

Modulation of Fibroblast Growth Factor-2 Receptor Binding, Signaling, and Mitogenic Activity by Heparin-Mimicking Polysulfonated Compounds

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ABSTRACT

Basic fibroblast growth factor (FGF-2) interacts with high-affinity tyrosine-kinase fibroblast growth factor receptors (FGFRs) and low-affinity heparan sulfate proteoglycans (HSPGs) in target cells. Both interactions are required for FGF-2-mediated biological responses. Here we report the FGF-2 antagonist activity of novel synthetic sulfonic acid polymers with distinct chemical structures and molecular masses (MMs). PAMPS [poly(2-acrylamido-2-methyl-1-propanesulfonic acid)], (MM $\approx$ 7,000–10,000), PAS [poly(anetholesulfonic acid)], (MM $\approx$ 9,000–11,000), PSS [poly(4-styrenesulfonic acid)], (MM = 70,000), and poly(vinylsulfonic acid) (MM = 2,000), inhibited FGF-2 binding to HSPGs and FGFRs in fetal bovine aortic endothelial GM 7373 cells. They also abrogated the formation of the HSPG/FGF-2/FGFR ternary complex, as evidenced by their capacity to prevent FGF-2-mediated cell-cell attachment of FGFR-1-overexpressing, HSPG-deficient Chinese hamster ovary cells to wild-type HSPG-bearing cells. Direct interaction

of the polysulfonates with FGF-2 was demonstrated by their ability to protect the growth factor from proteolytic cleavage. Accordingly, molecular modeling, based on the crystal structure of the interaction of FGF-2 with a heparin hexamer, showed the feasibility of docking PAMPS into the heparin-binding domain of FGF-2. In agreement with their FGF-2-binding capacity, PSS, PAS, and PAMPS inhibited FGF-2-induced cell proliferation in GM 7373 cells and murine brain microvascular endothelial cells. The antiproliferative activity of these compounds was associated with the abrogation of FGF-2-induced tyrosine phosphorylation of FGFR-1. Moreover, the polysulfonates PSS and PAS inhibited FGF-2-induced activation of mitogen-activated protein kinase-1/2, involved in FGF-2 signal transduction. In conclusion, sulfonic acid polymers bind FGF-2 by mimicking heparin interaction. These compounds may provide a tool to inhibit FGF-2-induced endothelial cell proliferation in angiogenesis and tumor growth.

Angiogenesis is the process of generating new capillary blood vessels. In the adult, the proliferation rate of endothelial cells is very low compared with many other cell types in the body. Physiological exceptions in which angiogenesis occurs under tight regulation are found in the female reproductive system and during wound healing. Uncontrolled endothelial cell proliferation is observed in tumor neovascular-

ization and in angioproliferative diseases (Pepper, 1997). Several molecules have been shown to stimulate endothelial cell proliferation in vitro and in vivo, and among them heparin-binding basic fibroblast growth factor (FGF-2) was one of the first to be characterized (Presta et al., 1986). The single-copy human FGF-2 gene encodes multiple FGF-2 isoforms with molecular mass (MM) ranging from 18,000 to 24,000 (Florkiewicz and Sommer, 1989). High-MM isoforms are colinear NH₂-terminal extensions of the better characterized MM 18,000 protein (Florkiewicz et al., 1991). Both low- and high-MM FGF-2 isoforms show angiogenic activity in vivo and induce cell proliferation, chemotaxis, and protease production in cultured endothelial cells (Presta et al., 1986; Gualandris et al., 1994; Ribatti et al., 1995) by interacting with high-affinity tyrosine-kinase FGF receptors (FG-

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ABBREVIATIONS: FGF-2, basic fibroblast growth factor; CHO, Chinese hamster ovary; ERK, mitogen-activated protein kinase; FCS, fetal calf serum; FGFR, FGF receptor; GAG, glycosaminoglycan; HSPG, heparan sulfate proteoglycan; MBEC, mouse brain microvascular endothelial 10027 cell; PAMPS, poly(2-acrylamido-2-methyl-1-propanesulfonic acid); PAS, poly(anetholesulfonic acid); PSS, poly(4-styrenesulfonic acid); PVS, poly(vinylsulfonic acid); HS, heparan sulfate; MM, molecular mass; CAM, chorioallantoic membrane.

FRs; Johnson and Williams, 1993) and low-affinity heparan sulfate proteoglycans (HSPGs) as polysaccharide (Schlessinger et al., 1995).

The physiological effects resulting from the interaction of FGF-2 with cell-associated and free HSPGs are manifold. HSPGs protect FGF-2 from inactivation in the extracellular environment and modulate the bioavailability of the growth factor (Saksela et al., 1988; Edelman et al., 1993). At the cell surface, free and cell-associated HSPGs may play contrasting roles in modulating the dimerization of FGF-2 and its interaction with FGFRs. For instance, free heparin induces FGF-2-FGFR interaction in heparan sulfate (HS)-deficient cells (Yayon et al., 1991). This relies on the capacity of the glycosaminoglycan (GAG) to form a ternary complex by interacting with both proteins (Guimond et al., 1993; Rusnati et al., 1994). In apparent contrast with these observations, free heparin inhibits the binding of FGF-2 to FGFRs when administered to cells bearing surface-associated HSPGs (Ishihara et al., 1993). This is probably due to the competition of free GAGs with cell-associated HSPGs and FGFRs for the binding to FGF-2. Thus, the bioavailability and the biological activity of FGF-2 on endothelial cells strictly depend on the extracellular GAG milieu, indicating the possibility of modulating the angiogenic activity of FGF-2 *in vivo* by using exogenous GAGs. Recent findings on the capacity of low MM heparin fragments administered systemically to reduce the angiogenic activity of FGF-2 support this hypothesis (Norrby and Ostergaard, 1996).

A further implementation of this hypothesis is that synthetic molecules that are able to interfere with the HSPG/FGF-2/FGFR interaction may act as angiogenesis inhibitors. In particular, heparin-mimicking, polyanionic compounds that are able to compete with HSPGs for growth factor interaction may be expected to hamper the binding of FGF-2 to the endothelial cell surface with consequent inhibition of its angiogenic capacity. Among such compounds are suramin (Pesenti et al., 1992), several suramin analogs (Firsching et al., 1995), and pentosan polysulfate (Zugmaier et al., 1992). The most extensively studied, polysulfonated naphtylurea suramin has been used with some benefit in clinical trials in cancer patients (Myers et al., 1992).

We recently reported that the sulfonic acid polymers poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPS), poly(anetholesulfonic acid) (PAS), and poly(4-styrenesulfonic acid) (PSS) are more potent inhibitors of neovascularization than suramin and pentosan polysulfate in the chick embryo chorioallantoic membrane (CAM), whereas the polyanionic poly(vinylsulfonic acid) (PVS) was ineffective (Liekens et al., 1997). Also, these sulfonic acid polymers exerted an antiangiogenic effect in the *in vitro* rat aorta-ring assay and inhibited FGF-2-induced human umbilical vein endothelial cell proliferation. Interestingly, a significant correlation was found between the angiostatic activity of these compounds in the CAM assay *in vivo* and their capacity to inhibit the FGF-2-induced mitogenic response *in vitro*, thus suggesting that FGF-2 is a target for sulfonic acid polymers (Liekens et al., 1997).

In the present study we have investigated the capacity of sulfonic acid polymers to interact with FGF-2 and to affect its biological activity *in vitro*. The results indicate that sulfonic acid polymers mimic functional features of heparin/HS by binding to FGF-2 and preventing its interaction with endo-

thelial cell surface HSPGs and FGFRs and by hampering the formation of the HSPG/FGF-2/FGFR ternary complex. Depending on the MM and structural features of the polysulfonate being tested, this interaction may result in the inhibition of the FGF-2-dependent intracellular signaling and mitogenic response in cultured endothelial cells.

Experimental Procedures

Materials

Suramin was obtained from Bayer AG (Leverkusen, Germany). PAMPS (MM = 7,000–10,000) was purchased from Monomer-Polymer & Dajac Laboratories (Feasterville, PA). PSS sodium salt (MM = 70,000) was obtained from Acros (Geel, Belgium). PAS sodium salt (MM = 9,000–11,000) was obtained from ICN (Costa Mesa, CA) and PVS sodium salt (MM = 2,000) was obtained from Sigma Chemical Co. (St. Louis, MO). Other PVS and PSS derivatives with various MM ranges were provided by Dr. P. Mohan, College of Pharmacy, University of Illinois (Chicago, IL). For the chemical structures of these compounds, see Fig. 1. Conventional heparin (MM = 13,600) was obtained from a commercial batch preparation of unfractionated sodium heparin from beef mucosa (1131/900 from Laboratori Derivati Organici S.p.A., Milan, Italy), which was purified from contaminants according to standard procedures. Human recombinant FGF-2 was obtained from Pharmacia-Upjohn (Milan, Italy) and hyaluronic acid was provided by Dr. M. Del Rosso (University of Florence, Italy).

Cell Cultures

Fetal bovine aortic endothelial GM 7373 cells were obtained from the Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ). GM 7373 cells were grown in Eagle's minimal essential medium containing 10% fetal calf serum (FCS), vitamins, and essential and nonessential amino acids (Presta et al., 1991). BALB/c mouse brain microvascular endothelial 10027 cells (MBECs) were grown in Dulbecco's modified minimum essential medium supplemented with 10% FCS. This spontaneously immortalized cell line was identified as endothelial on the basis of different phenotypic markers (Bastaki et al., 1997). Chinese hamster ovary (CHO)-K1 cells and A745 CHO cell mutants (provided by J.D. Esko, University of Birmingham, AL) were grown in Ham's F 12 medium supplemented with 10% FCS. A745 CHO cells harbor a mutation that inactivates the xylosyltransferase that catalyzes the first sugar transfer step in GAG synthesis (Esko, 1991).

Cell Transfection

The IIIc variant of murine FGFR-1 cDNA (provided by A. Mansukhani and C. Basilico, New York University Medical Center, New

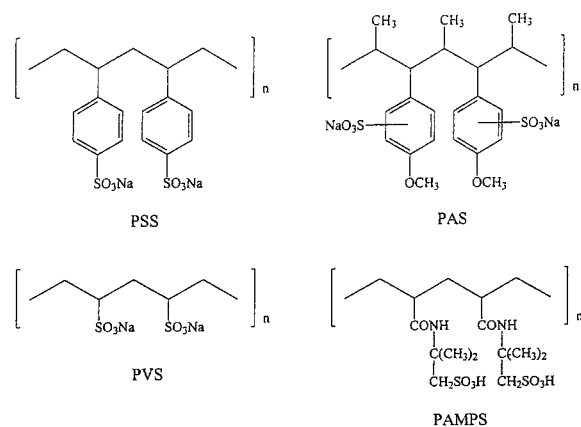


Fig. 1. Structural formulae of the sulfonic acid polymers.

York, NY) was cloned in the pCEP4 expression vector (Invitrogen BV, Leek, the Netherlands). Then, A745 CHO cells were transfected with the construct by the calcium phosphate precipitation protocol (Sambrook et al., 1989) and selected with 500 $\mu\text{g}/\text{ml}$ hygromycin. Resistant clones were tested for ^{125}I -FGF-2 binding capacity (Coltrini et al., 1994). The A745 CHO *flg*-1A clone, bearing about 30,000 FGFR-1 molecules per cell, was used for the FGF-2-mediated cell-cell adhesion assay.

The same FGFR-1 cDNA was also cloned in pZipNeoSV(X) and GM 7373 cells were transfected with the construct as above. Resistant clones were tested for ^{125}I -FGF-2 binding capacity and the GM 7373 *flg*-AA clone, bearing about 70,000 FGFR-1 molecules per cell, was used for FGFR-1 tyrosine phosphorylation experiments.

Tyrosine Phosphorylation of FGFR-1

GM 7373 *flg*-AA cells grown to 80 to 90% confluence in 35-mm-diameter dishes were treated for 10 min with 10 ng/ml of FGF-2 after preincubation for 30 min at room temperature with or without the polysulfonated compound under test (final concentration of 100 μM), without changing the medium. At the end of the incubation, cells were washed briefly with ice-cold PBS, lysed in reducing SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, sonicated at 50 W for 10 s, and boiled. All the samples were then subjected to SDS-PAGE under reducing conditions. Proteins were transferred electrophoretically onto a PolyScreen PVDF transfer membrane (Du Pont de Nemours, Cologno Monzese, Italy) and immunoblotted with antiphosphotyrosine antibody (4G10, Upstate Biotechnology Inc., Lake Placid, NY) or antiphospho-mitogen-activated protein kinase (ERK)-1/2 antibody (New England Biolabs, Inc., Beverly, MA). Membranes were incubated sequentially with horseradish peroxidase-conjugated secondary antibodies and with Renaissance chemiluminescence reagents (Du Pont de Nemours) according to manufacturer's instructions, and then exposed to Du Pont de Nemours Reflection films. Quantitation of the intensity of the bands was performed by soft-laser scanning of the film.

^{125}I -FGF-2 Binding Assay

Human recombinant FGF-2 was labeled with Na^{125}I (37 GBq/ml; Amersham International, Amersham, UK) using Iodogen (Pierce Chemical, Rockford, IL) as described (Coltrini et al., 1994). Twenty-four hours after plating in 24-well dishes at a density of 70,000 cells/cm², cells were washed three times with ice-cold PBS and incubated for 2 h at 4°C in binding medium (serum-free medium containing 0.15% gelatin, 20 mM HEPES, pH 7.5) with 10 ng/ml of ^{125}I -FGF-2 in the absence or in the presence of increasing concentrations of the compound being tested. Then, after a PBS wash, cells were washed twice with 2 M NaCl in 20 mM HEPES buffer (pH 7.5) to remove ^{125}I -FGF-2 bound to low-affinity HSPGs and twice with 2 M NaCl in 20 mM sodium acetate (pH 4.0) to remove ^{125}I -FGF-2 bound to high-affinity FGFRs (Coltrini et al., 1994). Nonspecific binding was measured in the presence of a 100-fold molar excess of unlabeled FGF-2 and subtracted from all values.

FGF-2-Mediated Cell-Cell Adhesion Assay

This assay was performed as described (Richard et al., 1995) with minor modifications. Briefly, wild-type CHO-K1 cells were seeded in 24-well plates at 52,000 cells/cm². After 24 h, cell monolayers were washed with PBS and incubated with 3% glutaraldehyde in PBS for 2 h at 4°C. Fixation was stopped with 0.1 M glycine and cells were washed extensively with PBS. Then, A745 CHO *flg*-1A cells (52,000 cells/cm²) were added to CHO-K1 monolayers in serum-free medium plus 10 mM EDTA with no addition or with 30 ng/ml FGF-2 in the absence or in the presence of increasing concentrations of the polysulfonate being tested. After 2 h of incubation at 37°C, unattached cells were removed by washing twice with PBS and A745 CHO *flg*-1A cells bound to the wild-type CHO monolayer were counted under an inverted microscope at 125X magnification. Data are expressed as

the mean of the cell counts of three microscopic fields chosen at random. All experiments were performed in duplicate and were repeated twice with similar results.

Proteolytic Digestion of FGF-2

The protective effect of the polyanions on tryptic digestion of FGF-2 was evaluated as described by Coltrini et al. (1993). Briefly, 1- μg aliquots of the growth factor were incubated at 37°C for 5 min in 50 mM Tris/HCl pH 7.5, in the presence of increasing concentrations of the test compounds. Then 60 ng of trypsin were added and digestion was allowed to proceed at 37°C for 3 h. In some experiments, FGF-2 was denatured at 90°C for 2 min before trypsin digestion, a treatment which completely abolishes FGF-2 biological activity. At the end of the trypsin digestion, all samples were supplemented with SDS-PAGE reducing sample buffer, heated at 100°C for 2 min, and subjected to SDS-PAGE (15%), after which the gels were silver-stained. The amount of undigested protein in a given lane was estimated by computerized image analysis.

Computer Modeling of FGF-2/PAMPS Complexes

Models for 14 mers of PAMPS were created using MacroModel version 5.0 (Mohamadi et al., 1990) and subjected to energy minimization and molecular dynamics using the AMBER force field contained in the MacroModel package in an attempt to find stable, low-energy conformations. The crystal structure of FGF-2 complexed with a heparin sulfate hexamer (Protein Database code 1BFC; Faham et al., 1996) was used as the starting point for a manual procedure to dock the PAMPS models to FGF-2 using MidasPlus (Ferrin et al., 1988). Models were constructed where the sulfonate groups of PAMPS mimicked the sulfate groups of the heparin hexamer and these models were subjected to a simple energy minimization procedure using AMBER (Pearlman et al., 1995) with the FGF-2 constrained to the structure adopted in the bFGF/heparin complex. Figure 5 was created using Bobscript (Esnouf, 1997), a version of MolScript (Kraulis, 1991), and rendered with Raster3D (Merritt and Murphy, 1994).

Cell Proliferation Assays

GM 7373 cells. Cells were seeded in 24-well plates at 70,000 per cm². After overnight incubation, cells were incubated for 24 h in fresh medium containing 0.4% FCS and 10 ng/ml of FGF-2 in the presence of increasing concentrations of the test compounds. At the end of the incubation, cells were trypsinized and counted in a Burkert chamber. Control cultures incubated with no addition or with 10 ng/ml of FGF-2 undergo 0.1 to 0.2 and 0.7 to 0.8 cell population doublings, respectively (Presta et al., 1991).

MBECs. Cells were seeded in 24-well plates at 5,000 per cm². After 16 h, cells were incubated in fresh medium with 10% FCS and 10 ng/ml of FGF-2 in the presence of increasing concentrations of the test compounds. Medium was changed after 2 days. On day 5, cells were trypsinized and counted (Bastaki et al., 1997).

Western Blot Analysis of ERK-2 in MBECs

Twenty-four hours after plating in 35-mm dishes at a density of 50,000 cells/cm², MBECs were incubated for 1 h in fresh medium containing 10% FCS and the test compounds. Cells were then treated for 20 min with 20 ng/ml FGF-2, washed with PBS, and lysed in 200 μl lysis buffer [20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 2 mM EDTA (pH 7.4), 1% Triton X-100, 10% glycerol, 1 mM sodium vanadate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 25 mM glycerophosphate, and 10 $\mu\text{g}/\text{ml}$ leupeptin]. Lysates were centrifuged for 10 min at 15,000g, and the protein concentration was determined. SDS-PAGE and Western blot analysis of the cell lysates were performed as described previously (Besser et al., 1995) using anti-ERK-2 antibodies (provided by Dr. Y. Nagamine, Friedrich Miescher Institute, Basel, Switzerland). Phosphorylation of ERK-2 was evidenced as a mobility shift on the gel (Besser et al., 1995).

Results

Effect of Sulfonic Acid Polymers on FGF-2 Binding to Low- and High-Affinity Receptors. Recently, a significant correlation was found between the antiangiogenic activity of a new class of sulfonic acid polymers (PAMPS, PAS, PVS, and PSS) and their capacity to inhibit FGF-2-induced mitogenic response in human umbilical vein endothelial cells. Based on this knowledge, we assessed the ability of these compounds to interact with FGF-2 and to prevent its interaction with cell surface binding sites.

In a first set of experiments, PAMPS, PAS, PVS, and PSS were evaluated for their capacity to affect FGF-2 binding to low-affinity HSPGs and high-affinity tyrosine kinase FGFRs in endothelial GM 7373 cells. As shown in Fig. 2, these molecules inhibited ^{125}I -FGF-2 binding to low- and high-affinity sites in GM 7373 cells with a different potency, PSS

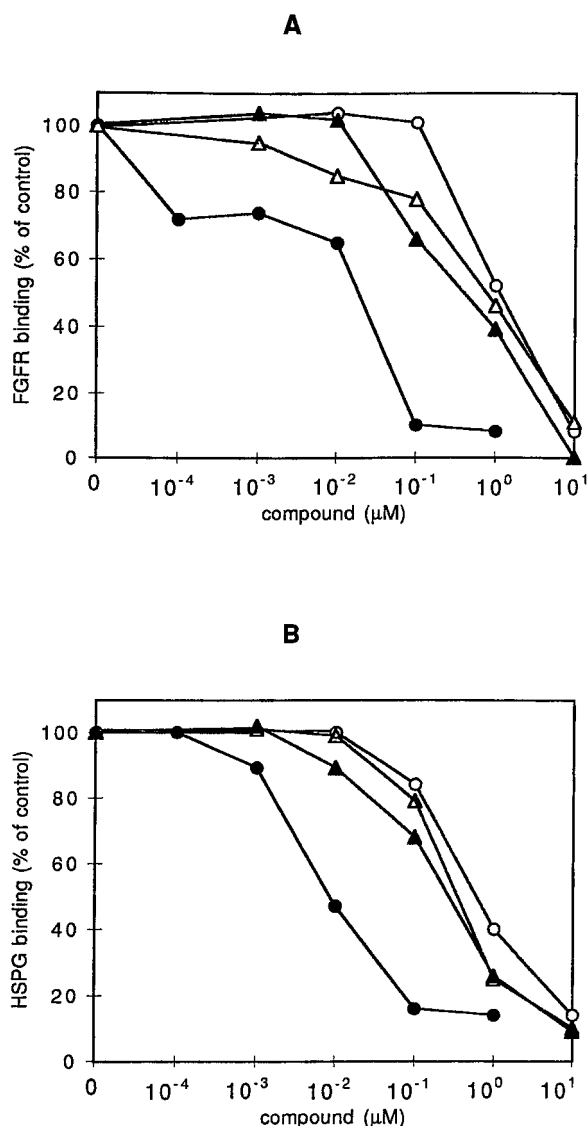


Fig. 2. Effect of polysulfonated compounds on receptor binding of FGF-2: GM7373 cell cultures, incubated with 10 ng/ml ^{125}I -FGF-2 in the presence of increasing concentrations of PSS (●), PAS (○), PAMPS (▲), or PVS (△). After 2 h of incubation at 4°C, the radioactivity associated with FGFRs (A) and HSPGs (B) was measured. Data are expressed as a percentage of the binding in the absence of compounds. Similar results were obtained in three independent experiments.

being the most effective. For each compound, the concentrations causing 50% inhibition (ID_{50}) of FGF-2 binding to FGFRs and HSPGs were comparable (0.02 μM versus 0.01 μM for PSS; 0.39 μM versus 0.27 μM for PAMPS, 0.75 μM versus 0.59 μM for PAS, and 1.11 μM versus 0.34 μM for PVS), suggesting that the antagonist activity of sulfonic acid polymers depends on their direct interaction with FGF-2 rather than with FGF-2 binding site(s) on the cell surface.

In a second set of experiments, the sulfonic acid polymers were evaluated for their capacity to prevent the formation of the HSPG/FGF-2/FGFR ternary complex. For this purpose, we used an experimental model in which the disruption of the complex abolishes FGF-2-mediated cell-cell attachment of HSPG-deficient CHO mutants stably transfected with FGFR-1 to wild-type CHO-K1 cells expressing HSPGs but not FGFR (Richard et al., 1995). In this model, a significant number of FGFR-1 transfectants adhere to the HSPG-bearing monolayer in the presence of 30 ng/ml FGF-2 (140 ± 20 cells per field) but not in the absence of the growth factor (20 ± 4 cells per field). Specificity of the cell-cell interaction was demonstrated by the inability of CHO mutants non-transfected with FGFR-1 to adhere to the monolayer in the presence of FGF-2 (15 ± 3 cells per field).

Again, all the molecules studied exerted an inhibitory activity on FGF-2-mediated cell-cell adhesion, PSS being the most effective (ID_{50} equal to 0.001 μM , 0.06 μM , 0.12 μM , and 0.07 μM for PSS, PAMPS, PAS, and PVS, respectively; Fig. 3). From the data presented in Figs. 2 and 3, it is evident that sulfonic acid polymers prevent the interaction of FGF-2 with its low- and high-affinity receptors. Interestingly, PSS appears to be the most potent among the polysulfonates studied, its activity being similar to that exerted by conventional heparin at equimolar doses and at least 1000-fold more potent than that exerted by the hexasulfonate and FGF-2 antagonist suramin when tested under the same experimental conditions (ID_{50} of 0.001 and 0.005 μM for PSS and heparin, respectively, versus 19 μM for suramin).

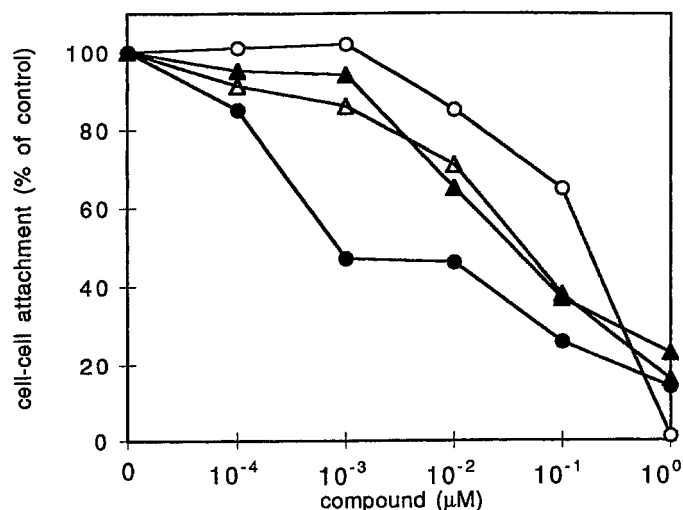


Fig. 3. Effect of polysulfonated compounds on FGF-2-mediated cell-cell adhesion. HSPG-deficient FGFR-1 transfectants were added to wild-type CHO monolayers in serum-free medium with 30 ng/ml FGF-2 in the presence of increasing concentrations of PSS (●), PAS (○), PAMPS (▲), or PVS (△). After 2 h of incubation at 37°C, the bound cells were counted under an inverted microscope. Experiments were performed in duplicate and were repeated twice with similar results.

Sulfonic Acid Polymers Prevent Trypsin Digestion of FGF-2. Previous experiments have demonstrated the capacity of heparin and suramin to protect FGF-2 from trypsin digestion by binding to the growth factor molecule (Coltrini et al., 1993). On this basis, to assess the possibility of a direct interaction of sulfonic acid polymers with the growth factor, 1- μ g aliquots of FGF-2 were incubated with trypsin in the presence of decreasing concentrations of the test compound. After 3 h incubation at 37°C, the amount of undegraded FGF-2 was quantified by SDS-PAGE followed by silver staining of the gel and computerized image analysis of the corresponding band. As shown in Fig. 4, all of the test compounds protected FGF-2 from proteolytic cleavage. This protective effect was dose-dependent, PSS being the most effective, and all the compounds possessed a molar potency higher than that of suramin. Under our experimental conditions, one molecule of PSS or PAMPS was able to protect from proteolytic cleavage up to ten molecules of FGF-2 whereas one molecule of PAS or of PVS was able to protect only one molecule of the growth factor. Furthermore, none of the compounds was able to protect heat-denatured FGF-2 from trypsin digestion at concentrations that completely prevented the degradation of the native molecule. This rules out the possibility that sulfonic acid polymers may inhibit the activity of trypsin and confirms the requirement of a proper three-dimensional conformation of FGF-2 for its recognition by polysulfonates.

Computer Model for the Interaction of Sulfonic Acid Polymers with FGF-2. The above described findings demonstrate that the sulfonic acid polymers, having heterogeneous chemical structures and sizes, are able to interact with native FGF-2 and to mimic functional properties of heparin, thus preventing the proteolytic cleavage of the growth factor and inhibiting its receptor binding activity. Faham et al. (1996) demonstrated the presence of a heparin-binding domain on the surface of the FGF-2 molecule when examining

the crystal structure of a heparin-derived hexasaccharide complexed to FGF-2. This model was used as a framework to investigate the interaction of the sulfonic acid polymers (i.e., PAMPS) with the heparin-binding site of FGF-2.

During polymerization, each 2-acrylamido-2-methyl-1-propanesulfonic acid monomer develops an asymmetric center at the middle carbon of each acrylamido unit, giving rise to a vast number of possible PAMPS oligomers depending on the absolute configuration at each chiral center. Several models of PAMPS oligomers were created and it was noted that a stable intramolecular hydrogen bond could be formed between adjacent acrylamido units when they had equivalent absolute configurations. In this case, a chain of hydrogen-bonded amide units could form around a central hydrocarbon chain, producing a helical structure with the sulfonate groups evenly spaced on the surface and about 9 to 10 Å apart.

Helical PAMPS models of either handedness could be docked into the heparin-binding site of FGF-2 by simple rearrangements of adjacent *t*-butylsulfonate groups without distorting the stable helical structure. However, there seemed to be a slight preference for the right-handed helix. In the preferred model (Fig. 5), the sulfonate groups interact with the same residues as the heparin sulfate groups, although one of the sulfonate groups adopts a different orientation and forms a strong hydrogen bond with the main chain nitrogen of K136.

Effect of Sulfonic Acid Polymers on the Biological Activity Exerted by FGF-2 on Cultured Endothelial Cells. The capacity of sulfonic acid polymers to affect the interaction of FGF-2 with endothelial GM 7373 cell surface and to prevent the formation of the HSPG/FGF-2/FGFR ternary complex (see above), prompted us to assess the effect of these polysulfonates on FGF-2-induced endothelial cell proliferation in vitro. As shown in Fig. 6, PSS, PAS, and PAMPS caused a dose-dependent inhibition of the mitogenic activity

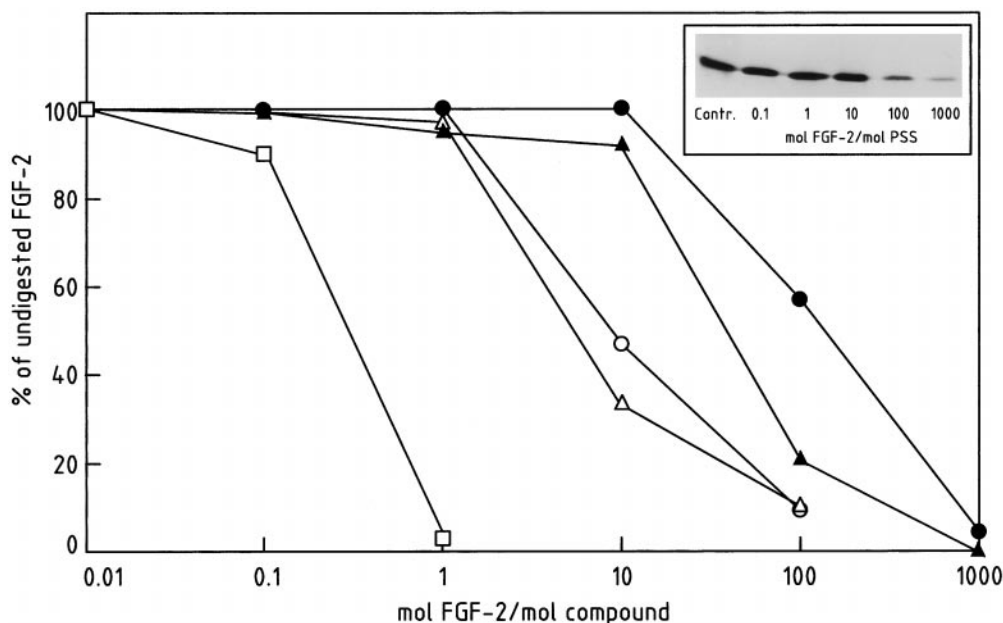


Fig. 4. Effect of different polysulfonates on FGF-2 tryptic cleavage. Aliquots (1 μ g) of FGF-2 were incubated at 37°C for 3 h with trypsin in the presence of decreasing concentrations of PSS (●), PAS (○), PAMPS (▲), PVS (Δ), or suramin (□) to obtain the indicated molar ratios. Samples were then analyzed by SDS-PAGE. Undigested FGF-2 in each lane of the gel was estimated by computerized image analysis. Inset: SDS-PAGE of FGF-2/PSS at increasing molar ratios.

of FGF-2 in GM 7373 cells with ID_{50} values equal to 0.004, 0.09, and 3.3 μ M, respectively. In keeping with its lack of antiangiogenic activity in vivo (Liekens et al., 1997), PVS did not exert a significant inhibition of FGF-2-induced cell proliferation at concentrations that were sufficient to cause an almost complete inhibition of 125 I-FGF-2 interaction with GM 7373 cells in the receptor binding assays. Similar results were obtained when the sulfonic acid polymers were evaluated for their capacity to inhibit FGF-2-induced cell proliferation in MBECs (Fig. 7A).

Next, we investigated the effect of these compounds on FGF-2-induced tyrosine phosphorylation of FGFR-1 and of intracellular proteins involved in FGF-2 signal transduction. For this purpose, FGFR-1 overexpressing GM 7373 *flg*-AA cells were preincubated with the different polysulfonated compounds followed by exposure to FGF-2. The cells were subsequently lysed, subjected to SDS-PAGE, and immunoblotted with antiphosphotyrosine antibody (Fig. 8, A and B) or anti-phospho-ERK-1/2 antibody (Fig. 8, C and D). As shown in Fig. 8, A and B, all compounds, except PVS, inhibited FGF-2-induced FGFR-1 phosphorylation. However, only the aromatic compounds PSS and PAS reduced ERK-1/2 activation (Fig. 8, C and D). Similar results were obtained in MBECs in which ERK phosphorylation, evaluated as a mobility shift of ERK-2 in SDS-PAGE (Besser et al., 1995), was inhibited by PSS and PAS whereas PAMPS and PVS were ineffective (Fig. 7B).

Previous reports have shown the importance of the size of the polysaccharide chain in the interaction of heparin/HS with FGF-2 and in the modulation of the biological activity of the growth factor. Indeed, even though the minimal FGF-2-binding sequence in HS has been identified as a pentasaccharide, a dodecasaccharide chain is required for the modulation of the biological activity of FGF-2 (Walker et al., 1994).

Similarly, both the very low-MM PVS and high-MM PSS bind to FGF-2 but only the latter is able to affect the mitogenic activity of the growth factor in cultured endothelial cells. Therefore, PVS and PSS polymer fractions of different size were evaluated for their ability to inhibit the FGF-2-induced mitogenic response in MBECs. As shown in Fig. 9A, the inhibitory activity of PSS fractions with MM decreasing from 70,000 to 1,800 was progressively reduced as a function of size, MM 1,800 PSS being devoid of any antiproliferative activity. Within the PVS series, fractions from 2,000 to 11,500 did not confer any FGF-2-antagonist activity (Fig. 9B).

Discussion

A dual receptor mechanism has been proposed for FGF-2 in which interaction of the growth factor with nonsignaling low-affinity HSPGs is required for its binding to the high-affinity tyrosine kinase FGFRs. FGFR occupancy will then trigger an intracellular signal cascade leading to multiple biological responses, including cell proliferation, migration, differentiation, protease production, and angiogenesis (Schlessinger et al., 1995).

Our data suggest that polysulfonated compounds with distinct chemical structures and sizes act as FGF-2 antagonists by mimicking some of the functional features of soluble heparin/HS. This is shown by the capacity of polysulfonates to impair the binding of FGF-2 to both HSPGs and FGFRs in cultured endothelial cells. Likewise, PSS, PAS, PAMPS, and PVS were found to abrogate FGF-2-mediated attachment of HSPG-deficient FGFR-1-transfected CHO mutants to a monolayer of wild-type HSPG-bearing CHO-K1 cells, which indicates that polysulfonates prevent the formation of the HSPG/FGF-2/FGFR ternary complex.

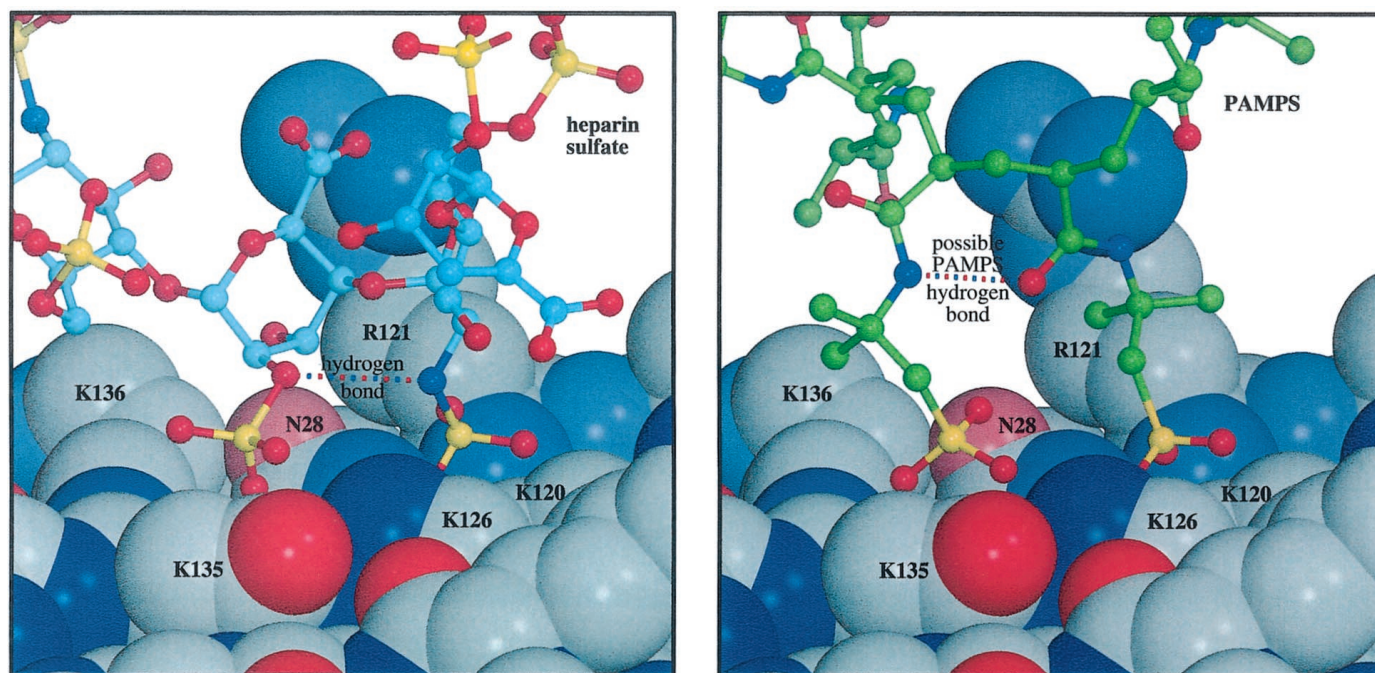


Fig. 5. The crystal structure for the FGF-2/heparin hexamer complex (Faham et al., 1996; left) and our model for the interaction between PAMPS and FGF-2 (right). FGF-2 is shown as an atom-colored space-filling model (with key residues in the binding site labeled), and the heparin sulfate and PAMPS models are shown as atom-colored ball-and-stick models with pale blue and green carbon atoms, respectively. Possible hydrogen bonds within the ligands, which may contribute to the stability of the complexes, are shown.

Polysulfonates interact directly with FGF-2, as shown by their ability to protect FGF-2 from trypsin digestion. It must be pointed out that polysulfonates failed to bind heat-denatured FGF-2, implying that the native conformation of the growth factor is required for the interaction. Similar results were obtained previously for the FGF-2/heparin interaction (Coltrini et al., 1993), where negatively charged sulfate groups of heparin were found to bind to basic amino acid residues of FGF-2. Indeed, X-ray crystallography has identified a cluster of noncontiguous positively-charged amino acids that form a "basic region" in the three dimensional structure of FGF-2. This cluster is able to interact with one to two sulfate groups, thus representing a putative heparin-binding region (Eriksson et al., 1993; Thompson et al., 1994). Recently, a specific heparin binding site within FGF-2 has been identified unambiguously by analysis of the crystal structures of heparin-derived hexasaccharides complexed with FGF-2 (Faham et al., 1996). Based on the coordinates of the FGF-2/hexasaccharide complex, we built a computer model for the FGF-2/PAMPS complex. The results suggest that the sulfonate groups of PAMPS may mimic sulfated heparin in its interaction with FGF-2. Although it would not be feasible to attempt to model all possible conformations of a molecule as large and complex as PAMPS, the modeling study showed the possibility of forming stable conformations that might then bind to the heparin-binding site of FGF-2 without distorting the structure of the protein. Even though extended regular helical structures are unlikely to be present in PAMPS, a molecule that probably contains a mixture of absolute conformations at the asymmetric carbon atoms, our model suggests that sulfonate groups within limited helical portions of the PAMPS molecule may bind FGF-2. Alternatively, other low-energy conformations of mixed stereochemistry may also present sulfonate groups in the proper position to enable complex formation. Taken together, our data provide compelling evidence that polysulfonates interact with the basic heparin-binding domain of FGF-2 via their sulfonate groups.

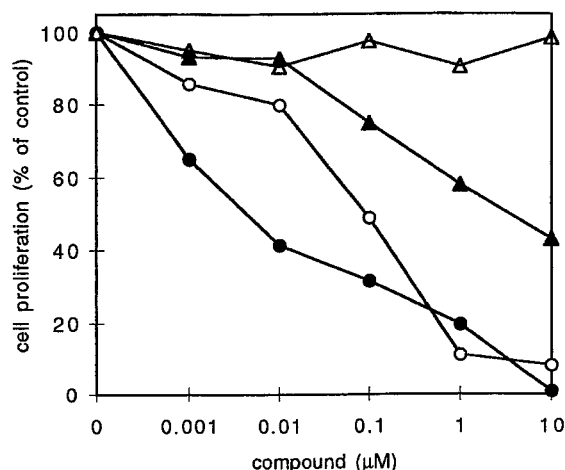


Fig. 6. Effect of the polysulfonates on FGF-2-induced proliferation in GM 7373 cells. GM 7373 cells were incubated with 10 ng/ml FGF-2 in the presence of increasing concentrations of PSS (●), PAS (○), PAMPS (▲), or PVS (Δ). After 24 h of incubation, the cells were trypsinized and counted in a Burkner chamber. Data are expressed as a percentage of proliferation compared with control cultures. Experiments were performed in duplicate and were repeated twice with similar results.

Despite the fact that all the polysulfonates tested are able to bind to FGF-2, they differ significantly in their FGF-2 antagonist activity. PSS, PAMPS, and PAS were found to prevent the binding of ^{125}I -FGF-2 to FGFR at 4°C with a potency similar to that shown for the inhibition of the FGF-2-mediated mitogenic response in GM 7373 and MBECs. In contrast, PVS did not affect FGF-2-induced mitogenesis even at concentrations that abolished the binding at 4°C of the growth factor to its receptors. Accordingly, PSS, PAS, and PAMPS drastically reduced short-term FGF-2-induced FGFR-1 phosphorylation in FGFR-1 overexpressing GM 7373 *flg*-AA cells whereas PVS was ineffective. However, inhibition of FGFR-1 phosphorylation did not result in the abrogation of ERK-1/2 activation in PAMPS-treated cells. In theory, the inhibitory effect exerted by these antagonists on FGF-2/FGFR interaction reflects a decrease in the initial rate of binding of FGF-2 to FGFR with no decrease in the total amount of ligand bound to the receptor at equilibrium. Indeed, heparin decreases the rate of binding of FGF-2 to FGFR-1 and FGFR-2 to about 30% of the rate observed in the absence of the antagonist, with no effect on the total amount of FGF-2 bound at equilibrium (Moscatelli, 1992). Thus, our observations indicate that a reduced rate of interaction of FGF-2 with its receptor may differently affect mitogenesis,

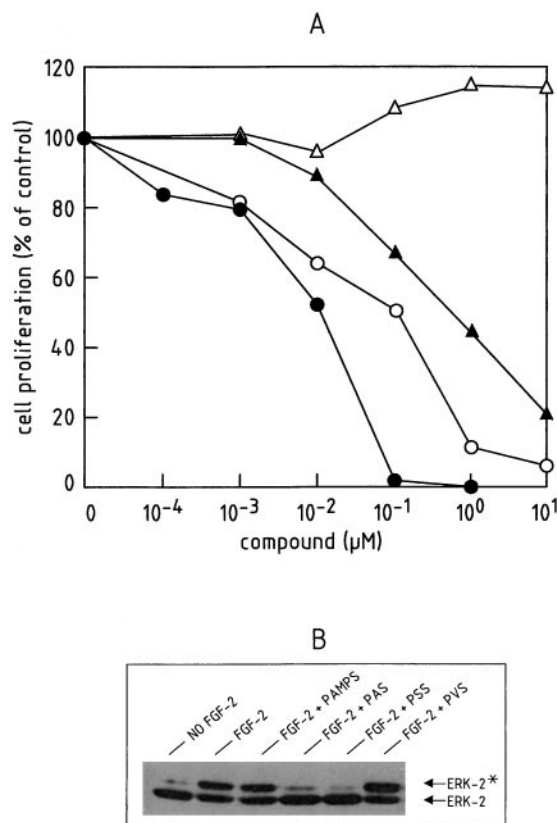


Fig. 7. Effect of polysulfonates on FGF-2 activity in MBECs. MBECs were incubated in the presence of 10 ng/ml FGF-2 and increasing concentrations of PSS (●), PAS (○), PAMPS (▲), or PVS (Δ). Medium was replaced every other day and cells were counted on day 5. Data are expressed as a percentage of proliferation compared with nontreated cell cultures. Similar results were obtained in three independent experiments (A). Cell extracts from control and FGF-2-treated cultures either in the absence or the presence of PSS (1 μM), PAS (10 μM), PAMPS (10 μM), or PVS (10 μM) were analyzed by Western blot using antibodies against ERK-2. Activation (phosphorylation) of ERK-2 is evidenced as a mobility shift on the gel (B). * indicates the phosphorylated isoform.

short-term phosphorylation of FGFR-1, and/or ERK-1/2 activation depending upon the antagonist being tested. The different FGF-2 antagonist activity exerted by PSS, PAMPS, PAS, and PVS may therefore reflect distinct chemical characteristics of the polysulfonate, including size, backbone structure, and presentation of the sulfonate group(s) to the growth factor, which may result in temporal and conformational differences in the ability of the ligand to interact with its receptor.

The distinct role of the different sulfate groups of heparin in FGF-2 interaction and in the modulation of the biological activity of the growth factor has been reported. Studies using selectively desulfated heparins revealed an absolute requirement for *N*- and 2-*O*-sulfate groups in FGF-2 binding, whereas 6-*O*-sulfates may be involved in FGFR binding and formation of the HSPG/FGF-2/FGFR ternary complex (Rusnati et al., 1994). This was supported by the fact that 6-*O*-desulfated heparin retains the capacity to bind FGF-2 without affecting FGF-2-induced mitogenesis (Guimond et al., 1993). Moreover, the size of the heparin fragment is of importance for the modulation of FGF-2-induced biological responses. Heparin-derived hexamers bind FGF-2 and inhibit FGF-2/HSPG interaction, whereas the smallest biologically active heparin fragment contains at least 8 to 12 saccharide residues (Guimond et al., 1993; Ishihara et al., 1993; Walker et al., 1994). These data can be interpreted on the basis of the capacity of GAGs to form ternary complexes by interacting with both ligand and receptor proteins. It is also possible that the dodecasaccharide interacts with two FGF-2 molecules, thereby stabilizing a FGF-2 dimer (Herr et al., 1997), which,

in turn, may facilitate FGFR dimerization, *trans*-phosphorylation, and signaling (Thompson et al., 1994; Spivak-Kroizman et al., 1994).

We investigated whether the FGF-2 antagonist activity of the polysulfonates depends on their size. For this purpose, different MM fractions of PSS were assessed for their ability to abolish FGF-2-induced mitogenesis. A decrease in the MM of PSS was paralleled by a decreased capacity to inhibit FGF-2 activity, the minimum active fraction having a MM of 5,400. A PSS fragment of MM 1,800 was completely ineffective, thus confirming the importance of the size of the polysulfonate in mediating FGF-2 antagonist activity. On the other hand, PVS fractions with MM as high as 11,500 did not acquire an anti-FGF-2 activity, indicating that the lack of antagonist activity of this polysulfonate also depends on chemical features other than size and the mere presence of sulfonate groups. For instance, stiffness of the molecule and the presence of aromatic rings may represent additional structural features required to interfere with FGF-2 activity. Indeed, PSS and PAS, which bear aromatic rings, abolished FGF-2-induced FGFR-1 phosphorylation, significantly reduced ERK-1/2 activation, and were the most potent in inhibiting FGF-2-induced mitogenesis. In contrast, PAMPS, a nonaromatic compound, was less effective as a FGF-2 antagonist and did not prevent ERK-1/2 activation.

We have demonstrated previously that PAMPS, PAS, and PSS inhibit microvessel formation in the rat aorta-ring assay and prevent the vascularization of the chick embryo CAM. In contrast, PVS did not exert antiangiogenic activity in either system (Liekens et al., 1997). It must be pointed out that

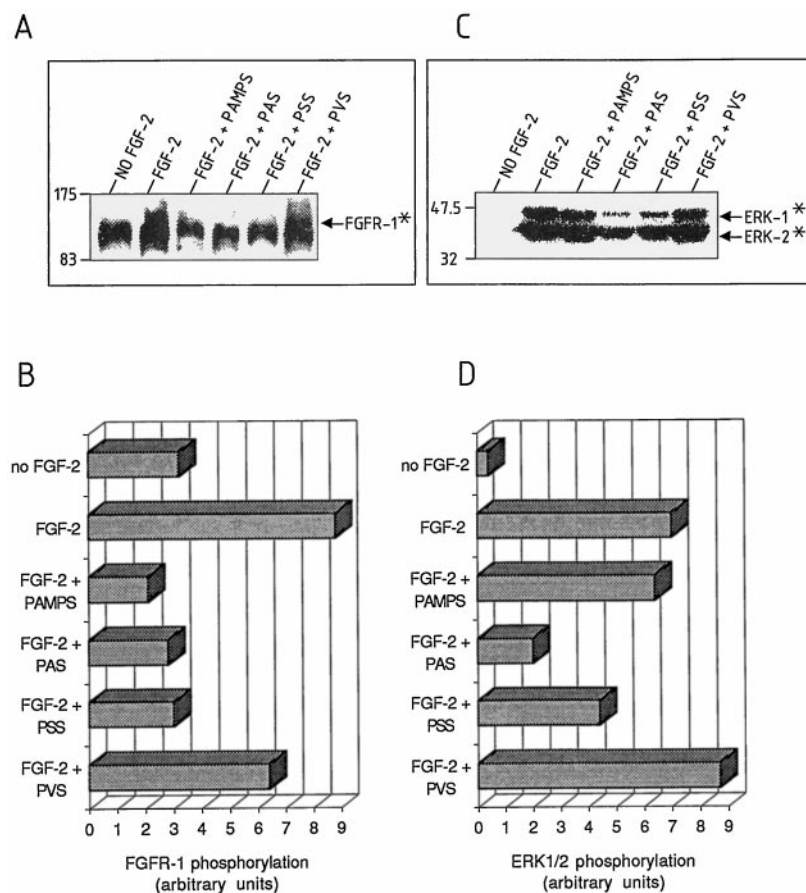


Fig. 8. Effect of sulfonic acid polymers on FGF-2-induced phosphorylation of FGFR-1 (A and B) and ERK-1/2 (C and D) in GM 7373 *flg*-AA cells. Subconfluent cells were exposed for 30 min to the polysulfonated compounds (all at 100 μ M) before adding 10 ng/ml of FGF-2. The cells were lysed and the cell extracts were subjected to SDS-PAGE and immunoblotted with antiphosphotyrosine antibody (A) or anti-phospho-ERK-1/2 (C). Quantitation of the intensity of the bands (B and D) was performed by soft-laser scanning of the film. Experiments were performed twice with similar results. The position of MM markers (in kilodaltons) is indicated.

angiogenesis occurs in the absence of exogenously added molecules both in the rat aorta-ring assay and in the CAM assay, indicating that polysulfonates act on endogenously released angiogenic factors. Among them, endogenous FGF-2 is a likely candidate. Indeed, neutralizing anti-FGF-2 antibodies decrease significantly the angiogenic process in both experimental models (Villaschi and Nicosia, 1993; Ribatti et al., 1995).

We have also demonstrated that polysulfonates inhibit cell proliferation induced by FGF-2 in cultured human umbilical vein endothelial cells (Liekens et al., 1997). Here we extend these observations by demonstrating the capacity of these compounds to interact directly with FGF-2 and to affect its receptor-binding capacity and biological activity in cultured endothelial cells of different origin. Taken together, our data identify FGF-2 as a target for the antiangiogenic action of polysulfonates. It must be pointed out, however, that various angiogenic factors besides FGF-2 (including other members of the FGF family, isoforms of vascular endothelial growth factor and placenta growth factor, hepatocyte growth factor, angiogenin, interleukin-8, and HIV-1 Tat protein) share the

capacity to interact with heparin and may thus be assumed to interact with polysulfonates as well. Further studies are required to assess the susceptibilities of the various heparin-binding angiogenic factors to this class of compounds.

The potent FGF-2 antagonist and antiangiogenic activity of PSS, PAS, and PAMPS suggest their possible use as drug candidates in the therapy of angiogenesis-related diseases and solid tumors.

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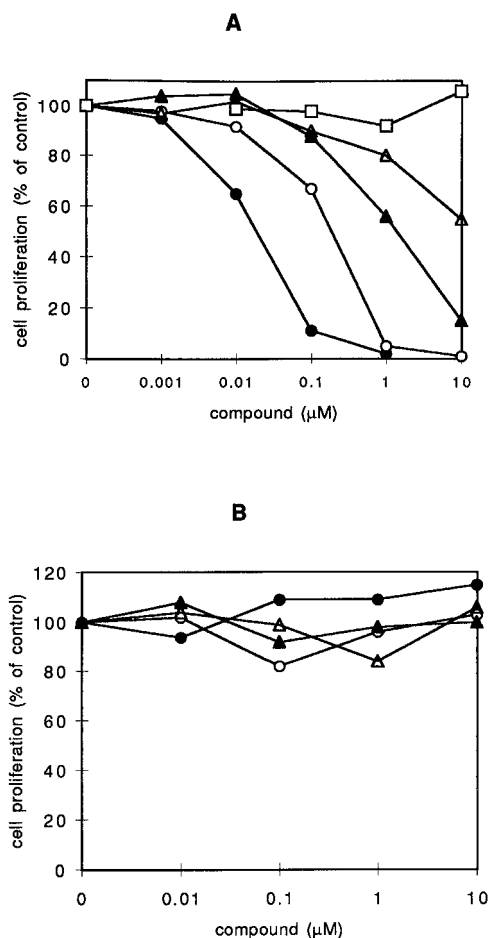


Fig. 9. FGF-2-mediated proliferation of MBECs in the presence of different MM fractions of PSS (A) and PVS (B). MBECs were incubated in the presence of 10 ng/ml bFGF and increasing concentrations of PSS [MM 70,000 (●), 18,000 (○), 8,000 (▲), 5,400 (Δ), or 1,800 (□)], PVS [MM 10,000–13,000 (●), 7,000–10,000 (○), 3,500–7,000 (▲), or 2,000 (Δ)] fractions. Medium was changed every other day. On day 5, cells were trypsinized and counted. Data are expressed as a percentage of proliferation compared with nontreated cells. Similar results were obtained in three independent experiments.

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