# The effects of glycosaminoglycans on thrombopoietin-induced megakaryocytopoiesis

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Background and Objectives. The extracellular matrix plays an essential role in normal hematopoiesis. Proteoglycans and glycosaminoglycans (GAG) are major components of the matrix. In this study, the effects of various GAG on the proliferation and differentiation of CD34+ megakaryocytic progenitor cells (CFU-Meg) were evaluated *in vitro*.

Design and Methods. CD34+ cells were highly purified from steady-state human peripheral blood. The GAG tested were hyaluronic acid (from humans, pigs and roosters), keratan sulfate, heparan sulfate, chondroitin sulfate (from whale, shark or squid cartilage) and dermatan sulfate (DS).

Results. When used alone, none of the GAG supported the clonal growth of CFU-Meg; however, in cultures stimulated by recombinant human thrombopoietin, human hyaluronic acid, whale chondroitin sulfate and DS significantly enhanced such growth. In particular, the addition of DS resulted in increases of about 1.3-fold, 1.6-fold and 2.0-fold in the numbers of total cells, megakaryocytes and CFU-Meg, respectively, compared with the control culture stimulated by thrombopoietin alone after 9-12 days of serum-free liquid culture. Furthermore, DS induced the generation of hyperploid megakaryocytes and promoted pro-platelet formation. Chemical fragmentation and desulfation of DS showed that a chain of at least 12 saccharides is required for colony-promoting activity and that the sulfate groups play an essential role.

Interpretation and Conclusions. DS acts on an immature population of CD34+ cells, stimulates the proliferation of CFU-Meg, and enhances the terminal maturation of megakaryocytes and thrombopoiesis. These results suggest that DS has a wide spectrum of action in promoting megakaryocytopoiesis and thrombopoiesis.

Key words: glycosaminoglycans, thrombopoietin, CFU-Meg, megakaryocytes, dermatan sulfate.

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Normal hematopoiesis is regulated by a complex network of soluble physiological regulatory factors, stromal cells and the extracellular matrix.[1-3] Stromal cells such as fibroblasts, endothelial cells, preadipocytes, macrophages and bone-lining mesenchymal cells produce and secrete various soluble factors and matrix molecules (fibronectin, laminin and collagen). Proteoglycans and extracellular matrix polysaccharides, termed glycosaminoglycans (GAG), are found on the surfaces of stromal cells and are major components of the extracellular matrix. They have been implicated in binding and directing the localization of hematopoietic cells and growth factors in the hematopoietic microenvironment, and their roles in hematopoiesis have been well established in previous studies.[4-7] Almost all GAG, including hyaluronic acid, chondroitin sulfate, dermatan sulfate, heparin and heparan sulfate, have been reported to stimulate murine megakaryocytopoiesis *in vitro* and *in vivo*.[8-11] Administration of low-molecular-weight heparin to mice increases both the blood platelet count and the numbers of single megakaryocytes and megakaryocytic progenitor cells (colony-forming unit megakaryocytes, CFU-Meg) in the bone marrow.[9] Furthermore, Tajika *et al.*[11] have reported that sulfated GAG (chondroitin sulfate, DS, dextran sulfate, heparin and heparin sulfate) promote pro-platelet formation by murine megakaryocytes and participate in thrombopoiesis in a manner different from that of cytokines such as interleukin-6 and interleukin-11. The ligand for the cytokine receptor c-mpl has been cloned and shown to encode a protein that promotes the proliferation of CFU-Meg *in vitro* and *in vivo*. This protein has been termed Mpl ligand, thrombopoietin, or megakaryocyte growth and development factor.[12-14] However, little is known about the effects of interactions between GAG and thrombopoietin on human megakaryocytopoiesis and thrombopoiesis. In this study, the effects of GAG from various sources, including hyaluronic acid, keratan sulfate, heparan sulfate, chondroitin sulfate and DS, on the proliferation and differentiation of CD34+ CFU-Meg prepared from steady-state human peripheral blood and stimulated by recombinant human thrombopoietin were examined *in vitro* in semisolid and liquid cultures.

## Design and Methods

### Reagents

Recombinant human thrombopoietin and human stem cell factor were purchased from Biosource (Tokyo, Japan) and used at concentrations of 50 ng/mL and 100 ng/mL, respectively. Hyaluronic acid, sodium salt (from human umbilical cord, 80-120×$10^4$ Da; from pig skin, 4-6×$10^4$ Da; from rooster comb, 60-120×$10^4$ Da, Artz®), keratan sulfate, sodium salt (from bovine cornea, 1.3×$10^4$ Da), heparan sulfate (from bovine kidney, 2-3×$10^4$ Da), chondroitin sulfate, sodium salt (chondroitin 4-sulfate in N-acetyl-D-galactosamine (GalNAc) from whale cartilage, 2.5-5×$10^4$ Da; chondroitin 6-sulfate in GalNAc from shark cartilage, 4-8×$10^4$ Da; chondroitin 6-sulfate in GalNAc and 2-sulfate in D-glucuronic acid from shark cartilage, 3×$10^4$Da; chondroitin 4- and 6-sulfate in GalNAc from squid cartilage, 2×$10^4$ Da) and dermatan sulfate (chondroitin 4- and 6-sulfate in GalNAc and 2-sulfate in L-iduronic acid from hog skin, 1.1-2.5×$10^4$ Da) were purchased from Seikagaku Kogyo (Tokyo, Japan). Each GAG was dissolved in $Ca2^+$ and $Mg2^+$-free phosphate-buffered saline (PBS) and the dose of each GAG in the culture was determined beforehand in a pilot study. The following fluorescence-labeled monoclonal antibodies were purchased from Beckman-Coulter-Immunotech (Marseille, France): fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 (FITC-CD34) FITC-conjugated anti-human CD41 (FITC-CD41) and FITC-conjugated anti-human CD42a (FITC-CD42). Mouse IgG1-FITC and mouse IgG2a-FITC (Beckman-Coulter-Immunotech) were used as isotype controls.

### Purification of CD34$^+$ cells

This study was approved by the Committee of Medical Ethics of Hirosaki University School of Medicine (Hirosaki, Japan). After obtaining informed consent from normal human blood donors, peripheral blood was collected at the Red Cross Blood Center (Hokkaido and Aomori). The buffy-coat was prepared from whole-blood by this center and was supplied to us. Low-density mononuclear peripheral blood cells were separated from the buffy coat by centrifugation for 30 min at 300×g on a cushion of Lymphosepar I (1.077 g/mL; IBL, Fujioka, Japan) and washed three times with PBS containing 5 mM EDTA. The CD34$^+$ cell content was enriched by magnetic cell sorting (Miltenyi Biotec, Germany), performed according to the manufacturer's instructions except that the cells were applied to three magnetic bead columns to achieve high CD34$^+$ cell purity. Finally, purified CD34$^+$ cells were obtained, being 0.1% of all cells present in the low density mononuclear cells. Expression of the CD34$^+$ phenotypes in those cells, measured using a fluorescence cell analyzer (EPICS-XL, Beckman-Coulter, Tokyo, Japan), was within the range 88 - 95%.

### Assay for CFU-Meg

CFU-Meg were assayed using a soft agar culture or a plasma clot culture technique, because some GAG destroy a matrix of plasma clot. The culture medium contained freshly prepared (day 0) or pre-cultured (day 3 to 15) CD34$^+$ cells, 10-15% human platelet-poor AB plasma and growth factor(s) in Iscove's modified Dulbecco's medium (IMDM; GIBCO BRL, Grand Island, USA) with additives of 100 U/mL penicillin (GIBCO BRL), 100 μg/mL streptomycin (GIBCO BRL), 1 mM sodium pyruvate (GIBCO BRL), 1% MEM vitamin (GIBCO BRL), 0.1 mM MEM non-essential amino acids (GIBCO BRL), 1×$10^5$ M thioglycerol (Sigma, St Louis, USA), 2 μg/mL L-asparagine (Wako Pure Chemicals, Tokyo, Japan), 74 μg/mL $CaCl_2$ (Wako Pure Chemicals), 0.2% bovine serum albumin (BSA; Sigma) and/or 0.2% agar (Difco, Detroit, USA). The medium (0.3 mL) was plated into the wells of 24-well culture plates (Falcon, Becton Dickinson Biosciences, Franklin Lakes, USA) and incubated at 37°C in a humidified atmosphere containing 5% $CO_2$ for 11-12 days.

### Liquid culture

Peripheral blood CD34$^+$ cells (5×$10^3$ cells/mL in serum-free medium plus thrombopoietin, with or without DS; total volume 0.5 mL/well) were placed into 24-well plates (Falcon) and cultured in serum-free IMDM supplemented with BIT9500 (StemCell Technologies, Vancouver, Canada), a serum substitute for serum-free cultures. The cultures were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. Every 3 days, the cells were harvested and the number of viable cells was determined by trypan blue exclusion. The total number of megakaryocytes was calculated from the total number of cells harvested from the culture and the proportion of CD42$^+$ among the harvested cells. The cells from these aliquots were assayed for the number of CFU-Meg using a plasma clot culture supplemented with thrombopoietin plus stem cell factor. The total number of CFU-Meg was calculated from the total number of cells harvested and the number of colonies per well.

### Immunofluorescence staining to identify megakaryocyte colonies

Each well was fixed twice for 15 min with a 2:1 mixture of acetone and methanol. The plates were dried in an airflow overnight and kept at -20°C until staining. For staining, the plates were removed from the freezer and returned to room temperature. PBS containing 0.5% bovine serum albumin (PBS-B) was then added to soften the agar. After discarding the solution, FITC-CD41 monoclonal antibody, diluted 1:100 in PBS-B, was added and the plates were incubated for 1 h at room temperature, then washed once with PBS-B. The nuclei were counter-stained with propidium iodine (0.3 ng/mL; Sigma). After a final wash, the colonies were counted with a fluorescence microscope (Olympus, Tokyo, Japan) at ×100 magnification.

As well as megakaryocyte colony scoring, megakaryocytes with various morphologies representing different stages in the process of thrombopoiesis were observed. At each successive stage, the megakaryocytes showed short cytoplasmic processes, long cytoplasmic extensions, pro-platelet development and dispersal of CD41-positive particles, respectively. Therefore, single megakaryocytes distant from colonies showing pro-platelet formation, or releasing platelet-like CD41-positive particles in the culture, were counted under the same conditions as those

described for colony scoring. The percentage of pro-platelet formation was calculated from the number of megakaryocytes showing FITC-CD41-positive particles and the total number of megakaryocytes, FITC-CD41-positive cells, counted.

### Determination of megakaryocyte DNA ploidy

Megakaryocyte DNA ploidy was determined using a modified version of a previously described protocol.[15] CD34+ cells (either freshly isolated or obtained from the liquid culture) were pelleted by centrifugation at 250×g for 5 min. The cell pellets were resuspended in PBS-B containing 5 mM EDTA and incubated for 20 min at room temperature with FITC-CD41 monoclonal antibody. The cells were washed with PBS, repelleted, resuspended in modified CATCH medium[16] containing 3.5% bovine serum albumin and 0.5% Tween 20 (Wako Pure Chemicals) and incubated at 4°C for 1 h. The cells in the suspension were fixed for 5 min by adding an equal volume of CATCH medium supplemented with 1% paraformaldehyde (Wako Pure Chemicals). After washing with PBS, the cells were resuspended in 50 μg/mL propidium blood dissolved in a solution containing 0.7% citric acid and 0.6% NaCl, and the mixture was incubated for 1 h at 4°C. A further incubation with RNase (50 μg/mL; Sigma) was performed for 30 min at room temperature before the cells were passed through a 35-μm nylon mesh. Freshly prepared peripheral blood CD34+ cells were used as the standard for analyzing 2N DNA content, and the mean channel of the 2N peak was determined by flow cytometry.

### Immunological marker analysis

Cell surface antigen expression was analyzed by direct immunofluorescence flow cytometry using single-staining with monoclonal antibody combinations including FITC-CD34 and FITC-CD42. Briefly, the cells were incubated for 20 min at room temperature with saturating concentrations of the relevant monoclonal antibodies, washed, and analyzed by flow cytometry. For each experiment, a negative control was provided by using isotype-matched control monoclonal antibodies.

### Activity of DS after chemical fragmentation or desulfation

DS was treated with hydrazine/hydrazine sulfate and nitrous acid using the method of Maimone and Tollefsen[17] with modifications. The resulting oligosaccharides were applied to a BioGel P-10 (-400 mesh, Bio Rad) gel filtration column (1.8 cm internal diameter×112 cm), previously equilibrated with aqueous 10% ethanol containing 1 M NaCl. Each fraction was collected, and the lengths of the saccharides were assessed by their absorbance at 210 nm. The oligosaccharide content of each fraction was determined as uronic acid using the carbazole-sulphuric acid reaction.[18] Each fraction was assayed for its colony-promoting activity at a dose of 1 nmol. Desulfation of DS was performed using a combination of the acetyl chloride and Kantor-Schubert methods.[19]

### Statistical analysis

The significance of differences between the control and experimental groups was determined by Student's t test.

## Results

### Effects of GAGs on the clonal growth of CD34+ megakaryocytic progenitor cells from human peripheral blood

The effects of various GAG on the clonal growth of CD34+ CFU-Meg from steady-state human peripheral blood were examined *in vitro*. For this purpose, the freshly prepared cells were grown in soft agar cultures containing human plasma with recombinant human thrombopoietin (Figure 1). The GAG were added to the cultures at a concentration ranging from 50-200 μg/mL. When used alone, none of the GAG supported the clonal growth of CFU-Meg (*data not shown*). However, when thrombopoietin was used as a growth factor, addition of 50 μg/mL human hyaluronic acid, whale chondroitin sulfate or DS increased CFU-Meg clonal growth significantly, producing approximately 1.3-1.5-fold increases in the total number of megakaryocyte colonies compared with the control. Our previous study showed that pig hyaluronic acid and heparan sulfate promoted the clonal growth of thrombopoietin-stimulated human placental/umbilical cord blood CD34+ CFU-Meg, whereas the same batches of these GAG showed no promoting effects on the clonal growth of CD34+ CFU-Meg from steady-state human peripheral blood (*data not shown*). Under these culture conditions, no colonies other than CFU-Meg-derived megakaryocytes were detected, irrespective of whether or not GAG were added. DS was especially effective at all the concentrations tested. In addition, the morphology of megakaryocytes changed during the process of thrombopoiesis. Megakaryocytes at these stages showed cytoplasmic processes, pro-platelet development and dispersal of CD41-positive particles, respectively, and finally the nucleus became inconspicuous. The formation of numerous megakaryocyte-derived pro-platelets and/or gpIIbIIIa-positive platelet-like particles was estimated quantitatively (Figure 2). In the control culture, 5% of the total counted single megakaryocytes were observed to show pro-platelet formation, releasing platelet-like CD41-positive particles. Compared with the control, DS promoted an 8-fold higher level of pro-platelet formation (40%). However, human hyaluronic acid and whale condroitin sulfate showed no promoting effect on pro-platelet formation, and no greater effect was observed with multiple combinations. In order to investigate the megakaryocytopoietic and thrombopoietic properties of DS in more detail, further experiments were performed as described below.

### Action of DS in serum-free liquid culture

The megakaryocytopoietic and thrombopoietic properties of DS were tested in serum-free liquid cultures supplemented with thrombopoietin alone until day 15. Every 3 days, cells were harvested and the number of viable cells was counted (Figure 3). In the control culture containing thrombopoietin alone, the total cell count increased with the duration of culture until day 12, rising by approximately $1.3\times10^5$ to $1.8\times10^5$ cells (3.2- to 4.6-fold increase) from the initial input ($4\times10^4$ cells) by day 9 to 12. As a result of adding DS, approximately $1.9\times10^5$ to $2.4\times10^5$ cells (4.8- to

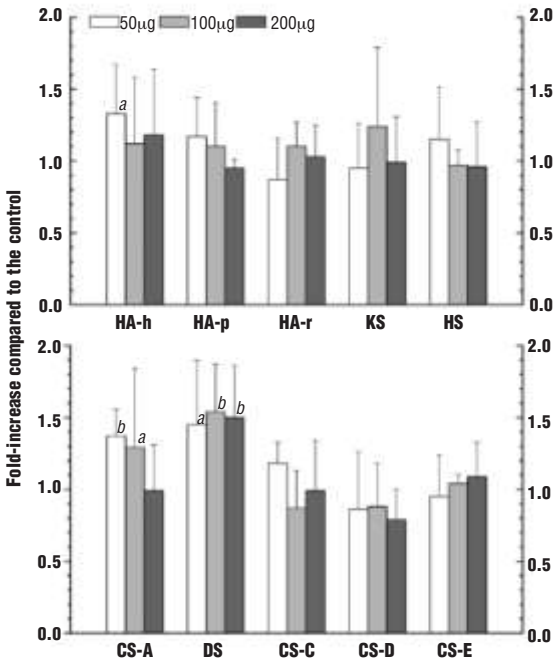


Figure 1. Effects of glycosaminoglycans (GAG) on the *in vitro* clonal growth of CD34⁺ megakaryocytic progenitor cells (CFU-Meg) from steady-state human peripheral blood. Highly purified CD34⁺ cells ($1\times10^3$ cells/mL) were incubated in soft agar culture with thrombopoietin (50 ng/mL) and various GAG at concentrations ranging from 50-200 μg/mL, for 11-12 days. Values represent the mean±S.D. of triplicate cultures in three separate experiments. HA-h, HA-p, HA-r: hyaluronic acid from human umblical cord, pig skin and rooster comb, respectively; KS: keratan sulfate; HS: heparan sulfate; DS: dermatan sulfate; CS: chondroitin sulfate (from A, whale; C and D, shark; E, squid cartilage). [a]$p<0.05$, [b]$p<0.01$ compared with thrombopoietin alone by Student's t test.

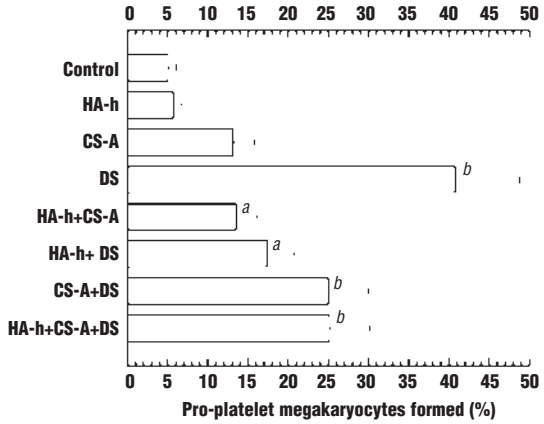


Figure 2. The percentage of *in vitro* pro-platelet megakaryocytes formed in GAG-containing cultures. Freshly purified peripheral blood CD34⁺ cells ($1\times10^3$ cells/mL) were cultured in soft agar culture containing 10% human platelet-poor AB plasma with thrombopoietin (50 ng/mL) plus combinations of human hyaluronic acid (HA-h; 50 μg), whale chondroitin sulfate (CS-A; 100 μg) and dermatan sulfate (DS; 100 μg) for 11-12 days. Values represent the mean±SD of six wells from two separate experiments. [a]$p<0.05$, [b]$p<0.01$ compared with thrombopoietin alone by Student's t test.

5.9-fold increase) were harvested, and by day 9 the difference between the thrombopoietin alone control culture and the thrombopoietin plus DS culture was significant. Phenotypic analysis of generated cells for the expression of CD34 and CD42 was performed using flow cytometry (Figure 4). The positivity rates were about 96.7% and 0.6%, respectively, of the initially input cells on day 0. After culture, the proportion of the CD34⁺ cells was found to decrease from 94.7% (day 3) to 6.3% (day 15) in the control culture, whereas the cells generated in the thrombopoietin plus DS culture decreased from 97.1% to 5.2% (*data not shown*). The proportion of CD42⁺ cells generated increased to 70.2% on day 12, indicating that megakaryocytes were becoming the major component of the generated cells by that time. There were no significant differences in the expression of these antigens between the two cultures, and DS did not affect their expression either. The relationship between the generation of megakaryocytes and culture period is shown in Figure 5. After liquid culture, the total number of megakaryocytes was calculated from the total number of cells harvested from the culture and the proportion of CD42⁺ cells among the harvested cells. In the control culture, the total number of megakaryocytes increased with the duration of culture until day 12, when approximately $1.4\times10^5$ cells (a 600-fold increase) were observed. Almost the same growth curve was obtained, reaching approximately $1.7\times10^5$ cells (a 700-fold increase), in the culture with thrombopoietin plus DS. At the same time, the total number of CFU-Meg was assessed using a plasma clot culture (Figure 6). In the control culture, the maximum increase in the total number of CFU-Meg was observed by day 9, when there were approximately $1.4\times10^3$ cells (a 4.6-fold increase). In the DS-culture, approximately $3.0\times10^3$ cells (a 10.1-fold increase) were harvested, this being a 2.2-fold increase compared with the control culture.To determine the effects of DS on the maturation of megakaryocytes, the distribution of DNA ploidy of these cells was analyzed on day 9 of culture (Table 1). The proportions of 2N megakaryocytes in the thrombopoietin-alone control culture and the thrombopoietin plus DS culture were 65.7% and 54.5%, respectively, this being a statistically significant difference between the two cultures. Hyperploid megakaryocytes (more than 8N ploidy) constituted 9.7% of the total megakaryocyte count in the control culture. While addition of DS increased the proportion of hyperploid megakaryocytes to 16.8% ($p<0.05$). Before preparing the samples for estimation of DNA ploidy, many large cells that appeared to be mature megakaryocytes and pro-platelet megakaryocytes were observed in the culture by microscopy. The presence of such cells was more evident in cultures with DS. However, the differences shown in Table 1 were less obvious than the findings obtained by microscopy during culture. One possible reason is that mature large megakaryocytes were damaged during the preparation for the estimation of DNA ploidy, perhaps due to pipeting stress. Taken together, these results indicate that DS promotes the generation of CFU-Meg from an immature population of CD34⁺ cells, stimulates the proliferation and differentiation of CFU-Meg, and enhances the terminal maturation of megakaryocytes and thrombopoiesis.

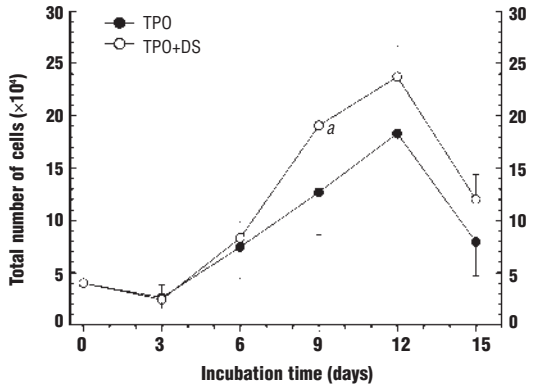


Figure 3. Relationship between incubation period and total number of cells generated in liquid culture. Highly purified CD34⁺ cells ($4\times10^4$ cells) were cultured in serum-free liquid medium supplemented with thrombopoietin (TPO; 50 ng/mL) alone or TPO plus dermatan sulfate (DS; 100 μg/mL). Every 3 days until day 15, the cells were harvested and the number of viable cells was determined by trypan blue exclusion. Values are the mean±SD of triplicate cultures in two to six separate experiments. [a]$p<0.05$ compared with thrombopoietin alone by Student's *t* test.

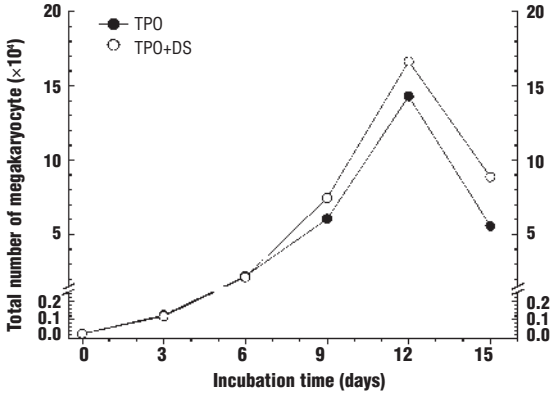


Figure 5. Relationship between incubation period and total number of megakaryocytes generated in liquid culture. The total number of megakaryocytes was calculated from the total number of cells harvested from the culture and the proportion of CD42⁺ among the harvested cells. The number of megakaryocytes in the initial input was about 30. Values are means for two separate experiments.

### *Effects of DS after chemical fragmentation or desulfation*

In order to determine the relationship between the structural characteristics of DS and its ability to promote clonal growth of CFU-Meg, chemical treatments were performed to fragment or desulfate DS. After fragmentation with hydrazine/nitrous acid, the resulting oligosaccharides were assayed for their colony-promoting activity (Figure 7). The minimum structural requirement for activity appeared to be a polysaccharide with a chain of at least 12 saccharides. When the sulfate groups, which are a structural feature of DS, were removed, the colony-promoting activities of DS disappeared completely.

## Discussion

Our previous study demonstrated that pig hyaluronic acid and heparan sulfate significantly promoted the clonal growth of thrombopoietin-stimulated cord blood CD34⁺ CFU-Meg.[20] In the present study of thrombopoietin-stimulated peripheral blood CD34⁺ CFU-Meg, neither of these compounds showed any clonal growth-promoting effects, whereas human hyaluronic acid, whale chondroitin sulfate and DS did so (Figure 1) in spite of the fact that both experiments were performed using of the same batches of GAG. It is difficult to explain these different responses in terms of the structures and characteristics of these five GAG. One possible reason is differences in the target cells. There are considerable differences in adhesion molecule expression and proliferation ability among the CD34⁺ cells contained in cord blood, bone marrow, granulocyte colony-stimulating factor-mobilized peripheral blood and steady-state peripheral blood.[21–23] In particular, in a healthy individual, the hematopoietic stem/progenitor cells circulating in the peripheral blood are more mature than their counterparts in the bone marrow and cord blood.[24,25] Although more detailed studies of the mechanisms involved in the relationship between GAG and target cells are required, differences between target cells may play a key role in the observed actions of GAG. In liquid culture, thrombopoietin alone stimulated an approximately 4.6-fold increase in CFU-Meg (Figure 5). Although no significant differences in the expression of these antigens were observed between cells cultured with or without DS, addition of DS to this culture resulted in an approximately

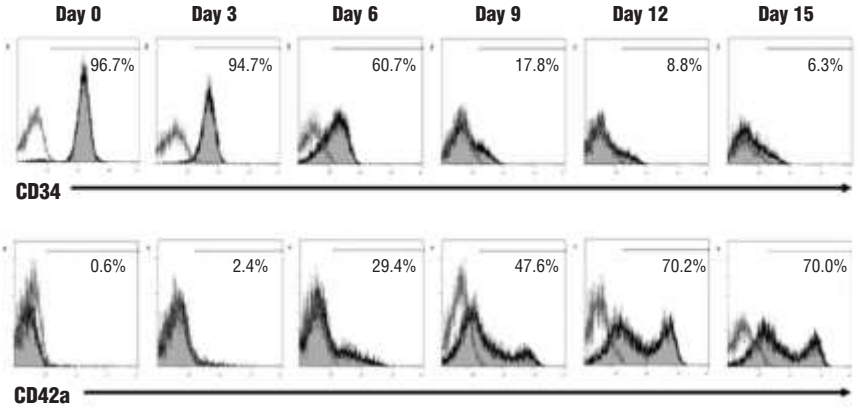


Figure 4. Flow cytograms of freshly prepared peripheral blood CD34⁺ cells and cells generated during liquid culture. The cells were treated with fluorescence-labeled anti-human monoclonal antibodies (FITC-CD34 and FITC-CD42). Representative cytograms are shown.

**Table 1. DNA ploidy distributions of megakaryocytes produced in culture.**

| Treatments | DNA ploidy (%) 2N | 4N | 8N | 16N | 32N |
|---|---|---|---|---|---|
| Initial input cells | 92.6±8.9 | 7.4±1.4 | 0 | 0 | 0 |
| Control (thrombopoietin alone) | 65.7±5.8 | 24.6±1.6 | 6.9±3.6 | 2.4±1.1 | 0.4±0.3 |
| Thrombopoietin + dermatan sulfate | 54.5±5.9[a] | 28.7±3.1 | 11.8±1.7 | 4.5±1.5 | 0.5±0.1 |

*Cells harvested from day 9 of culture were treated with FITC-CD41 monoclonal antibody and propidium iodide, and then each sample was analyzed for DNA ploidy distribution by flow cytometry. Values are means ± SD from four separate experiments. [a]p<0.05 compared with the thrombopoietin alone control by Student's t test.*

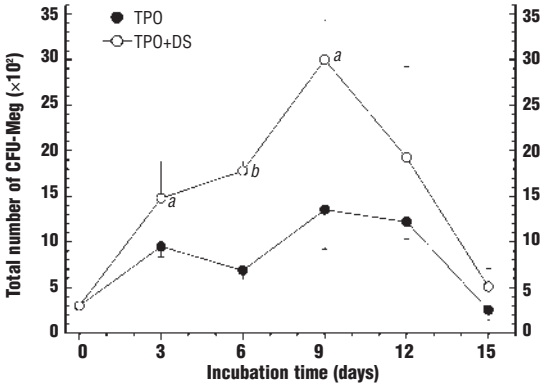


**Figure 6. Relationship between the incubation period and total number of CFU-Meg generated in liquid culture. The total number of CFU-Meg was calculated from the total number of cells harvested and the number of colonies per culture. Each aliquot of harvested cells was assayed for CFU-Meg. The number of CFU-Meg in the initial input was 300±84. Values are means±SD of triplicate cultures in two to six separate experiments. [a]p<0.05, [b]p<0.01 compared with thrombopoietin alone by Student's t test.**

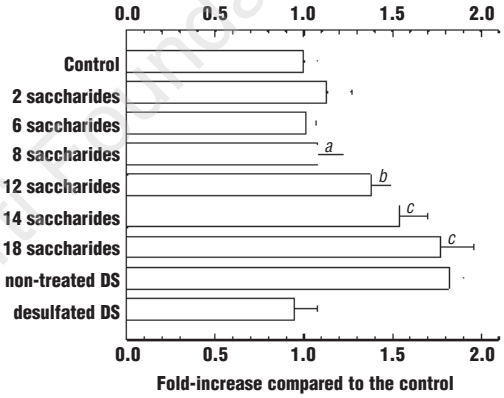


**Figure 7. Effects of DS after chemical fragmentation and desulfation on the *in vitro* clonal growth of CFU-Meg. Oligosaccharides (1 nmol) prepared from DS by the hydrazine/ hydrazine sulfate and nitrous acid method were assayed for their colony-promoting activity in a semi-solid culture system stimulated with thrombopoietin. Non-treated dermatan sulfate (DS; 50 μg) and desulfated DS were also tested. Values are means ± SD of triplicate cultures in three separate experiments. [a]p<0.05, [b]p<0.01 and [c]p<0.001 compared with thrombopoietin alone by Student's t test.**

10.1-fold increase in the number of CFU-Meg compared to the control cultures containing thrombopoietin alone. Furthermore, DS induced the generation of hyperploid megakaryocytes (Table 1) and promoted pro-platelet formation (Figure 2), suggesting that the action of DS extends from immature CFU-Meg to the terminal stages of differentiation of megakaryocytopoiesis and thrombopoiesis. A previous study demonstrated that various hematopoietic growth factors, cytokines and chemokines are secreted by $CD34^+$ cells and other hematopoietic progenitor-derived cells in an autocrine and/or paracrine manner.[26] The results of our flow cytometric analysis showed that the number of mature megakaryocytes increased dramatically under the influence of thrombopoietin and DS (Figures 4 and 5), implying that cytokines are produced to some extent by the various cells generated in this liquid culture system. We are now investigating whether DS acts directly or indirectly. In a preliminary quantitative determination of interleukin-6, interleukin-3 and granulocyte-monocyte colony-stimulating factor concentrations in harvested culture media (*data not shown*), there were no significant differences in the concentrations of these factors in conditioned media harvested from cultures grown with or without DS. Although we cannot completely exclude the possibility that DS acts indirectly on $CD34^+$ cells, these results suggest that it may have a direct promotional effect on megakaryocytopoiesis and thrombopoiesis *in vitro*. DS is a sulfated linear polysaccharide that consists of repeated units of GalNAc and uronic acids, mainly L-iduronic acid, but also some D-glucuronic acid.[27] Although in the present study chemical treatment failed to identify clearly the active fragment(s) of DS, it became apparent that the presence of a polysaccharide chain containing at least 12 saccharides was required for CFU-Meg-promoting activity (Figure 6). In addition, when the sulfates which are a structural feature of DS were removed, the colony-promoting activity was abolished (Figure 7). These results suggest that the sulfates contained in DS play an essential role in thrombopoietin-stimulated megakaryocytopoiesis. In the severe thrombocytopenia observed in patients who have undergone human bone marrow or placental and umbilical cord blood transplantation, and following moderately dose-intensive chemotherapy in patients with advanced cancer, concentrated platelet transfusion is the only therapy effective for reducing the duration of thrombocytopenia; however, frequent platelet transfusions often cause a refractory state if platelet concentrates that are HLA-incompatible with the recipient are used. The well-known mpl ligand thrombopoietin, has been shown to be the central physiological regulator of megakaryocytopoiesis and platelet production, and clinical trials of its effectiveness in thrombocytopenia have now been carried out in many countries.[28,29] On the other hand, the effect of thrombopoietin on chemotherapy-induced thrombocytopenia was less than expected on the basis of previous experiments performed *in vitro* and in animal models, since numerous preclinical studies have shown that thrombopoietin is highly effective in improving thrombocytopenia.[29] It is therefore possible that thrombopoietin alone is

insufficient to improve the thrombocytopenia that occurs after various forms of oxidative stress, such as irradiation and chemotherapy. Additional factors might be required to alleviate these problems. In the present study, GAG were evaluated in a culture containing thrombopoietin alone because our intention was to evaluate its activities in a simple system. Further studies will be necessary to evaluate the potential role of GAG under other conditions such as multiple cytokine combinations, given that various kinds of cyokines are present in normal peripheral blood.[30-33]

*IK, YT, TAT, KT: conceived the study.*

*The study was performed by Ikuo Kashiwakura's group, who also performed all the pilot experiments and most of the analyses. Tomoe Teramachi, Ikuko Kakizaki and Ikuo Kashiwakura performed the bench work and obtained all data for this study. Ikuo Kashiwakura and Keiichi Takagaki wrote the manuscript with contributions from the other authors. The authors declare that they have no potential conflict of interest.*

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