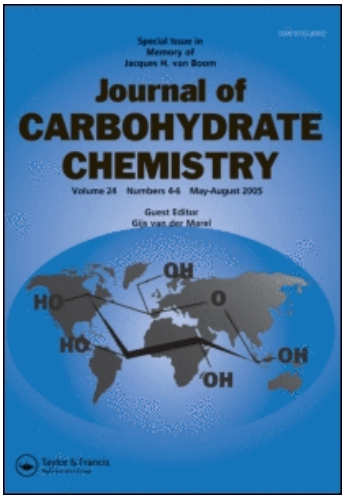

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MOLLIFICATION OF CYTOTOXICITY OF SULFATED POLYSACCHARIDES BY FIBROBLAST GROWTH FACTORS[1]

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ABSTRACT

Sulfated polysaccharide which had a relatively high degree of sulfation showed cytotoxicity to 3T3-L1 fibroblasts. Acidic and basic fibroblast growth factors inhibited the cell damage caused by the sulfated polysaccharides, while epidermal growth factor and platelet growth factor had no effects.

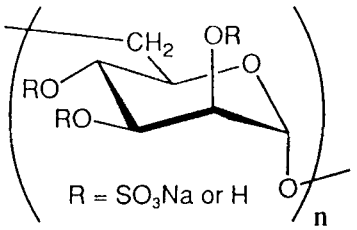
INTRODUCTION

Acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) are the prototype members of a family of heparin-binding growth factors. They exhibit pleiotropic biological activities and a strong affinity for heparin.[2,3] On the other hand, epidermal growth factor (EGF)[4] and platelet derived growth factor (PDGF)[5] do not interact with heparin. Heparin protects FGFs from denaturation and enzymatic degradation.[6,7] Heparin is also a potent modulator of FGFs' biological activity.[8] Heparan sulfate proteoglycans (HSPGs) protect FGF from proteolytic degradation[9] and modulate the activity of FGF.[10] HSPGs are essential for aFGF activity and bind with high affinity to the growth factor.[11,12]

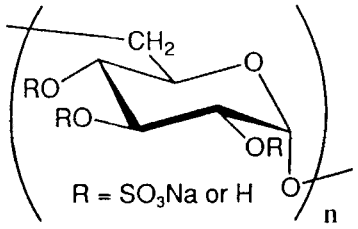
aFGF and bFGF lack the signal sequences for secretion. However, significant amounts of FGFs are found in the extracellular matrix.[13] Since no defined mechanism for the release of FGFs has been described, it has been believed that FGFs are released from dead or damaged cells.[14,15] Release of FGFs caused by cell death or damage may represent a rescue mechanism. Araki et al. demonstrated that active death (apoptosis) of

TABLE. Characteristics of sulfated polysaccharides.

Sulfated polysaccharide	Number of sulfate groups per sugar unit	Number average molecular weight x 10^{-4}
Heparin	1.5	1.1
Dextran sulfate	2.4	0.5
MPS_0	0	30.2
$MPS_{0.98}$	0.98	9.3
$MPS_{1.56}$	1.56	16.4
$MPS_{1.99}$	1.99	10.7



(1→6)-α-D-Mannopyranan sulfate



Dextran sulfate

endothelial cells inhibits FGF.[16] Moreover, it was reported that bFGF rescues neurons from the cell death caused by oxygen radical.[17]

Heparin is a linear and highly sulfated polysaccharide consisting of alternating uronic acid and D-glucosamine residues. It is difficult to assign structure-function relationships based on studies with native heparin, due to the complex chemical structure of heparin. Several studies have demonstrated that the size and the degree of sulfation was important for the effects of heparin.[18,19] It was also reported that dextran sulfate,[20] derivatized dextran,[21] and large number of polyanions[22] mimic the effects of heparin for FGFs.

In this study, we used synthetic (1→6)-α-D-mannopyranan sulfate with various degree of sulfation (MPS_X: x = number of sulfate groups per sugar unit). (1→6)-α-D-mannopyranan sulfate prepared by ring-opening is stereoregular sulfated polysaccharide with high molecular weight. Since MPS_X has a simple and well-defined structure,[23] the clear experimental results are expected and the clear structure-function relationships can be discussed. In this investigation, we report a rescuing effect of FGFs on fibroblast from the cell damage which was caused by sulfated polysaccharides with a high degree of sulfation.

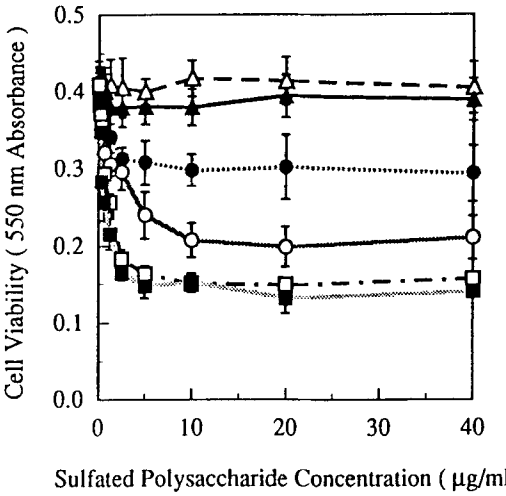


FIG. 1. Effect of sulfated polysaccharide for 3T-3-L1 fibroblasts (○: heparin; ●: dextran sulfate; △: MPS_0; ▲: $MPS_{0.98}$; □:$MPS_{1.56}$; ■:$MPS_{1.99}$). 3T3-L1 cells were plated at 8000 cells/well on a polystyrene 96 well multiplate and cultured for 66 h in a serum free medium supplemented with insulin and transferrin. Cell viability was measured by MTT assay.

RESULTS AND DISCUSSION

The chemical structures of synthetic mannopyranan sulfate and dextran sulfate are illustrated below, and the characterization of the sulfated polysaccharides is summarized in the TABLE.

Sulfated polysaccharides with a relatively high degree of sulfation were cytotoxic to 3T3-L1 fibroblast *in vitro* as shown in FIG. 1. Cell viability was measured by MTT assay (cf. EXPERIMENTAL).

Cell injury involving membrane destruction was also observed by phase-contrast microscopy. The cytotoxicity of dextran sulfate was low possibly due to the low molecular weight of the polysaccharide. The non-sulfated mannopyranan (MPS_0) and mannopyranan sulfate having a low degree of sulfation ($MPS_{0.98}$) did not show the cytotoxicity. These findings indicated that the cytotoxicity of sulfated polysaccharides is dependent on the degree of sulfation and the molecular weight.

Effects of the addition of rbFGF and PDGF on the cytotoxicity caused by sulfated polysaccharides are shown in FIG. 2. The addition of rbFGF relieved the cytotoxicity caused by heparin or dextran sulfate. On the other hand, the effect of PDGF which stimulates 3T3-L1 fibroblast proliferation was ambiguous. There is no report about the

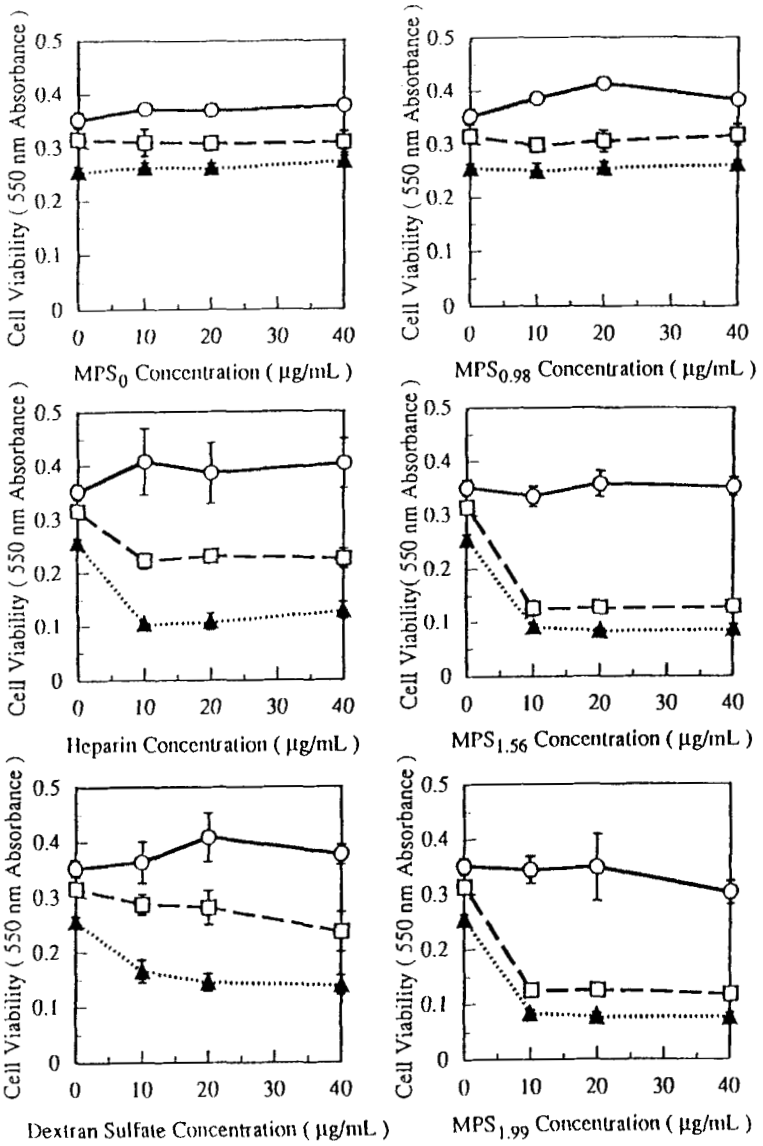


FIG. 2. Effects of rbFGF and PDGF on the cytotoxicity caused by sulfated polysaccharides (▲: absence of growth factor; O: rbFGF, 5 ng/mL; □: PDGF, 10 ng/mL) (37°C, 5% CO_2, 66 h).

interaction between PDGF and heparin. When the synthetic mannopyranan sulfates with higher degree of sulfation ($MPS_{1.56}$ and $MPS_{1.99}$) were used as sulfated polysaccharide, the addition of growth factors was more discriminative, resulting in a rescuing effect of rbFGF but no effect of PDGF. It is clear that this effects of rbFGF is due to not only mitogenic activity but also protective effect, compared with PDGF.

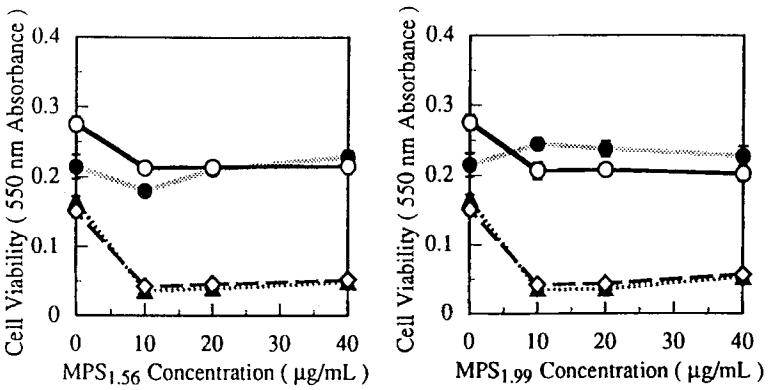


FIG. 3. Effects of aFGF, rbFGF, and EGF on the cytotoxicity caused by $MPS_{1.56}$ and $MPS_{1.99}$ (▲: absence of growth factor; ●: aFGF, 10 ng/mL; O: rbFGF, 1 ng/mL; ◇: EGF, 10 ng/mL) (37°C, 5% CO_2, 66 h).

Effects of aFGF, rbFGF, and EGF on the cytotoxicity caused by the highly sulfated mannopyranan sulfates ($MPS_{1.56}$ and $MPS_{1.99}$) are shown in FIG.3. aFGF, as well as rbFGF, relieved the cytotoxicity of $MPS_{1.56}$ and $MPS_{1.99}$. On the other hand, EGF, which has no effects on 3T3-L1 fibroblast proliferation and does not have a heparin-binding site, did not rescue the fibroblasts from the cell damage.

The similarity between two kinds of fibroblast growth factors (aFGF and rbFGF) in their effects on cytotoxicity caused by sulfated polysaccharide may relate to the finding that aFGF and rbFGF seem to interact with the same cell surface receptor.[24]

EXPERIMENTAL

Sulfated polysaccharides. A stereoregular (1→6)-α-D-Mannopyranan sulfate (MPS) was prepared by ring-opening polymerization of 1,6-anhydro-2,3,4-tri-*O*-benzyl-β-D-mannopyranose followed by debenzylation and sulfation of the obtained polymer.[23] Heparin (Grade I; H3125; anticoagulant activity: 180 UPS unit/mg) and dextran sulfate (D3037) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The number of sulfate groups per sugar unit in MPS was calculated from elemental analysis data. The number-average molecular weight of MPS was approximated by gel permeation chromatography (columns, Tosoh TSK gel; eluent, 66.7 mM phosphate buffer, pH 6.86) using standard dextran as reference.

Growth factors. Human recombinant bFGF (rbFGF)[25] was generously provided by Takeda Chemical Industries, Ltd. (Osaka, Japan). Natural aFGF from

bovine brain was purchased from BioScience Products AG (Emmenbürcke, Switzerland) and EGF from mouse submaxillary glands was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Natural human PDGF was purchased from Promega Co. (Madison, WI, U.S.A.).

Cell culture. 3T3-L1 fibroblasts were subcultured in tissue culture flasks (75 mm; Corning 25110; Corning Lab. Sci. Co. (NY, U.S.A.)) at subconfluent cell densities in Eagle's MEM supplemented with 10% fetal bovine serum (Gibco Lab. Life Tech., Inc. (Gaithersburg, MD, U.S.A.)), kanamycin, and L-glutamine. Cultures were maintained at 37 °C in a humidified tissue culture incubator in a 5% CO_2 / 95% air environment, and were used for experiments between passage 4 and 12.

Cell survival assay. The sulfated polysaccharide and the growth factor were added to the serum-free MEM supplemented with GMS-A (insulin 10 μg/mL, transferrin 5.5 μg/mL, sodium pyruvate 0.11 μg/mL, and sodium selenite 6.7 ng/mL: Gibco) and bovine serum albumin (0.4 mg/mL). 3T3-L1 fibroblasts were plated at 7000 cells/well on a polystyrene 96 well multiplate (Corning 25860) and cultured for 66 h. Cell viability was measured by MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide) assay.[26] The colorimetric MTT assay is based on the reaction that tetrazolium ring of MTT is cleaved in active mitochondria to formazan product. The amount of formazan generated is directly proportional to the cell number. MTT assay can estimate the vitality of cells or the degree of activation.

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