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RESEARCH PAPER

The Effect of Human Bone Marrow Stroma-Derived Heparan Sulfate on the *Ex Vivo* Expansion of Human Cord Blood Hematopoietic Stem Cells

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ABSTRACT

Purpose In order to address cell dose limitations associated with the use of cord blood hematopoietic stem cell (HSC) transplantation, we explored the effect of bone marrow stroma-derived heparan sulfate (HS) on the *ex vivo* expansion of HSCs.

Methods Heparan sulfate was isolated and purified from the conditioned media of human bone marrow stromal cells and used for the expansion of cord blood-derived $CD34^+$ cells in the presence of a cocktail of cytokines.

Results The number of myeloid lineage-committed progenitor cells was increased at low dosage of HS as illustrated by an increase in the total number of colony-forming cells (CFC) and colonies of erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) precursors. Notably, the stroma-derived HS did not alter the growth of $CD34^+$ HSCs or negatively affect the levels of various HSC phenotypic markers after expansion.

Conclusions This study shows that HS secreted into solution by stromal cells has the capacity to support hematopoietic cytokines in the maintenance and expansion of HSCs. The incorporation of stroma-derived HS as a reagent may improve the efficacy of cord blood HSC transplantation by enhancing the number of committed cells and accelerating the rate of engraftment.

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INTRODUCTION

Cord blood (CB) hematopoietic stem cell (HSC) transplantation plays a growing role in the treatment of a wide variety of malignant and non-malignant disorders such as leukemia, lymphoma, lymphoproliferative disorders and bone marrow failures (1,2). Cord blood, as a source of HSCs, widens the pool of potential donors compared to bone marrow and peripheral blood stem cells due to its ease of harvest, availability, less stringent HLA matching criteria and lower graft-*versus*-host disease. However, despite the advantages, the number of CB HSC transplantations recorded in a 2008 survey in Europe is only 7% of the total allogeneic HSC transplantations (3). This is due to the low number of cells collected per unit of CB that restricts its use to children and lightweight recipients. This cell dose limitation leads to a lower success rate in adult recipients, marked by a delay in engraftment and vulnerability to infectious morbidity (2). In order to address cell dose limitations, differing strategies to expand CB HSCs *ex vivo* have been proposed. Importantly, *ex vivo* expansion is geared not only to increase the number of transplanted cells, but the number of lineage-committed progenitor cells that can accelerate the engraftment process and reduce the risk of infection. Thus far, only one study has shown a marked improvement in the engraftment rate of *ex vivo* expanded CB HSC in a phase 1 clinical trial (4).

One of the attempts to improve HSC expansion *ex vivo* includes the incorporation of stromal components in culture to recreate the hematopoietic microenvironment in which stroma-derived extracellular matrix (ECM) and stem cells

provide complex molecular cues to support hematopoiesis (5–9). Up to two decades ago, it was believed that direct physical contact between HSC and stromal components was required for HSC maintenance (10,11). However, more recent studies show that stromal cell-derived conditioned media, in which various cytokines and proteoglycans are found, is sufficient to maintain HSCs (7,12). Furthermore, the complex role of glycosaminglycans (GAG) in supporting hematopoiesis, particularly those GAGs that originate from marrow-derived proteoglycans, is increasingly being elucidated; GAG can bind and regulate the distribution of various hematopoietic cytokines and maintain long-term culture-initiating cells (LTC-IC) (7,13,14). The positive effect of marrow-derived GAGs toward LTC-IC maintenance is attributed to heparan sulfate (HS) that is present as a minor component (10 to 12%) of the total GAG in the stroma-conditioned media (7).

Heparan sulfate has been long known to facilitate various physiological functions through its interaction with proteins (15–17). Our lab has shown temporal changes in HS proteoglycan profiles during osteogenesis (17,18). Moreover, the isolation, purification and subsequent reintroduction of these HSs from different stages of growth and osteogenic differentiation can differentially regulate cell growth (17). In a separate study, we have also shown that purified HS with affinity for FGF-2 can sufficiently support mesenchymal stem cell (MSC) proliferation in the absence of exogenously added FGF-2, presumably by supporting the activity of endogenous FGF-2 (19). The use of HS instead of FGF-2 for MSC expansion proved to be more beneficial, since the continuous use of exogenous FGF-2 during MSC osteogenic differentiation inhibits mineralization.

In the current work, we explored the effect of HS derived from HSC-supportive human bone marrow stromal cells towards the expansion of human CB HSCs and how it compares to the effect of total GAG. We hypothesize that human bone marrow-derived HS, herein referred to as HS5, can better maintain and enhance the proliferation of CB HSC in the presence of hematopoietic cytokines compared to total GAG. Total GAG and HS5 were isolated from the human bone marrow stromal cell line HS-5, herein referred to as BMS5, as this cell line has been shown to support the growth of hematopoietic stem cells both in co-culture systems and in the presence of its conditioned media (12). We examined the effect of total GAG or HS5 toward the expansion of purified $CD34^+$ cells from human CB units and $CD34^+$ $CD38^-$ subpopulation in the presence of a combination of hematopoietic cytokines: stem cell factor (SCF), FMS-like tyrosine kinase 3 ligand (Flt-3L) and thrombopoietin (TPO). Hematopoietic stem cell phenotypic marker expression and the formation of myeloid-colony-forming cells were assessed to reveal the pluripotency and state of differentiation of the cells. The effect of HS5 toward CB HSC expansion was also compared to heparin derived from porcine mucosa, an HSC non-supportive cell source. Heparin shares identical disaccharide unit building blocks with HS, though is more extensively sulfated compared to the latter and, hence, binds a greater number of cytokines than HS. We show that HS at low dose, but neither the total GAG nor heparin, has the ability to increase the number of myeloid lineage-committed progenitor cells despite having no effect towards overall expansion of HSCs and the more primitive, quiescent $CD34^+$ $CD38^-$ cell population. Thus, bone marrow-derived HS may have the potential to accelerate engraftment time by facilitating the expansion of committed cells from CB HSCs.

MATERIALS AND METHODS

Materials

All cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. All fluorescently labeled antibodies against the various cluster of differentiations (CD) investigated and their isotype-matched controls used in FACS analysis were purchased from BD Biosciences (San Jose, CA, USA). Commercial porcine intestinal mucosa-derived heparin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

The immortalized human bone marrow stromal cell line (HS-5, ATCC CRL-11882), herein referred to as BMS5, was maintained in DMEM, 10% FCS (Lonza Group Ltd., Basel, Switzerland), 100 U/mL penicillin/streptomycin, 4 mM L-glutamine and 1.5 g/L $NaHCO_3$. BMS5 cells were plated at 3×10^4 cells/cm^2 for all experimental analyses.

Hematopoietic stem cells were isolated from umbilical cord blood (UCB) units collected from the Singapore Cord Blood Blank (SCBB). Mononuclear cells were isolated from UCB by density gradient centrifugation using Ficoll-Paque Plus. Briefly, UCB was pre-diluted in PBS and gently layered on top of Ficoll. The sample was centrifuged, and the interphase layer was collected. The cells were washed with PBS twice before resuspension in PBS (pH 7.2) containing 2 mM EDTA and 0.5% BSA. $CD34^+$ hematopoietic stem cells (HSCs) were isolated from the cell suspension using CD34 MicroBead kit (MiltenyiBiotec, Bergisch Gladbach, Germany) according to manufacturer's specification. Isolated $CD34^+$ HSCs were cultured at 4×10^4 cells/mL on non-tissue culture-treated 24-well plates and expanded for

12 days in QBSF-60 Stem Cell serum-free medium (Quality Biological, Inc., Gaithersburg, MD, USA) supplemented with 50 ng/mL of SCF, Flt-3L and TPO (R&D Systems, Minneapolis, MN, USA) in the presence/absence of BMS5-derived total GAG or HS at indicated concentrations prior to analysis.

Cell Proliferation Assay

Total and viable cell numbers were counted at specified time points using Guava® Flex-reagent and the Guava® PCA-96 benchtop flow cytometer as specified by the manufacturer (Guava Technologies, Hayward, CA, USA). BMS5 cells were plated as described above and counted every other day for 11 days. Expanded CD34$^+$ HSCs were counted every 4 days during its 12-day expansion.

BMS5 Growth Factor Profiling at the Transcript Level

Total RNA was isolated at day 3 using NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) and reverse transcribed using Superscript III® First-Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's specifications. The expression of target genes was determined by amplifying the cDNA using AmpliTaq Gold® DNA Polymerase with Buffer II and $MgCl_2$ (Applied Biosystems) and dNTP (Invitrogen, Carlsbad, CA, USA). The primer sequences used are as outlined in Table I. The following reaction setup was employed: 1) activation step: 94°C for 10 min; 2) amplification step (30 cycles): 94°C for 45 s, 58°C for 45 s, 72°C for 45 s; and 3) final extension step: 72°C for 7 min.

BMS5 Cytokine Profiling at the Protein Level

The cells were seeded at 3×10^4 cells/cm^2 in maintenance medium. Conditioned medium from the culture was collected at an indicated time and stored at −80°C prior to quantification. The amount of cytokines present in the conditioned medium was assayed using their respective Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's specification. The values obtained were blanked using basal non-conditioned medium.

Total Glycosaminoglycan (GAG) and Heparan Sulfate (HS) Extraction and Purification

The BMS5 cells were seeded at 3×10^4 cells/cm^2 in a 15-cm dish in maintenance medium. One day after seeding, the medium was replaced with serum-free medium and the medium collected every other day up to day 11 and pooled. At each collection point, the medium was centrifuged at 5000 rpm for 10 min at 4°C and filtered using 0.45 μm filter to remove cell debris prior to storage at 4°C. The purification of HS from the cell-conditioned medium (herein referred to as HS5) and its disaccharide analysis was performed as previously described (20).

Table I List of Primers Used in PCR

Cytokines	Accession ID	F Primer	R Primer
Kit-L	NM_000899.3	CGGAGCCTCCAGTCTGTCATTA	AGGACTCACCCCTAAGGAGTGA
IL-8	NM_000584.2	TCCTTGTTCCACTGTGCCTTG	TGCTTCCACATGTCCTCACAAC
IL-6	NM_000600.1	AATTCGGTACATCCTCGACGG	GGTTGTTTTCTGCCAGTGCCT
IL-3	NM_000588.3	CCAATCCATATCAAGGACGGTG	GCTCAAAGTCGTCTGTTGAGCC
LIF	NM_002309.2	TCCCAACAGCAAGACGAGGAT	CAAGCTAAGCCGGATGAAGCAG
TPO	NM_000460.2	CTTCGTGACTCCCATGTCCTTC	CCCAAGCTAAAGTCCACAGCAG
M-CSF	NM_000757.3	GCAGCTGCAGGAACTCTCTTTG	TGACCTTCTCCAGCAACTGGAG
MIP1α	M24110.1	CTCTGCAAAACCCCCCAAAT	CAACTGCGGAGAAAGGAGAGAA
Flt3-L	NM_001459.2	AAGATGCAAGGCTTGCTGGA	AGATGTTGGTCTGGACGAAGCG
G-CSF	X03438.1	GCTTCCTGCTCAAGTGCTTAGA	GCACACTCACTCACCAGCTTCT
GM-CSF	NM_000758.2	AAGTTCTCTGGAGGATGTGGCT	TCATTCATCTCAGCAGCAGTGT
IL-1α	NM_000575.3	AAGGCTGCATGGATCAATCTGT	TCCCGTTGGTTGCTACTACCAC
IL-1β	NM_000576.2	AGGCGGCCAGGATATAACTGA	TTCTGTTCCCTTTCTGCCAGC
IL-11	NM_000641.2	GAAGTCCAAGAGTTCGAGACCG	CAGCATGCAGTGGTTTTGTAGC
IL-1RA	NM_000877.2	ACAAAATTGGCCAGAGAGTGG	CCATTGATTCTTGTCCCTCCTT
SDF-1	NM_199168.2	GCCAACGTCAAGCATCTCAAA	CCTGAATCCACTTTAGCTTCGG
Perlecan	NM_005529	TGGACACATTCGTACCTTTCTGA	CCTCGGACACCTCTCGAAACT
18sRNA	AY248756.1	TTCGAGGCCCTGTAATTGGA	GCAGCAACTTTAATATACGCTATTGG

High-Performance Size-Exclusion Chromatography of HS

A Dionex High-Performance Liquid Chromatography (HPLC) system was used to equilibrate a prepacked Superdex 200 HR column (10×300 mm, GE healthcare) at 0.5 ml/min in 10 mM HEPES buffer, 150 mM NaCl (pH7.2). The anion-exchange chromatography purified HS samples were fractionated to check the intact full chains. The column void (Vo) and total (Vt) volumes were determined using blue dextran 2000 and sodium dichromate, respectively. The Superdex 200 column was calibrated using gel filtration high and low molecular weight protein calibration marker proteins (1. Ferritin; 2. Aldalose; 3. Conalbumin; 4. Ovalbumin; 5. Carbonic Anhydrase; 6. Ribonuclease A; and 7. Aprotin). The elution volumes (Ve) of protein standards were converted into a calibration chart of Kav against molecular mass [$Kav = (Ve-Vo)/(Vt-Vo)$]. A line of best fit was fitted to the calibration data, and the equation of this line was used to estimate mass according to observed Kav.

Hematopoietic Stem Cell Phenotyping Using FACS Analysis

Hematopoietic stem cells were removed from culture after 12 days expansion, washed in PBS and resuspended in FACS buffer (2% FCS and 0.01% NaN_3 in PBS) before aliquoting into a round-bottomed 96-well plate at 1×10^5 cells/well. Cells were pelleted and triple-stained with FITC-conjugated CD38, PE-Cy5-conjugated CD34 and PE-conjugated CD44, CD90 or CD184 pre-diluted antibodies in ice for 20 min. Subsequently, cells were washed twice in FACS buffer before fixing in 4% PFA for 20 min at 4°C. Finally, cells were washed twice and resuspended in FACS buffer before analysis on FACSCalibur (Becton-Dickinson, San Jose, CA, USA). Isotype-matched controls were used for gating $CD34^+$ $CD38^-$ cell population and the PE-stained phenotypic markers within the population.

Colony-Forming Cell (CFC) Assay

Hematopoietic stem cell-colony-forming cell assay and colonies classification were performed using Methocult® H4434 Classic (StemCell Technologies Inc, Grenoble, France) according to manufacturer's specification. Briefly, $CD34^+$ HSCs that had been expanded for 12 days were resuspended in Iscove's MDM supplied in the kit with 2% FCS and mixed at a 1:10 (v/v) ratio with Methocult® H4434. The cells were plated at 500 cells per 35 mm dish and incubated at 37°C/5% CO_2 for 14 days prior to colony counting and classification. Colonies were classified as burst-forming unit-erythroid (BFU-E), a precursor to erythrocytes, and colony-forming unit-granulocyte macrophage (CFU-GM), a precursor to granulocytes and macrophages.

Statistical Analysis

Mean differences between samples were analyzed by performing homogeneity of variance test, followed by ANOVA. Differences of $p<0.05$ were considered significant.

RESULTS

Total GAG and HS Collection from BMS5

In order to determine the appropriate time point for GAG collection, BMS5 was cultured for up to 11 days, during which proliferation, viability and cytokine production were monitored. BMS5 continued to replicate after reaching 100% confluency around day 7, and its viability was maintained above 85% throughout the culture period (Fig. 1). At the transcript level, BMS5 was found to express various types of cytokines that are involved in HSC maintenance and expansion at day 3 (Fig. 2). The protein production of some of the cytokines that were used to support HSC culture in this study was expressed as early as day 1 in BMS5 (Fig. 2). Based on the protein synthesis profile, we believe that BMS5 would express GAGs that support the activity of these cytokines as early as day 1 as well. Coincidentally, the soluble form of HS, perlecan, was abundantly synthesized at the transcript level by BMS5 at day 3 (Fig. 2).

Thus, total GAG, likely containing a mixture of HS, chondroitin sulfate and dermatan sulfate, was isolated from BMS5 conditioned media at alternating days from day 1 to 11. Heparan sulfate (HS5) was purified from the total GAG mixture in order to determine whether this GAG species alone can influence cytokine activity to enhance HSC expansion. The isolated HS5 is heterogeneous in molecular weight with three predominant molecular weights of 7.5, 29 and 75 kDa present (Fig. 3). It is composed of 22.11% of non-sulfated, 38.79% of 6-O-sulfated, 4.20% of N-sulfated and 4.53% of 6-O-sulfated/N-deacetylated disaccharide units, while the remaining compounds were not defined.

Isolation and Purification of $CD34^+$ Hematopoietic Progenitor Cells

In order to test the effect of bone marrow-derived GAG and HS on CB HSC expansion, CB units were isolated from five different donors and purified for hematopoietic progenitor cells. CD34 serves as a marker for early hematopoietic progenitor cells and is widely used to purify HSCs from bone marrow and CB mononuclear cells. In the

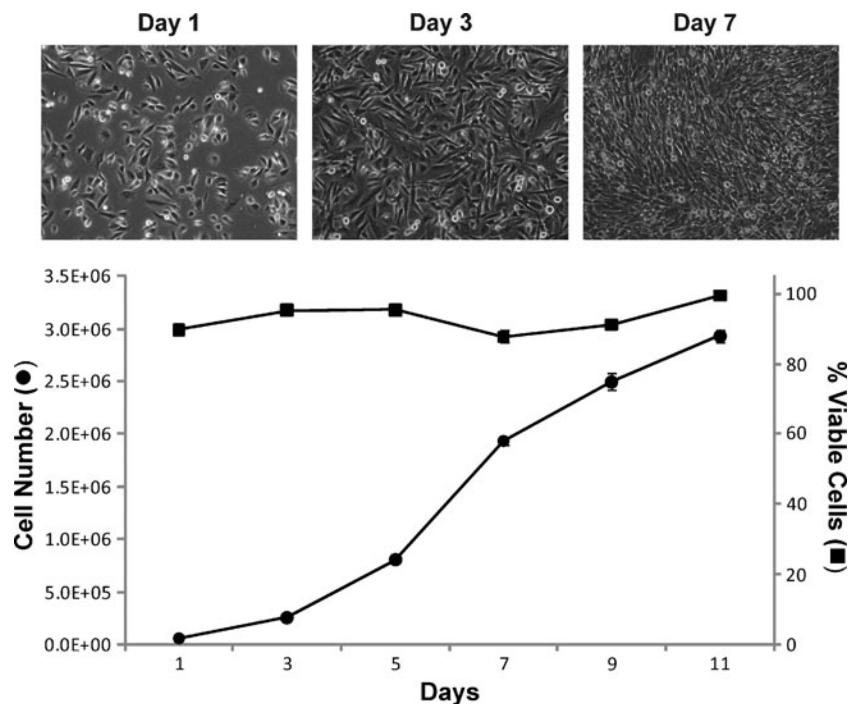

Fig. 1 BMS5 growth analysis. BMS5 seeded at 30,000 cells/cm^2 continue to expand up to 11 days. Cell viability was maintained above 80% throughout the culture period.

current study, we purified CD34$^+$ cells from fresh CB units at greater than 85% purity, as represented in Fig. 4. For this particular sample, 12% of the freshly isolated mononuclear cells are CD34$^+$ CD38$^-$ cells, which constitute the more immature subpopulation of the CD34$^+$ cells. The percentage of CD34$^+$ CD38$^-$ cells freshly isolated from CB varied greatly among the different samples, ranging from 12% to 62% and an average of 37.4%. After 12 days *ex vivo* expansion in the presence of SCF, TPO and Flt-3L, the percentage of CD34$^+$ CD38$^-$ cell population generally decreased with an average of 27.3% of the total mononuclear cells, ranging from 18.7% to 37.1%) (Fig. 5B).

The Effect of Total GAG or HS5 on HSC Expansion and Phenotypic Marker Expression

A wide variability was observed in the fold increase of total mononuclear cells among the different samples after 12 days *ex vivo* expansion. The cells treated with only cytokines increase in number by an average of 92-fold and a range of 11–286-fold increase. The magnitude in the fold increase seemed to correspond to the percent CD34$^+$ CD38$^-$ cell populations found in each expanded HSCs (data not shown). However, neither total GAG nor HS5 enhances HSC expansion above control for each donor (Fig. 5A). The percentage of CD34$^+$ CD38$^-$ cells after 12-day expansion was also not affected by the addition of total GAG or HS5 (Fig. 5B).

Additionally, the effect of total GAG and HS5 toward HSC phenotypic markers in expanded HSC was assessed. CD90 surface marker in CD34$^+$ cell population that is present in low percentage has been associated with colony-forming capacity (21), while CD184 and CD44 surface markers have been implicated in CD34$^+$ cell homing into the bone marrow (22,23). None of these surface markers were affected by the addition of total GAG or HS5 into the expansion culture medium as represented in Table II.

Clonogenic Potential of HSC Expanded in the Presence of Total GAG or HS5

Despite a lack of effect on the expansion of HSCs, we were interested in whether BMS5-derived total GAG and HS5 had any effect on the clonogenic potential of HSCs and the number of committed HSCs in the population. We found that total GAG had no effect on the clonogenic potential of HSC. However, low dose of HS5 (100 ng/mL or less) increased the clonogenic potential of HSC (Fig. 6). The number of committed HSC toward the erythroid (BFU-E) and granulocyte (CFU-GM) lineage also increased at low dose of HS5.

DISCUSSION

Since the concept of the stem cell niche was first introduced three decades ago by Schofield (24), the knowledge behind the role of stromal components toward HSC maintenance has evolved extensively. Both stromal-derived cytokines and proteoglycans have been shown to play an equally important role in hematopoiesis. The role of proteoglycans expressed by stromal cells has been extended to supporting endogenously expressed hematopoietic cytokines not only

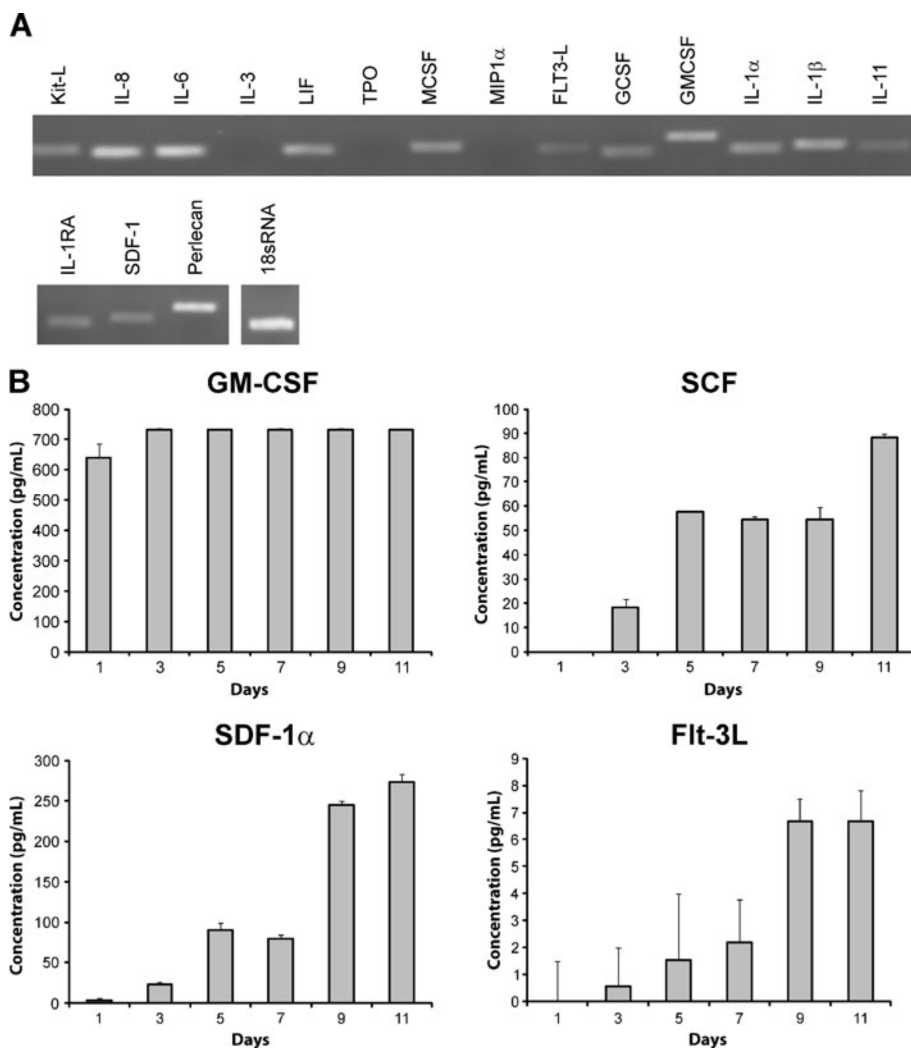

Fig. 2 BMS5 secretes various cytokines and growth factors at the (**A**) mRNA transcript and (**B**) protein level at different periods of growth. Cytokines that support hematopoietic stem cell expansion and maintenance were secreted as early as day 3 in culture.

on the surface of stromal cells and ECM, but also in solution (7). Indeed, 70% of total GAG synthesized by stromal cells is present in solution rather than on the stromal layer (25). This observation suggests a greater role for proteoglycans than merely modulating the localization of hematopoietic cytokines on the stromal layer. Here, we observed that purified HS5 from bone marrow-conditioned media performed better than total GAG in maintaining the clonogenic potential of *ex vivo* expanded HSCs. Gupta *et al.* (7) have also attributed HS as the GAG subset within bone

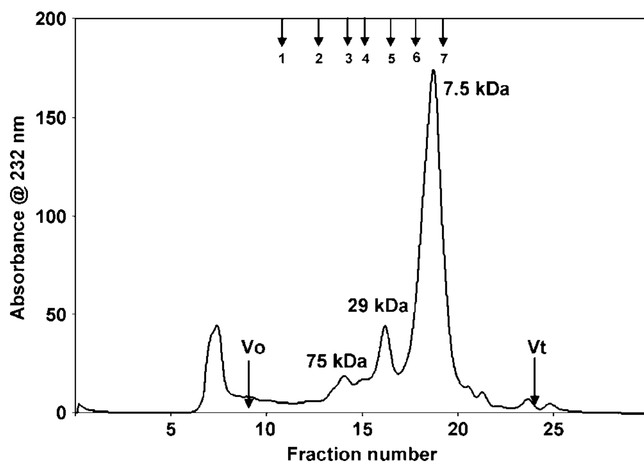

Fig. 3 HS5 molecular weight was distributed at 7.5, 29 and 75 kDa. HS5 molecular weight was assessed using a Superdex 200 column. The column was calibrated using gel filtration high and low molecular weight protein calibration marker-proteins to determine the molecular weight of HS5. Arrow indicates elution position of HMW and LMW protein standards (1.Ferritin; 2. Aldalose; 3. Conalbumin; 4. Ovalbumin; 5. Carbonic Anhydrase; 6. Ribonuclease A; and 7. Aprotin). *Vo* void volume; *Vt* total volume.

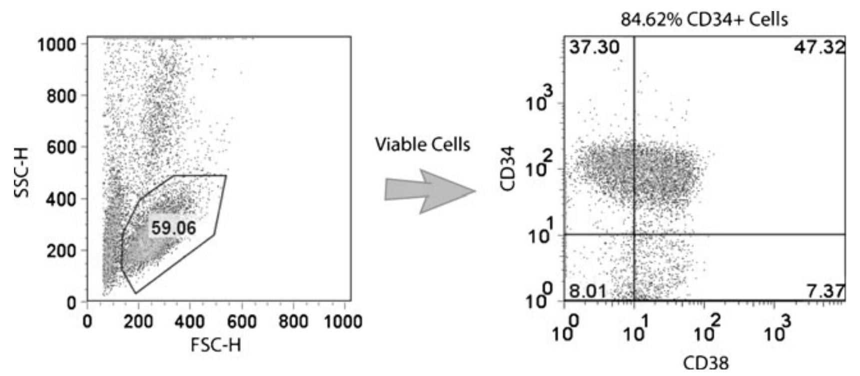

Fig. 4 Hematopoietic progenitors were isolated from umbilical cord blood using a $CD34^+$ progenitor cell isolation kit at a purity >80%.

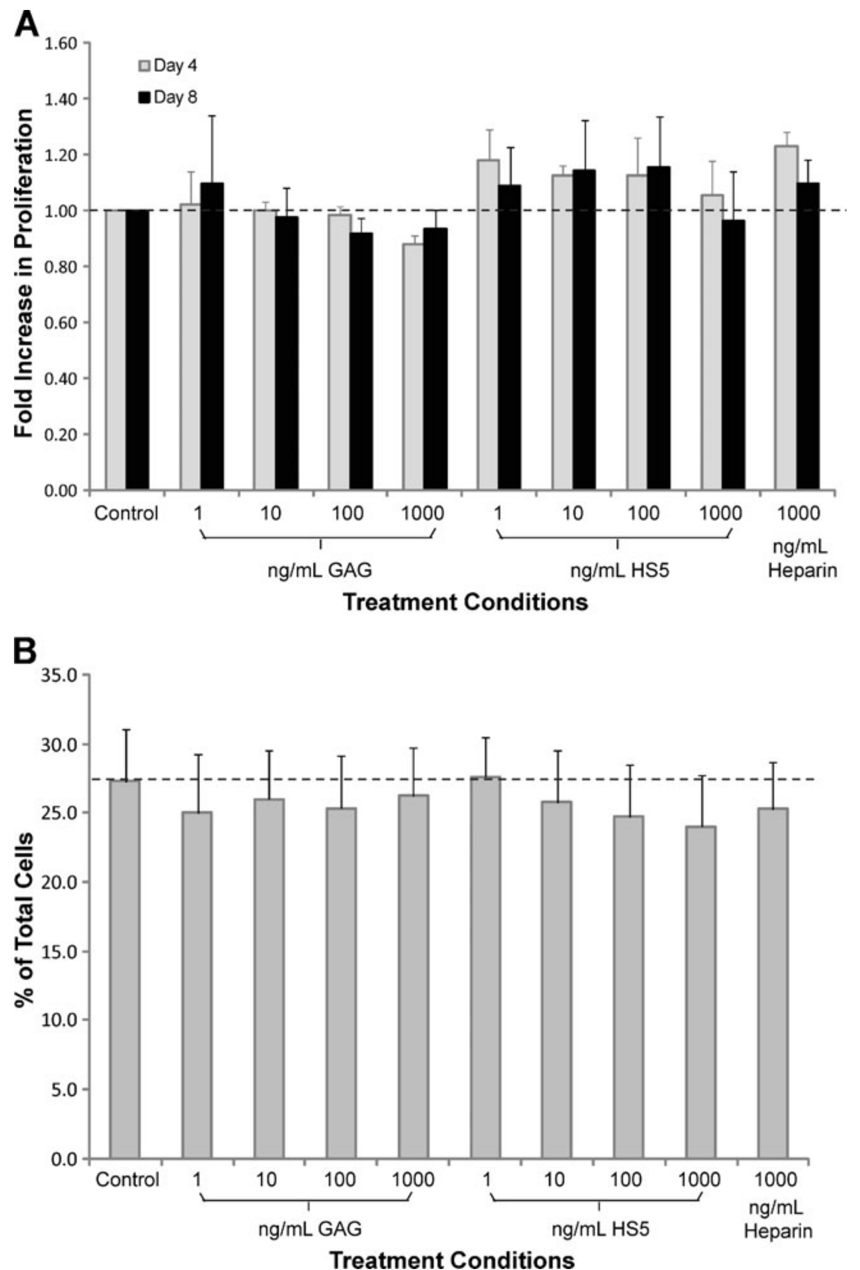

Fig. 5 HS5 and total GAG have no effect on (**A**) hematopoietic stem cell expansion nor (**B**) of the percent $CD34^+$ $CD38^-$ cells in the population after 12 days culture.

Table II Total GAGs and HS5 Have Little Effect Toward Phenotypic Markers of Hematopoietic Stem Cells

Treatment Group	CD44	CD90	CD184
Control	99.97%	3.42%	9.77%
1 ng/mL GAG	99.92%	4.37%	10.62%
10 ng/mL GAG	100.00%	3.98%	10.48%
100 ng/mL GAG	99.94%	3.65%	8.25%
1000 ng/mL GAG	99.94%	2.27%	8.04%
1 ng/mL HS5	100.00%	5.36%	16.86%
10 ng/mL HS5	99.97%	5.57%	12.81%
100 ng/mL HS5	99.94%	4.03%	11.84%
1,000 ng/mL HS5	99.94%	2.50%	8.95%
1,000 ng/mL Heparin	99.94%	4.16%	13.23%

marrow-conditioned media that can maintain LTC-IC for extended culture. Heparan sulfate has been implicated in various roles outside the hematopoietic niche, including facilitating protein dimerization, ligand-receptor interaction and protein stabilization (26–28). Consequently, it may also hold an important role in modulating various physiological and pathological processes within the hematopoietic niche. Stromal-derived HS has been identified as a component of the stromal ECM that promotes the maturation of promyelocytic leukemia cells *in vitro* (29).

The protein-binding capacity and function of HS are dictated by the length of the polysaccharide chain and the degree and pattern of sulfation of its disaccharide units (28,30,31). Gupta *et al.* (32) compared the sulfation profile of HS derived from a hematopoiesis-supportive and non-supportive cell line and found that 6-O sulfation is highly expressed in supportive HS. Interestingly, HS5 that was purified from BMS5 in the current study possesses a high

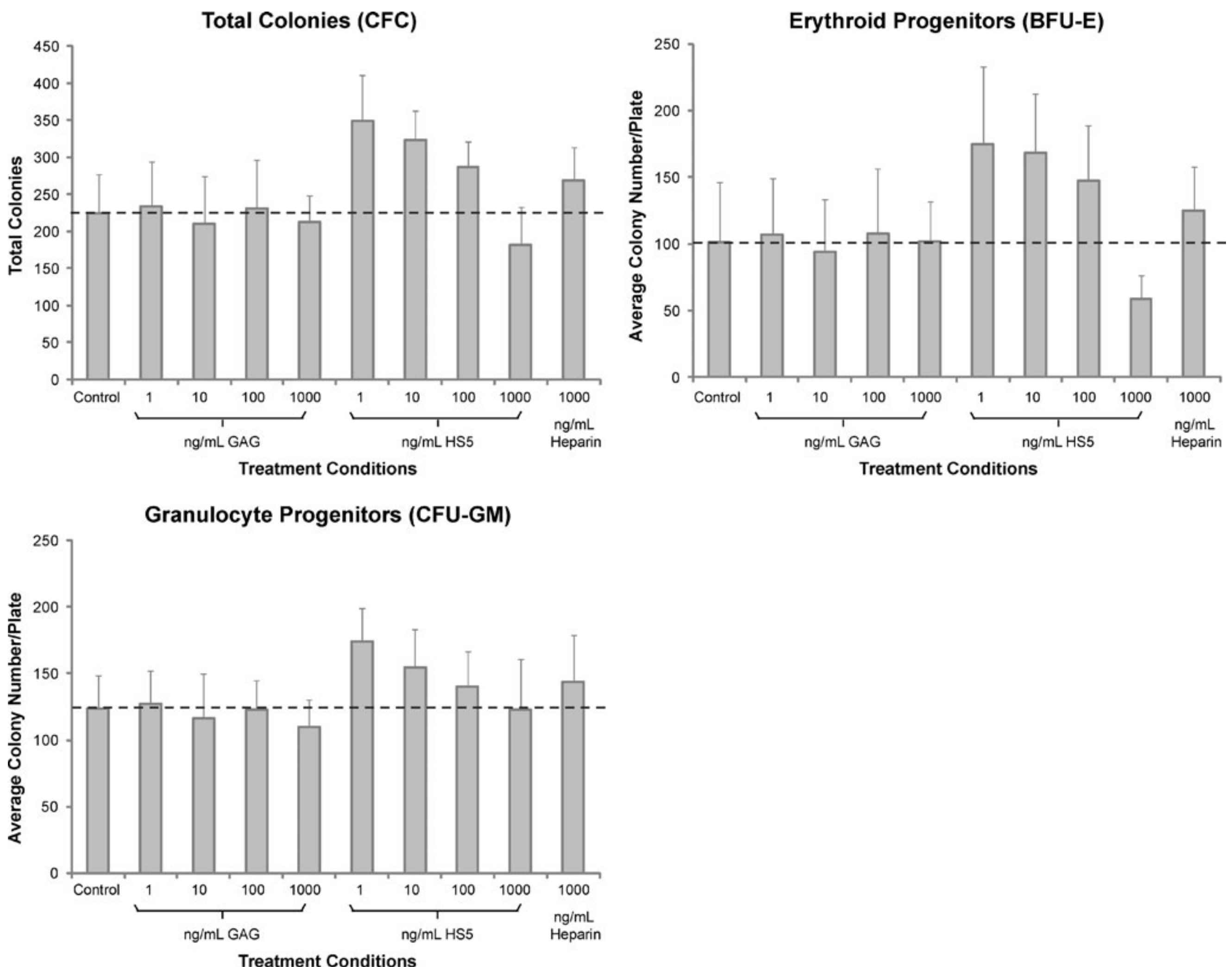

Fig. 6 HS5 but not total GAG markedly increased the clonogenic potential of HSCs and the formation of erythroid progenitors (BFU-E) and granulocyte progenitors (CFU-GM), especially at low dose.

percentage of 6-O sulfation as well. The highly 6-O sulfated HS has been previously shown to bind to hematopoietic cytokines such as interleukin-3, macrophage inflammatory protein-1α (MIP-1α), and thrombospondin (32,33). Emphasizing its importance further, the removal of 6-O-sulfation has been found to reduce the ability of heparin, a more extensively sulfated form of HS, to maintain LTC-IC in extended culture. Since O-sulfation was also found to be crucial for $CD34^+$ cell interactions with heparin, it was speculated that HS proteoglycans exert their effect on hematopoiesis by bringing HSC, stromal cells, ECM components and cytokines within proximity to one another. Given that our study shows that soluble HS5 influences the number of committed HSCs in the absence of its protein core, it is possible that stromal cell HS affects hematopoiesis by modulating protein conformation and presentation as well.

The purification of HSC through $CD34^+$ cell sorting generates a heterogeneous subpopulation of cells containing a mixture of primitive HSC, committed hematopoietic progenitors of the myeloid and lymphoid lineage and endothelial precursor cells (34,35). The heterogeneity of this subpopulation of cells can be subsequently influenced by storage and culturing conditions. The SCF and Flt-3L cytokines used in the current study are known to stimulate the growth and survival of primitive HSC (36,37), while the addition of TPO synergistically stimulates clonogenic growth (38). Interestingly, HS5 did not further encourage cytokine-induced $CD34^+$ or $CD34^+$ $CD38^-$ cell expansion but did affect the heterogeneity of the cell population by enriching the number of lineage committed cells. It is possible that HS5 acted on potentiating TPO to synergize with SCF and FLT-3L to influence the increase in clonogenic growth in the $CD34^+$ $CD38^-$ subset, while having no effect on the activity of SCF and FLT-3L in inducing the proliferation of primitive cells. It would be crucial to isolate HS5 interacting partners in the current culture condition and investigate the manner in which they potentiate colony formation.

Ex vivo expansion of CB HSCs generally increases the growth of committed cells at the expense of the more primitive $CD34^+$ $CD38^-$ cells, an HSC subset that is required for the reconstitution of long-term multilineage hematopoiesis. Hence, *ex vivo* expanded CB HSC has been coupled with unmanipulated CB for clinical transplantations (4,39–41). This co-transplantation method serves two purposes. First, it serves to increase the total number of nucleated cells (NC) transplanted. Second, it serves to balance the early engraftment that is anticipated from the more committed expanded CB populations, with a long-term immune reconstitution that can be derived from primitive cells found in the unmanipulated CB fraction. Heparan sulfate (HS5) acts favorably toward increasing the number of committed clonogenic cells in the expanded HSC population. Since colony-forming cell (CFC) content in CB HSCs is touted as a better indicator of survival rates in transplant patients compared to total NC (42), the inclusion of HS5 during HSC *ex vivo* expansion may potentially reduce mortality in CB transplant patients.

The results here have strengthened the importance of bone marrow stroma-derived HS within the hematopoietic niche in facilitating hematopoiesis and the feasibility of its use in HSC *ex vivo* expansion, especially in the expansion of committed progenitor cells. Since an optimal cytokine combination and concentration has not yet been established and 6-O sulfated HS has the ability to bind to various hematopoietic cytokines, it is necessary to further explore the optimal cytokine combination whose activity can be substantially enhanced by stroma-derived HS. Additionally, the role of HS in promoting the activity of hematopoietic cytokines needs to be clarified further in order to effectively introduce HS as a component of HSC *ex vivo* expansion culture condition. On the whole, the use of human bone marrow stromal cell-derived HS provides an appealing approach to CB HSC *ex vivo* expansion that may prove suitable for clinical application.

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