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The effect of growth factors on the cytotoxicity of sulphated polysaccharides

Megumi Kunou and Kenichi Hatanaka*

Department of Biomolecular Engineering, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 226, Japan

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The cytotoxicity of sulphated polysaccharides on 3T3-L1 fibroblasts was investigated. Dextran sulphate with a molecular weight of more than 100 000 and with a number of sulphate groups per sugar unit (degree of sulphation) of 2.4 showed strong cytotoxicity to 3T3-L1 fibroblasts. The synthetic (1→6)-α-D-mannopyranan sulphate having a degree of sulphation of more than 1.0 and with a molecular weight of approx. 100 000 also exhibited cytotoxicity. The results indicated that the cytotoxicity of sulphated polysaccharides strongly depended on their molecular weight and degree of sulphation. FGF-1 (aFGF) and FGF-2 (bFGF) protected the cell from the damage caused by the sulphated polysaccharide, while PDGF, EGF, and HGF had no such effect. Therefore, it is suggested that the rescuing effect is one of the special biological activities, and is shown only by FGFs. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Acidic and basic fibroblast growth factors (FGF-1 and FGF-2, respectively), FGFs, are the prototype members of a family of heparin-binding growth factors (Burgess & Maciag, 1989; Basilico & Moscatelli, 1992). They exhibit pleiotropic biological activities and a strong affinity to heparin. FGFs stimulate proliferation, migration, and differentiation of many kinds of cells (Fernig & Gallagher, 1994). Two classes of FGF receptors have been detected on the surface of target cells. One is a low affinity receptor, cell surface heparan sulphate proteoglycans (HSPGs) (Gallagher, 1994); the other one is a high affinity receptor, a tyrosine kinase, proto-oncogene product (Reiland & Rapraeger, 1993).

FGF-1 and FGF-2 lack the signal sequence for secretion. However, significant amounts of FGFs are found in the extracellular matrix (ECM) (Bashkin *et al.*, 1989). Although no defined mechanism for the release of FGFs has been described, it has been reported that FGFs are released from dead or damaged cells (McNail *et al.*, 1989; Haimovitz-Friedman *et al.*, 1991). Release of FGFs caused by cell death or damage may represent a rescue mechanism. In fact, FGFs act as survival factors (Tamm *et al.*, 1991) and are responsive to radiation damage (Haimovitz-Friedman *et al.*, 1991) and wound healing (McNail *et al.*, 1989; Gajdusek & Carbon, 1989). Moreover, not only are FGFs indispensable in the development of neurons (Nurcombe *et al.*, 1993), but they also rescue neurons from cell death caused by oxygen radicals (Enokido *et al.*, 1992), cerebellar ischemia (Lippoldt *et al.*, 1993) and excitotoxic damage (Mayer *et al.*, 1993).

FGFs are stored in a stable form by binding to HSPGs in the ECM *in vivo* (Rifkin & Moscatelli, 1989). Heparin protects FGFs from denaturation and enzymatic degradation (Gospodarowicz & Cheng, 1986; Mueller *et al.*, 1989). Moreover, heparin and HSPGs potentiate the biological activity of FGFs (Lindahl *et al.*, 1994; Ruoslahti & Yamaguchi, 1991). Heparin or HSPGs are essential for both mitogenic activity of FGFs and binding of FGFs to the high affinity receptor on the cell surface (Yayon *et al.*, 1991). The heparin binding domain of FGFs (Thompson *et al.*, 1994; Arakawa *et al.*, 1994) and effects of heparin on FGF oligomerization (Spivak-Kroizman *et al.*, 1994; Ornitz *et al.*, 1995) have also been reported.

Several studies have demonstrated that the degree of sulphation and the molecular size of heparin are important for the potentiation of FGF's activity

*To whom correspondence should be addressed.

(Aviezer *et al.*, 1994; Ishihara, 1994; Maccarana *et al.*, 1993) It has likewise been reported that dextran sulphates (Kajio *et al.*, 1992), derivatized dextran (Tardieu *et al.*, 1992) and a large number of polyanions (Volkin *et al.*, 1993) mimic the effect of heparin on the activity of FGFs.

We have previously reported the cytotoxicity of sulphated polysaccharides and FGFs' rescuing effects on cytotoxicity caused by sulphated polysaccharide (Kunou *et al.*, 1995). In this study, we present further information on the cytotoxicity of sulphated polysaccharides, and, the rescuing effect of FGFs. We prepared several amounts of linear (1→6)-α-D-mannopyranan sulphate (MPS_x, where x = number of sulphated groups per sugar unit) with various degrees of sulphation. Since MPS_x has a simple and well-defined structure, clear experimental results are expected and the structure–function relationships can be discussed thoroughly. Furthermore, we also used heparins and dextran sulphates with different molecular weights to evaluate the effects of molecular size.

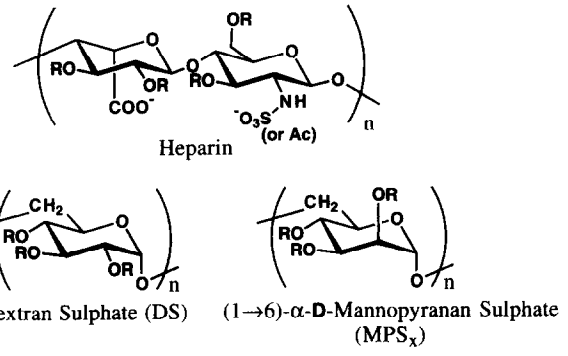

Fig. 1. Structures of sulphated polysaccharides (R = SO_3^- or H).

MATERIALS AND METHODS

Sulphated polysaccharides

Characterization of sulphated polysaccharides are summarized in Table 1, and chemical structures are illustrated in Fig. 1. The number, which is at the end of the compound's name, indicates molecular size[$\times 10^3$], and the subscript shows the degree of sulphation.

Heparins and dextran sulphates with different molecular weight were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The number of sulphate groups per sugar unit was provided by Sigma. The number-average molecular weight ($\overline{M_n}$)) of heparins and dextran sulphates was determined by gel-permeation chromatography (GPC, JASCO Model-800, Tokyo, Japan) using Ultrahydrogel 1000 and Ultrahydrogel 500, 250 columns (Japan Waters Ltd., Tokyo, Japan; eluent, 0.1 M sodium nitrate) with a multiangle light scattering detector (Dawn-DSP, Wyatt Technology, Santa Barbara, CA, USA) and RI detector (JASCO, Model 830-RI).

Stereoregular (1→6)-α-D-mannopyranan sulphate (MPS_x, x = number of sulphated groups per sugar unit) was prepared by ring-opening polymerization of 1,6-anhydro-2,3,4-tri-*O*-benzyl-β-D-mannopyranose with phosphorus pentafluoride as initiator in dichloromethane at −60°C under high vacuum and subsequent debenzylation and sulphation of the polymer obtained (Hatanaka *et al.*, 1991). The number of sulphate groups per sugar unit in mannopyranan sulphate was calculated by elemental analysis which was performed by Toray Research Center, Inc. (Kamakura, Japan). The number-average molecular weight ($\overline{M_n}$) of mannopyranan sulphate was determined by GPC (columns, Tosoh TSK gel (Osaka, Japan); eluent, 66.7 mM phosphate buffer, pH 6.86) using standard dextran as reference. The reactivity of hydroxyl groups for sulphation was in the order, 3-OH > 2-OH > > 4-OH. Thus, $MPS_{0.98}$ was relatively rich in residues monosubstituted at C-3, while $MPS_{1.56}$ and $MPS_{1.99}$ contained mainly of disubstituted C-2 and C-3 (Hatanaka *et al.*, 1991).

Table 1. Characteristics of sulphated polysaccharide

Sulphated polysaccharide	Number of sulphate groups per sugar unit	$\overline{M_n} \times 10^{3a}$
Heparin2.5 (H2.5)	1.6	2.5
Heparin5.0 (H5.0)	1.6	5.0
Heparin15 (H15)	1.5	14.6
Dextran sulphate4.5 (DS4.5)	2.2–2.6	4.5
Dextran sulphate6.2 (DS6.2)	2.3	6.2
Dextran sulphate11 (DS11)	2.4	10.9
Dextran sulphate151 (DS151)	2.3	151
Dextran sulphate396 (DS396)	2.4	396
MPS_0	0	302
$MPS_{0.98}$	0.98	92.7
$MPS_{1.56}$	1.56	164
$MPS_{1.99}$	1.99	107

[a]Number-average molecular weight (Dalton).

Growth factors and other chemicals

Human recombinant FGF-2 (Seno *et al.*, 1988; Seno *et al.*, 1990) was generously provided by Takeda Chemical Industries, Ltd. (Osaka, Japan). Human recombinant FGF-1 was purchased from Upstate Biotechnology (New York, USA). EGF from mouse submaxillary glands was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Natural human PDGF (PDGF-AB) was purchased from Promega Co. (Madison, WI, USA). Human recombinant HGF was kindly provided by Snow Brand Milk Products Co., Ltd. (Tochigi, Japan). Poly-L-lysine hydrobromide ($\overline{M_n} = 4.15 \times 10^4$, $\overline{M_w}/\overline{M_n} = 1.10$) was purchased from Sigma. Polystyrene sulphonate ($\overline{M_n} = 4.13 \times 10^4$, $\overline{M_w}/\overline{M_n} = 1.63$) was prepared by radical polymerization of *p*-styrene sulphonate.

Cell culture

3T3-L1 fibroblasts (CCL92.1) were subcultured in tissue culture flasks (75 cm^2, Corning 25110, Corning Lab. Sci. Co.; NY, USA) at subconfluent cell densities in Eagle's MEM supplemented with 10% fetal bovine serum (Gibco BRL, Life Tech., Inc.; NY, USA), 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 in experiments between passages 4 and 12.

Cell proliferation assay

Sulphated polysaccharide or ionic polymer and growth factor were introduced in serum-free Eagle's minimum essential medium supplemented with ITS-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 polystyrene 96 well multi-plate (MS-8096F, Sumitomo Bakelite Co., Ltd; Tokyo, Japan) and cultured for 48 h. Cell proliferation was measured by MTT activity (Mosmann, 1983). The colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay is based on the tetrazolium ring of MTT which was cleaved in active mitochondria to formazan product. The amount of formazan generated is directly proportional to the cell number. In other words, absorbance at 550 nm is directly proportional to the cell number in the colorimetric MTT assay. MTT assay can estimate the vitality of cells or the degree of activation. Cellular morphology was observed by Olympus CK2 phase-contrast microscope.

Cell attachment assay

3T3-L1 fibroblasts were plated in the same manner as proliferation assay except that two equal conditions of dishes were prepared every run. After 4 h incubation, cell attachment was measured as cell viability by the MTT method involving a medium change. The first series of dishes directly determined the cell number by the MTT method. The floating cells were removed by a medium change. With the other series, we performed additional medium change and rinsing twice by the same medium just before measurement of cell number, in order to estimate the degree of attachment of the cells on the substratum. The cells which were weakly attached on the dishes were removed by this procedure.

RESULTS AND DISCUSSION

Cytotoxicity of sulphated polysaccharides

Only polysaccharide was introduced into the medium. The low molecular weight heparin (H2.5) was not cytotoxic (Fig. 2). On the other hand, in the presence of heparin with relatively high molecular weight (H15), cell viability was remarkably low. Dextran sulphates have high degrees of sulphation. In the case of dextran sulphates having smaller molecular size, i.e., less than 11 kDa, no significant effect on cell viability was observed (Fig. 3). A dextran sulphate having a molecular weight of more than several hundred thousand exhibited strong cytotoxicity. Therefore, it is clearly indicated that molecular size is an important factor affecting the cytotoxicity of sulphated polysaccharides to fibroblasts.

Figure 4 shows the cytotoxicity of mannopyranan sulphate, MPS. Each MPS has a high molecular weight and various degrees of sulphation. The subscript

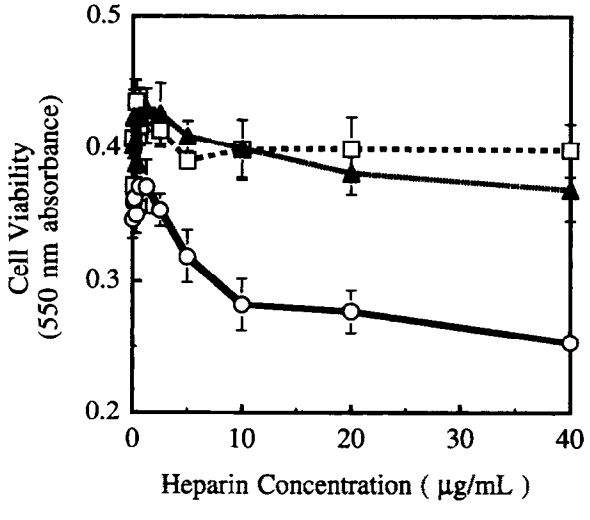

Fig. 2. Effect of heparin on 3T3-L1 fibroblast viability. □, H2.5; ▲, H5.0; ○, H15. 3T3-L1 fibroblasts were plated at 7000 cells/well on a polystyrene 96-well multi-plate and cultured for 48 h in a serum-free medium supplemented with insulin and transferrin. Cell viability was measured by MTT assay.

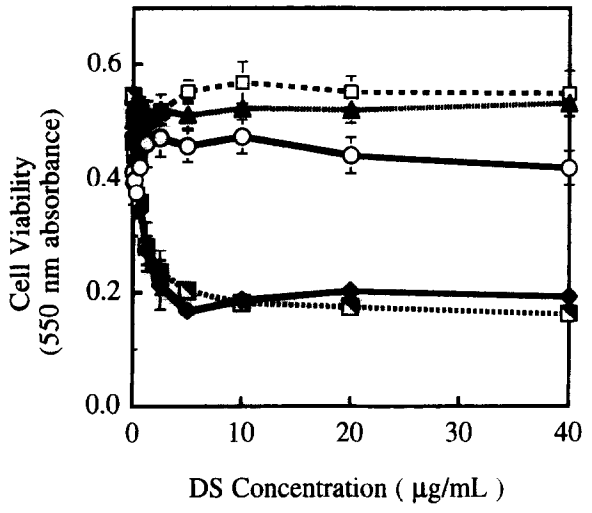

Fig. 3. Effect of dextran sulphate (DS) on 3T3-L1 fibroblast viability (37°C, 5% CO_2, 48 h). ○, DS4.5; ▲, DS6.2; □, DS11; ◆, DS151; ◪, DS396.

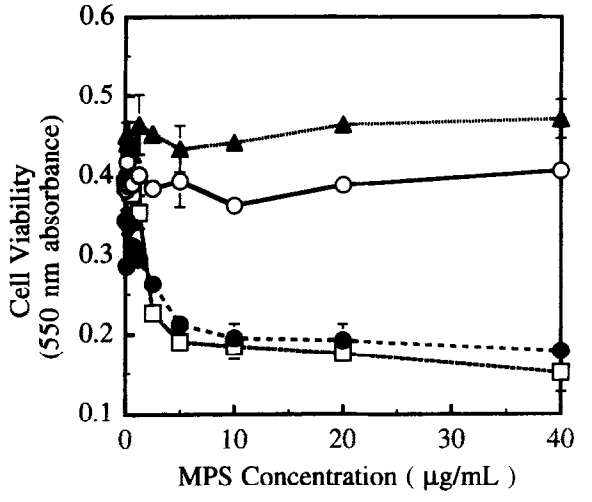

Fig. 4. Effect of mannopyranan sulphate (MPS_x) on 3T3-L1 fibroblast viability (37°C, 5% CO_2, 48 h). ○, MPS_0; ▲, $MPS_{0.98}$; •, $MPS_{1.56}$; □, $MPS_{1.99}$.

indicates the number of sulphate groups per sugar unit. MPS with a degree of sulphation greater than 1.0 caused a decrease in cell viability, indicating that the degree of sulphation of polysaccharides is also an important factor affecting cytotoxicity.

Sulphated polysaccharides including heparin showed cytotoxicity to 3T3-L1 fibroblasts. The cytotoxicity of sulphated polysaccharides strongly depended on their molecular weight and the degree of sulphation.

The rescue effect of growth factor on the cells damaged by sulphated polysaccharides

In order to compare the biological activity of FGFs, various types of growth factors were used, such as FGF-1, FGF-2, hepatocyte growth factor (HGF) (Mizuno & Nakamura, 1993; Zarnegar & Michalopoulos, 1995), platelet derived growth factor (PDGF (Meyer-Ingold & Eichner, 1995)), and epidermal growth factor (EGF; Carpenter, 1987). FGF-1, FGF-2, and HGF bind strongly to heparin (Lyon *et al.*, 1994; Mizuno *et al.*, 1994). On the other hand, PDGF and EGF bind weakly or almost do not interact with heparin (Jessell & Melton, 1992; LaRochell *et al.*, 1991).

FGF-1, FGF-2 and PDGF have proliferative activity on 3T3-L1 fibroblast, while EGF and HGF have no effect (Fig. 5). In serum-free medium supplemented by insulin, FGFs and PDGF have almost the same proliferative activity to 3T3-L1 fibroblasts.

Figure 6 shows the rescue effect of FGF-2 against the cytotoxicity of DS396, which exhibited the strongest cytotoxicity. HGF, PDGF, and EGF did not inhibit the cytotoxicity of DS396. Only FGF-2 was found to have a clear rescue effect.

When $MPS_{1.56}$ was introduced into the medium, FGF-1 and FGF-2 exhibited a rescue effect (Fig. 7). In the absence of FGFs, cell injury caused by membrane destruction was also observed by phase-contrast microscope.

PDGF as well as FGFs showed mitogenic activity (Fig. 5). However, in the presence of sulphated

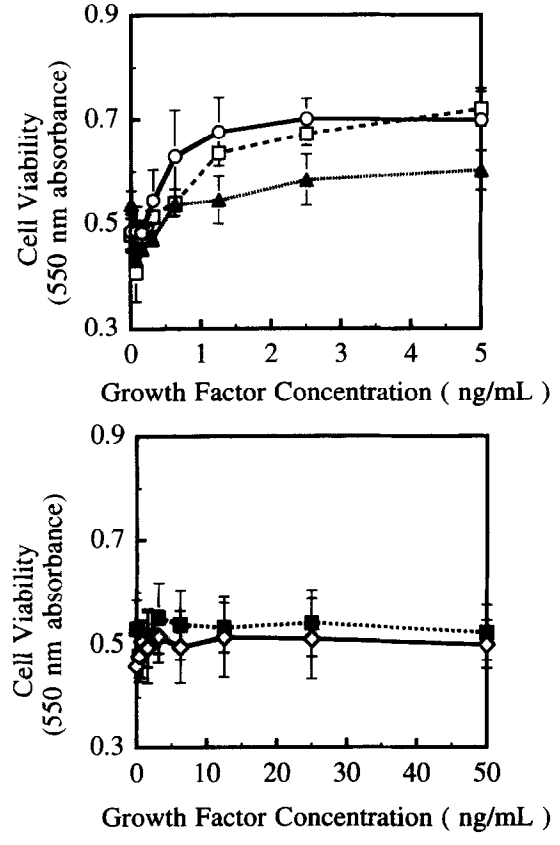

Fig. 5. Effect of growth factors on 3T3-L1 fibroblast proliferation (37°C, 5% CO_2, 48 h). □, FGF-2; ○, PDGF; ▲, FGF-1; ■, EGF; ◇, HGF.

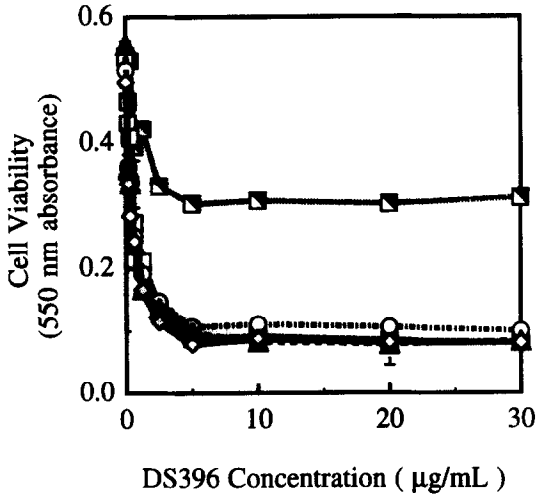

Fig. 6. Effect of growth factors on cytotoxicity caused by DS396 (37°C, 5% CO_2, 48 h). ◇, DS396 only; □, DS396 + EGF (50 ng/ml); ▲, DS396 + PDGF (5 ng/ml); ○, DS396 + HGF (50 ng/m); ◪, DS396 + FGF-2 (2.5 ng/ml).

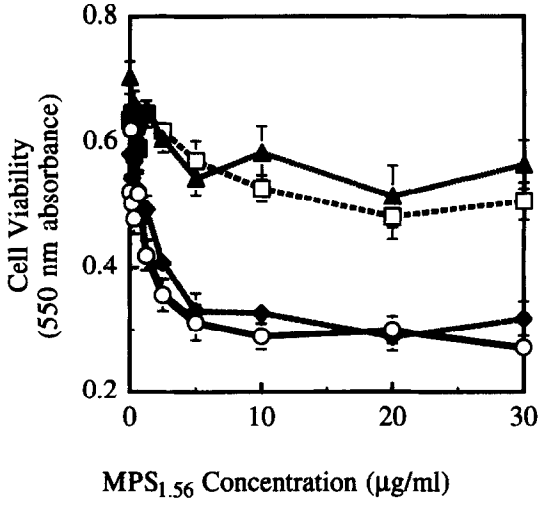

Fig. 7. Effect of FGF-1, FGF-2 and HGF on cytotoxicity caused by $MPS_{1.56}$ (37°C, 5% CO_2, 48 h). ○, $MPS_{1.56}$ only; ◆, $MPS_{1.56}$ + EGF (50 ng/ml); ▲, $MPS_{1.56}$ + FGF-1 (5 ng/ml); □, $MPS_{1.56}$ + FGF-2 (2.5 ng/ml).

polysaccharides that have both a high molecular weight and high degree of sulphation, cell viability was almost the same as in the absence of PDGF. FGF-1 and FGF-2 protected the cell from the damage caused by the sulphated polysaccharide, while PDGF had no such effect. It is unclear whether FGFs have a different mode of mitogenic action from PDGF or if FGFs have a peculiar signal pathway that is activated after cell damage. Because FGFs always act together with sulphated polysaccharides, this rescue effect is probably an inherent property of FGFs. Presently, whether the rescuing character of FGFs is to protect damaged cells from cell death or to activate the proliferation rate of cells that survived has yet to be determined (although the latter is unlikely to occur, since the results showed that the number of cells that survived appeared constant).

Hepatocyte growth factor (HGF) has heparin binding sites, and binds to the low affinity receptor on the cell surface, that is, HSPGs (K_d = 260–400 pM) (Tajima *et al.*, 1992; Naldini *et al.*, 1991). This is somewhat similar to that of FGFs. FGFs also interact with low affinity receptor, HSPGs (K_d = 2–200 nM) (Roghani *et al.*, 1994). However, the high affinity receptors for binding to HGF (*c-met* proto-oncogene product) are absent on the cell surface of the fibroblasts (Tajima *et al.*, 1992). If cytotoxicity of sulphated polysaccharide depends on their concentrated negative charge, neutralization of the strong charge of sulphated polysaccharide by growth factors might reduce cytotoxicity. However, it is rather unlikely that HGF or FGFs directly bound to the sulphated polysaccharide and resulted in decreased cytotoxicity of sulphated polysaccharide because the amounts of sulphated polysaccharides were approximately 1000-times that of growth factors. From these facts, it can be speculated that the rescue effect of FGFs might be receptor-mediated biological activity.

When 3T3-L1 fibroblasts were plated on polystyrene culture dishes for experiments, fibroblasts were treated by trypsin–EDTA solution in order to detach them from tissue culture flasks for subculture. Cell surface proteoglycans can be cleaved by trypsin (Rapraeger & Bernfield, 1985; Bame, 1993). In that situation, the cell membrane also undergoes mechanical damage. It is reasonable that cytotoxicity of sulphated polysaccharides acts more strongly on trypsin-treated cells and the cells damaged by sulphated polysaccharides perished slowly (data not shown). Initially, cytotoxicity of sulphated polysaccharide acts and proceeds continuously.

The effect of growth factor on the cells damaged by other ionic polymers

In order to determine the rescue effect of FGFs, we studied the effect of other polymers having a strong charge. These are poly-L-lysine (polycation) and polystyrene sulphonate (polyanion) (Fig. 8 and Fig. 9). Both polymers have strong charges and cytotoxicity. However, FGF-2 appeared to have no effect on cell damage caused by poly-L-lysine or polystyrene sulphonate. The rescuing effects of FGFs only acted on cytotoxicity of sulphated polysaccharides within the limit of these experiments. If the rescuing effect of FGFs is effective against the negative charge, it should be effective for polystyrene sulphonate, unless the latter has detergent effects that act on membrane destruction.

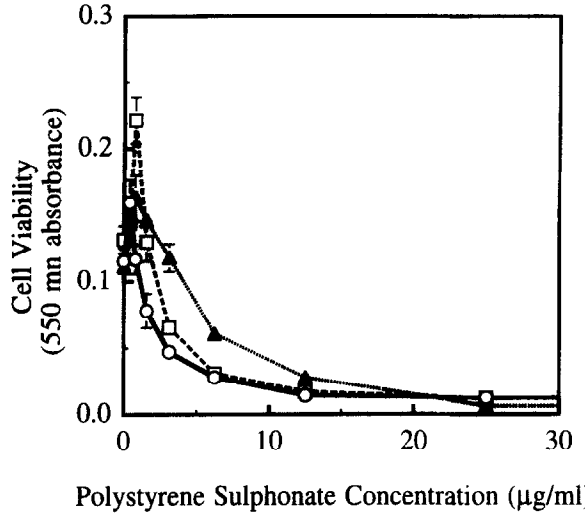

Fig. 8. Effect of polystyrene sulphonate on 3T3-L1 fibroblast (37°C, 5% CO_2, 48 h). ○, polystyrene sulphonate (PSS) only; ▲, PSS + FGF-2 (5 ng/ml); □, PSS + PDGF (10 ng/ml).

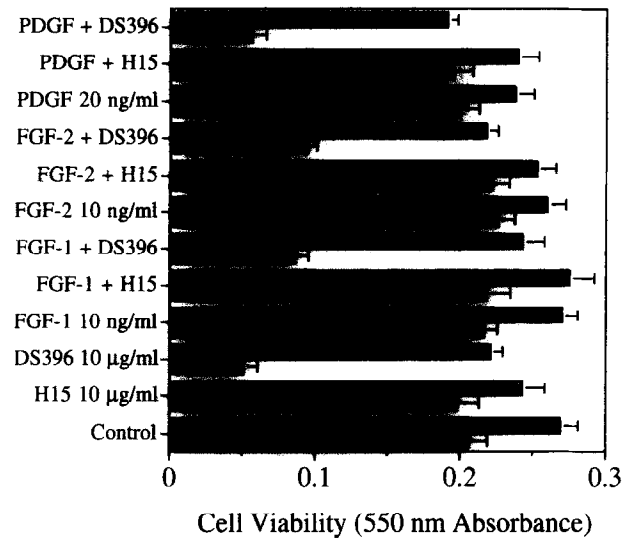

Fig. 10. Effect of sulphated polysaccharide on cell adhesion (37°C, 5% CO_2, 48 h). ■, before medium change; ■, after medium change.

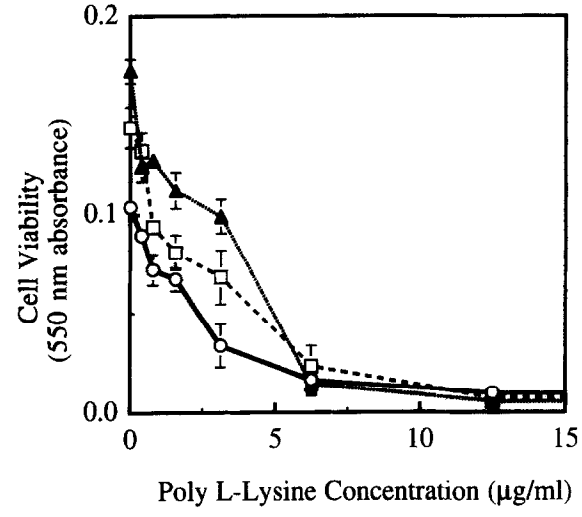

Fig. 9. Effect of poly L-lysine on 3T3-L1 fibroblast (37°C, 5% CO_2, 48 h). α, poly L-lysine (PLL) only; ▲: PLL + FGF-2 (5 ng/ml); □, PLL + PDGF (10 ng/ml).

Cell attachment assay

The presence or absence of sulphated polysaccharide in the medium has little or no effect at all on cell viability in initial time (Fig. 10). However, the force of cell attachments was significantly weakened by the addition of sulphated polysaccharide into the medium. The cytotoxicity of sulphated polysaccharide was not only by the action of cell membrane destination, but also by the reduction of the force of cell attachments. Addition of FGFs in the medium was not enough to recover the decrease of cell attachment force in initial time. Two hours pre-incubation in the medium containing 10% fetal bovine serum perfectly eliminated the cytotoxicity of sulphated polysaccharide (data not shown). In other words, the rescuing effects of FGFs did not take place rapidly.

CONCLUSION

The structure of heparan sulphate is highly heterogeneous, i.e., the total degree of sulphation is very low. However, it contains highly sulphated blocks (Turnbull & Gallagher, 1991). Heparan sulphate is not cytotoxic both *in vivo* and *in vitro*. On the other hand, heparin that is also present in a biological system (found in the liver or inside mast cells) exhibited slight cytotoxicity *in vitro* as mentioned. Moreover, based on the results of this study, highly sulphated polysaccharides, i.e., dextran sulphates and mannopyranan sulphates, exhibited cytotoxicity and the extent of cytotoxicity is dependent on the molecular weight and degree of sulphation.

Preliminary results demonstrated that the cytotoxicity of sulphated polysaccharides acts more strongly on damaged cells. When tissues are damaged, FGFs are probably released from the ECM by ECM-degradable enzymes, together with heparan sulphates. The highly sulphated regions of heparan sulphate that interact with FGFs (Walker *et al.*, 1994) may likewise be cytotoxic to injured cells. At that time, it may be evident that FGFs exhibit mitogenic activity and a rescuing effect. This is apparently a normal self-preserving mechanism of the living body. FGF-1 and FGF-2 displayed rescuing effects on the cytotoxicity caused by the sulphated polysaccharides. On the other hand, PDGF, HGF, and EGF did not show such effect. Therefore, the protecting effect seems to be a

characteristic unique to FGFs. Moreover, the rescue effect of FGFs is apparently effective for anions that can interact with FGFs (publication in progress). In this study, we have tried to present one of the multiple functions of FGFs, highlighting their potential, in terms of the rescuing effect. The mechanism of FGFs' rescuing effect is still unknown. Further investigation is necessary to elucidate this phenomenon.

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