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Modelling of ex vivo expansion/maintenance of hematopoietic stem cells

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Abstract In this study, we described the modelling of the expansion/maintenance of human hematopoietic stem/progenitor cells from adult human bone marrow. CD 34^+-enriched cell populations from bone marrow were cultured in the presence and absence of human stroma in serum-free media containing bFGF, SCF, LIF and Flt-3 ligand for several days. The cells in the culture were analysed for expansion and phenotype by flow cytometry. Although significant expansion of bone marrow cultures occurred in the presence and absence of human stroma, the results of expansion were effectively better in the presence of a stromal layer. In both situations the phenotypic analysis demonstrated a great expansion of CD 34^+38^- cells. The differentiative potential of bone marrow CD 34^+ cells co-cultured with human stroma was primarily shifted towards the myeloid lineage with the presence of CD 15 and CD 33.

Keywords Stem cells, Expansion, Hematopoeisis, Modelling

1 Introduction

Stem cells are usually considered to be cells having both the capacity for unlimited or prolonged self-renewal and the ability to differentiate into highly distinct cell lineages [1]. Usually, between the stem cell and its terminally differentiated progeny there are intermediate populations of committed progenitors with a limited capacity for proliferation and a restricted differentiation potential. Hematopoietic stem cell (HSC) biology research has been directed towards the identification of cell populations that possess stem cell characteristics and the study of mechanisms that regulate stem cell self-renewal and differentiation [2].

Because stem cells are rare, one of the major focuses in experimental hematology is the in vitro manipulation of HSC with the ultimate goal of expanding long-term transplantable HSC. Several studies [3, 4, 5, 6] have been performed in which the investigators have attempted to achieve ex vivo expansion of HSC, thus demonstrating the feasibility of in vitro culture of these cells in the presence or absence of stroma [3, 6]. Ex vivo expansion of hematopoietic stem/progenitor cells is highly desirable in a variety of clinical settings such as bone marrow transplantation, immunotherapy, gene therapy and production of mature blood cells [2].

It is generally held that, in the steady state, the majority of HSC are dormant in the G_0 phase of the cell cycle, and only a few actively cycling HSC supply all of the hematopoietic cells at a given time. To supply these cells, HSC must also be multipotential, i.e. capable of differentiating into all of the blood cell lineages. To accomplish this, HSC, in the presence of the appropriate growth factors, begin by first differentiating into multipotential progenitor cells. These cells are distinct from HSC because they no longer possess the ability to self-renew. The processes whereby stem cells self-renew and lineage commit, with the resultant progenitors giving rise to all of the differentiated mature blood cells that enter the circulation are collectively referred to as hematopoiesis (Fig. 1) [7].

Regardless of the source, the HSC content of the initial hematopoietic product can be enriched by selecting for cells expressing the surface antigen CD 34, which is found on many of the primitive HSC. However, it should be noted that CD 34 expression alone is not indicative of HSC.

The expansion and differentiation process, starting from the HSC and leading to the more mature blood cells, is complex and involves a series of steps, which, according to the growth factors that are present, can favour one or another of the possible outcomes. For the goal of obtaining the target differentiated cell type, it is crucial to be able to understand the complete process and to control the various steps involved.

Kinetic modelling, as it has been applied to many other biochemical systems, can provide a significant insight into the "limiting steps" involved in the scheme and on how the various cofactors that can be used can influence the

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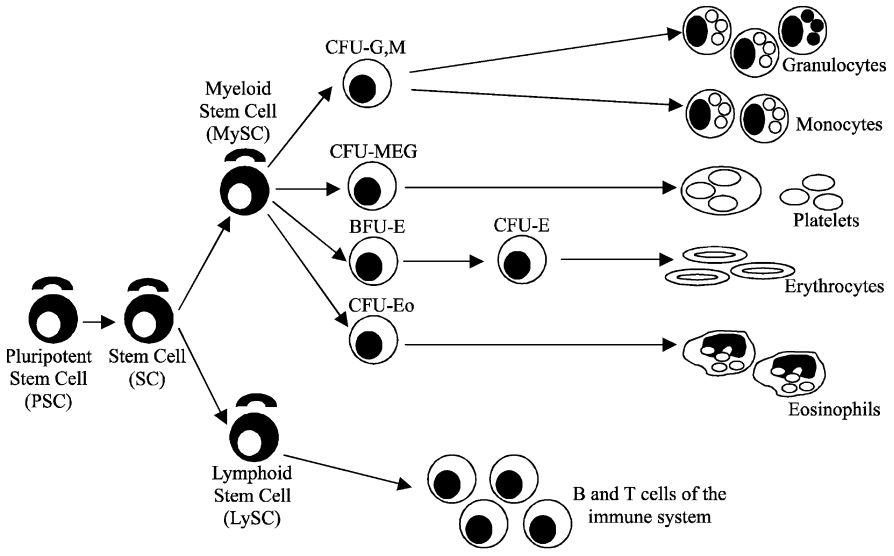

Fig. 1. Hematopoiesis (*CFU-GM*, colony-forming unit – granulocyte, macrophage; *CFU-MEG*, colony-forming unit – megakaryocyte; *BFU-E*, burst-forming unit – eryhtroid; *CFU-E*, colony-forming unit – erytroid; *CFU-E_0*, colony-forming unit – eosinophils)

outcome of the expansion process. If this kind of modelling is useful in the in vitro expansion, it will also prove to be extremely important in the design and operation of continuous reactors for these purposes.

In this work the analysis for expansion and phenotype of hematopoietic stem cells (CD 34^+) is undertaken. The experimental results are modelled based on a kinetic model, taking into account cell expansion, differentiation and death.

2
Materials and methods

2.1
Cell preparations

Heparinized adult human bone marrow (BM) was obtained from healthy donors (n=2) by the School of Medicine, University of Nevada, Reno, USA. Low-density (<1.077 g/cm^3) mononuclear cells (BM MNCs) were isolated from BM by centrifugation of the initial suspension on Ficoll (Sigma, St. Louis, USA), and the cells were then washed twice in Iscove's modified Dulbecco's medium (IMDM, GIBCO Laboratories, Grand Island, USA). BM MNCs were enriched for CD 34^+ cells using magnetic cell sorting (Minimacs, Milteny Biotec, Auburn, USA) [4, 8]. The purity of the CD 34^+ enriched cells ranged from 60 to 80%.

2.2
Cell expansion

The CD 34^+ enriched cells were resuspended in serum-free media, QBSF-60 (Quality Biological, Gaithersburg, USA), with SCF (100 ng/ml), LIF (10 U/ml), Flt-3 ligand (100 ng/ml) and bFGF (5 ng/ml) (Peprotech, Rocky Hill, USA) at a final concentration of 10^6 cells/ml, in the presence or absence of stroma. 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. The ex vivo expansion of the purified population was determined at each time point by counting.

2.3
Proliferation and phenotype analysis

The content of cells in each culture was evaluated by microscopy and viability checked by trypan blue dye exclusion. Phenotype was determined by flow cytometry using monoclonal antibodies against CD 3, CD 7, CD 14, CD 15, CD 19, CD 33, CD 34 and CD 38 (Becton Dickinson Immunochemistry Systems, San Jose, USA) and analysed in a FACScan.

2.4
Statistics and data analysis

The experimental results are presented as the mean plus/minus the standard error of the mean (SEM) for a confidence level of p<0.05.

3
Modelling

A kinetic model was developed [7] based on the scheme presented in Fig. 1, which computes the concentration of each type of cells as a function of time. Unlike the material balances that are written for chemical reactions (the actual "reactants", which would be the nutrients for the cells, are not explicitly accounted for in this model), the equations describing the change in the concentration of cells with time involve self-expansion terms. Although a balance has to be written specifically for each cell type, all the balances have a similar structure, containing four terms:

- a self-expansion term, with a rate constant k_e^X, where X represents the type of cell under consideration and which is corrected with a factor. As the total cell expansion is limited, either by volume constraint or due to nutrient depletion, a factor γ was taken into account. This factor is used to multiply all the expansion terms, and takes the form

$$\gamma = \frac{C_{\max} - \sum_{\text{all types of cells}} [X]}{C_{\max}}, \qquad (1)$$

where C_{max} is the maximum concentration of cells. In the computation of γ, the concentrations $[X]$ of all cells, including nonviable ones, are considered. This term reduces the rate of expansion as the concentration of cells increases, and is zero (no growth) when the total concentration of cells equals the maximum concentration allowed.

- a term for cell death, with a rate constant k_k^X;
- a term accounting for the differentiation, which includes:
  - a generation term that is dependent on the concentration of the parent cell type and has a rate constant k_d^X (differentiation rate into cell type X);
  - a consumption term involving the differentiation of cells X, themselves, into Y (the next cells along the differentiation line),with a rate constant k_d^Y. This term may have several components if X is able to differentiate into various cell types.

Thus, a general balance equation assumes the following structure:

$$\frac{d[X]}{dt} = k_e^X \gamma[X] + k_d^X[P_x] - \sum_Y k_d^Y[X] - k_k^X[X], \qquad (2)$$

where $[P_X]$ represents the concentration of the parent cell type, and the final summation is carried out on all Y, the cell types that are originated by X on its differentiation process.

For instance, the balance written for the original pluripotent stem-cell (PSC) (Fig. 1) is

$$\frac{d[PSC]}{dt} = k_e^{PSC}\gamma[PSC] - k_d^{SC}[PSC] - k_k^{PSC}[PSC], \qquad (3)$$

where k_e, k_d and k_k are the coefficients for expansion, differentiation into SC (Fig. 1) and death, respectively. In this particular case, there is no term corresponding to generation from a previous cell type.

Another example, the balance for the SCs, is

$$\frac{d[SC]}{dt} = k_e^{SC}\gamma[SC] + k_d^{SC}[PSC] - k_d^{MySC}[SC] - k_d^{LySC}[SC] - k_k^{SC}[SC]. \qquad (4)$$

The terms now refer to the facts that SC can multiply themselves, that they are produced by differentiation from PSC, that they, themselves, can evolve into MySC and LySC and that they can die.

Equations were written for all cell types, and the set of differential equations thus obtained was integrated by the Euler method in an Excel (Microsoft, USA) spreadsheet.

The results obtained from the simulation were fitted to all the available experimental data (total cells, total viable cells and population with specific phenotype) by a least-squares regression method. Minimization of the sum of the residuals by varying the relevant model parameters (kinetic constants and maximum concentration for the γ factor) was performed using the Solver tool in Excel. The integration step for the Euler method was selected in order to minimize the integration error (<1%).

4 Results and discussion

4.1 Expansion of hematopoietic stem/progenitor cells: stroma-containing versus stroma-free BM MNC cultures

Stroma-containing and stroma-free cultures of CD 34^+-enriched cell populations from bone marrow are compared in serum-free media containing bFGF, SCF, LIF and Flt-3 ligand for 22 days, each one starting with a population of 1.8×10^6 cells. Typical fittings of this model to experimental data are depicted in Fig. 2. The discontinuities observed in the raw data are due to a periodic harvesting/feeding procedure. In order to better visualize the expansion of cells, the data can be corrected to account for this procedure. Total expansion results are depicted in Figs. 3 and 4, showing a more efficient expansion in the presence of a stromal layer (Fig. 3), in terms of both the total number of cells and the increase, caused by the endogenous growth factors provided by the stromal cell layer during incubation.

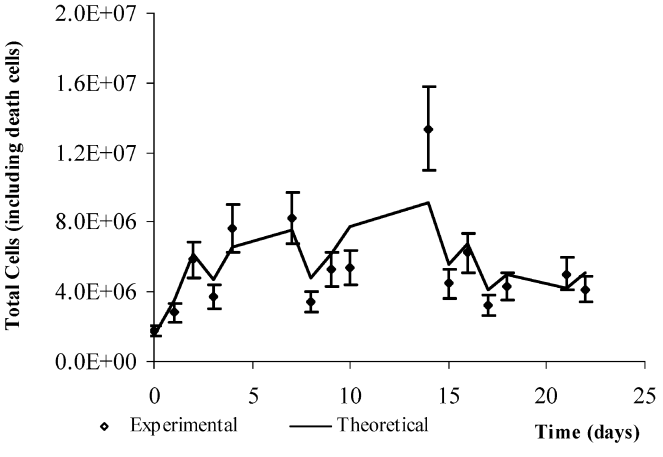

Fig. 2. Time course of total cell (BM MNCs) expansion: (♦) experimental values; (—) theoretical values

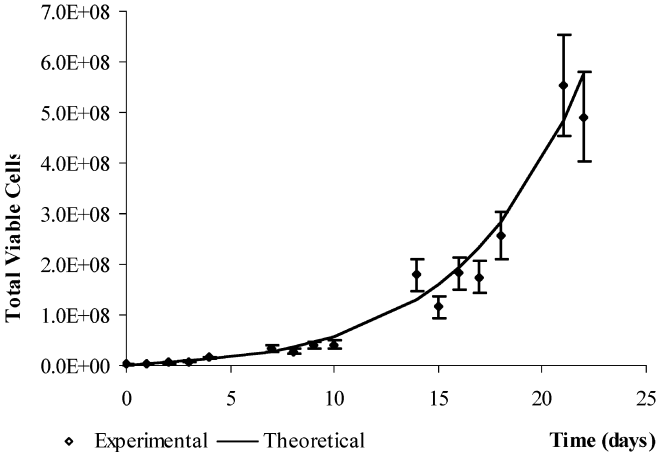

Fig. 3. Ex vivo expansion of viable BM MNCs in the presence of stroma: (♦) experimental values; (—) theoretical values

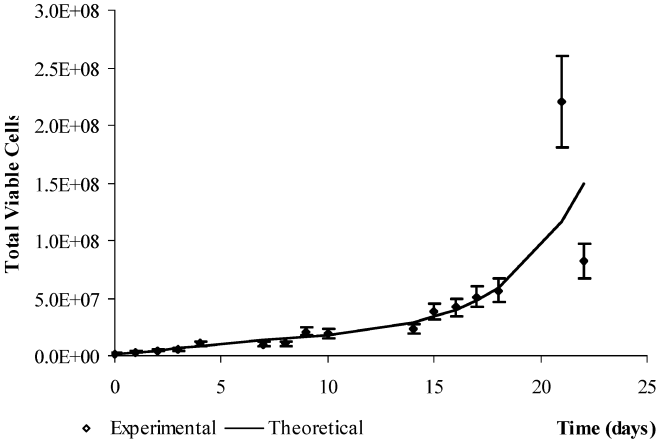

Fig. 4. Ex vivo expansion of viable BM MNCs in the absence of stroma: (♦) experimental values; (—) theoretical values

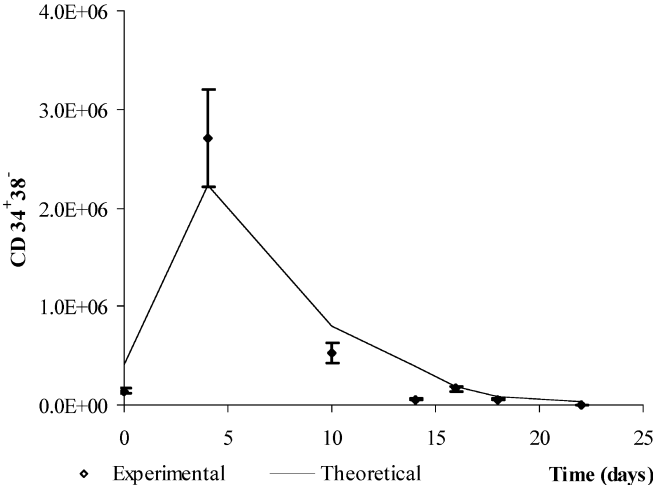

Fig. 6. Time course of CD $34^{+}38^{-}$ cell culture: (♦) experimental values; (—) theoretical values

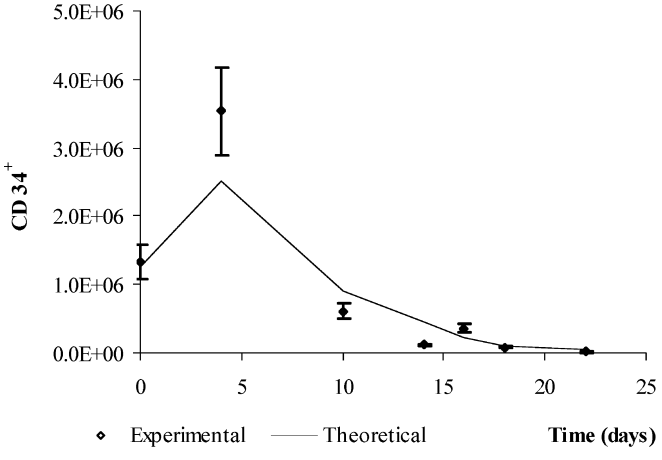

Fig. 5. Time course of CD 34^{+} cell culture: (♦) experimental values; (—) theoretical values

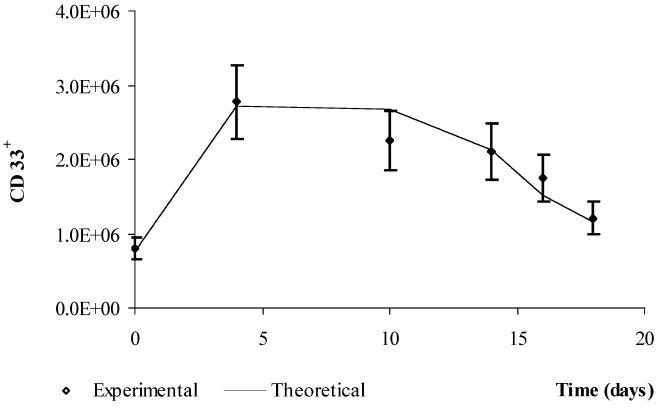

Fig. 7. Time course of CD 33^{+} cell culture: (♦) experimental values; (—) theoretical values

4.2 Expansion/differentiation pathways of hematopoiesis

The differentiative potential of bone marrow CD 34^{+} cells co-cultured with and without human stroma was primarily shifted towards the myeloid lineage with the presence of CD 15 and CD 33. The loss of expression of lymphoid markers (CD 3 and CD 19) was observed early in the culture. Although it was possible to maintain a CD $7^{+}14^{+}$ population in the culture (CD 7 is an early marker for lymphoid lineage, and CD 14 is a mature marker for myelo-monocyte lineage), this interesting result should be the subject of future analysis (data not shown).

From the kinetic model, the relative amounts of the different phenotypic cells (e.g. CD 34^{+}, CD $34^{+}38^{-}$, CD 15^{+}, CD 33^{+}) can also be estimated, with the aim of predicting the hematopoietic cell pathways for expansion/

Table 1. Rate constants for self-expansion (k_e^X), death (k_k^X) and dierentiation (k_d^X) for the various types of cells analysed and the maximum concentration of cells possible to obtain; values given by the modelling of the expansion of BM MNCs in the presence and absence of stroma

X	Rate constants (day^{-1})					
	k_e^X		k_k^X		k_d^X	
	(+) stroma	(–) stroma	(+) stroma	(–) stroma	(+) stroma	(–) stroma
PSC	3.6	3.8	9.4×10^{-5}	9.5×10^{-4}	–	–
SC	9.0×10^{-2}	1.3	9.9×10^{-4}	8.2×10^{-4}	3.7	4.2
MySC	7.3×10^{-1}	2.8×10^{-2}	1.9×10^{-3}	1.5×10^{-4}	8.3×10^{-2}	1.6
LySC	1.1×10^{-2}	1.0×10^{-1}	1.9×10^{-4}	1.4×10^{-3}	1.0×10^{-1}	3.3×10^{-2}
CFU-GM	7.4×10^{-1}	2.1×10^{-1}	8.7×10^{-2}	1.0×10^{-1}	1.3	9.1×10^{-1}
CFU-MEG	2.1×10^{-1}	1.7×10^{-3}	8.1×10^{-2}	2.9×10^{-3}	4.5×10^{-4}	1.4×10^{-1}
BFU-E	3.4×10^{-1}	4.2×10^{-3}	9.6×10^{-2}	3.2×10^{-1}	3.7×10^{-4}	1.2×10^{-1}
$CFU\text{-}E_0$	1.0×10^{-1}	4.9×10^{-1}	1.0×10^{-1}	9.1×10^{-2}	2.0×10^{-4}	4.4×10^{-2}

differentiation and identifying the key steps in the production of specific types of cells.

Typical fittings of this model to experimental data are depicted in Figs. 5 and 6 for more primitive cells, CD 34^+ and CD 34^+38^-, and for more mature cells, CD 33^+ (Fig. 7). The fitting of the model to the CD 15^+ cells was similar to the CD 33^+ (data not shown). The SEM values for the experiments of expansion and differentiation were similar to those obtained by other authors [9].

5 Conclusions

The results shown in Figs. 3 and 4 demonstrate that the model describes adequately the total cell number in the stroma-containing and stroma-free cultures, respectively. Both experimental and theoretical results indicate that the stroma layer is useful in the efficient ex vivo expansion/maintenance of stem/progenitor cells from human bone marrow.

Although the results obtained from the modelling are only related to one set of experiments, we can extract some preliminary conclusions. By the analysis of the constants given in Table 1, it can be observed that the introduction of the stromal layer:

- enhances the expansion of the majority of the more mature cells;
- reduces the death rate constant for the more primitive cell, PSC;
- reduces the differentiation for the more mature cells.

These observations are in accordance with the general expectation for the role of stroma in bone marrow, maintaining the progenitor cells and favouring the expansion, but not the differentiation, so that bone marrow can respond to whatever emergency the body faces. The establishment of reliable kinetic models will be useful in the development of bioreactor systems capable of being tuned for the production of specific blood products.

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