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Application of proteoglycan extracted from the nasal cartilage of salmon heads for *ex vivo* expansion of hematopoietic progenitor cells derived from human umbilical cord blood

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Abstract Highly purified proteoglycan (PG) extracted from the nasal cartilage of salmon heads was applied to the *ex vivo* expansion of hematopoietic progenitor cells prepared from human umbilical cord blood in serum-free cultures supplemented with the combination of early-acting cytokines, thrombopoietin (TPO), interleukin-3 (IL-3) and stem cell factor (SCF). PG showed no promoting effects on the cell proliferation rate; however, they promoted the generation of progenitor cells for granulocyte-macrophages, erythrocytes and/or megakaryocytes in culture with TPO alone or SCF plus TPO. However, no promoting effect was observed in a combination of IL-3 plus SCF, which showed the highest cell proliferation rate. PG failed to promote the generation of mixed colony-forming units (*i.e.* the relatively immature cells in hematopoiesis). These results suggest that PG acts on the relatively mature stem/progenitor cells, and may function as a regulatory factor in the differentiation pathway of hematopoiesis.

Keywords Proteoglycan · *Ex vivo* expansion · $CD34^+$ cells · Hematopoietic progenitor cells

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Introduction

The hematopoietic system is supported by the capacity of a relatively small population of hematopoietic stem cells for self-renewal and multipotency [1, 2]. Normal hematopoiesis is regulated by a complex network of hematopoietic stem cells, soluble physiological regulatory factors, stromal cells and extracellular matrices (ECM) [3–5]. Proteoglycan (PG) contains glycosaminoglycan (GAG) side chains that are attached to the polypeptide protein chain that forms the core of the molecule [4]. PG has been implicated in binding and directing the locations of both hematopoietic cells and growth factors in the hematopoietic microenvironment. Their roles in hematopoiesis have been well established in previous studies [6–9]. Although the importance of cellular interactions between hematopoietic stem/progenitor cells and marrow stromal cells is well established, the precise definition of the nature of many of these interactions at the molecular level is lacking and it still remains as objectives of fundamental importance to understanding hematopoietic regulation [10]. ECM molecules of all three categories (collagens, proteoglycans and glycoproteins) have been identified as part of this microenvironment, suggesting that these matrix molecules, in the combination with cytokines, are crucial for the compartmentalization of bone marrow [11]. Furthermore, recent studies have shown that PG/GAG is not only a component of ECM, but it also plays an extremely important role in hematopoietic regulatory system. Especially, since the stromal cell layers such as mesenchymal cells have been applied for *ex vivo* expansion of hematopoietic stem/progenitor cells, stromal sulfated glycoconjugates, PG/GAG, are thus expected to be potentially useful for the maintenance or expansion of hematopoietic stem/progenitor cells. To date, it has been very difficult

to apply PG as a physiological regulatory factor for an *in vitro* model because of its expense. Recently, we have succeeded in extracting PG from the nasal cartilage of salmon heads in an efficient and economical manner [12]. In this study, the capability of this PG for *ex vivo* expansion of hematopoietic progenitor cells from human umbilical cord blood (CB) was examined under serum-free culture conditions supplemented with early-acting cytokine combinations.

Materials and methods

Reagents

Recombinant human thrombopoietin (TPO), human interleukin-3 (IL-3), human stem cell factor (SCF) and human granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from Biosource (Tokyo, Japan). Recombinant human granulocyte colony-stimulating factor (G-CSF) and erythropoietin (Epo) were purchased from Sankyo Co. Ltd. (Tokyo, Japan). The amounts of these factors added per ml of medium are as follows: TPO, 50 ng; IL-3, 50 ng; SCF, 100 ng; GM-CSF, 10 ng; G-CSF, 10 ng; and Epo, 4 U. The following fluorescence-labeled monoclonal antibodies (MoAbs) were purchased from Beckman-Coulter-Immunotech (Marseille, France): fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 (FITC-CD34), phycoerythrin (PE)-conjugated anti-human CD41 (PE-CD41) and phycoerythrin-cyanin 5.1 (PC5)-conjugated anti-human CD45 (PC5-CD45). Mouse IgG1-FITC, mouse IgG1-PE and mouse IgG1-PC5 (Beckman-Coulter- Immunotech) were used as isotype controls.

Extraction of PG and its characteristics

PG is purified from the nasal cartilage of salmon heads according to the previous report [12]. In brief, PG was prepared from salmon nasal cartilage by extraction with 4% acetic acid and by precipitation with ethanol and dialysis against water. The molecular mass of the material was estimated to be 344 kDa, based on the results of a SDS/PAGE. The protein content of PG was approximately 7% and was consistent with that of PG from bovine nasal cartilage [13] and Swarrn rat chondrosarcoma [14]. The major amino acids in PG were aspartic acid, serine, glutamic acid, glycine and alanine, which together accounted for approximately half of the total amino acids present. The GAGs of PG also contained hexosamine, hexuronic acid and sulfate in a ratio of 1.00:0.99:0.67. Most of the GAGs from the nasal cartilage PG were of the monosulfated disaccharide structure (Table 1). PG was dissolved in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) at a concentration 0.05, 0.5 and 5 mg per ml, respectively.

Collection of CB and $CD34^+$ cell purification

This study was approved by the Committee of Medical Ethics of Hirosaki University School of Medicine (Hirosaki, Japan). After obtaining informed consent from the mothers, CB was collected at the end of full-term deliveries using a sterile collection bag containing the anticoagulant citrate-phosphate dextrose according to the guidelines of the Tokyo Cord Blood Bank. Low-density mononuclear CB cells were separated by centrifugation on Ficoll-Paque (1.077 g/ml, Amersham Pharmacia Biotech AB, Uppsala, Sweden) for 30 min at 300×g and washed three times with PBS containing 5 mM EDTA (EDTA-PBS) (Wako Pure Chemicals, Tokyo, Japan). They were then processed for $CD34^+$ cell enrichment according to the manufacturer's instructions. Magnetic cell sorting (Miltenyi Biotec, Germany) was used for the positive selection of $CD34^+$ cells. Finally, purified $CD34^+$ cells were achieved from 0.13–0.66% of all cells present in the light density mononuclear cells. The expression of the $CD34^+$ phenotypes in those cells, measured using a fluorescence cell analyzer (EPICS-XL, Beckman-Coulter, Tokyo, Japan), was within the range of 90–95%.

Ex vivo expansion

The $CD34^+$ cells ($1–2\times10^3$ cells/ml in medium with or without PG; total volume 0.5 ml/well) were placed into 24-well plates (Falcon) and cultured in serum-free Iscove's modified Dulbecco's medium (IMDM, Gibco BRL, Grand Island, USA) supplemented with BIT9500 (StemCell Technologies Inc, Vancouver, Canada), a serum substitute for serum-free culture. The cytokines were used in various combinations including TPO, IL-3 and SCF. The dose of PG in *ex vivo* expansion cultures was determined by the results obtained from the dose-response curve of an *in vitro* CB $CD34^+$ megakaryocyte colony-forming units (CFU-Meg) proliferation assay. PG-containing PBS, at concentrations of 0.05, 0.5 and 5 mg per ml, was added to the culture at 1% (v/v). Final concentrations of PG in the cultures were 0.5, 5 and 50 μg per ml, respectively.

The cultures were incubated at 37°C in the humidified atmosphere containing 5% CO_2. After 14 days of culture, the cells were harvested and the number of viable cells was

Table 1 GAG composition of PG extracted from the nasal cartilage of salmon heads

Molecular kind of GAG	Contents (%)
6-sulfated unsaturated disaccharide units	58.2
4-sulfated unsaturated disaccharide units	26.1
Non-sulfated unsaturated disaccharide units	8.7
Di-sulfated unsaturated disaccharide units	7.0

Each value was analyzed by chondroitinase ABC digestion and HPLC.

determined by trypan blue dye exclusion with a hemocytometer. The cells from these aliquots were assayed for the number of hematopoietic progenitor cells. Erythroid burst-forming units (BFU-E), granulocyte-macrophage colony-forming units (CFU-GM) and mixed colony-forming units (CFU-Mix) were assayed using a methylcellulose culture technique. The number of CFU-Meg was measured using a plasma clot culture technique. The total number of each type of progenitor cells was calculated from the total number of cells harvested and the number of each type of colony per well, respectively.

Methylcellulose cultures

The colony-forming cells including BFU-E, CFU-GM and CFU-Mix were assayed by methylcellulose culturing using MethoCult® (StemCell Technologies Inc.). Freshly prepared $CD34^+$ cells (day 0) or expanded cells (day 14) were plated into each well of 24-well culture plates (0.3 ml per well) in the culture medium containing SCF, IL-3, G-CSF, GM-CSF and Epo as colony-stimulating factors. Each dish was incubated at 37°C in the humidified atmosphere of 5% CO_2 for 14 days. The colonies consisting of more than 50 cells were counted with an inverted microscope.

Plasma clot cultures

The CFU-Meg was assayed by the plasma clot technique using human plasma. The culture medium contained freshly prepared $CD34^+$ cells (day 0) or expanded cells (day 14) plus 10–15% human platelet-poor AB plasma and TPO plus SCF in IMDM with the following additives: 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), 74 μg/ml $CaCl_2$ (Wako Pure Chemicals) and 0.2% bovine serum albumin (BSA, Boehringer Mannheim GmbH, Germany).The medium was plated into each well of 24-well culture plates (0.3 ml per well) and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 11–12 days.

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 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% BSA (PBS-B) was then added to soften the agar. After discarding the solution, FITC-CD41 MoAb 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 (PI, 0.3 ng/ml; Sigma). After a final wash, the colonies were counted with a fluorescence microscope (Olympus, Tokyo, Japan) at ×100 magnification.

Immunological marker analysis

The expression of cell surface antigens was analyzed by direct immunofluorescence flow cytometry, using triple-staining combinations of MoAbs including FITC-CD34, PE-CD41 and PC5-CD45. Briefly, the cells were incubated with the relevant saturated-concentration MoAbs for 20 min at room temperature, then washed and analyzed by flow cytometry. A negative control was provided by using an isotype-matched control MoAbs for every experiment.

Statistical analysis

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

Results

Effects of cytokine combinations plus PG on the expansion of total mononuclear cells

To avoid the problems arising from the inclusion of animal serum, BIT9500-IMDM was used as a serum-free medium for *ex vivo* expansions in this study. Various combinations of the early-acting cytokines TPO, SCF and IL-3 were used as hematopoietic growth factors to expand the hematopoietic stem/progenitor cells on the basis of our previous study [15]. The isolated $CD34^+$ cells were incubated in the presence of TPO alone, TPO plus SCF and IL-3 plus SCF, and were combined with PG (0.5, 5 and 50 μg/ml). After 14 days of culture, the cells were harvested and the number of viable cells was counted (Fig. 1).Whether PG had been added or not, TPO alone stimulated an approximate 80-fold increase in the total number of cells in comparison with the initial input. The other two combinations induced a 150- to 250-fold increase in the total number of cells. However, PG did not affect the total number of cells in these cultures. The expressions of $CD34^+CD45^+$ (the relatively immature cells which have a leucocyte common antigen) and $CD41^+CD45^+$ cells (the megakaryocytes which have a leucocyte common antigen) among those harvested from the culture were analyzed using a flow cytometer, respectively (Fig. 2). The expressions of the cell-surface antigens in the freshly prepared $CD34^+$ cells before culturing were 94.1 and 1.2%, respectively. The proportions of the $CD34^+CD45^+$ cell type after culturing with TPO alone, SCF plus TPO and

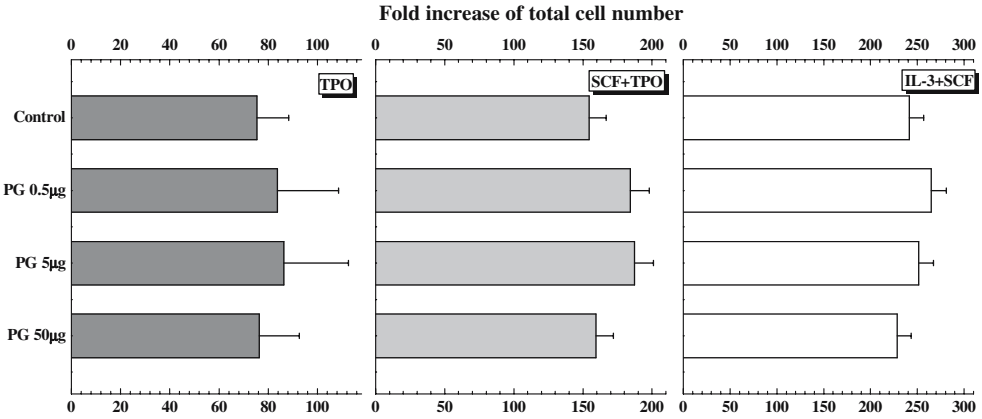

Fig. 1 Total number of mononuclear cells in liquid culture. Freshly prepared CB $CD34^+$ cells were cultured in a serum-free liquid medium stimulated with TPO alone, SCF plus TPO, IL-3 plus SCF, combined with PG at concentrations of 0.5, 5 and 50 μg/ml. On day 14, cells were harvested from each culture and the viable cells were counted using trypan blue. Values are the means ± S.D. of three separate experiments in triplicate cultures

IL-3 plus SCF were 0.52, 3.73 and 3.83%, respectively, thus showing a decrease in the proportion of $CD34^+$ cells (*i.e.* the relatively immature cells in hematopoiesis). In contrast, $CD41^+CD45^+$ cells were increased to 61.4, 5.3 and 17.3%, respectively. The megakaryocytes increased in the generated cells. However, no significant differences in the expression of these antigens were observed in the cells harvested from the cultures supplemented with, or without PG (data not shown).

Effects of various combinations of cytokines with PG on the expansion of hematopoietic progenitor cells

After *ex vivo* expansion, the total number of CFU-GM, BFU-E and CFU-Mix was assayed by a methylcellulose culture, respectively. In the culture containing all cytokine combinations, the total CFU-GM count increased with the duration of culture until day 14, rising by an approximately a 4- to 90-fold increase from the initial input (Fig. 3a). When 5 μg of PG was added to the culture supplemented with TPO alone and SCF plus TPO, the significant increase was observed in the total CFU-GM count in comparison to the control. In the case of BFU-E, the combinations without PG amplified the number of BFU-E by an approximate 10- to 150-fold increase from the initial input (Fig. 3b). Adding PG (0.5 μg) to the culture with SCF plus TPO resulted in the significant increase in the total number of BFU-E. In contrast, each dose of PG (5, 50 μg) showed the suppressive effects on the *ex vivo* expansion of CFU-Mix in the culture with SCF plus TPO and IL-3 plus SCF (Fig. 3c), respectively. At the same time, the total number of CFU-Meg was also assessed using a plasma clot culture (Fig. 4). The cytokine combinations without PG amplified the number of CFU-Meg by a 12- to 26-fold increase from the initial input. When 5 μg of PG was added to the culture with TPO alone, a significant difference was observed in

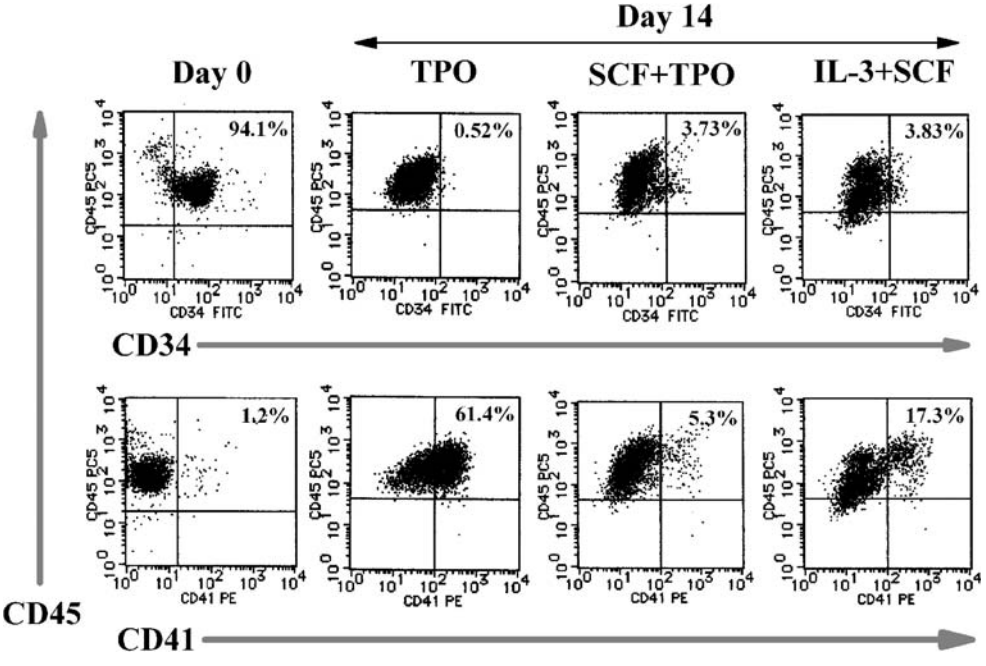

Fig. 2 Flow cytograms of cells harvested from cultures stimulated by a combination of TPO alone, SCF plus TPO and IL-3 plus SCF on day 14 of the culture. The cells were treated with fluorescence-conjugated anti-human CD34, CD41 and CD45 monoclonal antibodies. The expression of each surface antigen was analyzed using a flow cytometer

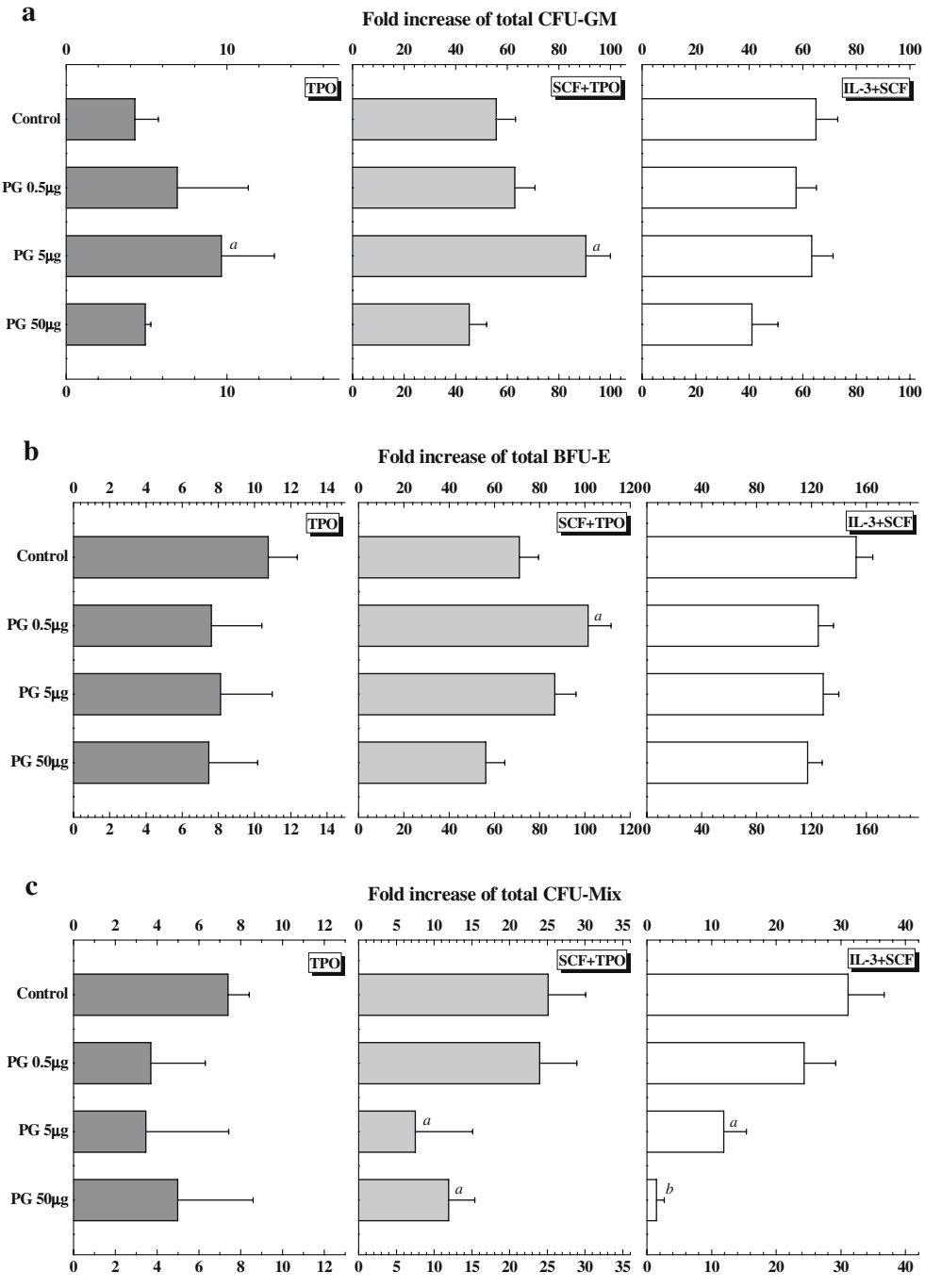

Fig. 3 Proportional increase in the total number of CFU-GM generated in the liquid culture (**a**). Proportional increase in the total number of BFU-E generated in the liquid culture (**b**). Proportional increase in the total number of CFU-Mix generated in the liquid culture (**c**). Freshly prepared CB $CD34^+$ cells were cultured in a serum-free liquid culture stimulated with the cytokine combinations listed in the legend of Fig. 1, combined with PG at concentrations of 0.5, 5 and 50 μg/ml. On day 14, cells harvested from each culture were plated into a methylcellulose culture supplemented with SCF, IL-3, G-CSF, GM-CSF and Epo. The culture was incubated for 14 days. Values are the means ± S.D. of three separate experiments in triplicate cultures. $^aP<0.05$, $^bP<0.001$

the increase of the total CFU-Meg. In contrast, the interaction of PG with a combination of IL-3 plus SCF resulted in the significant decrease in the development of CFU-Meg.

Discussion

In the present study, PG prepared from the nasal cartilage of salmon heads has shown no promoting effects on the cell proliferation rate in the presence of cytokine combinations (Fig. 1). The number of $CD34^+$ cells dramatically decreased in all cultures (Fig. 2). However, PG has promoted the generation of CFU-GM, BFU-E and CFU-Meg in the culture with TPO alone or a combination of SCF plus TPO, respectively (Figs. 3a, b and 4). PG failed to promote CFU-Mix (*i.e.* the relatively immature cells in hematopoiesis) production in all of the combinations (Fig. 3c). Each dose of PG (5, 50 μg) has shown suppressive effects on the *ex vivo* expansion of CFU-Mix in a combination of SCF plus TPO and IL-3 plus SCF, respectively, and it has suggested that PG may act as a suppressive or regulatory factor

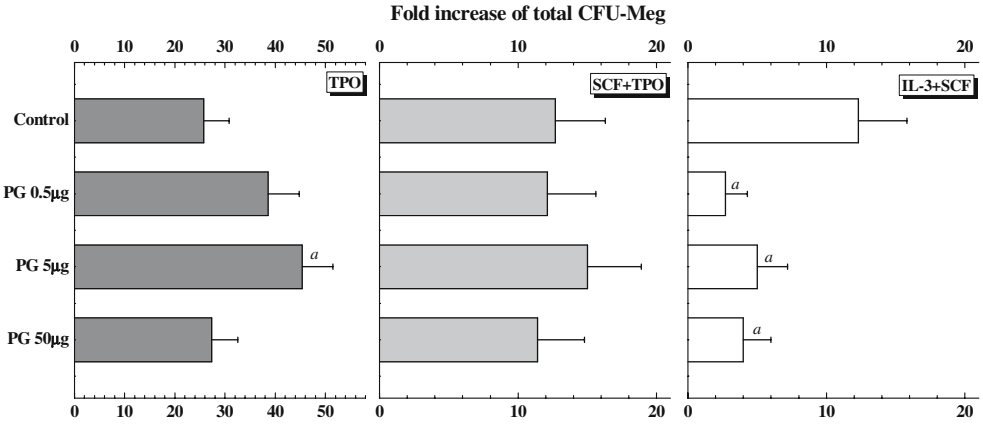

Fig. 4 Proportional increase in the total number of CFU-Meg generated in the liquid culture. Freshly prepared CB $CD34^+$ cells were cultured in a serum-free liquid culture stimulated with the cytokine combinations listed in the legend of Fig. 4. On day14, cells harvested from each culture were plated into a plasma clot culture supplemented with TPO plus SCF. The culture was incubated for 11 days. Values are the means ± S.D. of three separate experiments in triplicate cultures. $^aP<0.05$

depending on the cytokines. The results described above also suggest that PG may act on the relatively mature stem/progenitor cells through the hematopoietic differential pathway from stem cells to mature cells.

Stem cells localize in "stem cell niches" in the hematopoietic microenviroment, where they interact with stromal cells, specific ECM macromolecules and cytokines. Gupta *et al.* have demonstrated a role for marrow stroma-derived heparan sulfate (HS) GAG in the long-term *in vitro* maintenance of primitive human long-term culture-initiating cells (LTC-IC) [16]. They also suggested that HS are responsible for the juxtaposition of target cells, hematopoietic progenitors, with microenvironmental cells, cytokines, and ECM macromolecules [17]. Recently, highly *O*-sulfated stromal cell-derived HS GAGs have also been shown to help mediate the formation of such "stem cell niches" by colocalizing specific heparin-binding proteins with hematopoietic progenitor cells, thereby orchestrating the controlled growth and differentiation of stem cells [18]. In their studies, the supportive HS specifically contains higher 6-*O*-sulfation on the glucosamine residues and its molecular weight is significantly larger. In agreement with these observations, both purified 6-*O*-sulfated heparin and highly 6-*O*-sulfated bovine kidney HS similarly could maintain LTC-IC. In contrast, no supportive effect was observed in completely desulfated heparin, *N*-sulfated heparin, nor unmodified heparin. On the other hand, we have found that a GAG 4-*O*-sulfated dermatan sulfate (DS) promoted the generation of megakaryocytic progenitor cells in an *ex vivo* expansion culture with TPO alone. However, no effects were observed in a 6-*O*-sulfated GAG. When the sulfates were removed, DS activity disappeared [19]. The DS are prepared from bovine kidney containing a high proportion of 4-*O*-sulfation on their structure [20]. Since the variations in GAG levels may play a significant role in the regulation of hematopoiesis [9, 21, 22], the sulfation level may be an important feature for the *ex vivo* expansion of hematopoietic progenitor cells. The salmon PG used here was composed of 58% 6-*O*-sulfated unsaturated disaccharide units, 26% 4-*O*-sulfated unsaturated disaccharide units, 8.6% nonsulfated unsaturated disaccharide units and 7% disulfated unsaturated disaccharide units which contained sulfate at position C-6 of *N*-acetylgalactosamine and position C-2 of uronic acid as is evident from HPLC after chondroitinase ABC digestion (Table 1) [12]. Cellulose-acetate strip electrophoresis of pronase digests from the PG showed only one band, which migrated to the same position as that found for standard chondroitine 6-sulfate. Previous reports have indicated that the disaccharides of HS in particular can be differentially sulfated, which creates a large heterogeneity of HS [23, 24]. Although it is not clear how the core protein of salmon PG contribute in *ex vivo* human hematopoiesis, the present results suggest that sulfated GAGs domains of salmon PG may play an critical role in hematopoiesis. In addition, GAGs may also modulate the activity of localized cytokines on hematopoietic stem/progenitor cells [25–28], and can directly activate some growth factor receptors, thus suggesting that GAGs are required for the binding of several growth factors [16, 29, 30]. Taken together, the role of salmon PG may thus play a role in the optimal binding affinity and the kinetics of interactions with early-acting cytokines and matrix components.

Although a combination of IL-3 plus SCF resulted in the highest cell proliferation rate, no promoting effects were observed on the expansion of hematopoietic progenitor cells by PG (Figs. 1, 3 and 4). It is well known that the biological activity of IL-3 and SCF is related to the survival, maintenance and stimulation of proliferation in multi-lineage hematopoietic progenitors [31, 32]. Bruno et al. demonstrated that IL-3 or SCF biologically interacts with HS-PG, and that each interaction argues against the localization of hematopoietic progenitor cells [29]. In addition, Conget and Minguell reported that IL-3 regulates the amount of membrane-associated PG in multipotent hematopoietic cells [33]. Therefore, it is possible that

interactions in the structure of salmon PG, or the action of both factors and characteristics of hematopoietic progenitor cells may affect hematopoiesis *in vitro*. A further analysis of salmon PG structure-function may reveal important implications regarding the *ex vivo* expansion of both stem and progenitor cells.

The *ex vivo* expansion of hematopoietic stem cells with cytokines is an attractive and practical approach that has already led to shorten or abrogate the period of post-transplantation neutropenias [34]. Several previous studies have suggested that expanded mature or post-progenitor cells may make contributions to early hematopoietic recovery [35–37]. On the other hand, since cytokine combinations alone on the *ex vivo* expansion of hematopoietic stem/progenitor cells indicates a limited existence of hematopoiesis *in vivo*, many other additional factors may be required to be found. PG from the nasal cartilage of salmon heads is an attractive molecule in *ex vivo* expansion for generating hematopoietic stem/progenitor cells because PG acts as a promoting factor for generating some types of hematopoietic progenitor cells in our culture system [18]. More detailed studies are required to create a better process and to clarify the mechanisms of salmon PG structure regarding both the cytokine actions and the characteristics of the target cells.

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