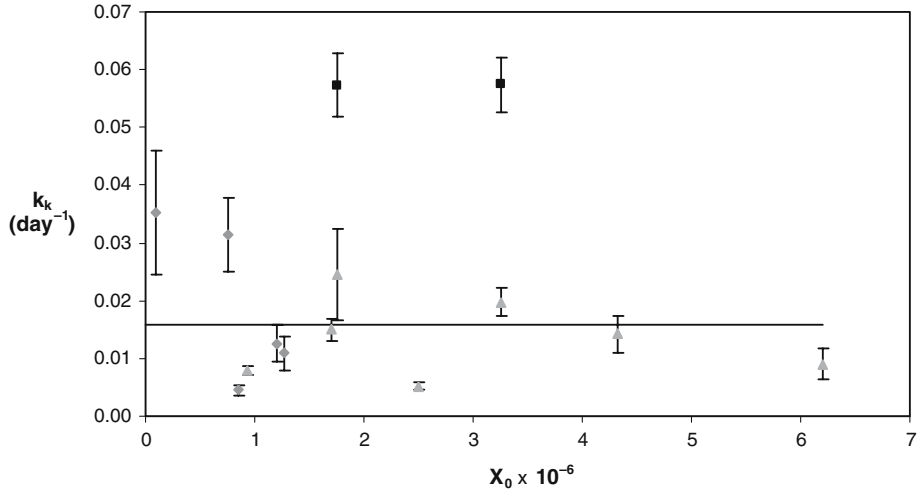

Fig. 4. Death rates as a function of the initial cell number (X_0) for the cultures over irradiated feeder cells. (◇) CB with stromal layer, (■) BM with no stromal layer and (△) BM with stromal layer. Horizontal line corresponds to the average of the values for all experiments with a stromal layer. Vertical bars correspond to estimated errors in the parameters as obtained by the bootstrap technique.

cell death dependency on the environment in which the cells are cultured.

The model presented here only addresses expansion and death of the total pool of hematopoietic cells in culture and does not address the phenotype distribution of the culture over time, namely the fraction of more primitive cells in the total population. Nevertheless, this two-parameter model can be used to extract quantitative information, through the estimation of meaningful kinetic parameters, from experiments in which the *ex vivo* expansion/maintenance of HSC is studied.

Acknowledgements

This work was supported by Fundação para a Ciência e a Tecnologia (project POCTI/EQU/38063/2001, and grants SFRH/BPD/6253/2001,

SFRH/BD/6209/2001 and SFRH/BD/6210/2001 awarded to B.S. Ferreira, R. Gonçalves and C. Lobato da Silva), respectively. The authors gratefully acknowledge CB sample supply from the Hospital D. Estefânia in particular the nursery team of Serviço de Ginecologia e Obstetrícia.

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