Design, characterization and application of a minibioreactor for the culture of human hematopoietic cells under controlled conditions

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Abstract

The *in vitro* culture of human hematopoietic cells has recently received considerable attention due to its clinical importance. Most studies of the culture and expansion of hematopoietic cells have been performed in static cultures but only very few reports exist on the use of bioreactors where strict control of environmental variables is maintained. In this work, the design, characterization and application of a fully instrumented minibioreactor for the culture of human hematopoietic cells from umbilical cord blood is presented. The system consists of a stirred-tank reactor where cells are maintained in suspension in an homogeneous environment and without the need of a stromal feeding layer. The minibioreactor was coupled to a data acquisition and control system which continuously monitored pH, dissolved oxygen and redox potential. When operated at 75 rpm with a hanging magnetic bar (impeller-to-tank diameter ratio of 0.57), the dead and mixing times were 120 and 80 s, respectively, and the maximum response rate and volumetric oxygen transfer coefficient were $0.8 \text{ mM O}_2 \text{ hr}^{-1}$, and 1.8 hr^{-1} , respectively. Such characteristics allowed a tight control of pH (until day 11) and dissolved oxygen at predetermined set-points, and up to a 7-fold expansion of hematopoietic progenitors was possible in cultures maintained at 20% dissolved oxygen with respect to air saturation. Growth phase and cell concentration could be inferred on-line through determinations of oxygen uptake rate and culture redox potential. Oxygen uptake rate increased during exponential growth phase to a maximum of $40 \mu\text{M hr}^{-1}$. Such an increase closely followed the increase in concentration of hematopoietic progenitors. In contrast, culture redox potential decreased during exponential growth phase and then increased during death phase. The designed system permits not only the maintenance of controlled environmental conditions and on-line identification of fundamental culture parameters, but also the application of control strategies for improving expansion of hematopoietic cells.

Abbreviations: CFU – colony forming units; CRP – culture redox potential; t_d – dead time of the system; DO – dissolved oxygen; HHC – human hematopoietic cells; MNC – mononucleated cells; MRR – maximum response rate; t_m – mixing time; OUR – oxygen uptake rate; $k_L a$ – volumetric oxygen transfer coefficient

Introduction

In recent years, *in vitro* culture of human hematopoietic cells (HHC) has experienced a growing interest which has been fostered by various applications where the expansion of stem and progenitor cells or

the generation of mature cells, such as erythrocytes and platelets, is required. A special attention has received the blood from umbilical cord due to its important advantages over other HHC sources (Mayani and Lansdorp, 1998), such as bone marrow and peripheral blood. Most of the information in the field of expansion of HHC has been generated using static

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cultures, usually multiwell plates, dishes, and flasks of culture volumes ranging between 0.1 to 10 mL. Particularly, the so called 'long term culture' has been widely used as an experimental tool for the study of hematopoiesis *in vitro* (Mayani et al., 1990, 1993, and 1998). However, the utility of all such systems for quantitative kinetic and metabolic studies is limited due to the reduced volume available and the impossibility of maintaining controlled and homogeneous conditions of environmental variables, including pH and dissolved oxygen (DO).

Expansion of HHC in various bioreactor configurations has been recently demonstrated. For instance, Sardonini and Wu (1993) reported the expansion of HHC in small-scale, non-instrumented air-lift, microcarrier, and agitated suspension bioreactors. In particular, their results showed that only the suspension bioreactor gave a better expansion than static cultures. Zandstra et al. (1994) also found that the use of microcarriers did not improve the performance of suspension cultures. Encapsulation in alginate beads (Levee et al., 1994) or immobilization in hollow fibers (Sardonini and Wu, 1993) has also been explored for human bone marrow cultures. However, separation of cells from these systems, as well as from microcarriers, can represent an important problem when harvesting HHC. Perfusion has been a preferred mode of operation, as depleted nutrients are continuously provided to the cells while metabolized medium is removed (Koller et al., 1993; Sandstrom et al., 1996). Although perfusion systems have been shown to perform adequately in the presence or absence of a stromal layer, several limitations and disadvantages persist. For instance, costly medium is inefficiently used and the systems are inherently heterogeneous, which can result in concentration gradients and difficulty in monitoring, controlling, cell sampling, and cell harvesting.

In contrast to static cultures and immobilized bioreactor systems, instrumented stirred-tank bioreactors are especially suited for maintaining a constant and homogeneous environment through the application of simple control algorithms. Nevertheless, very scarce information exists on the use of computer-controlled and instrumented stirred-tank bioreactors for the culture of HHC, probably due to concerns of cell fragility and complications when a stromal layer is required. To our knowledge, the only report of the culture of HHC in a stirred-tank bioreactor system is a recent study by Collins et al. (1998). A similar control system was used by Pierson et al. (1996) for the *ex*

vivo expansion of human natural killer cells. In addition to the inherent concerns of using stirred-tank systems, for routine experimental purposes, the high cost of culture medium (as high as \$2 mL⁻¹, if costs of cytokines are included) needed for HHC expansion makes prohibitive the use of commercially available stirred-tank bioreactors since relatively large volumes are required. In this work, the design characterization, and application of an instrumented stirred-tank minibioreactor for the culture of HHC maintained in an homogeneous suspension without a stromal layer, is presented. The system advantages include simple automatic measurement and control of the main culture variables through a computerized algorithm, and direct cell sampling and cell harvesting. The reactor volume was minimized in order to serve as an experimental tool where costly culture medium is optimized but enough volume remains to perform detailed kinetic characterizations. The utility of the developed system is exemplified by the expansion of HHC from umbilical cord blood cells. It is shown that on-line monitoring of oxygen uptake rate and culture redox potential can give fundamental information of the stage of the culture and cell concentration.

Materials and methods

Design considerations

The number of nucleated cells recovered from an umbilical cord is very limited, typically in the range of 40 to 200 × 10⁶ cells. Accordingly, the main design consideration was to keep the bioreactor volume as small as possible in order to have an initial mononucleated cell concentration of at least 0.5 × 10⁶ cells mL⁻¹. Previous reports have shown the importance of initiating cultures with a critical minimum inoculum concentration (Levee et al., 1994; Collins et al., 1996). A minimum bioreactor volume would also optimize expensive media usage. On the other hand, the bioreactor should be large enough in order to accommodate commercial-size autoclaveable pH, DO, and redox potential sensors, and to allow repeated medium sampling for detailed analytical characterization. Based on such constraints, a bioreactor was constructed in a local workshop with a nominal volume of 500 mL and a working volume in the range of 75 to 250 mL. The resulting liquid height to vessel diameter ratio was between 0.45 to 1.2. The vessel (7 cm diameter, 13.5 cm total height) was constructed

Figure 1. Schematic diagram of the minibioreactor and data acquisition and control system used for expansion of human hematopoietic cells.

in borosilicate glass and the head plate in stainless steel. Ports (including those for electrodes) were made from Swagelok-type fittings.

Bioreactor characterization

The bioreactor mixing time (t_m) was determined with a pH sensor (Ingold; 20 s response time), following the response curve after acid and base pulses. The t_m was defined as the time needed to reach 95% of final pH reading after acid or base additions. The volumetric oxygen transfer coefficient ($k_L a$), maximum response rate (MRR), and dead time of the system (t_d) were determined from the response curve of the system as measured from DO readings upon a step change in

the oxygen partial pressure in the reactor headspace, as described elsewhere (Court, 1988). The physical characterization of the bioreactor was performed in cell-free medium with agitation rates between 0 and 175 rpm, using a magnetically driven suspended bar (impeller-to-tank diameter ratio of 0.57).

Cells and culture medium

Umbilical cord blood, obtained from the Regional General Hospital No. 1 (Instituto Mexicano del Seguro Social) was processed in a Ficoll gradient as described by Koller et al. (1993), to separate mononucleated cells (MNC). MNC were seeded in 25 cm^2 T-flasks and placed in an incubator (Precision) for

Figure 2. Effect of agitation rate on dynamic parameters of the system. A) dead time and B) maximum response rate. Continuous lines drawn only to show trend.

3 days at 37 °C and 5% CO₂, prior to their inoculation into the bioreactor or the control culture. Such a procedure was implemented as a shortened quarantine to screen for possible microbial or fungal contamination in UCB samples. The medium used was IMDM (Sigma) supplemented with 10% fetal bovine serum (Gibco), 1 ng mL⁻¹ interleukin-3 (IL-3, Promega), 5 ng mL⁻¹ stem cell factor (SCF, Peprotech), 1 ng mL⁻¹ granulocyte-macrophage colony stimulating factor (GM-CSF, Probiomed), 3 U mL⁻¹ erythropoietin (EPO, Cilag), 0.1 mg mL⁻¹ streptomycin, 100 U mL⁻¹ penicillin, and 0.25 µg mL⁻¹ of amphotericin B (Sigma). Control cultures consisted of static 1-mL wells of a 24-well plate, inoculated from the same umbilical cord blood sample used in the bioreactor and performed simultaneously to the bioreactor cultures.

Bioreactor cultures

Batch cultures were performed using a working volume of 120 mL, agitated at 75 rpm, and maintained at 37 °C. DO was measured with a polarographic sensor (Ingold) and is reported as percentage with respect to air saturation. DO was controlled at a predetermined value of 20% with respect to air saturation by a PID algorithm which varied the oxygen composition in inlet gas through mass flow controllers for N₂, O₂ and CO₂ (Brooks Inc.) while maintaining the total gas flow rate constant at 250 mL min⁻¹. pH was measured with an Ingold electrode and was controlled at 7.2 by manipulation of CO₂ partial pressure in the inlet gas flow by a proportional control algorithm. Culture redox potential was measured with a platinum sensor (pHoenix). DO, pH, CRP, and individual gas flow rates were acquired on-line with a Macintosh II-SI computer via a MacADIOS 411 (GW Instruments) A/D D/A inter-

Figure 3. Effect of agitation rate on: A) percentage of suspended microcarriers (1 g L^{-1} Cytodex-1); B) mixing time; and C) volumetric oxygen transfer coefficient. The gas flow was fixed to 250 mL min^{-1} . Continuous lines drawn only to show trend. Data points correspond to average of two determinations. Error bars, except in one data point, are smaller than data symbols.

Figure 4. Typical behavior of a batch culture at 20% DO of human hematopoietic cells from umbilical cord blood. On-line acquired variables: A) DO; B) Inlet gas composition (% with respect to total gas flow rate of 250 mL min^{-1}); C) CRP and pH profiles.

phase. The control algorithms for DO and pH were written in Quick Basic® (Microsoft) and executed through the same A/D D/A interphase. Oxygen uptake rate (OUR) was determined on-line from a mass balance in the liquid phase according to Equation (1):

$$\text{OUR} = \frac{k_L a}{2} [(C_i^* + C_{i+1}^*) - (C_{L_i} + C_{L_{i+1}})] - \frac{C_{L_{i+1}} - C_{L_i}}{t_{i+1} - t_i} \quad (1)$$

where C_L and C^* are the oxygen concentration in the liquid phase and in equilibrium with the gas phase, respectively, t is time and i is the time discrete element.

C_L is given directly by the DO electrode, while C^* can be calculated from the O_2 partial pressure in the bioreactor headspace. Detailed description of assumptions leading to Equation (1) can be found elsewhere (Palomares and Ramírez, 1996; Higareda et al., 1997).

Analytical methods

Clonogenic assay for progenitor cells

Number of hematopoietic colonies was determined from methylcellulose-based semisolid cultures (Metho Culture; StemCell Technologies) containing 50 ng mL^{-1} SCF, 10 ng mL^{-1} IL-3, 10 ng mL^{-1} GM-CSF, and 3 U mL^{-1} EPO. Plates were inoculated with

Figure 5. Typical behavior of a batch culture at 20% DO of human hematopoietic cells from umbilical cord blood. A) viable mononuclear cell concentration (○) and total progenitor concentration (●); B) oxygen uptake rate; C) glucose (○) and lactate (●) concentration.

10 000 to 40 000 cell mL^{-1} and incubated for 14 days at 37 °C and 5% CO_2 . Hematopoietic colonies were classified as described previously (Mayani et al., 1990). A detailed description of the assay can be found elsewhere (Mayani et al., 1998).

Total cell concentration was determined in a Coulter Counter (Multisizer, Coulter Electronics) and viability by Trypan Blue exclusion using an hemacytometer. Glucose and lactate were determined from culture supernatants using a YSI 2700 (Yellow Spring Instruments) analyzer.

Results and discussion

In Figure 1, a schematic of the minibioreactor/data acquisition and control system designed is shown. The glass vessel included two side ports for inoculation and sampling, and two side arms for medium addition and removal for optional continuous or perfusion operation. The small volume used and the relatively large gas flow rate to volume ratio, needed for accurate OUR determinations (see Palomares and Ramírez, 1996; and Higareda et al., 1997), resulted in large amounts of medium being evaporated. This was prevented with a humidifying chamber placed at the inlet gas stream. For applications requiring the generation

Figure 6. Static control culture performed in 1-mL wells of a 24-well-plate. Culture performed simultaneously and with same inoculum and medium as that in Figures 4 and 5. Viable mononuclear cell concentration (○) and total progenitor concentration (●). Total MNC concentration not determined.

of mature cells, such as transfusions of erythrocytes or platelets (McAdams et al., 1996b) or research involving mast cells (Saito et al., 1996), the stirred-tank configuration offers the advantage of a straightforward scale-up. In cases where the goal is transplants of hematopoietic cells in adults, then an expansion of at least 2×10^9 mononucleated cells is required (calculated from Gluckman et al. 1989 and Kohli-Kumar et al., 1993). As shown below, based on the maximum oxygen transfer rate of the minibioreactor and the specific oxygen uptake rate of HHC, a single 250-mL bioreactor culture would be enough to generate the number of mononucleated cells required for an allogeneic transplant. Accordingly, for transplant applications, translation of the process to a clinical stage will not necessarily imply a volumetric increase of the culture, but rather warranting acceptability in safety and validation issues, including reproducibility. Such aspects are particularly satisfied in an homogeneous stirred-tank configuration.

Bioreactor characterization

The degree of homogeneity and the maximum oxygen transfer capacity are fundamental parameters of bioreactor performance. Therefore, a dynamic characterization of the minibioreactor was performed. The effect of agitation rate on the dead time of the system (t_d) and the maximum response rate (MRR), is shown in Figure 2. MRR and t_d indicate the rate at which the system responds, and the lag time before the system

responds, respectively, to a perturbation. It can be seen that above 75 rpm, t_d decreased from around 110 s, to about 40 s, and then remained relatively constant at such a value (Figure 2a). In contrast, MRR linearly increased as agitation rate increased (Figure 2b). Determination of MRR and t_d is important since such parameters define the PID control loop constants when using classical tuning methods such as the Ziegler and Nichols technique (Court, 1988). For an agitation rate of 75 rpm, the PID tuning resulted in 2.2, 216 s, and 54 s, for the gain, integral, and derivative constants, respectively. Interestingly, the gain is similar to values reported for *E. coli* cultures, whereas the integral and derivative constants were about 1 order of magnitude higher than typical bacterial cultures (Court, 1988).

To investigate if the impeller configuration chosen was enough to maintain the cells in suspension, 1 g L⁻¹ of Cytodex-1 (Pharmacia) was placed in the minibioreactor and the percentage of suspended microcarriers was measured as a function of agitation rate. As seen in Figure 3a, 25% of the microcarriers remained settled at 75 rpm, while above 125 rpm all microcarriers were suspended. The effect of agitation rate on the mixing time (t_m), and volumetric oxygen transfer coefficient (k_La) is shown in Figures 3b and c, respectively. It can be seen that t_m decreased from 140 to 50 s as agitation rate increased from 25 to 125 rpm. Similarly to MRR, the k_La linearly increased as agitation rate increased, reaching values as high as 3.5 hr⁻¹ at 150 rpm. These k_La values compare very

favorably with those reported for animal cell culture agitated-bioreactors in the 1- to 2-L scale range (Hu et al., 1986; Lavery and Nienow, 1987; Palomares and Ramírez, 1996). For a culture maintained at 20% DO using an O₂-enriched air source, the $k_L a$ of 1.8 hr⁻¹ obtained at 75 rpm is enough to maintain a concentration of at least 8×10^6 cell mL⁻¹ of cells respiring at a specific rate of 0.6×10^{-10} mmol cell⁻¹ h⁻¹. This specific oxygen uptake rate corresponds to the maximum value obtained in this work for total nucleated cells (see below). From the results shown in Figures 2 and 3, an agitation rate of 75 rpm was selected to perform batch cultures of HHC. Such agitation rate corresponded to an acceptable threshold value of homogeneity and dynamic characteristics of the system, but was low enough to minimize any possible damage to cells caused by excessive agitation, although this was not tested. Nevertheless, by comparing the lag phase and progenitor cells expansion data of static and bioreactor cultures, shown in the following sections, a possible hydrodynamic damage in the stirred-tank system can be discarded.

On-line monitoring and control of HHC cultures

The variables acquired on-line for a typical batch culture of HHC are shown in Figure 4. As seen in Figure 4a, the PID tuning constants derived from data in Figure 2 proved to be adequate for maintaining a tight control in DO, where only very minor perturbations were observed, usually related to external factors such as sampling. DO has been shown to be a fundamental variable which can determine differentiation of progenitor cells, proliferation of colony-forming cells, and production of mature cells and progenitors of different lineages, among other effects (Bradley, et al., 1978; Koller et al., 1992; McAdams et al., 1996a). However, very few reports, as the work shown here, have performed HHC cultures at a constant DO using a feedback control algorithm. Collins et al. (1998) and Pierson et al. (1996) have previously expanded HHC and human natural killer cells, respectively, in stirred-tank bioreactors maintained at constant DO. It should be noted that most of the studies of the effect of DO are based on maintaining a gaseous overlay of various O₂ concentrations and without any actual measurement of DO (Bradley, et al., 1978; Koller et al., 1992). However, this is not sufficient to maintain the DO neither at a constant nor at a predetermined value. Consequently, most literature reports can only assume that a 'low' or 'high' DO exists in the HHC cultures,

but the actual DO value and its variations remain unknown. Accordingly, the system presented here constitutes an important experimental tool for investigating the effect of constant DO on HHC cultures.

The inlet gas flow rates of O₂, N₂ and CO₂ are shown in Figure 4b, while the culture redox potential (CRP) and pH profiles are shown in Figure 4c. It can be seen that during the lag phase (first 3 days) the gas composition and CRP remained relatively constant. At the fourth day, upon initiation of the exponential growth phase (see progenitor cells in Figure 5a), the control loop automatically increased the O₂ flow rate in order to maintain the DO at the desired set-point and in response to the increased oxygen demand. After reaching a maximum at 7 days of culture, the O₂ flow rate decreased to 4.0% towards the end of the culture. Between days 4 and 11, accumulation of organic acids, particularly lactate, could be inferred from the decrease in CO₂ flow rate as the controller acted to maintain pH at the desired set-point (see also Figure 5c). After CO₂ depletion, control in pH was lost and decreased by almost 1.0 units. Base addition at this moment should prevent such a pH drop. This important decrease emphasizes the need for pH control, such as the one used here, as most HHC cultures performed in static or non-instrumented bioreactors are maintained in a constant CO₂ atmosphere and lack any type of pH control. In particular, it has been demonstrated that an optimum pH range exists for differentiation of HHC to specific lineages (McAdams et al., 1996a). Accordingly, the system presented here constitutes also an important tool for assessing the effect of pH in HHC cultures, and represents one of the few automatic feedback pH control system reported to date for such cells. It should be noted that HHC and human natural killer cells have previously been maintained at controlled pH in stirred-tank bioreactors by Collins et al. (1998) and Pierson et al. (1996), respectively. Finally, N₂ flow rate changed throughout the culture to maintain a constant total gas flow rate.

The various growth phases could also be identified from changes in the CRP profile. Initiation of the exponential growth phase could be inferred from a decrease in the CRP. For the culture illustrated in Figure 4, CRP decreased from -50 to -81 mV during the exponential growth phase and then increased during the death phase to a maximum of +34 mV. Similar characteristic variations in CRP and its correlation with viable cell concentration have been reported previously for hybridoma cultures at constant pH and DO (Hwang and Sinskey, 1991; Eyer and Heinzle,

1996; Higareda et al., 1997). In particular, Higareda et al. (1997) demonstrated the utility of simultaneous measurements of CRP and oxygen uptake rate (OUR) for discriminating real metabolic events from operational failures. To our knowledge, this is the first time that CRP measurements are determined, and its utility demonstrated, for a HHC culture. It should be noted that when DO and pH are maintained constant, changes in CRP solely reflect the metabolic activity of the cells. Hwang and Sinskey (1991) reported that the characteristic CRP variations could be attributed to thiol groups being excreted by animal cells. Thus, the results shown in Figure 4c are consistent with the idea that thiol production is a generalized phenomena in mammalian cell cultures. Furthermore, as established by Nernst Equation, CRP is inversely related to pH. Thus, the increase in CRP after the 11th day of culture is partially related to the decrease in pH.

In Figure 5, growth kinetics, OUR, glucose consumption, and lactate production of the same HHC culture as the one shown in Figure 4, are presented. The static control culture is shown in Figure 6. Both cultures showed a long lag phase (ca. 8 days if the preincubation in T-flask is considered). Such long lag phases have also been observed by others (Sardonini and Wu, 1993). The apparent absence of a lag phase for the progenitor cells is due to the limited number of assays performed during the first 5 days of culture. Only a small increase of mononucleated cells (MNC), was observed in the bioreactor (from 0.55 to 0.7×10^6 cell mL⁻¹) and control culture (from 0.55 to 1.01×10^6 cell mL⁻¹). Viability remained above 98% since inoculation until the end of exponential growth phase, whereas it remained between 94 and 80% during stationary phase. During death phase, viability decreased continuously until less than 20% at the end of the culture. Although cell expansion was very low for the cultures illustrated in Figures 4, 5, and 6, an expansion of MNC as high as 14-fold was possible in the minibioreactor system using a different cytokine combination (data not shown). Hematopoietic progenitors were determined for only a fraction of the samples. As seen in Figure 5a, progenitors concentration increased from 3 to 18×10^3 CFU mL⁻¹ during the first 7 days of culture, and then rapidly decreased to undetectable levels towards the end of the culture. A similar behavior has been reported by Collins et al. (1997) for a variety of non-instrumented cultures of peripheral blood and umbilical cord blood. It should be noted that even though only a ca. 1.3-fold expansion in total cells was observed, a 6-fold expansion of progenitor cells

was attained (Figure 5a). Furthermore, expansion of progenitor cells in the static control culture was only 3.6 fold (Figure 6), i.e., lower than in the stirred-tank system. Thus, caution should be taken when evaluating the performance of a cultivation system only on the basis of total cell expansion. For instance, a total cell expansion as high as 27-fold has been reported for human bone marrow in a suspension bioreactor, but only less than 3-fold expansion of progenitor cells could be attained (Sardonini and Wu, 1993).

OUR, as determined on-line by Equation (1) from DO and inlet gas composition data, is shown in Figure 5b. As it can be seen, OUR remained relatively constant during the first 4 days, corresponding to the lag phase of the culture. During exponential growth phase, OUR increased exponentially from $12 \mu\text{M hr}^{-1}$ to a maximum of $42 \mu\text{M hr}^{-1}$, closely following the concentration of total hematopoietic progenitors. As hematopoietic progenitors concentration decreased, OUR also decreased to a final negligible value. A similar behavior of OUR with respect to the various growth phases of the culture has been previously reported by our group for hybridoma (Higareda et al., 1997) and insect cell cultures (Palomares and Ramírez, 1996). The importance of results shown in Figure 5 are several-fold. Firstly, OUR monitoring can be used as a very convenient tool for on-line assessment of the various growth phases of the culture. In addition, OUR has been shown to be an effective tool for application of nutrient supply strategies for optimizing hybridoma and insect cell cultures (Palomares and Ramírez, 1996; Higareda et al., 1997). Accordingly, similar strategies could also be applied in HHC cultures by using OUR as the feed-back control variable. Finally, due to their close and direct correlation, OUR can be used as a simple technique for assessing hematopoietic progenitors concentration. Collins et al. (1998) have recently shown that maximum specific oxygen uptake rate closely corresponded with the maximum percentage of progenitor or colony-forming cells. It should be noted that determination of hematopoietic progenitors is performed by a time-consuming and laborious clonogenic assay (14 days). Thus, on-line and real-time estimation of hematopoietic progenitors concentration is a very valuable tool for control and optimization of HHC cultures.

Only very scarce information of OUR determinations in HHC cultures is available. For instance, Sardonini and Wu (1993) were not able to detect OUR in hollow fiber perfusion bioreactors due to the small DO differences between the inlet and outlet

streams. Peng and Palsson (1996), measured OUR in a specially designed six-well plate insert. Although accurate values were measured, the dynamic method used only allowed discrete OUR determinations for a limited number of samples during the whole culture period. Furthermore, due to the small volume of the system, each determination originated from a different well which had to be sacrificed. Similarly, Kimura et al. (1987) also determined OUR by the dynamic method from samples of a HHC line taken from a 100-mL spinner flask. Such determinations suffer from the same limitations associated to OUR measurements by the dynamic method (Higareda et al., 1997). Peng and Palsson (1996) have also determined OUR of HHC in a radial-flow parallel-plate perfusion system, but no continuous OUR data was reported. The specific OUR values, corresponding to the OUR data shown in Figure 5 (0.4 to 0.6×10^{-10} mmol cell⁻¹ hr⁻¹), compare very well with data reported by Collins et al. (1998) and Peng and Palsson (1996) for other HHC cultures but are 2 to 3 times lower than the values given by Kimura et al. (1987) for a permanent HHC cell line which may have altered metabolic responses. Likewise, specific OUR for HHC obtained here are 2 to 5 times lower than for other mammalian and insect cell cultures (Palomares and Ramírez, 1996; Higareda et al., 1997).

Finally, the onset of the stationary phase with respect to MNC and sharp decrease in total progenitors occurred at glucose and lactate concentrations of 3 and 0.7 g L⁻¹, respectively (Figure 5c), and coincided with the sudden decrease in OUR (ca. 6.8 days). Thus, such a decrease in OUR cannot be attributed to glucose limitation. Furthermore, Kimura et al. (1987) have shown that specific OUR is not affected by lactate concentrations as high as 13 g L⁻¹. Accordingly, a possible inhibitory effect on OUR by the lactate concentrations obtained in this work can be ruled-out. The OUR behavior can therefore be indicative of another substrate or toxic by-product limiting HHC cultures at around the 7 day of culture, but this remains to be proved. Collins et al. (1997) have reported that specific lactate production rate, and thus the specific glucose consumption rate, was three orders of magnitude higher for hematopoietic progenitors than for cells that do not generate colonies. This suggests that OUR must be mainly influenced by the hematopoietic progenitors.

Conclusions

Blood from umbilical cord represents an exceptional source of HHC for various clinical applications if a suitable *in vitro* expansion and differentiation system is available. The minibioreactor/data acquisition and control system presented here is an important experimental tool for studying cultures of HHC from umbilical cord blood, and can constitute a very attractive system for scale-up of such cultures to a clinical stage. The main advantages of the system include an homogeneous environment and the possibility of a strict control and on-line monitoring of the various environmental variables, including pH and DO, while optimizing medium usage. In particular, characteristic variations of oxygen uptake rate and culture redox potential were determined for HHC batch cultures maintained at a constant and predetermined pH and DO values. In addition, the utility of on-line determinations of OUR and CRP was demonstrated. On-going research in our laboratory has shown that expansion of CFU-GM, total CFU, and MNC, of 10-, 9.2-, and 14-fold can be achieved in the minibioreactor system presented in this paper, which compare very favorably with other systems reported in the literature.

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