A COMPARATIVE GENE-EXPRESSION ANALYSIS OF CD34+ HEMATOPOIETIC STEM AND PROGENITOR CELLS GROWN IN STATIC AND STIRRED CULTURE SYSTEMS

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Abstract: Static and stirred culture systems are widely used to expand hematopoietic cells, but differential culture performances are observed between these systems. We hypothesize that these differential culture outcomes are caused by the physiological responses of CD34+ hematopoietic stem and progenitor cells (HSPCs) to the different physical microenvironments created in these culture devices. To understand the genetic changes provoked by culture microenvironments, the gene expression profiling of CD34+ HSPCs grown in static and stirred culture systems was compared using SMART-PCR and cDNA arrays. The results revealed that 103 and 99 genes were significantly expressed in CD34+ cells from static and stirred systems, respectively. Of those, 91 have similar levels of expression, while 12 show differential transcription levels. These differentially expressed genes are mainly involved in anti-oxidation, DNA repair, apoptosis, and chemotactic activity. A quantitative molecular understanding of the influences of growth microenvironments on transcriptional events in CD34+ HSPCs should give new insights into optimizing culture strategies to produce hematopoietic cells.

Key words: CD34+ hematopoietic stem and progenitor cells, Ex vivo expansion, Culture microenvironment, SMART-PCR, cDNA array

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Abbreviations used: CB – cord blood; CFC – colony-forming cells; EDTA – ethylenediaminetetraacetic acid; HSPCs – hematopoietic stem and progenitor cells; IMDM – Iscove’s modified Dulbecco’s medium; MNC – mononuclear cells; PBS – phosphate buffer solution; ROS – reactive oxygen species; SDS – sodium dodecylsulfate; SSC – sodium chloride/sodium citrate
INTRODUCTION

Ex vivo expansion of cord blood (CB) cells, especially CD34+ hematopoietic stem and progenitor cells (HSPCs), is important for clinical practices of treating blood disorders [1]. Currently, static culture systems (i.e. well-plates, T-flasks and gas-permeating bags) and stirred ones (i.e. spinner flasks and stirred bioreactors) are successfully used to expand hematopoietic cells. In addition, it was reported that a stirred culture had a better proliferation potential than a static control [2, 3]. However, the underlying molecular mechanisms accounting for any differences remain poorly understood.

A stirred culture is characterized by a homogeneous culture environment, ease of monitoring and controlling culture parameters, and scaling up. However, stirring disturbs the interplay between HSPCs and their progeny, the extracellular matrix or growth factors, and introduces hydrodynamic forces produced by agitation, which have important roles in regulating the proliferation, differentiation, and apoptosis of HSPCs [4]. Thus, even if all the other culture conditions the HSPCs are exposed to are the same, local microenvironments may vary in different culture systems. The physiological responses of CD34+ HSPCs to their growth microenvironments determine culture performances. To better elucidate the effects of culture microenvironment on the biological behavior of HSPCs, we investigated the gene expression patterns of CD34+ HSPCs grown in static and stirred systems. Identifying differentially expressed genes could provide some valuable clues for modifying suboptimal hematopoietic cell-expansion culture protocols.

MATERIALS AND METHODS

CB collection

CB is the blood that remains in the umbilical cord and placenta at the time of birth. Using a sterile 250 ml blood bag containing anticoagulant, CB was collected shortly after a baby had been delivered. Briefly, the cord was clamped at both distal ends and cut. A needle was inserted into the umbilical vein at a prepared site to harvest the CB into the collection bag by gravity flow. Afterwards, the bag was gently rotated to mix the anticoagulant with the blood. The entire procedure was completed within 10-15 minutes of delivery. The collected CB was kept at room temperature.

Mononuclear cell (MNC) separation

The CB was processed to isolate MNC within 12 hours of collection. A density gradient centrifugation was done to separate MNC, using the lymphocyte separation medium (1.077 g/ml). This technique removed red blood cells and plasma, and isolated the MNC fraction, which contains hematopoietic stem and committed progenitor cells at different developmental stages. MNC were counted and cultured immediately after separation.
Cell culture
The CB MNC were inoculated at $1 \times 10^6$ cells/ml in static or stirred culture systems using Iscove’s modified Dulbecco’s medium (IMDM) (Gibco, Langley, VA, USA) supplemented with 20% fetal bovine serum, 50 ng/ml stem cell factor, 5 ng/ml interleukin-3, and 10 ng/ml interleukin-6. All the cytokines were purchased from PeproTech (PeproTech, Rocky Hill, NJ, USA). The static culture was done in 24-well plates (Nunc, Denmark) with 2 ml of culture medium per well. The stirred culture was done in at least 30 ml of culture medium in 125-ml spinner flasks (Techne, UK) with an agitation rate of 30 rpm. Both types of culture were maintained at 37°C in a humidified incubator with 5% CO₂. On day 3, the cells were fed: one half of the spent medium was disposed of, and the equivalent volume of fresh medium was added to the culture systems.

Colony-forming cell (CFC) assays and flow cytometric determination of CD34⁺ cells
After a 7-day culture, the CB cells were assessed for the expansion of total cells, CFC, and CD34⁺ cells, based on the initial inputs. CFC assays and flow cytometry were done to determine the HSPC expansion. Briefly, $1 \times 10^4$ cells were plated in semi-solid IMDM medium (Gibco) containing methylcellulose supplemented with the corresponding cytokines [3]. After at least 14 days of incubation, colonies containing > 50 cells were identified as CFC. In addition, $1 \times 10^6$ cells were labeled with 25 μl of PE-conjugated mouse-anti-human CD34-antigen antibody (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) following the manufacturer’s instructions. A Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) was used to distinguish the PE-conjugated antibody-tagged cells, and the results were analyzed using CellQuest software (Becton Dickinson).

The isolation and purification of CD34⁺ cells
The enrichment of CD34⁺ cells was positively selected using the MiniMACS magnetic separation system (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer’s protocols. Briefly, $1 \times 10^8$ MNC obtained after culture were suspended in 300 μl sorting buffer (1 x phosphate buffer solution (PBS)) supplemented with 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5% bovine serum albumin, 100 μl human immunoglobulin (Ig) FeR-blocking antibody, and 100 μl monoclonal hapten-conjugated CD34 antibody (clone QBEND/10; Miltenyi Biotec), and were incubated for 30 min at 4°C. After washing with 1 ml sorting buffer, the cells were centrifuged at 1000 rpm for 10 min, resuspended in 400 μl sorting buffer, and separated on a column exposed to the magnetic field of a MACS device (Miltenyi Biotec). The column was washed with sorting buffer until no cells effluxed, and was then removed from the separator. The retained cells were eluted using 600 μl sorting buffer. To
further purify the CD34+ cells, the harvested cells were subjected to a second round of column separation. The purity of the isolated CD34+ cells was > 95%.

cDNA pool construction by SMART-PCR
A total of 1.60 × 10^5 and 1.68 × 10^5 CD34+ cells were respectively harvested from the static and stirred cultures. The total RNA was isolated using Trizol reagent (Invitrogen, Burlington, ON, USA) according to the manufacturer’s instructions. The collected total RNA was digested with RNase-free DNase (Promega, Madison, WI, USA) to remove contaminant genome DNA. To overcome the limitation of the rarity of the RNA sample, SMART-PCR technology was used to generate sufficient cDNA. An aliquot of the purified total RNA was reverse-transcribed into cDNA using SMART II, CDS primers (Clontech, Palo Alto, CA, USA) and Superscript II reverse transcriptase (Clontech). cDNA pools were constructed by SMART-PCR amplification of cDNA using a SMART cDNA synthesis kit (Clontech).

cDNA array experiments and data mining
The cDNA pools generated as described above were purified with a PCR purification kit (Qiagen, Valencia, CA, USA). About 500 ng of purified SMART double-strand cDNA and the cDNA array gene-specific primer mixture were used to synthesize the probes with α-32P dATP and Klenow enzyme, as suggested by the suppliers. The probes were purified using a NucleoSpin Extraction kit (Clontech).
Membranes covering 588 genes (Clontech) were pre-hybridized in BD ExpressHyb buffer (Clontech) for 30 min with continuous agitation at 68°C, then hybridized overnight with the probes at 68°C, followed by 30 min washes in decreasingly stringent wash buffers (2 × sodium chloride/sodium citrate (SSC)/1% sodium dodecylsulfate (SDS), followed by 0.1 × SSC/0.5% SDS, followed by 2 × SSC). After the washing procedures, the membranes were exposed to a low-energy phosphorimaging screen overnight. The arrays were scanned with a Phosphor-Imager (Packard Instruments Co., Meriden, CT, USA) and analyzed using Optiquant software (Packard Instruments Co.). The results were exported as Excel files for further analysis. For each array, the background was calculated from the area between the probe grids, and subtracted from the signal intensity for individual genes. Signal intensities from different membranes were normalized using the global method, which is the most suitable for closely related samples. A gene with a signal intensity ≥ 3 times higher than the background was identified as significantly expressed. Those genes with ≥ 2-fold different expression levels were regarded as differentially expressed genes.

Confirmation by semi-quantitative PCR
To confirm the data from the cDNA arrays, semi-quantitative PCR was carried out using the cDNA templates generated above. The amount of cDNA was equalized based on the relative expression levels of the α-tubulin gene in the samples. The PCR conditions for the individual genes were determined by
RESULTS

The expansion of total cells, CFC, and CD34\(^+\) cells in static and stirred cultures

After 7 days, the expansions for the total cells, CFC, and CD34\(^+\) cells were calculated (Tab. 1). Although only a low proliferation level of total cells was observed, the number of HSPCs was significantly increased, which is important for the clinical settings of expanded hematopoietic cells. The results also revealed that a stirred culture offers proliferative advantages over a static culture in the expansion of CD34\(^+\) cells and CFC. This is consistent with the results of previous studies [2, 3]. The greater output of total cells in the static culture may be due to differential responses of HSPCs and their progeny to specific microenvironments in these systems.

Tab. 1. Expansion level for total cells, CFC, and CD34\(^+\) cells in static and stirred culture systems. The numbers are the multiple by which the level was higher

| Culture systems | Total cells | CD34\(^+\) cells | CFC  |
|-----------------|-------------|------------------|------|
| Static culture  | 1.47        | 2.44             | 8.10 |
| Stirred culture | 1.27        | 5.43             | 10.60|

The gene expression patterns of CD34\(^+\) HSPCs grown in different culture systems

The genes in the cDNA array consist of the most crucial cellular pathways and functions. In our preliminary work, we repeated the hybridization experiments. The results revealed that the signal intensity from the second hybridization was about 70% of the first. Therefore, only the first hybridization data is shown here. By the criteria described above, 103 and 99 genes were significantly expressed in the CD34\(^+\) cells from the static and stirred cultures, respectively. Of those, more than 88% of the expressed genes (91 out of 103 genes) showed similar transcriptional levels, suggesting that CD34\(^+\) HSPCs cultured in static and stirred systems share a similar genotype. In addition, 12 genes displayed differential expression levels in the macroarray analysis. Fig. 1 depicts the gene expression profiling of both CD34\(^+\) cell populations. The expressed genes are classified into 17 functional classifications, primarily involved in transcription regulation (16 genes), the cell cycle (12), oncogenes and tumor suppressors (10), stress response proteins (16), apoptosis-associated proteins (16), cell signaling and extracellular communication proteins (18), and intracellular transducers,
Fig. 1. The gene expression patterns of CD34⁺ HSPCs cultured in static (above) and stirred (below) culture systems. The gene expression array consists of 6 individual units, which are indicated by A through F. Each unit contains 7 columns and 14 lines, i.e. 98 genes. In total, 588 genes are arrayed on the membrane, and each gene is in duplicate. At the bottom of the membrane, there are reference genes. Their expression signals acted as a criterion to assess the validity of the hybridization results.
effectors and modulators (26). No signals of significant expression were detected in immune system proteins, extracellular transport/carrier proteins, membrane channels and transporters, extracellular matrix proteins, metabolic proteins, post-translational modification/protein folding, or RNA processing, turnover and transport (see Tab. 2). Further analysis showed that more than 17% of the genes (103 out of 588 genes) were expressed at levels ≥ 3 times higher than the background, and about 6% of the genes (36 out of 588 genes) were expressed at levels ≥ 10 times higher than the background. The numbers of high-activity genes (more than 10-fold over the background) expressed in the static and stirred cultures were respectively 34 and 36. These highly expressed genes are associated with transcription (8 genes), the cell cycle (5), stress response proteins (7), apoptosis-associated proteins (4), and cell signaling and extracellular communication proteins (7).

Tab. 2. Functional classifications of the expressed genes in CD34+ HSPCs from static and stirred cultures

| Functional classifications | S/W ≤ 0.5 | 0.5 < S/W < 2.0 | S/W ≥ 2.0 |
|---------------------------|-----------|-----------------|-----------|
| All genes                 | 11        | 91              | 1         |
| Cell surface antigen      | 1         | 1               | 0         |
| Transcription regulation  | 0         | 16              | 0         |
| Cell cycle                | 1         | 11              | 0         |
| Cell adhesion receptors/proteins | 0 | 4 | 0 |
| Oncogenes and tumor suppressors | 0 | 10 | 0 |
| Stress response proteins  | 5         | 11              | 0         |
| Trafficking/targeting proteins | 0 | 1 | 0 |
| Translation               | 0         | 1               | 0         |
| Apoptosis-associated proteins | 3 | 13 | 0 |
| DNA-binding and chromatin proteins | 0 | 1 | 0 |
| Cell receptors (by ligands) | 0 | 4 | 0 |
| Cell-signaling, extracellular communication proteins | 3 | 14 | 1 |
| Intracellular transducers, effectors and modulators | 1 | 25 | 0 |
| Protein turnover          | 0         | 5               | 0         |
| Cell receptors (by activities) | 1 | 3 | 0 |
| Cytoskeleton/motility proteins | 0 | 1 | 0 |
| DNA synthesis, recombination, and repair | 2 | 7 | 0 |

Threshold = 3; static culture, 103; stirred culture, 99. Due to the genes having been classified in multiple categories, the total number of genes listed in this table is not equal to the number of expressed genes (103). S/W: the ratio of gene expression intensity between the stirred and static culture systems.
A low agitation rate has little effect on the expression of surface molecules and receptors on CD34+ HSPCs

It was documented that the hydrodynamic forces produced by agitation influenced the transcriptional levels of cell surface receptors and changed the physiological response and proliferative potential, implying hydrodynamic force is an important operational parameter in the art of cell culture [5, 6]. Furthermore, the proliferation of hematopoietic cells is dependent on the agitation rate and impellor configuration [2]. To better understand the roles agitation plays in hematopoietic cell culture, we analyzed the gene expression levels of surface molecules and receptors on CD34+ cells cultured in different systems. In total, 14 genes encoding cell surface molecules were found to be significantly expressed. These genes showed similar expression levels, with the exception of an apoptotic receptor, TNFRSF1B (Tab. 3). The slight differences detected in the CD34+ cell samples from the two systems are likely to reflect the negligible influence of a low agitation rate (30 rpm) on the molecular phenotype of CD34+ HSPCs. This may partially explain the observation that a relatively low agitation rate supports the proliferation of hematopoietic cells (Tab. 1).

Tab. 3. The expression of cell surface molecules and receptors on CD34+ HSPCs

| Coordinate | Accession number | Gene name | Multiple (S/W) |
|------------|------------------|-----------|---------------|
| E3l        | M27492           | IL1R      | 1.22          |
| E2l        | M29696           | IL7R      | 1.09          |
| A2c        | U01134           | fms-related tyrosine kinase 1, FLT-1 | 1.01 |
| C1m        | M33294           | tumor necrosis factor receptor superfamily, member 1A, TNFRSF1A | 1.00 |
| E2m        | X01057           | IL2Ra     | 0.93          |
| E6n        | M15395           | integrin, β 2 , CD18 (p95) | 0.86 |
| E7k        | Y00796           | integrin, α L, CD11A (p180) | 0.81 |
| E2n        | M20566           | IL6R      | 0.80          |
| B1d        | X01060           | transferrin receptor, CD71 | 0.79 |
| E6m        | L12002           | integrin, α 4, CD49D | 0.78 |
| B4j        | U05875           | interferon γ receptor 2, IFNGR2 | 0.74 |
| E3g        | D11086           | IL2Rγ     | 0.69          |
| E7j        | X74295           | integrin, α 7 | 0.67 |
| C1c        | M32315           | tumor necrosis factor receptor superfamily, member 1B, TNFRSF1B | 0.49 |

Multiple: the multiple by which the level was different. S/W: the ratio of gene expression intensity between the stirred and static culture systems.
Growth microenvironments in different culture systems influenced the gene expression of CD34+ HSPCs

As shown in Tab. 4, a total of 12 genes were differentially expressed. Most of the genes showed differential expression levels of 2- to 3-fold, whereas BNIP3 was differentially expressed by 8-fold more. With the exception of that of chemokine CCL2, the expression levels were higher in the CD34+ HSPCs cultured in the static system. These results suggested that the growth microenvironments formed in static and stirred culture systems influenced the gene expression of CD34+ cells.

Tab. 4. Differentially expressed genes in CD34+ HSPCs from static and stirred cultures

| Coordinate | Accession number | Gene name | Multiple (S/W) |
|------------|------------------|-----------|---------------|
| F3a        | M24545           | chemokine (C-C motif) ligand 2, CCL2 | 2.61 |
| B7h        | L19185           | peroxiredoxin 2, PRDX2 | 0.50 |
| C3g        | X79389           | glutathione S-transferase theta 1, GSTT1 | 0.50 |
| C7b        | M13267           | superoxide dismutase 1, SOD1 | 0.41 |
| C7a        | M13194           | excision repair cross-complementing rodent repair deficiency, complementation group 1, ERCC1 | 0.43 |
| C6b        | M31899           | excision repair cross-complementing rodent repair deficiency, complementation group 3, ERCC3 | 0.41 |
| F2e        | K03515           | glucose phosphate isomerase, GPI | 0.48 |
| F3i        | M57502           | chemokine (C-C motif) ligand 1, CCL1 | 0.30 |
| D6n        | U15979           | delta-like 1 homolog, DLK-1 | 0.37 |
| D5k        | U02368           | forkhead box O1A, FOXO1A | 0.39 |
| C1c        | M32315           | tumor necrosis factor receptor superfamily, member 1B, TNFRSF1B | 0.49 |
| C4d        | U15174           | BCL2/adenovirus E1B 19kDa interacting protein 3, BNIP3 | 0.12 |

Only genes with at least 2-fold different expression levels are listed. Multiple: the multiple by which the level was different. S/W: the ratio of gene expression intensity between the stirred and static culture systems.

Confirmation by semi-quantitative PCR

To validate the data from hybridization, CCL2, ERCC1 and BNIP3 were selected to test the expression levels in the two CD34+ cell populations. The primers used in this study are listed in Tab. 5. As shown in Fig. 2, a significant up-regulation of CCL2 was found in the CD34+ cells grown in the stirred culture. No substantial expression of BNIP3 and ERCC3 was detected in CD34+ cells.
cells from the stirred culture, while weak signals were seen in CD34⁺ cells from
the static control under the same PCR conditions. These results further
corroborate the validity of the cDNA array analysis.

Tab. 5. The primers used in the experiment

| Gene  | Sequences (5’→3’) |
|-------|-------------------|
| BNIP3 | F: ATGGGATGGTGGTCAAGTCG  R: AAGGTGCTGGTGAGGTT |
| CCL2  | F: CTTCTCTGCTGCTGCTC  R: CCTGAACCACTTCTGCTT |
| ERCC3 | F: CTACATCGCCAAAGTCCA  R: TCTTCTGCAACCATCCCT |
| α-tubulin | F: TCACTGCTTCCCTGAGATT  R: CGTCACCACGGGTACAACA |

F: forward primer, R: reverse primer.

DISCUSSION

The gene expression patterns of fresh and cultured CD34⁺ cells were examined
in our laboratory using SMART-PCR and cDNA arrays [7]. We found that 45
genes displayed differential expression levels before and after culture; this is
responsible for the altered property of the expanded CD34⁺ cells to some degree.
In this study, we systematically analyzed the gene expression profiling of CD34⁺
HSPCs cultivated in static and stirred systems. Comparative gene expression
could aid in understanding the physiological responses of CD34⁺ cells to their
culture environments. In addition, it is necessary to investigate protein
expression to better evaluate the effects of ex vivo culture environments on the
biological activity of CD34⁺ cells.
The data indicated that CD34⁺ HSPCs share similar gene expression patterns
when they are cultured in different culture systems (Tabs 2 and 3). This is
probably due to their exposure to the same macroenvironments (i.e. accessory
cells, medium, and cytokine cocktails). To some extent, it also provides
a molecular-level explanation as to why a stirred culture can support the growth
of hematopoietic cells as a static culture does, thus giving a basis for developing
stirred bioreactors to produce hematopoietic cells.
Even when CD34+ HSPCs are grown under the same conditions but in static and stirred systems, the specific growth microenvironments in which they reside differ, which leads to the differential expression of a small number of genes (Tab. 4). Relative to the stirred culture, the static culture yields a higher local cell density because of sedimentation. Crowding cells compete for consumption of dissolved oxygen or nutrient substances, leading to the accumulation of metabolic by-products (i.e. oxygen free radicals). This may induce oxidative stress, which is mediated by the reactive oxygen species (ROS) in CD34+ HSPCs when they are grown in static culture. Oxidative stress is hostile to the biological activity of the cells, with effects including growth arrest, DNA damage, and cell apoptosis [8]. To decrease the damage caused by oxidative stress, CD34+ HSPCs initiate the relevant mechanisms to survive the oxidative environments, as evidenced by the enhanced expression of genes resistant to oxidative damage. PRDX2 encodes a member of the antioxidant enzyme family, which reduces hydrogen peroxide and alkyl hydroperoxides. It has a protective role in cells fighting against oxidative injury and apoptosis [9]. Similarly, as an anti-oxidative reagent, SOD1 is responsible for destroying free superoxide radicals in cells [10]. GSTT1 catalyzes the conjugation of reduced glutathione to a variety of compounds to lower the cellular damage induced by oxidation [11]. Both ERCC1 and ERCC3 are required for the excision repair of DNA double strand breaks when cells are exposed to stressful environments such as radiation and oxidative stress [12, 13]. TNFRSF1B plays a role in protecting cells from apoptosis by stimulating anti-oxidative pathways [14]. BNIP3 levels increase in response to hypoxia; its elevated expression induces cell death [15]. Notably, GPI is a hypoxia-induced cytokine that regulates the locomotion and metastasis of tumor cells to survive the environment of hypoxia [16]. It was recently reported that the interaction of the transcription factor FOXO and β-Catenin of the Wnt signaling was enhanced to tolerate the condition of oxidative stress [17], which is detrimental to stem cell self-renewal [18]. Taken together, it is proposed that oxidative stress occurs in CD34+ cells cultured in a static system, which induces CD34+ cells to recruit multiple mechanisms to endure the oxidative environment, such as overexpression of anti-oxidative enzymes, repair of damaged DNA, and regulation of apoptosis. The data also implies that oxidative stress may be associated with a lower level of expansion of HSPCs in static culture. The regulation of oxidative stress (i.e. the supplement of an anti-oxidant to the medium) could provide some insights into improving the manipulation of hematopoietic cell cultures in static systems. Elucidating the relationship between cell proliferation and ROS will be instructive in addressing differential performances in different culture systems.

The gene for DLK-1, a ligand for the Notch receptor, was expressed more in CD34+ cells expanded in the static culture. It blocks the differentiation of hematopoietic primitive cells by reinforcing the Notch signaling pathway [19], implying that growing CD34+ HSPCs in stirred systems could lead to greater differentiation when compared to the static controls. In fact, the results of
hematopoietic cell culture from our laboratory showed that a stirred culture induces differentiation at a faster rate than a static one (data not shown). Suppressing the factors inducing differentiation would be helpful for the performance of HSC expansion in stirred culture systems.

In conclusion, comparative gene expression profiling gives a glimpse of the effects of culture microenvironments on the genetic program of CD34+ HSPCs grown in different systems. The identification of differentially expressed genes could represent a new avenue to optimizing culture parameters to expand hematopoietic cells.

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