Ex vivo culture with human brain endothelial cells increases the SCID-repopulating capacity of adult human bone marrow

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ABSTRACT

Adult human bone marrow (ABM) is an important source of hematopoietic stem cells for transplantation in the treatment of malignant and non-malignant diseases. However, unlike the recent progress which has been achieved with umbilical cord blood, methods to expand ABM stem cells for therapeutic applications have been disappointing. In this study, we describe a novel culture method utilizing human brain endothelial cells (HUBEC) which supports the quantitative expansion of the most primitive measurable cell within the adult bone marrow compartment, the NOD/SCID repopulating cell (SRC). Co-culture of human ABM CD34\(^+\) cells with brain endothelial cells for 7 days supported a 5.4 fold increase in CD34\(^+\) cells, induced >95% of the CD34\(^+\)CD38\(^-\) subset to enter cell division, and produced progeny which engrafted NOD/SCID mice at significantly higher rates than fresh ABM CD34\(^+\) cells. Using a limiting dilution analysis, we found the frequency of SRC within fresh ABM CD34\(^+\) cells to be 1 in 9.9 x 10\(^5\) cells. Following HUBEC culture, the estimated frequency of SRC increased to 1 in 2.4 x 10\(^5\) cells. All mice transplanted with HUBEC-cultured cells showed B lymphoid and myeloid differentiation, indicating that a primitive hematopoietic cell was preserved during culture. Non-contact HUBEC cultures also maintained SRC at a level comparable to contact HUBEC cultures, suggesting that cell-to-cell contact was not required. These data demonstrate that human brain endothelial cells possess a unique hematopoietic activity which increases the repopulating capacity of adult human bone marrow.
Introduction

The development of ex vivo culture methods that promote the expansion of adult human bone marrow (ABM) stem cells would have direct application in clinical gene therapy and stem cell transplantation. However, results obtained from stroma-based \(^1\) and stroma-free ex vivo culture systems \(^2-^5\) have been disappointing, due to insufficient activation of primitive CD34\(^+\)CD38\(^-\) cells, cell differentiation, and a loss of repopulating capacity following short term culture.\(^6\) Moreover, increased CD34\(^+\) cell numbers, colony-forming cells (CFC), and long-term culture initiating cells (LTC-IC) are not quantitative indicators of in vivo repopulating potential.\(^7-10\) Therefore, the importance of evaluating ex vivo cultured cells in an in vivo repopulation model has been emphasized.\(^8\)

The NOD/SCID model system has been used to measure the long-term reconstitution potential of ex vivo expanded human lymphohematopoietic stem cells.\(^7-10\) SCID-repopulating cells (SRC) are enriched in human cord blood (CB) as compared to adult ABM and mobilized peripheral blood \(^11,12\) and are most highly concentrated within the CD34\(^+\)CD38\(^-\) population.\(^8\) SRC are considered to be biologically more primitive than assayable LTC-IC and CFC progenitors \(^1,9,10,13\) which are found in both the CD34\(^+\)CD38\(^+\) and CD34\(^+\)CD38\(^-\) pools.\(^8\) As further evidence, gene transduction studies have shown that LTC-IC and CFC are readily transduced but contribute little to NOD/SCID engraftment.\(^2,7\)
When human hematopoietic stem cells (HSC) are co-cultured in contact with bone marrow stroma or conditioned medium from stromal cultures, a percentage of LTC-IC and CFC can be expanded in vitro over several weeks. Similarly, bone marrow, umbilical vein, and yolk sac-derived endothelial cell cultures elaborate growth factors which regulate hematopoiesis and support the proliferation of myeloid, erythroid, and megakaryocytic progenitors. We have previously demonstrated that a porcine brain microvascular endothelial cell line (PMVEC) plus cytokines was capable of supporting a robust expansion of human CD34+CD38- progenitors while maintaining cells capable of repopulating SCID-Hu bone as well as cells capable of rescuing lethally irradiated baboons. In these studies, we did not quantify the frequency of repopulating cells in the transplanted grafts.

Recent studies utilizing rigorous limiting dilution analyses have demonstrated that both stroma-containing and stroma-free culture conditions can support the quantitative expansion of SRC within human CB. However, the ex vivo expansion of human ABM stem cells under similarly stringent conditions has not been demonstrated. In fact, a recent limiting dilution analysis demonstrated a 6-fold decline in SRC within human ABM during short-term culture with ABM stroma. In this study, using quantitative limiting dilution analysis, we demonstrate that the number of engraftable SRC within human ABM increases following co-culture with primary human brain endothelial cells (HUBEC). The HUBEC ex vivo culture system has potential application in the expansion of ABM stem cells for clinical transplantation and
will also be a new resource for the identification of molecules which affect stem cell self renewal.
Materials and Methods

Isolation of primary human brain endothelial cells (HUBEC)

Vessel segments (< 10 cm) from the central nervous system and outside the CNS (renal artery) were collected from cadavers within 12 hours post-mortem under an approved tissue procurement protocol. Vessel segments were placed in complete endothelial cell culture medium containing M199 (GIBCO/BRL, Gaithersburg, MD), 10% heat-inactivated FBS (Hyclone, Logan, UT), 100 µg/ml L-glutamine, 50 µg/ml heparin, 30 µg/ml endothelial cell growth supplement (Sigma, St. Louis, MO), 100 U/ml penicillin and 100 µg/ml streptomycin.

Vessels were incised longitudinally and oriented in such a fashion that the lumen side contacted the dish surface during in vitro culture. Well developed endothelial cell colonies were evident by day 14 and confluent monolayers were achieved by day 30 of culture. Colonies were fed weekly with complete medium and several passages of the primary cells were banked.

CD34+ cells plus HUBEC co-culture

Purified human ABM CD34+ cells were obtained from Poietics Technologies, Inc. (Gaithersburg, MD). HUBEC were subcultured at 1 x 10^5 cells/well in gelatin-coated 6-well plates (Costar, Cambridge, MA) as previously described. After 72 hours, HUBEC monolayers were washed with PBS and the spent medium was replaced with ex vivo expansion culture medium (5 mL/well) consisting of IMDM (GIBCO/BRL) containing 10% FBS, 200 µM L-glutamine, 2ng/mL GM-CSF, 5ng/mL IL-3, 5 ng/mL
IL-6, 120 ng/mL SCF, and 50 ng/mL flt-3 ligand (R & D Systems, Minneapolis, MN) to each well. Purified ABM CD34+ cells (1 x 10^5) were added to each well and cultures were maintained at 37°C in 5% CO2 atmosphere. After 7 days of culture, nonadherent cells were harvested by washing the monolayers gently with warm complete culture medium. In the HUBEC non-contact cultures, ABM CD34+ cells were plated in the upper compartment of the culture well, separated from HUBEC monolayers by transwell inserts (0.4 µm; Costar).

**Immunofluorescence staining and cell cycle analysis**

ABM CD34+ cells and cultured cells were stained with monoclonal antibodies against CD34-FITC and CD38-PE (Becton Dickinson-BD, San Jose, CA) and analyzed by FACS (Coulter Epics Elite, FL). Controls consisted of isotype-matched mAbs. We performed the surface, intracellular, DNA (SID) cell cycle analysis as previously described 29 using anti-CD34-APC (BD), CD38-PE (BD), Ki-67-FITC (Immunotech, ME), and 7-AAD (Sigma, MO). Isotype controls were performed in parallel for each sample.

**In vitro methylcellulose colony forming assays**

Purified ABM CD34+ cells and ex vivo cultured cells (5-500 x 10^2) were cultured in 35-mm culture dishes (Miles Laboratories, Naperville, IL) as previously described. 22 Culture media consisted of 1 mL of IMDM, 1% methylcellulose, 30% FBS, 10 U/mL erythropoietin, 2 ng/mL GM-CSF, 10 ng/mL IL-3, and 120 ng/mL SCF. At day 14, we evaluated triplicate cultures to determine the number of colonies (>50 cells) per dish.
NOD/SCID marrow cells were washed x 2 and placed (1 x 10^5) in methylcellulose containing culture media containing the above noted human cytokines and analyzed at day 14 for evidence of human colonies.

**Transplantation of fresh ABM CD34^+ cells and HUBEC-cultured cells in NOD/SCID mice**

NOD/SCID mice^30^ were transplanted with either fresh purified ABM CD34^+^ cells or the progeny of ABM CD34^+^ cells cultured with HUBEC supplemented with GM-CSF + IL-3 + IL-6 + SCF + flt-3 ligand over a range of doses designed to achieve no engraftment in a significant fraction. To avoid donor variability, HUBEC cultures were established with the identical ABM CD34^+^ cells as used for transplantation into mice designated as the “fresh ABM CD34^+^” group. Cells were transplanted via tail vein injection after irradiating the mice with 300 cGy using a ^137^Cs source as previously described.^31^ The mice received no CD34^-^ accessory cells or exogenous cytokines to facilitate engraftment. Mice were sacrificed at week 8 and marrow samples were obtained by flushing their femurs and tibias with IMDM at 4°C.

Flow cytometric analysis was performed as previously described using commercially available monoclonal antibodies against human leukocyte differentiation antigens to identify engrafted human leukocytes and discriminate their hematopoietic lineages.^31^ Immunofluorescence staining of marrow cells was performed following our previously published procedures.^31^
Statistical analysis

For purposes of our limiting dilution assays, we scored a transplanted mouse as positive if $\geq 1\%$ of the marrow cells expressed human-CD45 via FACS analysis, based upon the engraftment criteria established by Ueda et al. $^{27}$ We calculated the SRC frequency in each cell source using the maximum likelihood estimator as described previously by Taswell et al. for the single hit Poisson model. $^{12,27,32}$ $\chi^2$ provides a measure of the legitimacy of using pooled data and of the validity of applying the single hit model. $^{12,30}$ We calculated confidence intervals for the frequencies using the profile likelihood method. As a confirmation of the maximum likelihood estimator, we also applied a minimum $\chi^2$ estimator to the pooled data.
Results

HUBEC co-culture supports ABM progenitor cell proliferation and expansion

HUBEC displayed cobblestone morphology at confluence and >90% expressed von Willebrand Factor, but we did not detect CD34 or CD38 expression by flow cytometry (data not shown). The effects of HUBEC contact and non-contact co-culture, liquid suspension culture, and human non-brain endothelial cell culture on CD34+ cell expansion and CFC generation were compared. All cultures were supplemented with GM-CSF + IL-3 + IL-6 + SCF + flt-3 ligand because our previous studies indicated that this combination optimized the expansion of ABM CD34+ cells.21,22 HUBEC culture supported a 16.1-fold increase in total cells, a 5.4-fold increase in CD34+ cells, and a 212-fold increase in the CD34+CD38- subset (n=8) (Table 1). CD34+CD38- cells increased from 1.6% of the total population at day 0 to 21.6% at day 7 and constituted 64% of the day 7 CD34+ cell pool. HUBEC non-contact cultures supported a 16.4-fold expansion of total cells, a 2.8-fold increase in CD34+ cells, and a 35-fold increase in the CD34+CD38- population. In contrast, liquid suspension cultures and non-brain endothelial cell cultures supported similar increases in total cells and CD34+ cells, but neither maintained CD34+CD38- cells at day 7 (Figure 1A-E) (Table 1).

HUBEC co-culture supported a 15.1-fold increase in CFU-GM, a 5.2-fold increase in CFU-Mix, and an 8.0-fold increase in BFU-E compared to input values and this CFC activity was significantly greater than that produced by liquid suspension cultures and non-brain endothelial cell cultures (Table 1). Non-contact HUBEC
cultures supported an expansion of total CFC which was greater than either liquid suspension or non-brain endothelial cell culture, but optimal CFC production occurred in the HUBEC contact cultures.

HUBEC contact and non-contact cultures induced a high percentage of quiescent ABM CD34^+CD38^- cells to enter cell division by day 7. At day 0, 95.7% ± 3.9 of the CD34^+CD38^- population resided in G_0, 3.9% ± 3.2 in G_1, and 0.7% ± 0.7 in G_2/S/M phase. After 7 days of HUBEC co-culture, 62.3% ± 9.9 of the CD34^+CD38^- cells had entered G_1, 33.3% ± 7.6 were in G_2/S/M phase, and only 3.9% ± 2.7 remained in G_0 (Figure 2). Similarly, non-contact HUBEC cultures induced 49.1% ± 2.4 into G_1, 25.3% ± 6.2 into G_2/S/M phase, and only 25.1% ± 3.8 remained in G_0. In contrast, we could not perform cell cycle analysis of CD34^+CD38^- cells from liquid suspension and non-brain endothelial cell cultures due to the undetectable frequency of CD34^+CD38^- cells at day 7.

**HUBEC-cultured cells engraft NOD/SCID mice at a higher frequency than fresh ABM CD34^+ cells**

NOD/SCID mice were transplanted with either fresh ABM CD34^+ cells (n = 47) or the progeny of ABM CD34^+ cells cultured with HUBEC x 7 days (n = 47) over a range of doses which resulted in non-engraftment in a fraction of the mice.
Transplantation of $1 \times 10^5$ fresh ABM CD34$^+$ cells resulted in no engraftment in 10 mice, whereas transplantation of $5 \times 10^5$ to $1 \times 10^6$ ABM CD34$^+$ cells resulted in engraftment in only 10 of 22 recipients (45%) at low huCD45$^+$ cell levels (mean 3.3%) (Figure 3A-C). At a dose of $1.5 \times 10^6$ ABM CD34$^+$ cells, 7 of 7 transplanted mice showed human cell engraftment, suggesting that non-limiting numbers of SRC were present at that dose (Figure 3D). When the progeny of HUBEC cultures over the same dose range were transplanted, the rate of NOD/SCID engraftment increased (Fig. 3A-C). The progeny of $1 \times 10^5$ ABM CD34$^+$ cells cultured with HUBEC engrafted in 2 of 10 mice and the progeny of $5 \times 10^5$ to $1 \times 10^6$ ABM CD34$^+$ cells engrafted in 21 of 22 mice (96%) at high levels (mean 11.7% huCD45$^+$ cells) (Fig. 3A-C). Twelve mice transplanted with $5 \times 10^5$ to $1 \times 10^6$ fresh ABM CD34$^+$ cells showed no human cell engraftment, but all 12 mice transplanted with the HUBEC-cultured progeny of these ABM CD34$^+$ cells demonstrated engraftment of $\geq 1\%$ huCD45$^+$ cells. At a dose of $1.5 \times 10^6$ ABM CD34$^+$ cells, HUBEC–cultured progeny engrafted in 7 of 7 mice at levels of huCD45$^+$ cell engraftment equivalent to fresh ABM CD34$^+$ cells (Fig. 3D).

In order to assess the capacity of non-contact HUBEC cultures to maintain SRC, we also transplanted NOD/SCID mice with the progeny of ABM CD34$^+$ cells cultured in this manner. The progeny of $1.5 \times 10^6$ ABM CD34$^+$ cells plated in HUBEC non-contact cultures engrafted in 7 of 7 mice at a high levels (mean 62.8% huCD45$^+$) comparable to the engraftment observed with the progeny of contact HUBEC-cultured cells transplanted at the same dose (Figure 3E). As a control, we also transplanted mice
with the progeny of ABM CD34+ cells which were plated in liquid suspension cultures supplemented with the identical cytokines for 7 days. None of the 3 mice in this group showed human cell engraftment or human CFC activity (Figure 3E).

HUBEC-cultured cells engraft in NOD/SCID mice with multilineage differentiation

Figure 4A shows human CD45+ cell engraftment within a representative mouse transplanted with fresh ABM CD34+ cells (1 x 10^6) versus an animal transplanted with the progeny of the same dose of ABM CD34+ cells following HUBEC culture. Detailed FACS analysis demonstrated lymphoid and myeloid differentiation in mice transplanted with limiting doses of HUBEC-cultured cells (Figure 4B). The proportion of CD34+ cells, CD19+ B cells, and CD13+ myeloid cells was highly similar within mice transplanted with limiting doses of HUBEC-cultured cells as compared to mice engrafted using fresh ABM CD34+ cells, indicating that a highly primitive repopulating cell was sustained during HUBEC culture (Table 2). Detection of human CFC within NOD/SCID mice correlated closely with the hu-CD45+ cell engraftment which we observed. Mice transplanted with the progeny of 1 x 10^6 ABM CD34+ cells cultured with HUBEC demonstrated multilineage human CFC activity which was 41-fold greater than the human CFC activity within mice transplanted with the same dose of fresh ABM CD34+ cells (Table 2).
**HUBEC co-culture increases the frequency of SRC within human bone marrow**

For statistical analysis, we pooled data from the limiting dilution assays of fresh ABM CD34\(^+\) cells and HUBEC-cultured cells, according to methods previously described.\(^{12,27}\) We calculated the frequency of SRC using the maximum likelihood estimator.\(^{32}\) The value of \(\chi^2\) in all cases was not statistically significant (\(P > 0.10\)), demonstrating internal consistency in our assays and allowing pooling of the data. The frequency of SRC within fresh ABM CD34\(^+\) cells was 1 in 9.9 \(\times\) 10\(^5\) cells (95% Confidence interval [CI]: 1/650,000 – 1/1,600,000) (Figure 5A). The SRC frequency within HUBEC-cultured cells was significantly higher at 1 in 240,000 cells (CI: 1/140,000 – 1/410,000) (Figure 5B). Therefore, co-culture of adult human ABM CD34\(^+\) cells with HUBEC monolayers supported a 4.1-fold increase in SRC. As further confirmation of the validity of applying the single-hit Poisson model to our limiting dilution assay, we also estimated the frequency of SRC using the minimum \(\chi^2\) estimation.\(^{12}\) \(\chi^2\) was again not significant in all cases (\(P > 0.20\)).
Discussion

Bone marrow transplantation is a curative therapy for an increasing number of malignant and non-malignant diseases.\(^{33}\) However, the ex vivo expansion of adult bone marrow for application in gene therapy, immune tolerance induction,\(^{34}\) and other diseases has been unsuccessful due to the differentiation and cell death which occurs when these cells are exposed to cytokines.\(^{1,5}\) In this study, using a limiting dilution analysis, we have demonstrated for the first time that the SCID-repopulating cell numbers within adult ABM can be quantitatively increased via ex vivo co-culture with primary human brain endothelial cells (HUBEC). Co-culture of ABM CD34\(^+\) cells with HUBEC supplemented with GMCSF + IL-3 + IL-6 + SCF + flt-3 ligand induced >95% of the CD34\(^+\)CD38\(^-\) population to enter cell division and supported a 4.1-fold increase in SRC as compared to starting ABM CD34\(^+\) populations. Since stem cell division and maintenance of stem cell repopulating capacity are requirements for successful retroviral gene transfer, the HUBEC culture method may provide significant advantages for clinical gene therapy protocols. Although other factors, such as the expression of retroviral receptors on target stem cells,\(^{35}\) are also important predictors of the success of retroviral gene therapy, our previous investigations have shown that co-culture of human ABM CD34\(^+\) cells with a porcine brain endothelial cell line (PMVEC) increased the gene transfer efficiency into the CD34\(^+\)CD38\(^-\) subset in the absence of measurable increases in the expression of retroviral receptors.\(^{36}\) We are currently examining the
effect of HUBEC co-culture on the gene transfer efficiency into BM CD34⁺ CD38⁻ cells and long term repopulating cells in the NOD/SCID model.

In contrast to the results presented here, previous studies have indicated that the ex vivo culture of ABM stem cells results in a decline in repopulating capacity.¹,³⁷ Gan et al. reported that 1 week culture of human ABM mononuclear cells (MNC) with ABM stroma caused a 6-fold decline in SRC as compared to unmanipulated ABM MNC.¹ Studies of mouse ABM cultures have demonstrated similar losses in the recovery of long term repopulating cells after 3-4 weeks in culture.³⁷ Of note, both the murine studies and the studies by Gan et al. were performed in the absence of exogenous cytokines which would have been expected to drive differentiation.⁴-⁶ In our studies, we supplemented HUBEC monolayers with a cytokine combination which maximally induced progenitor cell division, and, despite this, we observed a measurable increase in SRC over time. Since exposure to GM-CSF + IL-3 + IL-6 + SCF + flt-3 ligand in the absence of HUBEC caused a decline in detectable SRC over 7 days of culture, we postulate that HUBEC may have protected ABM SRC from differentiation during exposure to cytokines while also supporting the self-renewal of this primitive population.

Although steady state ABM CD34⁺CD38⁻ cells are highly enriched for SRC,⁸ we observed no correlation between the observed increase in SRC (4.1-fold) and the larger increase in CD34⁺CD38⁻ cells during culture (212-fold). This may be explained, in part, by a down-modulation of CD38 expression on committed progenitors which may
have occurred during culture. Dorrell et al. demonstrated that a significant percentage of cord blood CD34^+CD38^+ cells acquired a CD34^+CD38^- phenotype during 4-day culture with fibronectin plus IL-6 + SCF + G-CSF + flt-3 ligand, and a depletion of retinoids within the culture may account for this result. In addition, apoptotic events associated with cytokine deprivation of transplanted CD34^+CD38^- cells may contribute to their loss of repopulating capacity. Similarly, Glimm et al. and others have demonstrated that cell cycle associated defects may adversely affect the ability of sub-populations of proliferating CD34^+CD38^- cells to contribute to in vivo engraftment. Our preliminary investigations indicate that co-culture of sorted ABM CD34^+CD38^- cells with HUBEC results in a mean 6-fold increase in CD34^+CD38^- cells, an expansion which correlates closely with the 4.1 fold increase in SRC which we have observed in this study (Chute et al., manuscript in preparation). These results suggest that newly generated SRC during 7-day HUBEC cultures are quite possibly CD34^+CD38^- cells. Cell sorting studies of HUBEC-cultured populations should help determine which population is enriched for SRC following culture and provide a more precise understanding of the effect of HUBEC culture on primitive ABM subsets.

There are several potential mechanisms through which HUBEC culture may have increased the SCID-repopulating capacity of adult ABM CD34^+ cells. First, contact with HUBEC may have triggered self-renewal divisions within a subpopulation of primitive marrow cells, resulting in an absolute increase in SRC. This result would be consistent with the Single hit Poisson model as it has been applied previously.
Alternatively, exposure to HUBEC may have positively altered the engraftment capacity of the limited number of SCID-repopulating cells within the steady state ABM CD34+ population. Alteration of adhesion receptor expression on primitive cells has been associated with enhanced engraftment in murine models \(^{44,45}\) and the upregulation of CXCR4 on CB cells during culture has been associated with increased engraftment in NOD/SCID mice.\(^{46}\) Engagement of the Jagged/Notch pathway also has been shown to promote the maintenance of primitive hematopoietic cells during culture.\(^{47}\) Finally, CD34− and CD34+CD38+ accessory cells contained within the HUBEC-cultured grafts may have facilitated the engraftment of SRC within the recipient NOD/SCID marrow.\(^{48,49}\) However, since liquid suspension cultured-grafts contained equivalent numbers of CD34− and CD34+CD38+ accessory cells and failed to engraft NOD/SCID marrow, this explanation alone is incomplete. Whether HUBEC culture causes an absolute increase in ABM repopulating cells or augments the engraftment capacity of repopulating cells within adult bone marrow, the clinical impact of this method for stem cell expansion protocols would be the same: increased delivery of competent repopulating cells to the marrow.

The data from our non-contact HUBEC cultures with human ABM CD34+ cells suggest that cell-to-cell contact may not be required for the maintenance of marrow SRC in this system. This result differs from previous studies which have demonstrated a requirement for either stroma cell contact or adhesion via integrins to the fibronectin–COOH domain for the maintenance of adult-source stem cells during exposure to
cytokines. In this study, non-contact HUBEC cultures maintained a percentage of CD34^+CD38^- cells at day 7 and, more importantly, the progeny of these cultures maintained SCID repopulating capacity. Although we did not perform a limiting dilution analysis to estimate the SRC frequency within non-contact HUBEC cultures, our results suggest a differential maintenance of SRC within non-contact HUBEC cultures as compared to liquid suspension cultures. The lack of requirement for cell-to-cell contact may also be clinically advantageous since endothelial cell contamination of hematopoietic grafts would be eliminated.

Since the clinical transplantation of cord blood CD34^+ cells is limited by low cell numbers and delayed neutrophil/platelet engraftment, we are currently testing the capacity of HUBEC culture to expand repopulating cells within this population. Additionally, we will be performing serial transplantation studies to confirm that HUBEC co-culture maintains cells with long-term repopulating capacity and we plan to test HUBEC culture with other cytokine combinations, such as SCF + flt-3 ligand + TPO + IL-6/sIL-6R, in order to further augment the expansion of SRC presented here. Finally, the concentrations of IL-3 (5 ng/mL), IL-6 (5 ng/mL), and GMCSF (2 ng/mL) which we utilized in this study were lower than other investigators have previously applied to induce stem cell proliferation in vitro. We will additionally test whether higher concentrations of these cytokines might further increase the SRC expansion observed here.
Although remarkable progress has been made recently in the ex vivo expansion of human cord blood SRC, this progress has not translated into successful methods for the ex vivo expansion of either bone marrow or peripheral blood mobilized stem cells. The limiting dilution analysis presented here demonstrates that long term repopulating cells within adult human bone marrow can be increased via exposure to human brain endothelial cells. This culture method may prove clinically useful for both the ex vivo expansion and genetic modification of adult human bone marrow stem cells.
Acknowledgments

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TABLE 1. HUBEC Co-culture promotes the expansion of human CD34+ subsets and CFC as compared to controls

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cell yield</th>
<th>No. of Cells Procured x 10^5</th>
<th>CD34(^+)</th>
<th>CD34(^+)CD38(^+)</th>
<th>CD34(^+)CD38(^-)</th>
<th>No. of CFC x 10^4</th>
<th>CFU-GM</th>
<th>BFU-E</th>
<th>CFU-Mix</th>
<th>Total</th>
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<tbody>
<tr>
<td>Input</td>
<td>5.0</td>
<td>5.0</td>
<td>4.91 ± 0.07</td>
<td>0.08 ± 0.04</td>
<td>3.7 ± 0.9</td>
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<td>HUBEC</td>
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<td>27.0 ± 1.1</td>
<td>9.0 ± 0.4</td>
<td>17.0 ± 0.5</td>
<td>56.0 ± 2.2</td>
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<td>(16.1 fold)</td>
<td>(5.4 fold)</td>
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<td>(21.6%)</td>
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<tr>
<td>HUBEC Non-contact</td>
<td>82.0 ± 3.7</td>
<td>14.0 ± 0.1</td>
<td>10.4 ± 0.3</td>
<td>2.8 ± 0.1</td>
<td>30.0 ± 1.3</td>
<td>6.0 ± 0.5</td>
<td>4.3 ± 0.9</td>
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<td>(16.4 fold)</td>
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<td>(13.0%)</td>
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<td>Stroma-Free</td>
<td>51.0 ± 1.3</td>
<td>15.1 ± 0.7</td>
<td>15.1 ± 0.6</td>
<td>0.02 ± 0.02</td>
<td>15.8 ± 4.5</td>
<td>0.7 ± 0.7</td>
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<td>(10.2 fold)</td>
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<td>27.7 ± 6.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10.4 fold)</td>
<td>(3.6 fold)</td>
<td>(34.8%)</td>
<td>(0%)</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Purified human ABM CD34\(^+\) cells (5 x 10^5) were plated per culture treatment (n=8) as described in the Materials and Methods. Non-adherent cells were harvested on day 7 of culture and analyzed via flow cytometry. Numbers in parentheses in the Cell yield and CD34\(^+\) columns reflect the fold increase in these populations following culture. Numbers in parentheses in the CD34\(^+\)CD38\(^+\) and CD34\(^+\)CD38\(^-\) columns indicate the percentage of each population as a subset of the total population. CFC values represent the total number of CFC per culture.
TABLE 2. HUBEC-cultured cells generate multilineage human cell differentiation and human progenitor cell colonies within the marrow of transplanted NOD/SCID mice

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Human CFC formed from ABM of NOD/SCID mice:</th>
<th>Percent of engrafted CD45⁺ cells expressing:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GM</td>
<td>E</td>
</tr>
<tr>
<td>Fresh ABM CD34⁺</td>
<td>0.8 ± 0.5</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>HUBEC</td>
<td>44.2 ± 15.9</td>
<td>3.4 ± 1.7</td>
</tr>
</tbody>
</table>

NOD/SCID mice were transplanted with either 1 x 10⁶ ABM CD34⁺ cells or the progeny of this dose of ABM CD34⁺ cells following 7 days of HUBEC-culture as described in Materials and Methods. The CFC content in each group was determined from triplicate cultures of 1 x 10³ NOD/SCID marrow cells per dish under procedures described in the Materials and Methods (n=11). The numbers shown under the CD34, CD19, and CD13 columns indicate the percentage of engrafted human CD45⁺ cells which expressed the particular differentiation antigen. For animals transplanted with Fresh ABM CD34⁺ cells, we were able to analyze only those mice with human cell engraftment ≥ 1%.
Figure 1

A)

B)
Figure 2

A

Day 0
FITC vs. 7AAD CD38

Day 7
FITC vs. 7AAD CD38

B
Figure 3

A)
B)

% Human Cell Engraftment

100

10

1

0.1

Fresh HUBEC-Cultured

BM CD 34⁺
C)

![Graph showing % Human Cell Engraftment vs. BM CD34+ and HUBEC-cultured samples.](image-reference)
D)

% Human Cell Engraftment

Fresh
BM CD34+

HUBEC-cultured

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E) 

![Graph showing % Human Cell Engraftment with data points for HUBEC-Transwell, GM-CSF + IL-3 + IL-6 + SCF + Flt-3 ligand.](image)

- % Human Cell Engraftment
- HUBEC-Transwell
- GM-CSF + IL-3 + IL-6 + SCF + Flt-3 ligand
Figure 4 A
Figure 4 B

I

Mouse IgG1 FITC

Mouse IgG1 FITC

II

CD45 perCP

m-CD45 FITC

III

Mouse IgG1 PE

Mouse IgG1 FITC

IV

CD34 PE

CD38 FITC

V

CD19 PE

CD3 FITC

VI

CD33 PE

CD13 FITC
Figure 5

A)

Fresh ABM CD34+ Cells

Fraction of negative mice

Number of transplanted BM CD34+ cells

990,000
Figure 1. Phenotypic analysis of bone marrow CD34\(^+\) cells following ex vivo culture. Purified human CD34\(^+\) cells were plated on confluent HUBEC monolayers, stroma-free liquid suspension cultures, non-brain endothelial cell monolayers, and non-contact HUBEC cultures in the presence of optimal concentrations of GM-CSF + IL-3 + IL-6 + SCF + flt-3 ligand for 7 days. (A) The phenotype of untreated purified bone marrow CD34\(^+\) cells at day 0. (B) HUBEC cultured bone marrow cells at day 7, demonstrating high percentage of CD34\(^+\) and CD34\(^+\)CD38\(^-\) cells. (C) Stroma-free liquid suspension cultures and (D) Non-brain endothelial cell co-cultures, demonstrating loss of CD34\(^+\)CD38\(^-\) phenotype cells. (E) HUBEC non-contact cultures, showing preservation of cells with CD34\(^+\)CD38\(^-\) phenotype. Harvested non-adherent cells were stained with FITC-conjugated CD34 mAb and PE-conjugated CD38 mAb and analyzed by FACS. Log fluorescence distribution of CD34 expression is shown along the X-axis and CD38 expression along the Y-axis. Cursor lines indicate the nonspecific staining levels of isotype matched control mAbs.

Figure 2. HUBEC culture induces cell division within quiescent ABM CD34\(^+\)CD38\(^-\) cells. (A) The dot plot shows the 7-AAD versus Ki-67 FITC profile for steady state bone marrow CD34\(^+\)CD38\(^-\) cells, with the majority of the population residing in G\(_0\). (B) CD34\(^+\)CD38\(^-\) cells were FACS sorted from 7 day HUBEC co-cultures and stained for 7-AAD versus Ki-67 FITC expression, demonstrating that
majority of the CD34⁺CD38⁻ population had entered cell cycle. Cell cycle analysis of CD34⁺ cells was performed using the SID method described in Materials and Methods.

**Figure 3.** Human cell engraftment in NOD/SCID mice transplanted with limiting doses of ABM CD34⁺ cells and HUBEC-cultured progeny. (A) 1 x 10⁵ ABM CD34⁺ cells (left) or their progeny following HUBEC-culture (right) were transplanted into NOD/SCID mice. The level of human cells present in the murine bone marrow at 8 weeks was determined by flow cytometric analysis of human CD45 expression. 5 x 10⁵ ABM CD34⁺ cells or their HUBEC-cultured progeny (B), 1 x 10⁶ ABM CD34⁺ cells vs. HUBEC-cultured progeny (C), 1.5 x 10⁶ ABM CD34⁺ cells vs. HUBEC-cultured progeny (D) are shown. (E) The engraftment of progeny of 1.5 x 10⁶ ABM CD34⁺ cells cultured with HUBEC non-contact cultures is shown (left) as well as the engraftment of the progeny of 3 x 10⁶ ABM CD34⁺ cells cultured with GMCSF + IL-3 + IL-6 + SCF + flt-3 ligand in the absence of HUBEC (right).

**Figure 4.** Phenotypic analysis of HUBEC-cultured cells engrafted in the bone marrow of NOD/SCID mice. (A) Expression of human CD45⁺ cells within the bone marrow of a control NOD/SCID mouse which was not transplanted (top), expression of human CD45⁺ cells within the bone marrow of a NOD/SCID mouse transplanted with 1 x 10⁶ fresh ABM CD34⁺ cells (middle), and expression of human CD45⁺ cells within the bone marrow of a NOD/SCID mouse transplanted with the progeny of 1 x 10⁶ ABM
CD34+ following co-culture with HUBEC (bottom). Isotype controls are shown at left. (B) Lineage distribution of engrafted human cells within a representative mouse transplanted with HUBEC-cultured cells. Panel I, murine marrow stained with isotype control IgG1-FITC and IgG1-PerCP. Panel II, staining of murine marrow with anti-human CD45-PerCP and anti-murine CD45-FITC, showing both human and murine populations. Panel III, isotype staining with IgG1-FITC and IgG1-PE. Panel IV, expression of human CD34-PE and CD38-FITC on engrafted cells within murine marrow. Panel V, staining with anti-CD19 and anti-CD3, demonstrating CD19 expression on engrafted human cells. Panel VI, expression of human CD33 and human CD13 on engrafted cells within the marrow.

**Figure 5.** HUBEC-culture increases the frequency of SRC within adult human bone marrow. (A) NOD/SCID mice (n = 47) were transplanted with fresh ABM CD34+ cells over a range of doses and the engraftment frequencies at each dose are plotted. The resultant curve indicates the estimated frequency of SRC within this population. (B) NOD/SCID mice (n = 47) were transplanted with the progeny of ABM CD34+ cells cultured with HUBEC plus GM-CSF + IL-3 + IL-6 + SCF + flt-3 ligand. The engraftment frequencies are plotted at each dose and the resultant curve indicates the frequency of SRC within this population.
Ex vivo culture with human brain endothelial cells increases the SCID-repopulating capacity of adult human bone marrow

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