Development of a fixed bed bioreactor for the expansion of human hematopoietic progenitor cells

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Abstract

The *ex vivo* expansion of hematopoietic progenitor cells is of great interest for a variety of clinical applications, e.g. bone marrow transplantation or gene therapy. Therefore it is of general interest to develop a culture system, able to mimic the *in vivo* hematopoiesis, which is a prerequisite for long-term hematopoietic culture. Our approach was to modify a continuously perfused bioreactor for cultivation and expansion of human hematopoietic stem cells. Therefore we immobilized stromal cells (human primary stromal cells or the murine cell line M2-10B4) in porous glass carriers in a fixed bed reactor and cocultivated human hematopoietic progenitor cells for several weeks. After inoculation of mononuclear cells derived from umbilical cord blood or peripheral blood stem cells both adherent and non adherent cells were harvested and analyzed by flow cytometry and short-term colony assays. During cultivation there was a permanent production of progenitor cells and mature blood cells derived from the immobilized cells in the carriers. We could demonstrate the immobilization of hematopoietic progenitor cells of the myeloid system detectable in short-term colony assays. Additionally we could observe the expansion of very early progenitor cells (CFU-GEMM) up to 4.2-fold and later progenitor cells (CFU-GM and BFU-E) up to 7-fold and 1.8-fold, respectively.

Abbreviations: BFU-E – burst forming unit-erythrocyte; CB – cord blood; CFU-GEMM – colony forming unit-granulocyte erythrocyte monocyte megacaryocyte; CFU-GM – colony forming unit-granulocyte monocyte; HC – hydrocortisone; IL – interleukin; KL – kit ligand or stem cell factor; LTC – long-term culture; MNC – mononuclear cell; PBSC – peripheral blood stem cell.

Introduction

One main topic of experimental hematology is the identification of cultivation conditions and systems for hematopoietic stem cell expansion. These transplantable stem cells are used for a wide range of clinical applications, including cancer treatment, gene therapy, and transfusion medicine. The development of a reactor system able to mimic the *in vivo* hematopoiesis is of general interest because of the potential use in

generating large amounts of cells for future transplantation protocols (McAdams et al., 1996b; LaIuppa et al., 1997; Kögler et al., 1998).

Several bioreactors have been evaluated for their application in hematopoietic cell culture (McAdams et al., 1996a).

Sardonini et al. (1993) examined static culture, suspension culture, microcarrier culture, airlift reactor and hollow fiber culture. Using mononuclear cells a CFU-expansion by a factor of 2.5 was reached in a suspension culture system. Perfusion cultures utilizing stromal layers with cord blood, CD34+ cells, or mononuclear cells have been reported (Koller et al., 1993, 1995). Up to 41-fold CFU-expansion was obtained by inoculating the bioreactor with CD34+

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purified cells. This approach is based on the consideration that the intimate contact between stromal cells and hematopoietic cells is essential for maintenance of hematopoiesis *in vitro*.

The most common used culture methods for expanding CFU-GM consist of relatively small scale liquid cultures in which hematopoietic cells are cultured in the presence of multiple cytokines instead of stromal cells. Growth factors especially IL-3, IL-6 and SCF are most often used and CFU-GM expansion by factor 50 has been reported (Haylock, et al., 1992).

Nevertheless best expansion rates of early progenitors cells (LTC-IC) have been achieved in stromal dependent systems (Koller et al., 1993).

By cocultivation of murine stromal cells and hematopoietic cells in open porous collagen microcarriers in a continuous perfused culture chamber Wang et al. (1995) tried to imitate the three dimensional *in vivo* environment of the bone marrow. In this system they could produce lymphoid cells and all stages of committed cells. A similar system was used for culturing human bone marrow cells (Mantalaris et al., 1998) with special focus on the erythropoiesis. No data for CFU-expansion have been reported there.

In this report we compare a fluidized bed bioreactor and a fixed bed system with respect to their capability to support the cultivation and expansion of human hematopoietic progenitor cells. In both systems porous glass carriers were used for cocultivation of human hematopoietic cells and human primary stromal cells or the murine cell line M2-10B4, respectively.

Materials and methods

Umbilical cord blood was collected, with the mothers' informed consent, from the umbilical cord of normal infants as previously described (Somville et al., 1996). Leukapheresis samples were obtained from healthy individuals donating cells for allogeneic transplantation. The cells were mobilized by the administration of G-CSF and the samples were collected in autologous plasma with citrate anticoagulation.

Blood and leukapheresis samples

Umbilical cord blood samples were separated using a LymphoprepTM-gradient (Nycomed Pharma, Oslo, Norway) (density 1.077 g/cm³) to obtain the mononuclear cell fraction (MNC). Low-density cells were recovered and washed twice in PBS. Leukapheresis

samples (hereinafter termed 'peripheral blood stem cells' PBSC) were used unseparated.

Stromal feeder cells

Human bone marrow-derived stromal cells were established as described previously (Oostendorp et al., 1997) without additional characterization. Mononuclear plastic-adherent BM cells were cultivated in 80 cm² culture flasks in Dexter-type long-term culture medium (LTC medium: McCoy's 5a medium supplemented with 10% fetal calf serum, 10% horse serum, 18 mM HEPES, 2 mM L-glutamine, 26 mM sodium bicarbonate, 1 mM sodium pyruvate, 0.4% MEM non essential aminoacid solution, 0.8% MEM essential aminoacid solution, 1% MEM vitamin solution (all solutions from Life TechnologiesTM, Eggenstein, Germany), 1×10^{-4} M α -thioglycerol, 1×10^{-6} M hydrocortisone) with half-medium change weekly. After 3 to 4 weeks the confluent cell layers were passaged and cultured for 2 more weeks. The stromal cells were collected by trypsinization and then immobilized on gelatine modified glass carrier (Lüllau et al., 1992) in a bioreactor. Characterization of the stromal cells was done by flowcytometric analysis using the anti-human fibroblast antibody DIA120 (Dianova, Hamburg, Germany) (data not shown). The murine stromal cell line M2-10B4 (gift from Dr. C. Eaves, Vancouver, British Columbia, Canada) was maintained in RPMI + 10% fetal calf serum (FCS). Cells were irradiated with 80 Gy in a ¹³⁷Cs gamma-source (Gamma-Cell, Ontario, Canada) and then immobilized on gelatine modified glass carrier in a bioreactor.

Cocultivation of stromal feeders with human hematopoietic cells in a bioreactor

The bioreactor was inoculated with human primary stromal cells ($1-2 \times 10^6$ per ml_{carrier}) or irradiated cells of the murine cell line M2-10B4 (5×10^6 per ml_{carrier}) in LTC medium, and was thereafter maintained at 37 °C. After the establishment of an intact feeder layer, the reactor was inoculated with peripheral blood stem cells (PBSC) or mononuclear cells derived from cord blood (CB). The cells were cocultivated at 37 °C with a pO₂ setpoint of 50% and perfusion rate of 0.5 VVD (Schwartz et al., 1991) in order to avoid glucose limitation. Five ml medium samples and 1 ml carrier samples were collected, and both non adherent and adherent cells were analyzed separately in colony assays and by flow cytometry. Adherent cells were collected 10 min after the addition of trypsin (colony assays) or collagenase/dispase (flow cytometry) to the

Figure 1. Schematic diagram of the fixed bed bioreactor system. In the reactor vessel porous glass carriers are laying on a metal sieve. Medium circulation from the top to the bottom of the reactor vessel is maintained by a pump in-between the recycling loop. Fresh medium is pumped into the system before the aeration vessel. For optimal temperature both glass vessels (reactor and aeration vessel) are connected to a water bath.

carrier sample. Each sample was washed twice with PBS and used for the different analyses.

Fixed bed and fluidized bed bioreactor

A fluidized bed reactor system as described previously (Thömmes et al., 1992) was compared with a fixed bed bioreactor, generated by adding a steel sieve with a pore size of 0.5 mm at the bottom of the glass tube (Figure 1). Additionally the direction of the circulation of the medium was reversed and therefore the carriers were laying on the sieve without movement. The linear flow velocity within the fixed bed was reduced to 14 cm/min (compared to 75 cm/min within the fluidized bed). Aeration occurred by diffusion through a silicone membrane, therefore no aeration rate could be specified. By shortening the length of the aeration tube in the external aeration vessel from 10 to 2 m, we were able to decrease the working volume of the reactor from originally more than 100 to 70 ml. Gelatine modified open porous glass carriers (borosilicate glass, SIRAN, Schott Glaswerke, Mainz, Germany) with a diameter of 400 to 700 μm , a pore size of 60 to 120 μm and a porosity of 50%, were used for cell immobilization. Viability and cell number were

determined by using trypanblue in a hemacytometer (Neubauer).

Colony-forming cell assay

The CFC assays were performed as previously described (Sutherland et al., 1990) using 10^3 to 10^4 cells per 1 ml of MethoCult media containing 10% agar-LCM (Terry Fox Laboratory, Vancouver). Growth factors like rh IL-3 (20 ng/ml) and rm KL (100 ng/ml) were added separately. Cultures were incubated at 37 °C in a humidified atmosphere and 5% CO_2 . Hematopoietic colonies (between 20 and 100 colonies per ml culture) were scored at day 14 using an inverted microscope (32-fold magnification).

Cytokines

rm KL produced in *E. coli* was a generous gift from Dr. R. Mailhammer (GSF, Munich, Germany). rh IL-3 was produced in our laboratory using a baculo expression system (gift from Dr. Mailhammer, GSF Munich).

Quantification of stromal cytokine release by ELISA

Crude conditioned medium collected at the stationary phase of stromal cells cultivated in a fluidized bed

Figure 2. Cocultivation of primary stromal cells with MNCs derived from PBSC: Cell numbers of adherent cells (carrier samples with squares) and non adherent cells (media samples with circles) as a function of time cultivated in two different reactor systems (— = fixed bed reactor with bold symbols; - - - = fluidized bed reactor with open symbols). The graphic shows two representative and independent experiments.

reactor was tested for the concentration of G-CSF, GM-CSF, IL-6, KL, M-CSF, MIP-1 α , TGF- β with commercially available ELISA kits (R&D Systems).

Results and discussion

Cultivation of primary stromal cells in a fluidized bed bioreactor

In our first experiments we used a fluidized bed reactor with gelatine modified glass carriers. A reactor with a total volume of 90 ml and a carrier volume of 25 ml was inoculated with 1×10^7 human primary stromal cells. About 60% of the cells were immobilized on the carriers. After five days a constant cell density of 6×10^5 (5×10^5 up to 3×10^6 cells per ml in other experiments, depending on the passage of the cells inoculated) was reached and maintained at a constant level (up to 50 days, data not shown). Therefore irradiation of the primary stromal cells for growth inhibition was not necessary. In the case of cocultivation new stimuli are present and starting of stromal cell growth could be possible, but there was no restart of stromal cell growth detectable (e.g. increasing feeding rate, colonies in short-term assays).

Cytokine expression of the stromal cell layer modulated by IL-1 β and hydrocortisone

We had to show whether the feeder layer established on glass carriers grown in a perfusion culture

Figure 3. Cocultivation of primary stromal cells with MNCs derived from PBSC: number of progenitor cells analyzed in colony-forming assays per ml carriers A) grown on the carriers in a fluidized bed bioreactor compared to B) cells on the carriers and C) cells in the medium grown in a fixed bed bioreactor.

Table 1. The cell specific production rate q_{SP} ($\mu\text{g}/(10^9 \text{ cells per d})$) of primary stromal cells in a fluidized bed reactor at a cell number of $3 \times 10^6 \text{ c/ml}_{\text{carrier}}$ calculated as follows:

$$q_{SP} = X_P \cdot \text{MFR} \cdot N_R^{-1}$$

where

X_P = product concentration [$\mu\text{g/l}$]

MFR = medium feed rate [ml/d]

N_R = total cell number in the reactor

Medium	G-CSF	GM-CSF	KL	M-CSF	MIP-1 α	TGF- β
w/o IL-1 β /HC	0	0.15	0.003	0.65	0	0
w IL-1 β /HC	3.01	2.49	0.075	5.73	0.2	0.02

system has the same functional capacity to support hematopoiesis as described for primary feeder layers cultivated in culture flasks and irradiated for growth inhibition (Eaves et al., 1991; Mayani et al., 1992; Quesenberry et al., 1993). Primary stromal cells were cultivated in LTC medium without hydrocortisone in a fluidized bed reactor as described above. During the stationary phase of the culture we took samples of medium and analyzed the concentration of different cytokines in the crude medium by ELISAs. Besides this, the influence of IL-1 β (100 U/ml) and hydrocortisone (1×10^{-6} M) on the cytokine expression of primary stroma was measured to investigate whether stromal cells cultivated in a fluidized bed reactor respond to the same supplements as stromal cells cultivated in culture flasks. Additionally, we wanted to know if cytokines important for hemopoiesis could be produced from the stromal cells in higher concentrations. We could show the increase of the cell specific production rate of all analyzed cytokines after the addition of IL-1 β and hydrocortisone to the medium (Table 1) as described previously for primary stroma cultivated in cell culture flasks (Marley et al., 1992). These results indicate that cultivation of primary stromal cells on microcarriers in a perfused cultivation system lead to comparable feeder cells as cultivated in a two-dimensional system.

Cocultivation experiments in a fluidized bed and a fixed bed bioreactor

After the establishment of a stromal feeder layer on porous glass carriers has been done successfully, we started to perform coculture experiments using PBSC. In first coculture experiments in a fluidized bed reactor we could show the immobilization of hematopoietic cells on the carriers, but during the cultivation the

hematopoietic cells detached from the carriers and were rinsed. As previously described by Coulombel et co-workers (1992) hematopoietic cells adhere to stromal cells but the adherence is not as strong as for stromal cells. During cultivation in a fluidized bed reactor the force of movement of the carriers inhibits adhesion of hematopoietic cells to stromal cells grown on the carriers and therefore none of the inoculated hematopoietic cells could stay on the carriers. Therefore we had to avoid the movement of the carriers and the reactor system was modified to a fixed bed system as described in Material and Methods. We suppose that by the action of the carrier collisions the cells are 'shaked out' from the pores. A similar behaviour appeared during cultivation of a hybridoma cell line (only one of about 20 different hybridomas used) and a murine mast cell line, which could be immobilized in a fixed bed but not a fluidized bed system (data not shown). In the following experiment both reactor types were inoculated with primary stromal cells derived from one donor in LTC medium. After 7 days 1×10^8 MNC of PBSC from one donor were added to the established stromal feeder layer. Both reactors had a working volume of 173 ml. The fluidized bed reactor contained 32 ml carrier in contrast to 20 ml carrier of the fixed bed reactor. The immobilization of the hematopoietic cells was comparable in both reactor types (37 and 41% of the inoculum). In the fluidized bed reactor the cell number in the carriers and in the medium was rapidly decreasing in contrast to the fixed bed reactor. As shown in Figure 2 in the fixed bed reactor the cell number reached a stable amount of 5×10^6 cells per $\text{ml}_{\text{carrier}}$ and $2-3 \times 10^4$ cells per $\text{ml}_{\text{medium}}$. Having a constant medium feed rate (MFR) of 36.4 ml per day and a reactor working volume of 173 ml, the culture medium has been exchanged six times within

A. Medium

B. Carrier

Figure 4. Frequency of different progenitor cells derived from CB A) in the medium and B) on the carriers cocultivated with irradiated M2-10B4 cells in a fixed bed bioreactor. The graphic shows one representative experiment.

28 days. Therefore at this stage of fermentation cells in the medium are only cells originating from the immobilized cell population and are not suspension cells remaining from day zero. The calculated cell production rate is $3.6\text{--}5.4 \times 10^5$ cells per ml carrier and day produced by an immobilized cell population of 5×10^6 cells per ml carrier.

Analyses of the expansion of hematopoietic progenitor cells in both bioreactors by using colony-forming assays

As shown in Figure 3A we could find a permanent decrease of immobilized progenitors in the fluidized bed reactor. The number of progenitor cells in the medium was not detectable anymore after day 2 (data not shown). In the fixed bed reactor we could find a constant number of progenitor cells immobilized on the carriers up to day 14 (Figure 3B). Additionally to that we found a nearly constant number of progenitors cells in the different medium samples (Figure 3C). Consequently there must be a permanent production of CFCs on the carriers and a permanent release of CFCs into the medium.

Expansion of progenitor cells derived from cord blood cocultivated with the murine stromal cell line M2-10B4 in a fixed bed bioreactor

We could show the establishment of a functional feeder layer on the porous glass carriers when we cultivated irradiated M2-10B4 cells in a fixed bed bioreactor. During a cultivation period of about 3 to 4 weeks the cell layer was stable without cell detachment (data not shown). After inoculation of a fixed bed reactor with 5×10^6 cells per ml_{carrier} the cells were cultivated at least for 24 h establishing a semiconfluent feeder layer. 1×10^8 MNCs derived from cord blood were cocultivated with the pre-formed layer for 2 weeks in LTC medium. On day 2, 5, and 8 samples of medium and carrier were collected. First of all the frequency of the non adherent progenitor cells in the medium compared to the adherent CFC on the carrier was determined. We could demonstrate that the frequency of the progenitor cells immobilized on the glass carrier maintained (3.3 to 2.7 CFU-E and 20 to 31 BFU-E per 10^4 MNC from day 0 to day 8) or even increased (42.7 to 251.3 CFU-GM and 6 to 21.3 CFU-GEMM per 10^4 MNC from day 0 to day 8) during the culture period (Figure 4B). In contrast to that the frequency of the progenitor cells in the medium only maintained (42.7 to 72.7 CFU-GM per 10^4 MNC) or even decreased (3.3 to 2.3 CFU-E, 20 to 3 BFU-E, and 6 to 2 CFU-GEMM per 10^4 MNC) (Figure 4A). All these data refer to the total reactor volume (medium and carriers). Additionally CFU-GM and early progenitor cells like CFU-GEMM could be expanded up to 7-fold and 4.2-fold respectively (Figure 5).

Figure 5. Fold increase of hematopoietic progenitor cells (attached plus suspension cells) derived from CB cocultivated with irradiated M2-10B4 cells in a fixed bed bioreactor (same experiment as in Figure 4).

Conclusion

In this study, we compared two perfusion systems, fixed bed and fluidized bed bioreactors, with respect to their capability to support the cultivation and expansion of human hematopoietic progenitor cells. In a fixed bed reactor hematopoietic cells could be immobilized in porous glass carriers, on which there was a preformed feeder layer of human primary stromal cells or the murine stromal cell line M2-10B4, supporting adhesion, survival, and self-renewal of early hematopoietic cells. Hematopoietic progenitor cells derived from two different sources (PBSC and CB) were expanded and during the whole cultivation period permanently released out of the carrier into the medium. In a fluidized bed the force of movement of the carriers inhibited adhesion of hematopoietic cells to stromal cells and therefore the inoculated hematopoietic cells could not stay on the carriers.

Our results indicate, that a fixed bed bioreactor is a valuable model for cultivation and expansion of human hematopoietic progenitor cells. Further examinations will be done concerning the development of very early progenitors like long-term culture initiating cells (LTC-IC) in this system. Other stromal cells like recombinant cytokine producing stromal cells will be tested as well as cytokine supplemented media.

Fluidized bed technology may be used in a later culture stage when the cells have already colonized the carriers. Although the performance of the system for CFU-expansion is not yet sufficient for a clinical application we are convinced that these systems can be further developed to become scalable bioreactors for production of large quantities of cells for transfusions.

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