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Synthetic polymers as xeno-free materials for stabilizing basic fibroblast growth factor in human mesenchymal stem cell cultures

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A R T I C L E   I N F O

A B S T R A C T

Series of sulfonated polymers were evaluated as additives in cell culture media. Some of the compounds, such as sulfated polyvinyl alcohol (PVA), prevented denaturation and loss of basic fibroblast growth factor during cell culture and enhanced human mesenchymal stem cell proliferation. These compounds are xeno-free alternatives of heparin, an animal-derived sulfated saccharide, often used as an additive. To the best our knowledge, this study is the first to show that chemically defined synthetic chemicals, such as sulfated polyvinyl alcohol, can be used for this purpose.

1. Introduction

Human mesenchymal stem cells (hMSCs) have clinical potential in regenerative medicine; therefore, efficient and low-cost culture systems that facilitate the large-scale expansion of quality-controlled hMSCs are required. However, the culture systems that have been developed to date are not suitable for clinical applications. Conventional culture methods use media, such as basal nutrient medium, supplemented with fetal bovine serum (FBS) [1], which contain ill-defined components and are undesirable for clinical applications. Various xeno-free culture systems have been developed, such as Stem Pro (Thermo Fischer Scientific), StemXVivo (R&D Systems), MSCM-sf (ScienCell), and MSC Medium-serum-free (Merck). These xeno-free culture systems usually require expensive recombinant human proteins, such as basic fibroblast growth factor (bFGF), which is an essential factor for stem cell cultivation [2–4].

FGF is a multifunctional protein with mitogenic activity and regulatory, morphological, and endocrine effects [5,6]. One of the subtypes of the FGF family, bFGF (FGF-2), is often used as a supplement in stem cell cultures, but its denaturation, aggregation, and precipitation often lead to the loss of its activity [7,8]. Most serum-free media supplements, including bFGF, are recommended to be stored at −20 °C or below. It is also recommended that these media should be stored at 4 °C after the addition of supplements to the basal media, and be used within 1 week. Considerable efforts have been made in the delivery and formulation of

bFGF and other proteins to stabilize them for therapeutic purposes [9–11], but there has been limited research on stabilizing bFGF in the cell culture media.

Signal transduction by bFGF occurs after its association with its receptor tyrosine kinase and a proteoglycan, heparan sulfate, to form a specific complex on the cell surface. It is known that heparin protects basic and acidic FGF from acid- and heat-induced inactivation [12]. Efficacy of heparin addition to somatic cell cultures was tested previously [13], as was the bioconjugation with heparin [14] and the controlled release of bFGF-heparin complexes [15] for bFGF protection in the culture media. Heparin alters the biological properties of hMSCs [16]. Therefore, long-term heparin supplementation requires sufficient study before clinical use. Furthermore, heparin is incompatible with xeno-free requirements and undesirable for clinical applications involving stem cell cultures (e.g., with hMSCs).

In this study, a decrease in the performance of hMSC culture media related to bFGF concentrations was first clarified. Then, bFGF-containing hMSC culture media were supplemented with heparin and other several sulfated forms of polysaccharides, oligosaccharides, and synthetic polymers and their protecting effects against bFGF loss were evaluated. Polysaccharides were selected according to their structural analogy to heparin, but their structural characteristics, such as the structure of constituent sugars, and the substituents were different from those of heparin. In addition, oligosaccharides and synthetic polymers were used to assess structure-activity relationships. The minimum

structural requirement for the protection of bFGF and the enhancement in cell proliferation of hMSC were discussed.

2. Materials and methods

2.1. Materials

bFGF (catalog #: 064–05381: recombinant, animal-derived-free) and albumin solution (catalog #: 014–21543: human recombinant) were purchased from Fujifilm Wako Pure Chemical (Japan). Normal human bone marrow-derived hMSCs and MSCGM-CD media (SCGM-CD Blue Kit; catalog #:00190632) and a serum-free and chemically defined media for hMSC proliferation, were purchased from Lonza (USA). Dulbecco's modified Eagle's medium (DMEM) and FBS were purchased from Thermo Fisher Scientific (USA). Penicillin-streptomycin (100 U/mL, 100 μg/mL), polyvinyl sulfonate, and sulfur trioxide trimethylamine complex were purchased from Sigma-Aldrich (USA). Hoechst staining solution was obtained from Nacalai Tesque (Japan; catalog #:33258). All other chemicals were procured as follows: Heparin sodium and polyallylamine from Nacalai Tesque (Japan); dextran sulfate sodium (Mw 25,000, sulfur content 15–20%) and maltoheptaose from Fujifilm Wako Chemicals (Japan); fucoidan from Funakoshi (Japan); alginate, xanthan gum, and maltotriose from Tokyo Chemical Industry (Japan); cellulose from Merck (USA); polyvinylamine (Mw 25,000) from Polyscience (USA); sucrose octasulfate potassium from Carbosynth (USA); PVA(Mw 16,000, 98% hydrolyzed) from Acros Organics (USA); and dimethylformamide from Kanto Chemical (Japan).

2.2. Synthesis of sulfated materials

PVA (200 mg; Mw, 16,000, 98% hydrolyzed) was dissolved with 600 mg of sulfur trioxide trimethylamine complex in dehydrated N,N-dimethylformamide (6 mL), and the mixture was stirred overnight at 70 °C. The supernatant was removed by decantation, and acetone (20 mL) was added to the precipitate. After stirring, the precipitate was collected by filtration, and the solid obtained was dissolved in distilled water (2 mL). Subsequently, 1.5 mL of 30% aqueous sodium acetate was added, and the mixture was stirred at 25 °C for 2 h. After adding ethanol (12 mL) to the mixture, the precipitate was collected by filtration and dissolved in distilled water (3 mL). The solution was then dialyzed overnight using a dialysis tubing (Spectra/Por MWCO 6000–8000; Spectrum Laboratories) against distilled water, and the dialysate was lyophilized to obtain 61 mg of a white solid. The identity of the compound was confirmed using ^{1}H-nuclear magnetic resonance spectroscopy at 400 MHz (Bruker Ultrashield, Bruker, USA). Other sulfated materials were synthesized similarly with slight modifications. For the synthesis of the sulfated polysaccharides and oligosaccharides, 1 g of sulfating reagents and 10 mL of solvent were used for 200 mg of saccharides, and for the other synthetic polymers, 2.5 g of sulfating reagents and 25 mL of solvents were used for 200 mg of polymers. For the purification of oligosaccharides, dialysis tubing of MWCO 1000 was used. Structure of all products was confirmed using nuclear magnetic resonance.

2.3. Culture medium and cell culture

MSCGM-CD medium was prepared according to the manufacturer's protocol (Lonza) provided with the complete synthetic medium kit. In the aging tests, 30–40 mL/bottle of MSCGM-CD medium was placed in 50 mL conical tubes. The tubes were incubated at 37 °C for 1, 4, and 7 days, and then stored in the refrigerator at 4 °C until further analyses. hMSCs were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO$_2$. The culture media were replaced once or twice per week. The cells were expanded until they occupied 90% of the dish and used between passages 3 and 8. The cells were seeded at 10,000–20,000 cells/

well in 24-well microplates containing medium (1 mL) following adaptations to the MSCGM-CD medium. The numbers of cells attached to microplate surfaces were quantitated using fluorometric DNA analyses, as described previously [17] and were correlated with the cell counts, measured using a hemocytometer. Since cell counts varied in each experiment, the data were provided in terms of fold change (%).

2.4. bFGF concentration measurement in culture media using enzyme-linked immunosorbent assay (ELISA)

bFGF concentrations in MSCGM-CD media were measured using a human bFGF ELISA kit (Ray Biotech, USA) according to the manufacturer's instructions. The absorbance of the ELISA samples was measured at 450 nm using a microplate reader (Corona Electric, Japan). In these assays, the minimum detectable amount of human bFGF was 50 pg/mL. Samples were prepared in triplicate and analyzed.

2.5. Addition of bFGF solution to the culture medium and evaluation of test compounds

Precisely 5 mL aliquots of PBS (−) containing 50 μg bFGF were mixed with 5 mL aliquots of albumin solution (10 mg). The mixtures were sterilized by filtration (filter: Mirex SLG VJ 1356; syringe: Terumo 5 mL syringe), and 12 μL aliquots were added to 1 mL aliquots of the MSCGM-CD medium in each well to achieve a final bFGF concentration of 60 ng/mL. Test compounds (heparin, dextran sulfate, fucoidan, alginate sulfate, cellulose sulfate, xanthan gum sulfate, maltoheptaose sulfate, maltotriose sulfate, sucrose octasulfate, PVA sulfate, polyvinyl sulfonate, polyvinylamine sulfonate, and polyallylamine sulfonate) were dissolved in PBS at a concentration of 25 mg/mL. They were diluted further with MSCGM-CD medium containing 60 ng/mL of bFGF to a concentration of 2.5 mg/mL. Ten-fold serial dilutions with the medium containing bFGF were performed to achieve the final concentrations of the test compounds (250 pg/mL–2.5 μg/mL). Test samples were incubated at 37 °C for 7 days in sealed Falcon^{TM} tubes and then stored overnight or longer at 4 °C. Subsequently, bFGF concentrations were measured using ELISA. Osmotic pressure and pH were measured on the preparation day, and pH was also measured on the 7th day to confirm that no significant changes occurred. hMSCs were cultured in 24-well plates as described above. After 6 days, the most suitable day for discriminating the differences in cell proliferation, the cell numbers were determined via fluorometric quantifications of cell DNA as described above.

2.5. Statistical analysis

All cell cultures were tested in triplicate, and significant differences were identified using Student's t-test in Microsoft Excel. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Confirmation of bFGF decrease in media

First, we investigated hMSC proliferation in MSCGM-CD medium stored at 37 °C and 4 °C. Cell proliferation rates decreased when the medium was stored at 37 °C (Fig. 1A). After 7 days of storage at 37 °C, cell counts after 6 days of culture were approximately 60% lower than those in fresh medium (day 0). Even after the storage at 4 °C for 6.5 months, a similar decrease in cell counts was observed. The bFGF concentrations that were measured using ELISA after hMSC cultivation are shown in Fig. 1B. After a 6-day culture in the fresh medium (Day 6 of 0-day storage), bFGF concentrations significantly decreased compared to the initial concentration (Day 0 of 0-day storage) and were comparable to those in the medium stored at 37 °C for 7 days, before cell culture. To confirm the effects of bFGF on proliferation, we supplemented aged
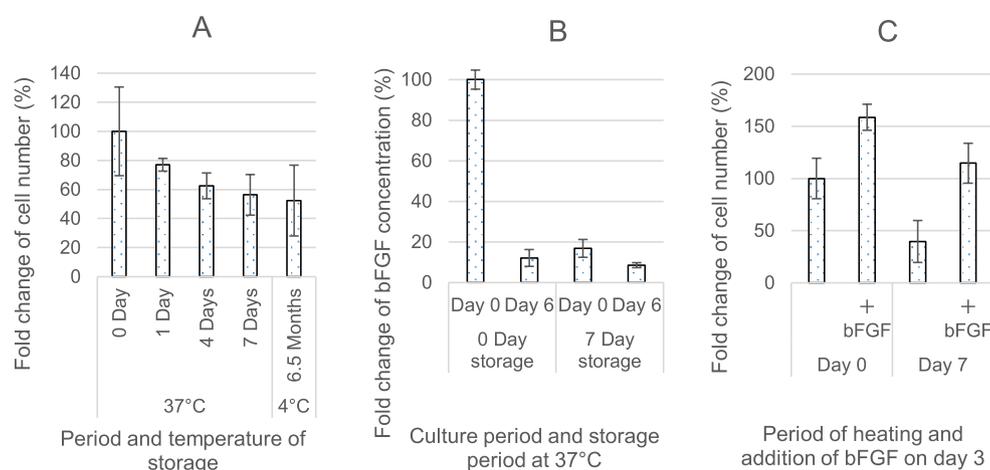
Fig. 1. Basic fibroblast growth factor (bFGF) concentration in culture medium and effects on human mesenchymal stem cell (hMSC) proliferation. A: Thermal treatment of culture media and its effects on hMSC proliferation hMSCs were seeded in 24-well microplates (10,000 cells/well) and cultured in the MSCGM-CD media that had been stored at 37 °C for 1, 4, or 7 days or at 4 °C for 6.5 months. Cell counts were measured on day 6. Data are presented as percentage cell counts in the freshly prepared medium.

B. bFGF concentrations in the culture medium following hMSC culture hMSCs were seeded in 24-well microplates (20,000 cells/well) and cultured in the MSCGM-CD media. bFGF concentrations in the medium were determined before cultivation and on day 6 using ELISA. Data are presented as percentage of bFGF concentrations in the freshly prepared medium. Samples were prepared in triplicate and analyzed.

C. Effect of bFGF supplementation during cell culture hMSCs were seeded in 24-well microplates (20,000 cells/well) and cultured in the MSCGM-CD media, which were stored at 37 °C for 7 or 0 days. Supplementary bFGF was added to the culture on day 3 at a final concentration of 60 ng/mL. Cell counts were measured on day 6. Data are presented as percentages of cell counts in the freshly prepared medium.

medium with 60 ng/mL bFGF on day 3 of the culture period. Recovery of the proliferation potency was observed in the culture medium stored at 37 °C for 7 days before cultivation, and enhanced proliferation was observed even in the culture with fresh medium (Fig. 1C). Since it was confirmed that the proliferation potency was dependent on bFGF concentration in the media and bFGF concentration decreased during storage at 37 °C as well as during cultivation, we surveyed materials for bFGF stabilization in the media.

3.2. Effect of materials on bFGF stabilization

As glycosaminoglycans, such as heparan sulfate and heparin, bind to and stabilize bFGF, we first tested the effect of heparin on the amount of bFGF measured using ELISA. MSCGM-CD medium containing 60 ng/mL of bFGF was supplemented with various concentrations (250 pg/mL –25 μg/mL) of heparin and then the solutions were incubated at 37 °C for 1 week. The percentage ratios of the remaining bFGF relative to bFGF concentrations in the medium stored at 4 °C were determined. Heparin was effective at concentrations as low as 2.5 ng/mL, and the
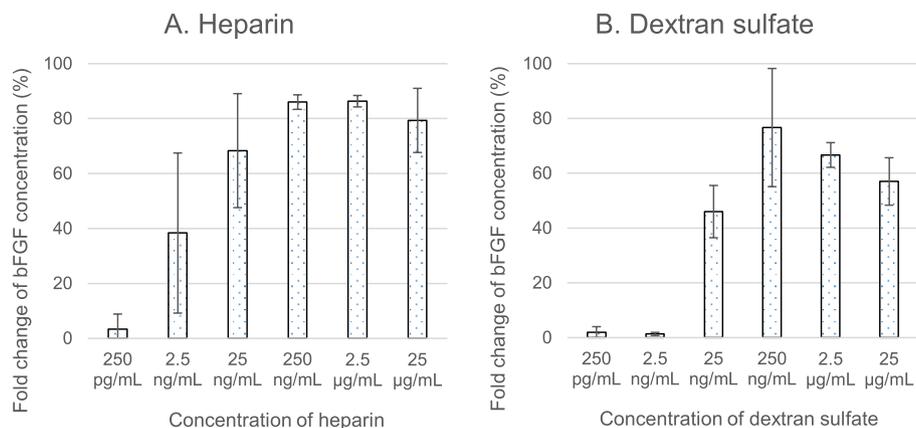

Fig. 2. Effects of heparin and dextran sulfate on basic fibroblast growth factor (bFGF) concentrations in MSCGM-CD media MSCGM-CD medium was stored at 37 °C for 1 week after the addition of (A) heparin or (B) dextran sulfate, and bFGF concentrations were then measured using enzyme-linked immunosorbent assay. Data are presented as percentages of bFGF concentrations in the medium stored at 4 °C for 1 week. Samples were prepared in triplicate and analyzed

stabilization effect was enhanced at higher concentrations (Fig. 2A).

The lowest effective doses for dextran sulfate were slightly higher (25–250 ng/mL) than those for heparin (2.5–25 ng/mL), but their effects were weaker than those of heparin by 10–25% and a decrease in the concentration of the remaining bFGF was observed at high concentrations (Fig. 2B). The average values at 25 ng/ml, 250 ng/ml, 2.5 µg/ml and 25 µg/ml for heparin were 68, 86, 86 and 79%, respectively, while those for dextran sulfate were 46, 77, 67 and 57%, respectively. Effects of other sulfated polysaccharides are summarized in Fig. 3A. The results were classified into four levels, namely, strong ($\geq$60%), moderate ($\geq$40%), weak ($\geq$20%), and none (<20%) based on the amount of remaining bFGF to clarify the simplified tendency of the effects by structures. The sulfated polysaccharides showed moderate activity compared to heparin or dextran sulfate at higher concentrations despite the difference in sugar backbones, degree, and position of sulfation. The sulfated oligosaccharides also showed a protective effect at higher concentrations. Among the compounds tested, longer oligosaccharides were more effective (Fig. 3B). Among the synthetic sulfated or sulfonated polymers, polyallylamine sulfonate showed only a weak effect, whereas the other three polymers showed strong to moderate effects at higher doses (Fig. 3C). Among the compounds tested, heparin, dextran sulfate, sucrose octasulfate, and PVA sulfate were chosen for further evaluation because of their characteristic structures and ease of availability.

3.3. Effect of materials on cell proliferation

To confirm the ability of the different materials for hMSC cultures, we tested their effects on cellular proliferation in MSCGM-CD medium after 6 days of continuous culture. Since some cytotoxicity was observed at higher concentrations, a concentration of 2.5 µg/mL or less was used. Cell counts in the test medium were compared with those in the fresh medium. Heparin dose-dependently enhanced cell proliferation from 2.5 to 250 ng/mL, but it was not statistically significant (Fig. 4A). Dextran sulfate significantly increased the cell growth at 25 ng/mL (p = 0.0291) compared to the positive control, which was MSCGM-CD

medium to which 60 ng/mL of additional bFGF was added at the start of cultivation. Sucrose octasulfate increased cell growth in a dose-dependent manner, with cell proliferation significantly higher at 2.5 µg/mL than that with no additive control (p = 0.0388). PVA sulfate significantly enhanced cell proliferation at 100 ng/mL (p = 0.0218) compared to the control. However, dose-dependent growth reductions were observed at higher concentrations of PVA sulfate (250 ng/mL to 2.5 µg/mL; Fig. 4B), possibility due to cytotoxicity.

4. Discussion

On measuring bFGF in the media using ELISA, a decrease in its levels during storage and cell culture was observed. This decrease was correlated with the suppression of cell proliferation. It should be noted that ELISA measures the immunoreactive molecular species of bFGF. Though it seemed that most bFGF was depleted upon storage at 37 °C, undetectable bFGF might still support the cell growth to some extent. It is known that bFGF binds to proteoglycans on the cell surface, and the sulfated compounds tested in this study could possibly bind to bFGF and protect it from denaturation and/or adsorption. In the ELISA experiments of the polysaccharides, dextran sulfate was the second most effective after heparin. Fucoidan, alginate sulfate, cellulose sulfate, and xanthan gum sulfate showed moderate protective effects. As shown in Fig. 2, the effect of heparin and dextran sulfate declined at higher concentrations although not significantly. The effect of cellulose sulfate and xanthan gum sulfate also decreased at high concentrations, as shown in Fig. 3A. These sulfated polysaccharides might affect ELISA by binding to bFGF or by interfering with the detection reaction. The structural difference between heparin and the other sulfated polysaccharides is that heparin contains a glucosamine moiety similar to proteoglycans, and this difference might be the key for the superior activity of heparin over other compounds. However, other factors, such as the size of the molecules and the degree of sulfation should be considered.

The sulfated oligosaccharides showed some effect at relatively higher concentrations than the sulfated polysaccharides and among the compounds tested; higher molecular weight resulted in higher efficacy. The
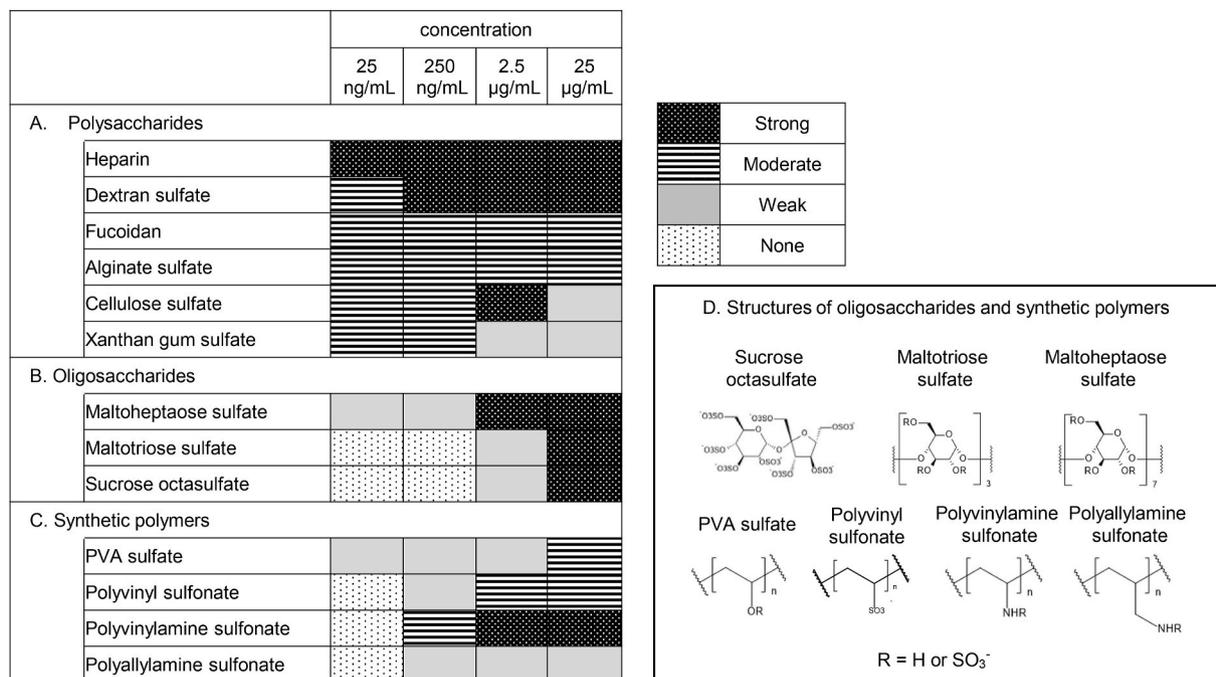

Fig. 3. Enzyme-linked immunosorbent assay (ELISA) results for the level of bFGF protection by test compounds from 25 ng/mL–25 µg/mL Based on the ELISA results shown in Fig. 2A and B, a protection value of 25 ng/mL to 25 µg/mL is indicated for polysaccharides (A), oligosaccharides (B), synthetic polymers (C). Strong: $\geq$60%; moderate: $\geq$40%; weak: $\geq$20%; none: <20%. The structures of oligosaccharides and synthetic polymers which are evaluated by ELISA are shown (D). R in the structure indicates H or SO_3.
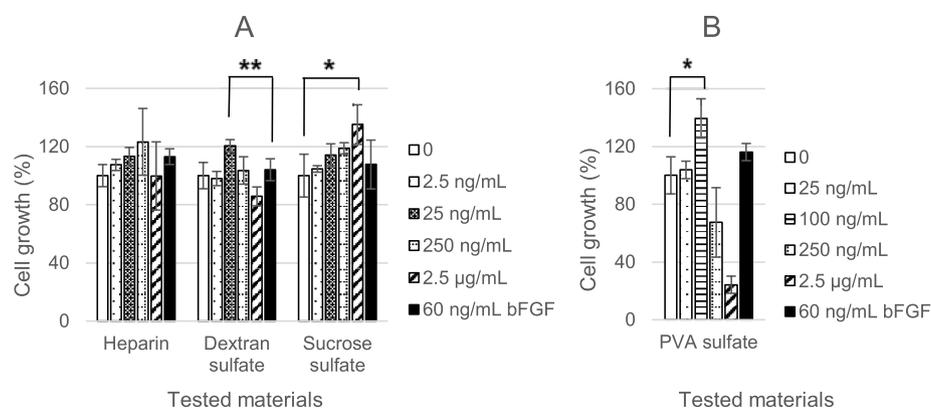
Fig. 4. Effects of various sulfated compounds on human mesenchymal stem cell (hMSC) proliferation. hMSCs were seeded in 24-well microplates (20,000 cells/well) and cultured with various supplements, i.e., (A) heparin, dextran sulfate, sucrose sulfate, or (B) PVA sulfate. The hMSC counts were determined on day 6. The black bar on the extreme right for each supplement indicates the positive control as 60 ng/mL bFGF (added initially to the test sample for reference). Differences between the test culture and negative control (0) values and positive controls were both considered significant when * and **p < 0.05, respectively.

sulfated forms of synthetic polymers were also effective in protecting bFGF in the medium. The sugar backbones were not necessary for this effect. Other materials, such as polysaccharides, chelating agents, antioxidants, and water-soluble polymers were also evaluated with ELISA; however, no protective effects were observed (data not shown), indicating that a cluster of sulfated or sulfonated groups is essential for protecting bFGF in MSC culture media.

After confirming the effects of the additives on bFGF protection in MSC culture media, their effects on the enhancement of cell proliferation were tested for four materials. Among the synthetic compounds, PVA sulfate was selected because of ease of availability. All four compounds showed increased cell proliferation in particular concentration ranges. Heparin and dextran sulfate showed effects at relatively low concentrations. Suppression of growth was observed at higher concentrations of these materials, except for sucrose octasulfate, possibly due to their toxicity to the cells.

In a previous study, heparin and dextran sulfate were reported to protect bFGF from denaturation [9], and the effective lowest protecting concentration for 5 μM of bFGF was reported as 5–25 μg/mL. In our results, the effective concentration of the dextran sulfate for cell proliferation was 25 ng/mL in a medium containing 60 ng/mL (3.5 nM, FW 17,000) of bFGF. The ratio of bFGF to dextran sulfate was similar to that reported in the previous study.

Sucrose octasulfate, a sulfated disaccharide, triggered a dose-dependent increase in cell proliferation without any growth inhibition at the concentration of 2.5 μg/mL, and the increase was significant. In contrast, the protective effect at this concentration, shown using ELISA, was weak. It was reported that sucrose octasulfate activates the bFGF signaling pathway [18]. In this report, the crystal structures of the ternary complex of dimeric FGF2, FGF-receptor (FGFR), and sucrose octasulfate were reported. The effect of sucrose octasulfate may be caused not only by stabilizing bFGF but also by interacting with bFGF and FGFR to form a complex and activating the signaling pathway for cell proliferation.

PVA sulfate strongly influenced cell growth at 100 ng/mL, while the effects observed at 25 ng/mL were weak, and those at 250 ng/mL were toxic; therefore, further investigation is warranted to identify the effective range. In addition, further studies are warranted to ascertain whether the effect of PVA sulfate observed is attributed only to the stabilizing action on bFGF, since sulfated materials are known to interact with extracellular matrices [19] and other biomolecules. PVA alone is not cytotoxic in stem cell cultures [20]. This toxicity may be due to the sulfated forms of PVA or impurities. The current report is the first to demonstrate the effect of PVA sulfate to stabilize bFGF and enhance cell proliferation of hMSCs. PVA sulfate can easily be synthesized and will be useful as a cell culture supplement at the proper concentration.

In conclusion, we found some simple compounds as supplements that

are useful for MSC culture media, such as MSCGM-CD. The highest non-toxic concentration for heparin is 250 ng/mL; dextran sulfate, 25 ng/mL; PVA sulfate, 100 ng/mL; sucrose octasulfate, 2.5 μg/mL. However, the effective dose, which should be determined for each compound, depends on the molecular structure, length, and an optimal concentration with no toxicity. Previous studies showed that MSC proliferation can be enhanced by the addition of other types of compounds, such as antioxidants [21] or endogenous proteins [22]. However, most of the compounds used in this study can be prepared as xeno-free compounds, and considerable economic and practical advantages with their application are expected.

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