ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Co-Culture of Stromal and Erythroleukemia Cells in a Perfused Hollow Fiber Bioreactor System as an In Vitro Bone Marrow Model for Myeloid Leukemia

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ABSTRACT: We have developed a hematopoietic co-culture system using the hollow fiber bioreactor (HFBR) as a potential in vitro bone marrow model for evaluating leukemia. Supporting stroma using HS-5 cells was established in HFBR system and the current bioprocess configuration yielded an average glucose consumption of 640 mg/day and an average protein concentration of 6.40 mg/mL in the extracapillary space over 28 days. Co-culture with erythroleukemia K562 cells was used as a model for myeloleukemic cell proliferation and differentiation. Two distinct localizations of K562 cells (loosely adhered and adherent cells) were identified and characterized after 2 weeks. The HFBR co-culture resulted in greater leukemic cell expansion (3,130 fold vs. 43 fold) compared to a standard tissue culture polystyrene (TCP) culture. Majority of expanded cells (68%) in HFBR culture were the adherent population, highlighting the importance of cell–cell contact for myelo-leukemic proliferation. Differentiation tendencies in TCP favored maturation toward monocyte and erythrocyte lineages but maintained a pool of myeloid progenitors. In contrast, HFBR co-culture exhibited greater lineage diversity, stimulating monocytic and megakaryocytic differentiation while inhibiting erythroid maturation. With the extensive stromal expansion capacity on hollow fiber surfaces, the HFBR system is able to achieve high cell densities and 3D cell–cell contacts mimicking the bone marrow microenvironment. The proposed in vitro system represents a dynamic and highly scalable 3D co-culture platform for the study of cell-stroma dependent hematopoietic/leukemic cell functions and ex vivo expansion.

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KEYWORDS: hollow fiber bioreactor; leukemia; hematopoietic, co-culture; stromal cell

Introduction

Extensive research has been pursued to understand hematopoiesis and the etiology of leukemia. Hematopoiesis, the process of blood formation, requires an intricate balance in hematopoietic stem cell (HSC) self-renewal and differentiation which critically depends on its specific microenvironment called the stem cell niche (Iwasaki and Suda, 2010; Li and Li, 2006; Schofield, 1978; Wilson and Trumpp, 2006). Failure in homeostasis results in leukemia where hematopoietic progenitors no longer differentiate and acquire instead an uninhibited capacity to proliferate. Though hematopoietic malfunction is often a consequence of genetic aberration, alternations in the hematopoietic microenvironment further contributes to the progression and/or sustenance of many hematological diseases.

The importance of the microenvironment for HSC function was revealed when a mutation in the gene encoding membrane-bound stem cell factor (SCF) present in a Sl/Sl^{d} mice (steel-Dickie mice), altered the HSC niche which eventually resulted in bone marrow failure and HSC maintenance in vivo (Barker, 1994; McCulloch et al., 1965). This study suggests that regulatory signals involve not only direct cell–cell contact, typically between stem cells and niche cells, but also indirect interactions mediated by the extracellular matrix (ECM) or cytokines (Torok-Storb et al., 1999). Besides functional hematopoietic cells residing in the bone marrow, the niche also consists of stromal cells which synthesize growth, differentiating, and inhibiting factors, along with ECM components to provide cues and signals needed for the maintenance and differentiation of hematopoietic stem and progenitor cells (Bramono et al., 2010; Breems et al., 1998; Jing et al., 2010; Naughton, 2003; Thalmeier et al., 1994; Wagner et al., 2007; Zuckerman, 1984).

Significance of supporting stromal cells in maintaining long-term hematopoietic cultures in vitro was first demonstrated in a feeder-layer-based co-culture technique by Dexter et al. (1977). This co-culture was established by inoculating mouse bone marrow cells onto a confluent irradiated stromal cell layer which will support

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hematopoietic cell culture. This technique proved to be superior in maintaining HSC stemness as opposed to cells cultured in the absence of a feeder layer or additional cytokines which terminally differentiated within the first 2 weeks of culture (Brandt et al., 1990; Naughton, 2003; Verfaillie, 1992) as a consequence of in vitro spontaneous differentiation (Sorrentino, 2004). However, static co-culture systems often suffer from decreased viability at 8–12 weeks and reduced cell proliferation over time (Toogood et al., 1980).

Culture improvements such as the utility of perfusion can be made to optimize conditions for furthering cellular growth. For large-scale expansion, bioprocess stability and scalability become yet more critical factors. This area has consequently led to the development of bioreactor systems in various configurations; perfusion chambers, stirred reactors, packed and fluidized bed reactors have been explored for ex vivo expansion (Cabral, 2001; Cabrita et al., 2003). Many scientists believed that the best approach in understanding hematopoietic biology and designing an ideal in vitro system for hematopoiesis is to replicate the bone marrow microenvironment. To this end, our goal is to develop an in vitro culture system that can replicate key aspects of the cell-stroma microenvironment found in the bone marrow. The bone marrow, as depicted in Figure 1A, consists of a vast vasculature of medullary sinuses surrounded by a packed density of hematopoietic and supporting cells. One method which we believe holds much promise in replicating cellular densities and fluid dynamicity inside the bone marrow is the hollow fiber bioreactor (HFBR). The discovery of HFBR system addressed many drawbacks in conventional static culture systems such as constraints in growth space and non-homogenous distribution of dissolved oxygen, pH, glucose, and cytokines (Collins et al., 1998). The HFBR is a continuous perfusion system that ensures a constant media exchange and optimized oxygen delivery (Fig. 1B). With a high surface area to volume ratio and three-dimensional (3D) structural contact, it can model in vivo tissue conditions and replicate cell-stroma and ECM interactions found in the stem cell niches (Fig. 1B). Furthermore, this system facilitates bioprocess scalability replacing the need for hundreds of tissue culture flasks while concentrating the secreted products by hundred times or more (Cadwell, 2004; Cadwell and Whitford, 2009).

In this study, a continuous co-culture system for leukemic cells using the HFBR is described. A supporting stroma culture using HS-5 stromal cells was first established and maintained for up to 28 days; the stroma culture was characterized by evaluating the kinetics of glucose uptake and protein production. Subsequently, the leukemic model was developed using K562 erythroleukemia cells for chronic myeloid leukemia (CML). To assess bioprocess efficacy of the HFBR as a large-scale co-culture system for hematopoietic/leukemic cells, the culture was evaluated for its ability to expand cells in vitro and support hematopoietic differentiation.

Materials and Methods

Cell and Culture Resources

An immortalized human bone marrow stromal cell line, HS-5 (ATCC) and human erythroleukemia cell line, K562 (ATCC, Manassas, VA) were used in this study. Cells were routinely propagated in tissue culture polystyrene (TCP)-flasks in complete medium at 37°C in a humidified incubator with 5% CO_2. Cells were passaged every 2–3

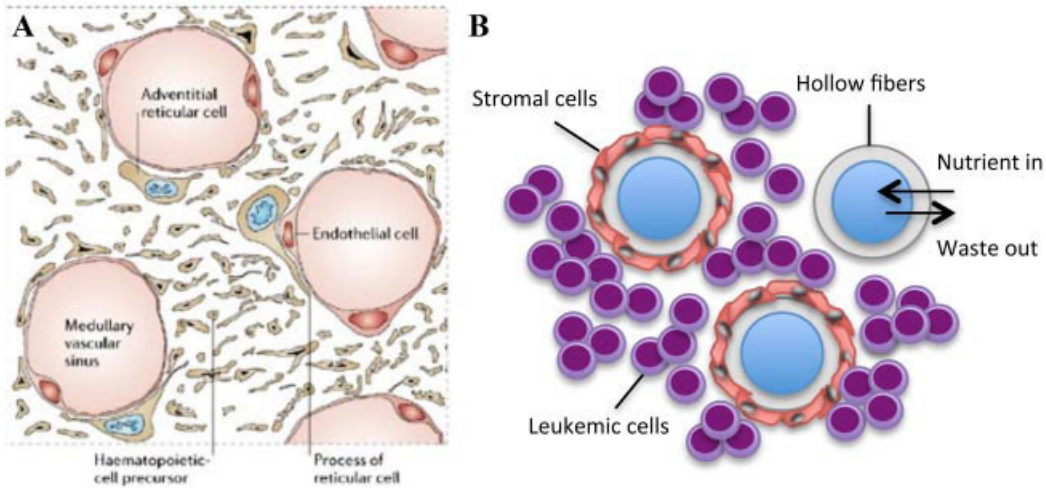

Figure 1. Schematic diagrams of the human bone marrow vasculature (**A** Adapted from Nagasawa) and the co-culture technology used in the hollow fiber bioreactor system (**B**). Stromal cells acting as feeder layers grow in the extracapillary space outside the fiber wall. Culture medium flows inside the fiber and due to the semipermeable nature of the fiber, nutrients and metabolic waste are allowed to diffuse across the fiber.

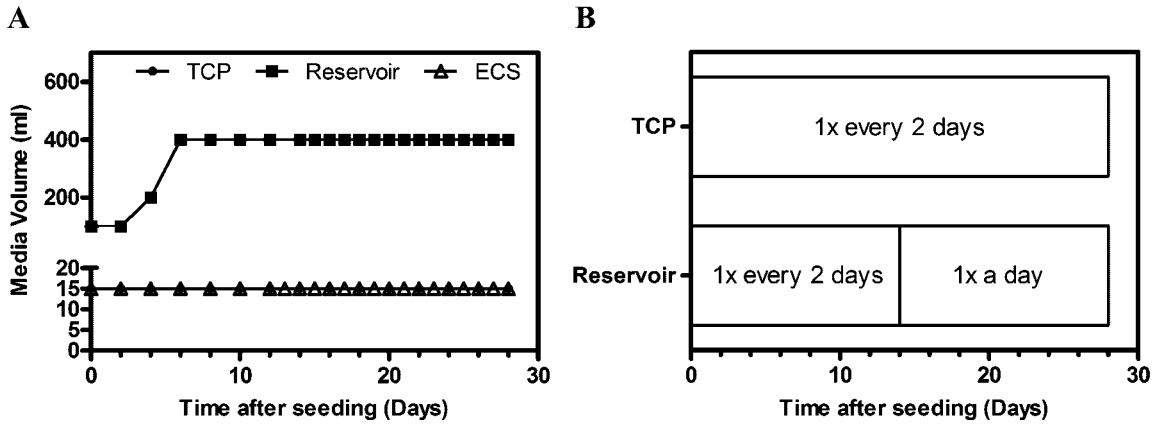

Figure 2. Feeding strategies implemented for HFBR and standard TCP cultures reflecting the total volume of media (**A**) and frequency of media change (**B**). Total volume of media used in HFBR starts at 100 mL and increased to 400 mL as cell number increases. The frequency of media chance in TCP was every 2 days and that in the HFBR was either every 2 days or everyday.

days on standard 2D TCP-flask cultures. Dulbecco's modified eagle's medium (DMEM) (Invitrogen) and Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, Life technologies, NY) were used for HS-5 and K562 cell culture respectively. Both medium containing 4.5 g/L glucose were supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 1.5 g/L sodium bicarbonate (Sigma, Aldrich) and 1% penicillin/streptomycin (A&E Scientific, PAA Laboratories GmbH, Austria) to constitute the complete medium.

Hollow Fiber Bioreactor (HFBR) System

The HFBR (medium sized cartridge C2011) used for this study was purchased from FiberCell Systems (FiberCell Systems Inc., Federick, MD), USA. The hollow fibers are made of polysulfone with a diameter of approximately 200 μm and have a molecular weight cut off (MWCO) of 20 kDa at 50%, retaining approximately half of a secreted protein of 20 kDa within the extracapillary space (ECS). Fibers in the bioreactor create a total surface area of 2,100 cm^2 within an ECS volume of 15 mL.

Inoculation and Cell Culture

Prior to cell inoculation, the HFBR system was circulated twice with phosphate buffed saline (PBS; Sigma-Aldrich), once with DMEM medium (Invitrogen) and finally once with complete DMEM medium. Each cycle lasted for at least 24 h. To establish a stroma culture in the HFBR, a total of 1×10^8 (as per manufacturer's recommendation) HS-5 cells were needed to initiate the culture due to the high surface area (2,100 cm^2) available in the bioreactor; cells were resuspended in 15 mL of DMEM complete medium and introduced into the ECS using a syringe through the left side port with both central end ports closed. To ensure a uniform distribution of cells in the ECS, cells were flushed back and forth three to four times through the side ports. Excess medium in the syringes were forced across the fiber membrane into the reservoir by reverse filtration. The culture was initiated with 100 mL of complete medium in the reservoir to support the high cell density culture and a media replacement was performed every 2 days by removing 75% of the waste medium. The reservoir volume was gradually increased from 100 to 400 mL (Fig. 2A). Media replacement strategy was performed according to manufacturer's guidelines while concurrently monitoring glucose concentration in the reservoir. During the first 2 weeks of culture, reservoir media was replaced every 2 days, subsequently this was performed everyday (Fig. 2B). Medium in the ECS is also regularly replaced as needed. For TCP stroma culture, 1.5×10^6 cells were inoculated into a standard T75 flask and a change of media was performed every two days using a total volume of 15 mL as per standard practice.

To characterize and demonstrate long-term performance of HS-5 stroma culture in the HFBR, we cultured only the stroma cell line up to 28 days and collected media samples to evaluate daily glucose consumption and protein levels in the HFBR. We further separated the proteins collected from our media samples and performed a Western blot analysis of the growth factor IL-6. To establish hematopoietic co-culture in the HFBR, stromal cells were first introduced and maintained in DMEM complete medium for 5 days prior to inoculation of K562 cells (5×10^5 cells) then the reservoir medium was switched to IMDM complete media. Full ECS medium replacement was done on day 4 of co-culture onwards. Spent ECS medium containing loose cells were centrifuged and inoculated back into the ECS. The K562 co-culture was maintained for 14 days. Static TCP suspension culture (control) and co-culture were established and used as a comparison.

Glucose and Protein Assays

Media samples of 100 μL were taken during each medium replacement and measured for glucose and protein concentrations. Glucose concentration in culture media was determined with a glucose assay reagent (Sigma-Aldrich) via phosphorylation of glucose to glucose-6-phosphate, followed by its oxidation to 6-phosphogluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD) over a 15 min incubation period. This oxidation causes an equimolar reduction of NAD to NADH, resulting in an increase in absorbance at 340 nm which is directly proportional to glucose concentration. Glucose consumption was calculated based on the difference in glucose levels to a reading taken from the previous day.

Concentration of solubilized protein was assessed using a dye-binding assay (Bio-Rad Laboratories), causing a differential color change of the dye in response to various protein concentrations. Subsequent to a 5 min incubation period, the colored reaction solution was measured spectrophotometrically at a wavelength of 595 nm. Relative protein concentrations were determined based on bovine serum albumin (BSA) standard curve. Protein production was calculated based on the difference in protein levels to a previous day reading.

Cell Harvesting

Cells were dislodged from the fibers using StemPro Accutase cell dissociation reagent (Invitrogen) with 30 min incubation at 37°C for each cycle and were harvested by flushing through the ECS using syringes. Two harvest cycles were performed for each experiment. For HFBR co-culture, cells removed from bioreactor with and without the presence of Accutase were termed as adherent and loose cells respectively. To separate K562 cells from HS-5 cells, cell suspension were reseeded into plates and left in the incubator for 1 h. Suspension K562 cells were then collected for analysis. Growth and viability of cells were assessed by the enumeration of live cells performed via trypan blue exclusion and manual counting using a hemocytometer.

Gel Electrophoresis and Western Blot Analysis

From each media sample, 50 μg of proteins were loaded onto each lane of the gel in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Protein samples were mixed with 2× SDS–PAGE sample buffer which consisted of 0.125 M Tris–HCl at pH 6.8, 4% SDS, 20% (v/v) glycerol, 10% β-mercaptoethanol, and 0.005% (w/v) bromophenol blue. The sample mixture was heated at 95°C for 10 min for protein denaturation and placed in ice prior to gel loading. A protein ladder consisting of a range of proteins from 10–250 kDa was included in each gel. One-dimensional SDS–PAGE was performed in the OmniPAGE mini vertical gel electrophoresis unit (Cleaver Scientific, Warwickshire, UK) initially at 90 V then a constant voltage of 150 V. Gels were stained with silver staining and visualized in the Chemidoc XRS (Bio-Rad) gel documentation imaging system.

To perform Western analysis, gels are blotted onto 0.2 μm nitrocellulose membranes. Protein blotting was performed in the OmniPAGE mini (Cleaver Scientific) at a constant current of 100 mA for up to 120 min. The blotted membrane was then air-dried for at least 1 h at room temperature prior to immunostaining. Western blot analysis of the growth factor IL-6 (Abcam, Cambridge, UK) was performed. Culture media were used as negative control 1 and K562 cell lysate was used as negative control 2. Protein membranes were then incubated overnight at 4°C in the primary antibody and 1 h at room temperature for the secondary antibody. The primary antibody dilution for IL-6 was 1:1,000. Secondary antibodies were conjugated to horseradish peroxidase (HRP) and a dilution of 1:3,000 was used. Proteins were detected using ECL chemiluminescent substrate (GE Healthcare, NJ). Western blot images were captured in a gel documentation imaging system.

Flow Cytometry

To monitor cell differentiation, harvested K562 cells (1×10^6) were incubated for 30 min in 4°C with a FITC-, PE-, or PerCP-conjugated antibodies against Gly-A (Becton Dickinson, NJ), CD71 (Becton Dickinson), CD13 (Becton Dickinson), CD45 (Becton Dickinson), and CD41 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA). To exclude non-specific binding of antibodies, FITC-conjugated mouse IgG_{2b} isotype control (Santa Cruz), and PE- and PerCP-conjugated mouse IgG_1 isotype control (Santa Cruz), PE-conjugated IgG_3 isotype control (Santa Cruz) were used. Cells were washed twice with PBS, resuspended in buffer containing PBS, 2% FBS, and 0.1% sodium azide (Sigma-Aldrich) and analyzed on a flow cytometer (Beckman Coulter, CA).

Wright–Giemsa Staining

For hematological identification, K562 cells were cytospined on clean glass slides at 100*g* for 3 min, stained with Wright–Giemsa (Merck Chemicals, Merck KGaA, Damstadt, Germany) and then visualized under a light microscope. Hematopoietic cell populations were distinguished and identified via their morphological appearances.

Data Analysis

Results obtained from flow cytometry data and cell fold expansion were analyzed and represented as the mean ± standard deviation of triplicates. Statistical analysis was performed using paired and unpaired Student's *t*-test for evaluating surface marker expression levels before and after 14-day cultures, and between different experimental groups.

Table I. Comparison of HS-5 stromal cell growth in TCP and HFBR systems.

System	Surface area (cm^2)	Total cells inoculated ($\times 10^6$)	Inoculum density ($cells/cm^2$) ($\times 10^4$)	Growth period (days)	Total cells harvested ($\times 10^6$)	Final cell density ($cells/cm^2$) ($\times 10^4$)
TCP	75	1.5	2	28	45.14	60.2
HFBR	2,100	100	4.76	28	442.24	21.1

Observed differences were regarded as statistically significant if the calculated two-sided *P*-value was less than 0.05.

Results and Discussion

Process Characteristics of Hollow Fiber Stromal Culture

Growth Kinetics of HS-5 Cells

Growth kinetics of HS-5 cells on TCP culture were per normal growth in vitro with a steady exponential increase during the first 16 days of culture before reaching a plateau when cells are space-inhibited for further expansion. The calculated average population doubling time was 2.86 days and the total harvested cell number was 4.5×10^7 cells (Table I). Growth kinetics of HS-5 cells in HFBR cannot be readily characterized however, under the same culture volume provided in the ECS (15 mL), final cell harvest in the HFBR system achieved a significantly higher total cell number with a minimum harvest of 4.4×10^8 cells. Initial seeding density in the HFBR is also significantly higher (1.0×10^8 vs. 1.5×10^6 in TCP). This is due to the high surface area to volume ratio (2,100 cm^2 in a 15 mL reactor) compared to conventional TCP which enables HFBR to support large cell number expansion. However, a complete harvest was not readily achievable due to the high packing density of cells and fibers. Use of collagenase improved cell dissociation but we believe that some cells remain trapped between fibers. Besides achieving a large cell harvest, good cell viability (>95%) was accomplished at the end of 28-day culture.

Glucose Consumption

No significant changes in glucose levels of the TCP culture was observed during the first 2 days but an exponential decrease began after day 2 and a final concentration plateau was reached on Day 10. The final glucose concentration maintained in TCP culture was 0.95 ± 0.08 mg/mL (Fig. 3A). In the HFBR, a more gradual decrease in glucose levels was achieved and reached steady state in 4 days (Fig. 3A). The final concentration at bioprocess stability was 1.5 ± 0.3 mg/mL (from day 4 to 20). In comparison, average physiological blood glucose level is around 1 g/L glucose and typical cell culture formulations contain glucose of 1 g/L up to 10 g/L. Average total cell glucose consumption in TCP culture was 45 mg/day while those in the HFBR was 640 ± 220 mg/day. Daily glucose consumption in our HFBR system is considered to be under "good" conditions of a healthy cell culture per manufacturer's recommendation (<1,000 mg/day). The focus on bioprocess monitoring should be emphasized to ensure a healthy cell culture in the bioreactor (Lim et al., 2007) as high glucose consumption induces high lactate production; the exposure to high amounts of lactate has been reported to inhibit cell growth (Strand et al., 1984). The advantage of perfusion in the HFBR system allows one to tune process operations in line with the needs to the culture and ensures an overall superior bioprocess stability than flask cultures (von Woedtke et al., 2002).

Protein Concentrations

Protein levels in TCP stroma culture averaged at 3.60 mg/mL while that in the HFBR averaged at 3.90 mg/mL in the reservoir and 6.40 mg/mL in the ECS (Fig. 4A). Protein levels in the ECS were higher than that in the reservoir

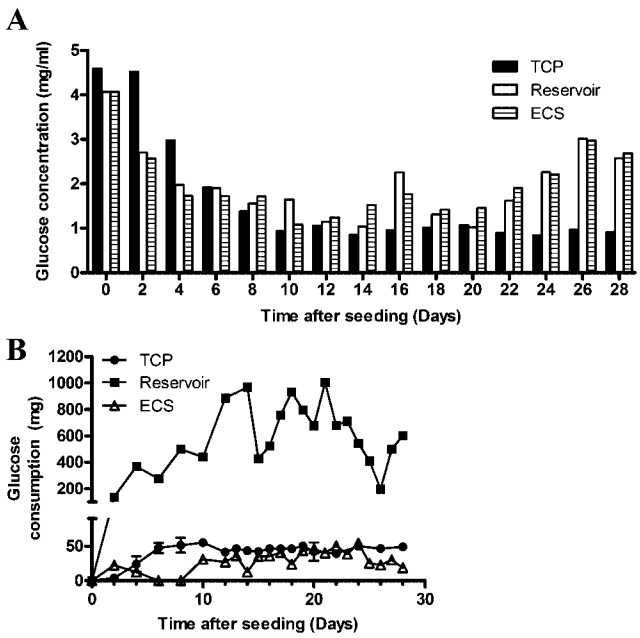

Figure 3. Comparison of glucose concentration (**A**) and glucose consumption (**B**) in medium of TCP and reservoir and ECS of hollow fiber system. A steady decline in glucose concentration was seen in TCP culture. Similar trend was also observed in HFBR culture until Day 22. Glucose concentration in reservoir and ECS was relatively comparable. Cells in HFBR system consumed more glucose as compared to TCP.

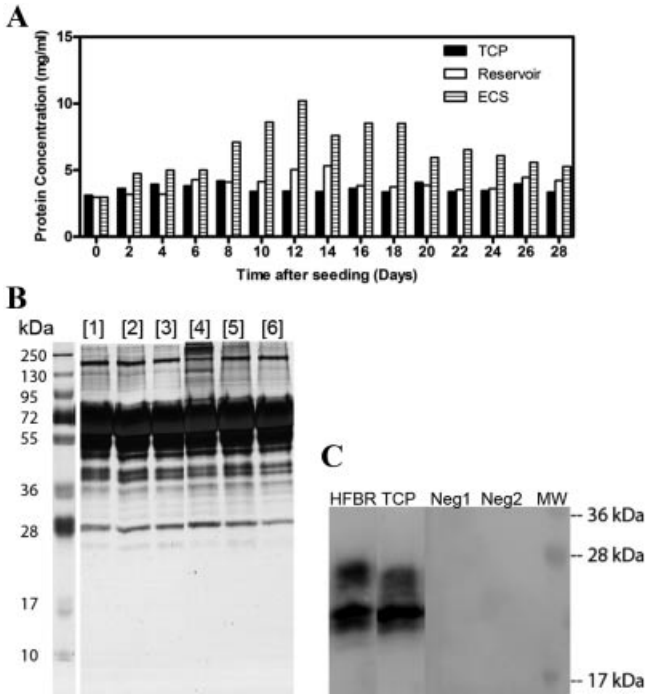

Figure 4. Total protein concentrations found in TCP and HFBR culture (**A**). Protein production started to decrease after 10 days of TCP culture whereas in HFBR culture, production of protein changes depending on its feeding rate. Gel electrophoresis of protein samples (**B**). Lanes 1–3 from TCP on Days 8, 14, and 21; Lanes 4–6 from HFBR on Days 8, 14, and 21. Western blot analysis of IL-6 of HFBR and TCP media samples (**C**). Negative controls were normal culture media (Neg1) and K562 cell lysate (Neg2).

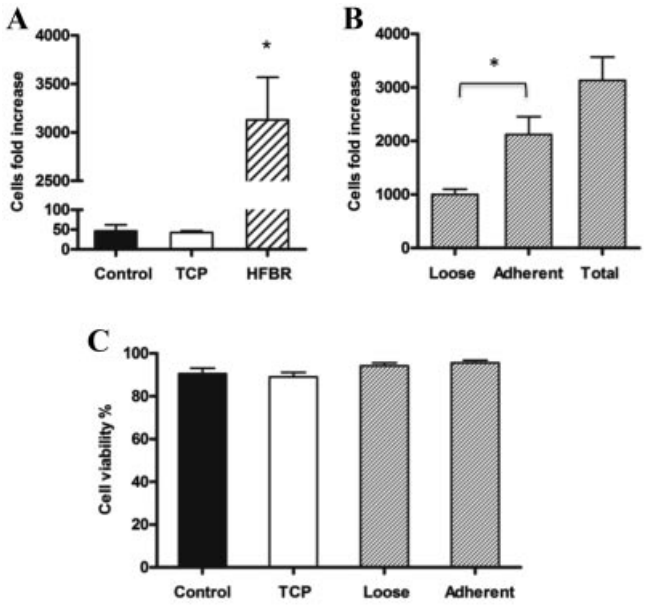

Figure 5. Total cell expansion and cell viability of K562 cells co-cultured in the presence of HS-5 stromal cells. Cells were co-cultured in TCP and HFBR systems for 14 days; harvested K562 cells were compared with control i.e. suspension culture in the absence of stromal cells in TCP system (**A**). Expansion in HFBR is significantly higher than both TCP cultures (* for P-value < 0.05). Harvested cells in HFBR co-culture were sorted into two populations: loose and adherent (**B**). Adherent cells showed significantly higher proliferation than loosely adherent cells (* for P-value < 0.05). Cell viability for control and TCP were determined using trypan blue exclusion (**C**).

indicating that some of the proteins produced by HS-5 stromal cells were retained and concentrated in the ECS. Previous research has shown that polysulfone fibers which is a hydrophobic material, readily adsorbs proteins (Norde, 1986; Patkar et al., 1993); this will therefore affect to some extent the recovery of secreted proteins. Pre-coating polysulfone fibers with another protein such as bovine serum albumin (BSA) or κ-casein have shown to solve this problem (Arnebrant and Nylander, 1986; Patkar et al., 1993). The low molecular weight cut-off (MWCO-20) permits only small molecules < 20 kDa to diffuse freely across the membrane. Larger proteins (>20 kDa) produced by stromal cells in the ECS will therefore not readily diffuse through the polysulfone membrane; these include growth factors such as IL-6 and LIF. Our protein gel (Fig. 4B) revealed that most of the proteins produced from HS-5 stromal cells and collected in TCP or ECS were above 20 kDa. Apparent bands between 28 and 36 kDa observed in protein samples from both cultures are likely growth factors typically produced by HS-5 such as IL-1α and IL-1β at around 31 kDa, M-CSF at 36.4 kDa, and glycosylated LIF which could range from 32 to 62 kDa. Strong bands at around 28 kDa and weak bands below 28 kDa are likely growth factors IL-11 or IL-6. Interlekin-11, a multi-functional cytokine which plays a key role in megakaryocyte maturation, has a predicted molecular weight of 19.3 kDa but under reducing conditions, appear on SDS gel at 24–28 kDa. Interleukin-6 which is a prototypic pleiotrophic cytokine is often variably glycosylated and will show as multiple bands between 22 and 27 kDa. Our Western blot analysis confirms the presence of various IL-6 monomers present in both TCP and HFBR cultures (Fig. 4C). Our observations suggest that exogenous growth factor production is preserved in the HFBR as produced in normal TCP cultures.

Co-Culture of Erythroleukemia Cells

In vitro proliferation of human K562 erythroleukemia cells in TCP was first evaluated in the presence of HS-5 stromal cells and in a stroma-free culture (control). Maximum growth of K562 cells in TCP on stroma supported versus stroma-free culture were 43- and 52-fold, respectively, but this comparison showed no statistical difference (Fig. 5A). Proteins secreted from stromal cells, both soluble and ECM proteins, may induce enhancing or inhibitory effects on hematopoietic and leukemia cells. In one study, conditioned media collected from HS-5 stromal culture imposed inhibitory effects on proliferation of K562 cells in vitro

(Lee et al., 2008) while in another study, a direct contact culture improved short-term proliferation and viability of primary AML cells in vitro by protection from apoptosis (Garrido et al., 2001). The competing effects between secreted soluble factors and stromal contact could have resulted in a net no-change effect of myeloid leukemia cell proliferation in our TCP experiments. Stromal cell numbers remained the same after 14 days of co-culture thus the ratio of stroma to leukemic cell contact becomes limiting as K562 cell number increases. This limitation is a main disadvantage of 2D TCP cultures.

In contrast, co-culture established in HFBR supported a prolonged continuous growth of K562 cells in vitro, reaching a fold increase of 3,130 (initial inoculum of 5×10^5 cells) after 2 weeks in culture compared to 43 folds in the TCP co-culture system (Fig. 5B). The average population doubling time of K562 cells cultured in the HFBR system was estimated at 29 h, which corresponds well to the normal doubling time of K562 cells of 24–30 h (Drexler, 2000) while that observed in TCP which also averaged at 30–31 h lasted only 1 week. Very little/slow growth was observed on week 2, as K562 cells cease to proliferate on TCP in vitro. This demonstrates an advantage of the HFBR system in sustaining normal and healthy cell division continuously throughout a 2-week culture period whereas in TCP culture, it is limited by an availability of space and sub-optimal physiological conditions slowed growth rates and resulted in an eventual cease of growth. Furthermore, low MWCO fibers allow the retention of many key proteins within the ECS (where K562 cells reside) while maintaining a continual exchange of nutrients and metabolites; this ensures a well-balanced physiological culture environment. We believe that the retention of key soluble proteins and growth factors within the ECS significantly improved the cultivation of K562 cells in vitro whereas in TCP culture, these viable proteins are continuously being removed during each change of culture media. Greater stromal contact is another reason in supporting sustained cell proliferation.

Harvested K562 cells from HFBR system were classified into two populations: loose and adherent. Loose cells were loosely adhering onto the HFBR co-culture which can be removed by flushing medium in a continuous repeated motion through the side ports of the cartridge. The adherent population were cells that would only detach by the action of Accutase. In this study, adherent K562 cells made up 68% of the total cell harvest accounting for over 2,000-fold increase in cell numbers thus suggesting that stromal cell surface is the predominant site of K562 cell proliferation. Our observations are therefore in congruent with the findings of others and support the notion of cell-stromal contact enhanced leukemic survival and proliferation. Due to the large surface area to volume ratio provided in the HFBR system, cell-stromal contact is maximized. This provides not only fertile grounds for a long-term stable co-culture but also the opportunity for interrogation of stromal effects due to its biomimicry in cell densities and 3D cell–cell contacts found in the bone marrow. A manual evaluation by trypan blue exclusion showed high cell viability of >90% in both TCP and HFBR co-cultures (Fig. 5C).

Morphological Observations

The cytoplasm and progressive condensation of nuclear chromatin were used as morphological evidence of cytoplasmic and nuclear maturation respectively. Early myeloid progenitors (myelocytes and myeloblasts) make up the majority of K562 cell population, and this was indicative of the K562 cell population pre-culture (Fig. 6A). These progenitors have a diameter of about 15–20 μm with a round/ovoid nucleus and a small cytoplasm to nucleus ratio. Erythroid progenitors, namely proerythroblasts and orthochromatic erythroblasts (labeled 3 in Fig. 6A–D), tend to have denser nuclei structure, indicating chromatin condensation. They also tend to have more rounded nucleus with a dark to light blue cytoplasm. Monocytes can vary in appearances and sizes which can range from 20 to 40 μm, and often have a lobed nucleus. The appearance of monocytes was greater after 14 days in co-culture (Fig. 6B–D). Neutrophilic granulocytes tend to have segmented nucleus (Fig. 6B). Megakaryocytes and its early progenitors which are generally larger cells with a variable size range of 30–100 μm appeared more prominent in HFBR cultures (Fig. 6C and D). They also have multiple nuclei and a cytoplasm full of granulation. The morphological analysis reflect the presence of monocyte, erythrocyte and megakaryocyte lineages, signifying the differentiating capacity of K562 cell line to a diverse myeloid population achieved in the HFBR system in vitro.

Immunophenotypic Analysis

Previous studies have shown that K562 cells can exhibit both erythroid and megakaryocyte markers in absence of any inducing agent (Rowley et al., 1992) and this is parallel to our results shown in Fig. 7. Surface marker expression of K562 cells at the start of a culture, Day 0, showed high percentages of Gly-A, CD71 and CD41 positive cells. Culture of cells in TCP, with or without stroma, displayed no considerable difference in the percentage of Gly-A (late erythroid marker) positive cells but a significant increase in CD13 positive cells was observed (Fig. 7A and B) in stroma-supported culture. This result suggests that stroma cell contact may be responsible for proliferation and differentiation of myeloid/monocytic progenitors, and could attribute from growth factors produced by HS-5 stromal cells, such as IL-1, IL-6, G-CSF, and LIF, which showed to induce differentiation to myeloid and macrophage/monocyte lineages (Cohen et al., 1992; Metcalf, 1989; Yamamoto-Yamaguchi et al., 1989). Besides the effects of endogenously produced factors, emerging evidence showed that cell–cell interactions, playing an essential role in HSC regulation of normal hematopoiesis, may be as equally important for stromal cell-mediated inhibition in leukemic cell

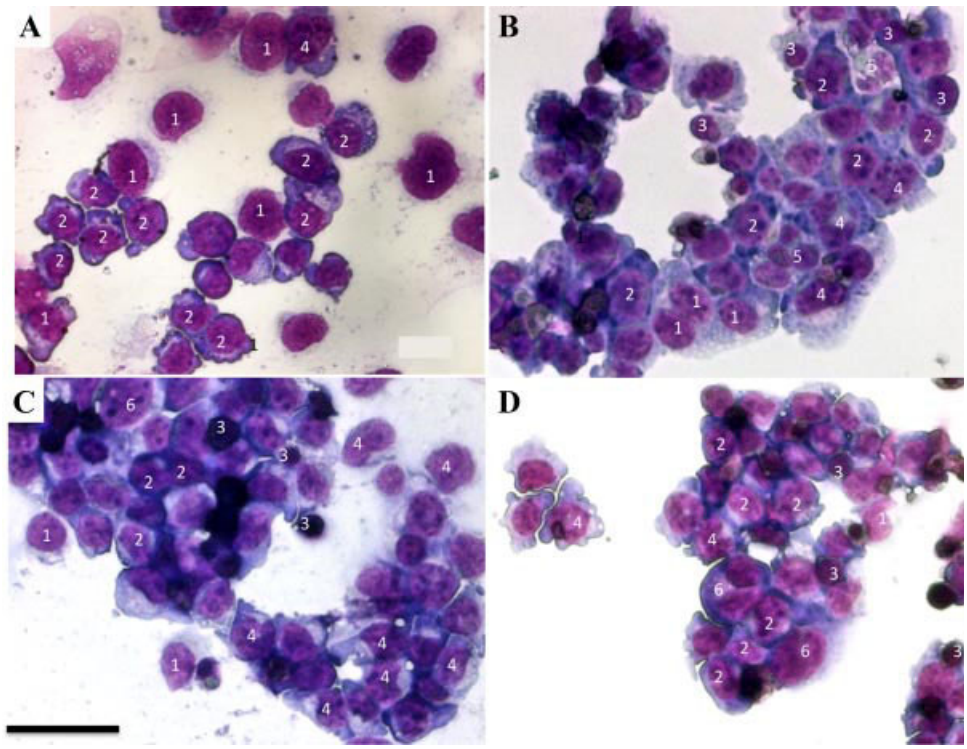

Figure 6. Morphological appearances of K562 cells at the start of the culture—Day 0 (**A**), 14 days in 2D co-culture (**B**), adherent population in HFBR culture after 14 days (**C**), and the loose population in HFBR culture after 14 days (**D**). Distinct populations of myelocytes [1], myeloblasts [2], erythroblasts [3], monocytes [4], neutrophilic granulocyte [5], and megakaryocytes [6] were identified in these images. Scale bar is 50 μm.

differentiation (Weber and Tykocinski, 1994). A difference in CD13 expression between the loose and adherent cell population (38.09% and 99.86% respectively) found in HFBR culture (Fig. 7C) lean toward this hypothesis. Loosely adherent cells which do not benefit from direct cell–cell contact with HS-5 stromal cells tend toward a more mature cell population which may have lost their adhesive properties. Thus a low percentage of CD13 surface expression in loosely adherent cells is preserved; their profile resembles suspension culture in TCP control (27.63%) (Fig. 7B) but loosely adherent cells from the HFBR culture show slightly greater numbers of CD13 positive cells due to the presence of exogenously secreted growth factors supporting myeloid/monocytic proliferation.

In normal hematopoiesis, bone marrow stromal cells express adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) (Clark et al., 1992; Simmons et al., 1992) that mediate the binding of hematopoietic progenitors. This type of interaction is responsible for homing and localization of primitive progenitors into their hematopoietic niches (Vermeulen et al., 1998) and in maintaining HSC self-renewal properties in vitro (Clark et al., 1992). As progenitors commit toward specific lineages, variable expression and functionalities of integrin and adhesive protein receptors would occur (Lund-Johansen and Terstappen, 1993). In erythroid maturation, variable integrin levels and adhesive capacities of erythroid progenitors to fibronectin were observed until a final loss of attachment at terminal erythroid differentiation (Eshghi et al., 2007; Vuillet-Gaugler et al., 1990). An ex vivo study performed by Zweegman et al. (2000) showed that stromal contact inhibited megakaryocyte maturation and non-adherent populations tend toward granulocytic and erythroid lineages. Distinction between loose and adherent cell populations is useful for evaluating the roles of cell-stroma contact on hematopoietic and leukemic cell differentiation. Although this can still be studied on TCP cultures, limitations in cell–cell contact and cellular densities may impact cell fate decisions on specific cell type lineages.

In our study, direct contact culture on TCP did not alter erythroid and megakaryocytic differentiation as observed in standard suspension culture. Both TCP cultures (stroma-free vs. stroma-supported) showed similar trends in CD41, CD71, and Gly-A expressions (Fig. 7). A closer analysis in the sub-populations of erythroid lineages revealed that erythroid maturation is mildly induced on TCP cultures leading to a decrease in Gly-A^+/$CD71^+$ (CFU-E and pronormoblasts) by ~20% and a corresponding increase ~25% in Gly-A^+/$CD71^-$ (reticulocyte or mature erythroid

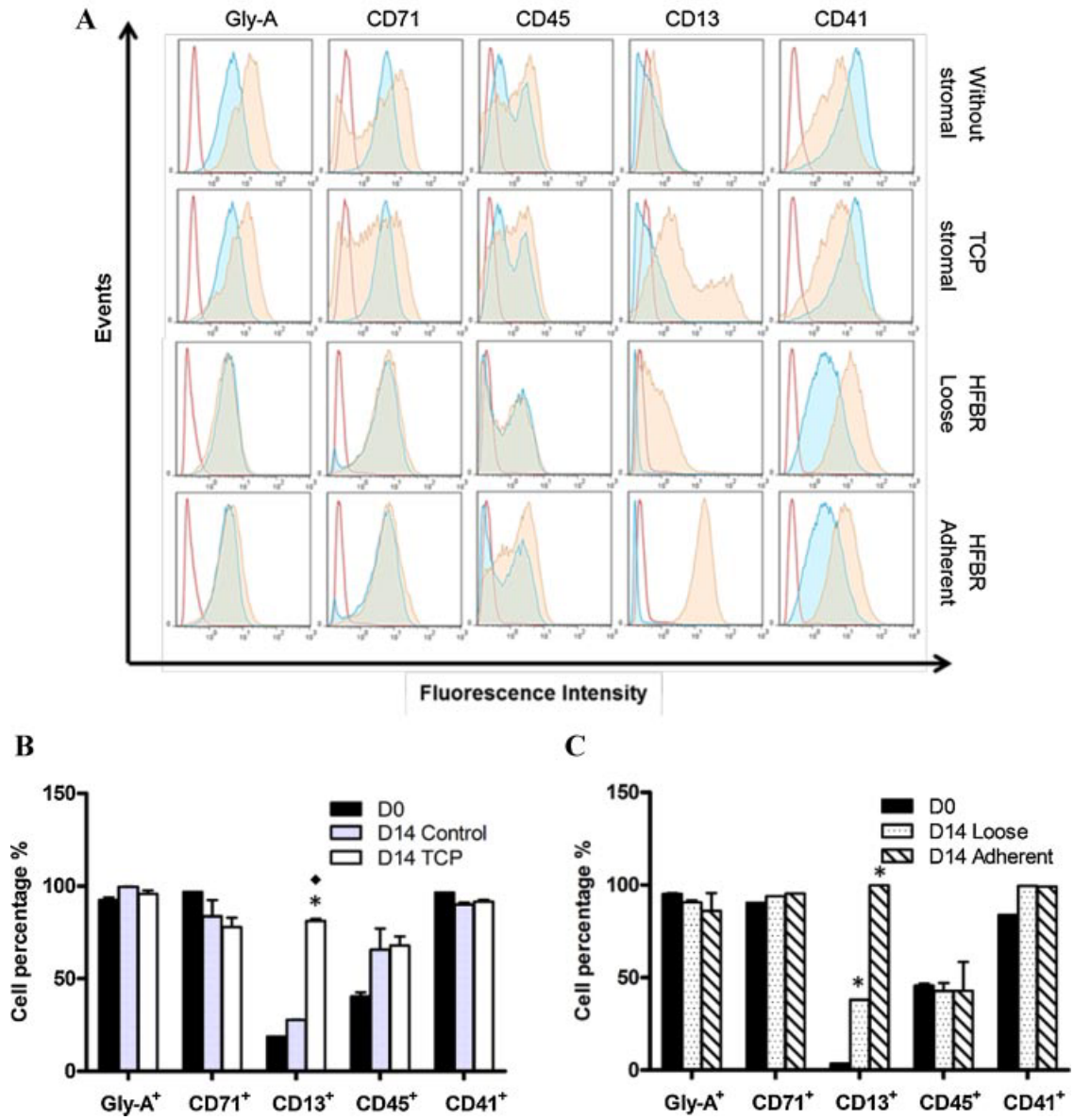

Figure 7. Surface marker expression of cells expanded in TCP and HFBR co-culture. Plots from FACS analysis of K562 cells with monoclonal antibodies directed against various hematopoietic cell surface markers (blue peaks for Day 0; orange peaks for Day 14) (**A**). An isotype control was included in each experiment to identify background fluorescence (red peaks). Number of cells analyzed (events) was plotted on the *y*-axis; intensity of staining was plotted on the *x*-axis. Percentage of cells expressing each marker in TCP culture (**B**). Percentage of cells expressing each marker in HFBR culture (**C**).

cells) population. In contrast, co-culture established in the HFBR did not induce erythroid maturation, that is, the same percentages of CFU-E and pronormoblasts (Gly-A^+/$CD71^+$) at ~90% were maintained as those observed at the start of the culture (Day 0) with very few mature erythroid cells (Gly-A^+/$CD71^-$) present. We believe that the tight cell–cell contact due to a high packing density in the HFBR system inhibited erythroid maturation. Overall, co-cultures in both TCP and HFBR favored monocytic progenitor cell proliferation and supported the survival of both erythroid and megakaryocytic cell lineages.

Conclusion

This study premiers the utility of a HFBR modeled as a bone marrow mimetic in vitro system for leukemic cell proliferation and differentiation. Our unique HFBR co-culture approach enables the extension of the well-established Dexter's culture in process scalability and biomimicry. Localization of leukemic cells to stromal cells which is significantly enhanced in the HFBR resulted in sustained cell growth over a 2-week culture as opposed to a TCP culture where cell growth ceased after one week. Dense

cellular contacts in the HFBR not only inhibited spontaneous erythroid differentiation but is likely to support better leukemic cell survival and provides closer cell physiological conditions mimicking the bone marrow. Thus we believe that the HFBR is a better in vitro model for testing leukemic cell survival and drug resistance than standard TCP cultures. Our data further suggest that the stromal cell surface remains the predominant site of K562 cell proliferation along with diversity in multi-lineage myeloid differentiation. With the advantage of achieving high cell densities and 3D cellular contact, the HFBR system recreates a closer mimicry of the cell-stroma microenvironment which can provide significant improvements to in vitro studies of stroma-dependent leukemic and hematopoietic cell functions.

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