Short Heparin Sequences Spaced by Glycol-Split Uronate Residues Are Antagonists of Fibroblast Growth Factor 2 and Angiogenesis Inhibitors

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Received February 11, 2002; Revised Manuscript Received May 17, 2002

ABSTRACT: Fibroblast Growth Factor-2 (FGF2) is a major inducer of neovascularization (angiogenesis). Heparin activates FGF2 by favoring formation of ternary complexes with its cellular receptors (FGFRs). Controlled 2-O-desulfation followed by exhaustive periodate oxidation/borohydride reduction has been used to generate sulfation gaps within the prevalent heparin sequences, building-up arrays of pentasulfated trisaccharides (PST, consisting of a 2-O-sulfated iduronic acid flanked by two N,6-disulfated glucosamines) spaced by reduced, glycol-split uronic acid (sU) residues. The structure of the prevalent sequences of the novel heparin derivative has been confirmed by mono- and two-dimensional NMR analysis. NMR spin−lattice relaxation times (T2) and nuclear Overhauser effects suggest that the sU residues act as flexible joints between the PST sequences and cause a marked distortion of the chain conformation of heparin required for formation of ternary complexes. Since the splitting reaction also occurs at the level of the essential glucuronic acid residue of the active site for antithrombin, the heparin derivative has no anticoagulant activity. However, it fully retains the FGF2-binding ability of the original heparin, as shown by its capacity to protect FGF2 from trypsin cleavage and to prevent the formation of heparan sulfate proteoglycan (HSPG)/FGF2/FGFR1 ternary complexes. However, when compared to heparin it showed a reduced capacity to induce FGF2 dimerization and to favor the interaction of [125I]FGF2 with FGFR1 in HSPG-deficient, FGFR1-transfected CHO cells. Accordingly, it was more effective than heparin in inhibiting the mitogenic activity exerted by FGF2 in cultured endothelial cells. Finally, it inhibited angiogenesis in a chick embryo chorioallantoic membrane (CAM) assay in which heparin is inactive.

Basic Fibroblast Growth Factor (FGF2)† is a potent angiogenic molecule belonging to a family of heparin-binding proteins (1, 2). FGFs use a dual receptor system to exert their cellular effects. The signal transducing component is a family of tyrosine-kinase FGF receptors (FGFRs). The other component is the glycosaminoglycan (GAG) moiety of a heparan sulfate proteoglycan (HSPG). FGF2 is inactive in normal tissue and becomes activated upon tissue injury, inflammation or tumor invasion. The enzyme heparanase releases the growth factor from HS chains and also generates heparin-like HS fragments that bind to (and activate) FGF2 (3, 4). Activation of FGF2 occurs through its dimerization (5, 6), which in turn facilitates FGFR dimerization and transmembrane signaling (7−9). FGF2 can be also activated by exogenous heparin (3, 8). Conceivably, favoring of FGF2 and FGFR oligomerization and mitogenic signaling are at the basis of the pro-angiogenic activity of heparin in a chick embryo chorioallantoic membrane (CAM) model in the absence of angiostatic substances such as corticosteroids (10).

The structure of heparin is largely comprised of regular trisulfated disaccharide (TSD) sequences made up of alternating essential glucuronic acid residue of the active site for antithrombin, the heparin derivative has no anticoagulant activity. However, it fully retains the FGF2-binding ability of the original heparin, as shown by its capacity to protect FGF2 from trypsin cleavage and to prevent the formation of heparan sulfate proteoglycan (HSPG)/FGF2/FGFR1 ternary complexes. However, when compared to heparin it showed a reduced capacity to induce FGF2 dimerization and to favor the interaction of [125I]FGF2 with FGFR1 in HSPG-deficient, FGFR1-transfected CHO cells. Accordingly, it was more effective than heparin in inhibiting the mitogenic activity exerted by FGF2 in cultured endothelial cells. Finally, it inhibited angiogenesis in a chick embryo chorioallantoic membrane (CAM) assay in which heparin is inactive.

† This work was supported in part by grants from the Associazione Italiana per la Lotta contro le Malattie (AIDS Project), National Research Council (Target Project on Biotechnology), Ministero dell’Università e della Ricerca Scientifica e Tecnologica (Centro di Eccellenza IDET, and Cofin 2000) to M.P. and Cofin 2000 to M.R.

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1 Abbreviations: FGF2, basic fibroblast growth factor; GAG, glycosaminoglycan; FGFR, tyrosine kinase receptor; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; IdoA, L-iduronic acid; IdoA2SO3, L-iduronic acid 2-sulfate; GlcA, D-glucuronic acid; GlcNAc, N-acetyl-D-glucosamine; GlcNAc6SO3, N-acetyl-D-glucosamin 6-sulfate; GlcN, D-glucosamine; GlcNSO3, D-glucosamine 6-sulfate; GlcN6SO3, 6-sulfated glucosamines; GlcNAc6SO3, N-acetyl-D-glucosamin 6-sulfate; PST, pentasulfated trisaccharide; p-PST-U, poly-pentasulfated trisaccharide; p-PST-sU, poly-pentasulfated trisaccharide sU; GPC-HPLC, gel permeation chromatography−high performance liquid chromatography; NMR, nuclear magnetic resonance; 1D, one-dimensional; 2D, two-dimensional; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; DQF, double quantum filter; TPPI, time-proportional phase incrementation; HSQC, heteronuclear single-quantum coherence; HMBC, heteronuclear multiple-bond correlation; INEPT, insensitive nuclei enhanced by polarization; CPMG, Carr-Purcell-Meiboom-Gill sequence; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; CAM, chicken chorioallantoic membrane.
nating, α,1,4-linked residues of 2-O-sulfated L-iduronic acid (IdoA2SO3) and N,6-disulfated D-glucosamine (GlcNSO3-6SO3). These sequences are generated by the action of several enzymes on the biosynthetic precursor N-acetylated heparosan. The TSD sequences of heparin are occasionally interrupted by nonsulfated uronic acids (either GlcA or IdoA) and by undersulfated hexosamines (GlcNSO3, GlcNac, GlcNac6SO3). 3-O-Sulfated glucosamines (GlcNSO3SO3 or GlcNSO3,6SO3) are minor but important constituents of heparin, since they are part of the pentasaccharide binding site for antithrombin (AT), essential for the expression of significant anticoagulant activity (11, 12). HS, which is also biosynthesized starting from N-acetyl heparosan, is more heterogeneous and less sulfated than heparin. It chiefly contains GlcA and GlcNac residues together with minor, heparin-like sequences (12).

The structure and minimal size of heparin (and HS) chains able to bind to FGFs (especially to FGF2) has been extensively investigated using both natural fragments (13–15) and synthetic oligosaccharides (16, 17). Whereas oligosaccharides as small as tetrasaccharide bind to FGF2 (5, 18, 19), chains longer than octa-decasaccharides are necessary for mitogenic activity (5, 8, 9, 20). Also some undersulfated heparin oligosaccharides (missing, i.e., 6-O-sulfate groups on aminosugar residues) are able to bind to FGF2 (13–15).

The X-ray structures of tetra- and hexasaccharide–FGF2 complexes indicate that the minimum FGF2-binding heparin structure consists of a NSO3 group (NS) and a 2-OSO3 group (25) on contiguous GlcN and IdoA residues, respectively (19). Molecular models clearly show NS/2S pairs on both sides of a heparin helix, irrespective of the conformation of the IdoA2SO3 residues (21). These pairs of sulfate groups are also evident when the primary structure of the “regular” sequences of heparin is depicted as repeating tetrascarachide units 1 (Figure 1), a representation that takes into account the trans orientation of alternate disaccharide units and shows at first glance which substituent groups are close to each other on adjacent residues. As shown by molecular modeling (8, 20, 22) and X-ray diffraction studies (7, 8, 23), relatively long arrays of NS/2S pairs are necessary for binding more than one FGF2 molecule and formation of signal-transducing heparin/FGF/FGFR complexes. It was of interest to investigate whether the structure of heparin could be modified such that, while retaining the ability of the polysaccharide to bind 1:1 to FGF2, dimerization of FGF2 and formation of FGF2/FGFR complexes could be largely inhibited. With the aim of obtaining FGF2 inhibitors, we have generated heparin/FGF/FGFR complexes. It was of interest to investigate whether the structure of heparin could be modified such that, while retaining the ability of the polysaccharide to bind 1:1 to FGF2, dimerization of FGF2 and formation of FGF2/FGFR complexes could be largely inhibited. With the aim of obtaining FGF2 inhibitors, we have generated sulfation gaps along the regular heparin sequences by selectively removing 2S groups to reach a ratio of about 1:1 between sulfated and nonsulfated uronic acid residues. To disrupt the original helical chain conformation, the C(2)–C(3) bonds of all nonsulfated uronic acid residues have been split, generating flexible joints along the heparin chains while minimizing cleavage of glycosidic bonds. The novel heparin derivative retains the FGF2-binding ability of the parent heparin. Although it is a poor inducer of FGF2 dimerization, it is a better inhibitor than heparin of the FGF2-induced growth of endothelial cells and is antiangiogenic in an established chick embryo chorioallantoic membrane (CAM) assay.

Materials. Porcine mucosal heparin sodium salt (170 IU/mg, FU IX) was prepared by Laboratorio Derivati Organici (Trino Vercellese, Italy). Sulfation pattern, by 13C NMR (24); IdoA2SO3 65.6%; GlcA + IdoA 34.4%; GlcN N-sulfation 86.0%; GlcN 6-O-sulfation 78.4%. Average molecular weights, by GPC–HPLC (25): Mn 15 500; Mw 17 000; polydispersity = 1.1. Beef lung heparin used as a reference for NMR relaxation measurements (Mw 12 000) was prepared by Hepar. N-Acetyl heparin was prepared as previously described (26). Human recombinant FGF2 was purified from Escherichia coli cell extract by heparin-Sepharose affinity chromatography as described by Isacchi et al. (27).

Preparation of Reduced Oxyheparin (RO-Heparin). RO-heparin was prepared essentially as described in ref 28. Briefly, 1 g of heparin dissolved in 25 mL of distilled water was added at 4°C of 25 mL of NaIO4 0.2 M; the solution was stirred in the dark for 20 h, added of ethylene glycol, desalted by tangential ultrafiltration, added of 400 mg of NaBH4, stirred for 3 h at room temperature, neutralized with diluted HCl and desalted by tangential ultrafiltration.

Preparation of Partially 2-O-Desulfated Heparin. Heparin selectively O-desulfated at C2 of the IdoA residue was

![Figure 1: Prevalent sequences in regular regions of heparin and chemically modified heparins.](image-url)
prepared by alkaline treatment of heparin following a modification of the methods of Perlin et al. (29, 30). Heparin (4 g) was dissolved in 25 mL of water at 60 °C. A total of 25 mL of 2 M NaOH were added to the solution. After stirring for 30–45 min at 60 °C, the reaction was stopped by cooling, and the solution brought to pH 7 with HCl. The product (heparin epoxide, with 21% epoxidation as determined by 13C NMR (31)) was isolated by freeze-drying, dissolved in 75 mL of water and heated for 48 h at 70 °C. (Yield: about 85%.)

Exhaustive Glycol-Splitting of Partially 2-O-Desulfated Heparin. Partially 2-O-desulfated heparin was periodate-oxidized and reduced essentially as described for heparin (28). The final product, chiefly p-PST-sU, Mw 9800 (see text), was recovered by freeze-drying. (Yield: about 65% as referred to the starting 2-O-desulfated heparin.)

NMR Spectroscopy. The 1D and 2D spectra were obtained at 500 MHz for 1H and 125 MHz for 13C, with a Bruker AMX spectrometer, equipped with a 5-mm H/X inverse probe, from D2O solutions (15 mg/0.5 mL D2O, 99.99% D), or, for long experiments, from buffer solutions (10 mM phosphate buffer, pH 7, in D2O). Chemical shifts, given in parts per million downfield from sodium-3-(trimethylsilyl)-propionate, were measured indirectly with reference to acetone in D2O (δ 2.235 for 1H and δ 30.20 for 13C). Experiments were performed at 45 °C on samples treated with EDTA (32) to avoid signal shifts and broadening caused by the presence of trace divalent cations. (EDTA was removed by gel filtration on Sephadex G25 before the spectral measurements.) The 1H, COSY, and TOCSY spectra were obtained with presaturation of the HOD signal. 13C spectra for monitoring reaction kinetics were obtained at 100 MHz with a Bruker AMX400 spectrometer equipped with a multinuclear 10-mm probe, from D2O solutions (100 mg/mL).

The DQF–COSY and 2D-TOCSY spectra were obtained with presaturation of the HOD signal and 16 scans/each free induction decays were used. The 1H/13C chemical shift correlation (HSQC) (33) and HMBC (34) spectra were performed using z-gradients for coherence selection. The HSQC spectrum was obtained with carbon decoupling during acquisition period in phase sensitivity-enhanced pureab-}

Computational Methods. Modeling calculations were performed with MacroModel V7.0 software package running on a Silicon Graphics SGO2 workstation, and the AMBER* force field was used for energy calculations (36). Conformational searching was made using the Pollack-Ribiere minimization procedure with 1000 trial structures, starting with the geometry of the glycosidic linkages reported for heparin (21).

Proteolytic Digestion of FGF2. The protective effect of heparin derivatives on tryptic digestion of FGF2 was evaluated as described by Coltrini et al. (37). Briefly, 1-μg aliquots of FGF2 were incubated at 37 °C with trypsin in the presence of the GAG and its integrity was quantified by SDS–PAGE followed by computerized image analysis of the gel.

[125I]FGF2 Dimerization. Human recombinant FGF2 was labeled with Na125I (37 GBq/mL; Amersham International, Amersham, U.K.) using iodogen (Pierce Chemical, Rockford, IL) as described (27). Two nanograms of [125I]FGF2 was incubated for 2 h at 37 °C in PBS in the absence or in the presence of increasing concentrations of heparin derivatives. Then, [125I]FGF2 dimers were cross-linked by adding 1 mM bis-[2-(sucinimido-oxycarboxyloxy)-ethyl]sulfone (BSO-COES, Pierce Chem. Co.). After 30 min, samples were added with reducing sample buffer, boiled, and loaded onto a SDS–10% polyacrylamide gel. Gels were dried and exposed to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) at −70 °C. The 36 kDa band, corresponding to [125I]-FGF2 dimer, was quantified by computerized image analysis of the autoradiography using a Magiscan Image Analyzer (Joyce-Loebl LTD, England) with the Genias 3.0 software package.

Cell Cultures. Transformed fetal bovine aortic endothelial GM 7373 cells, corresponding to the described BFA-1c 1BPT multilayered transformed clone (38) were obtained from the National Institute of General Medical Sciences, Human Genetic Mutant Cell Repository (Camden, NJ). Cells were grown in Eagle’s minimal essential medium containing 10% fetal calf serum (FCS), vitamins, and essential and nonessential amino acids. CHO-K1 cells and A745 CHO cell mutants (kindly provided by J. D. Esko, University of California, San Diego) were grown in Ham’s F 12 medium supplemented with 10% FCS. A745 CHO cells harbor a mutation which inactivates the xylosyltransferase that catalyzes the first sugar transfer step in GAG biosynthesis (39). The A745 CHO flg-1A clone, bearing about 30 000 FGFR1 molecules/cell, has been generated in our laboratory by transfection with the IIIC variant of murine FGFR-1 cDNA (40).

FGF2-Mediated Cell–Cell Adhesion Assay. This assay was performed as described (40). Briefly, A745 CHO flg-1A cells (52 000 cells/cm²) were added to gluteraldehyde-fixed CHO-K1 monolayers in serum-free medium plus 10 mM EDTA with no addition or with 30 ng/mL FGF2 in the absence or in the presence of increasing concentrations of the heparin under test. After 2 h of incubation at 37 °C, A745 CHO flg-1A cells bound to the monolayer were counted under an inverted microscope at 125× 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.
were incubated for further 6 h with [3H]thymidine (1 Ci/mL) without changing the medium. After 16 h, all cell cultures were treated with 30 ng/mL FGF2 in the absence or in the presence of increasing concentrations of the GAG under test. The radioactivities incorporated in the absence of added GAG was 20 and 50 ± 7 cpm/well for control and FGF2-treated cell cultures, respectively.

Cell Proliferation Assays. Cell proliferation assay on GM 7373 cells was performed as described (42). In our experimental conditions, control cultures incubated in 0.4% FCS with no addition or with 10 ng/mL FGF2 undergo 0.1--0.2 and 0.7--0.8 cell population doublings, respectively. Cells grown in 10% FCS undergo 1.0 cell population doublings (42).

DNA synthesis was evaluated on BAE cells seeded on 96 well plates at 2500 cells/well. After 24 h, cells were incubated in DMEM plus 0.5% FCS for a further 48 h. Then, cells were treated with 30 ng/mL FGF2 in the absence or in the presence of increasing concentrations of the GAG under test without changing the medium. After 16 h, all cell cultures were incubated for further 6 h with [3H]thymidine (1 µCi/mL) and radioactivity incorporated in TCA-precipitable material was counted. In our experimental conditions, radioactivity incorporated in the absence of added GAG was equal to 155 ± 20 and 507 ± 45 cpm/well for control and FGF2-treated cell cultures, respectively.

Chick Embryo Chorioallantoic Membrane (CAM) Assay. Fertilized White Leghorn chick eggs were incubated under conditions of constant humidity at 37 °C. On the third day of incubation, a square window was opened in the egg shell after removal of 2--3 mL of albumen so as to detach the developing CAM from the shell. The window was sealed with a glass of the same size, and the eggs were returned to the incubator. At day 8, 1 mm3 sterilized gelatin sponges (Gelfoam, Upjohn Company, Kalamazoo, MI) adsorbed with the heparin under test (50--100 µg/embryo) dissolved in 5 µL of PBS were implanted on the top of growing CAMs under sterile conditions (43). Sponges containing vehicle alone were used as negative controls. CAMs were examined daily under a Zeiss stereomicroscope SR equipped with the MC 63 Camera System (Zeiss, Oberkochen, Germany). On day 12, blood vessels entering the sponges within the focal plane of the CAM were recognized macroscopically at 50× magnification and counted by two observers in a double-blind fashion (44). On the same day, microvascular density at the gelatin sponge-CAM boundary was assessed histologically by a planimetric method of point counting exactly as described (43). Mean values ± SD were determined for each analysis.

RESULTS

Synthesis and Structure of the Heparin-Derived Poly-Pentasulfated Trissaccharide (p-PST•sU). Our aim was to quench the FGF2-activating properties of heparin while preserving its capability to bind the growth factor. Our strategy was to generate 2-O-sulfation gaps between short sequences of the polysaccharide containing the minimum disaccharide sequence GlcNSO3-IdoA2SO3 binding to FGF2 (19, 45), and then disrupt the original chain conformation by splitting the C(2)−C(3) bonds of all nonsulfated uronic acid residues with periodate (i.e., the original ones and those generated by 2-O-desulfation). Conditions for the glycol splitting reaction (with periodate, followed by reduction with borohydride of the resulting dialdehydes) (28, 46) were chosen such as to minimize concomitant cleavage of inter-residue glycosidic bonds, thus largely preserving the original size of the polysaccharide chains. Since the parent pig mucosal heparin already contained over 31% nonsulfated uronic acids (∼22% GlcA and ∼9% IdoA), removal of about 25% of its 2-O-sulfate groups was planned in a way to achieve a total of about 55% nonsulfated uronic acids. The reason of targeting slightly over 50% of total nonsulfated uronic acid residues susceptible to glycol splitting relied on the consideration that a significant proportion of GlcA residues of heparin is part of undersulfated, N-acetylated regions (“NA blocks”) rather than regularly substituting IdoA2SO3 residues in “regular” regions (12). Selective 2-O-desulfation of heparin can be accomplished by simple lyophilization of a basic solution of heparin (29) However, the lyophilization method commonly leads to totally 2-O-desulfated heparins. To perform graded O-desulfation, in the present work the alternative route of 2-O-desulfation in solution (30, 31) was chosen, and experimental conditions were adapted to obtain intermediate heparin epoxides of any desired degree of conversion. The isolated epoxide derivatives were analyzed by 13C NMR (Figure 2b), and experimental conditions were optimized (1 M NaOH, 35 min at
60 °C) to afford a product with about 32% of the IdoA2SO₃ residues in the original sequences 1 converted into 2,3-anhydro-guluronate (α-GulA) residues as in sequences 2 (Figure 1). This degree of conversion corresponds to about 22% of total uronic acids.) The product was then heated 1 h at pH 7 at 60 °C to convert the epoxidated iduronate rings into 2-O-desulfated uronic acid (α-L-galacturonic acid, GalA) residues, as confirmed by analysis of 1H and 13C NMR spectra in comparison with the spectra of the corresponding fully modified heparins (30, 31). Integration of relevant 13C signals confirmed that the product prevalently consisted of sequences 3 (GlcNSO₃6SO₃−IdoA2SO₃−GlcNSO₃6SO₃−GalA)ₙ, i.e., sequences of a pentasulfated trisaccharide (PST) followed by a nonsulfated uronic acid residue (U). Exhaustive periodate oxidation followed by borohydride reduction afforded a final product 4, Figure 1). The 13C spectrum of 4 is compared in Figure 2 with the spectra of the parent heparin (1), the intermediate epoxide (2), and the galactosyl-derivative (3). The spectrum of 4 is fully compatible with the proposed prevalent structure and with the expected uniform distribution of split residues. It is in fact remarkably clean, showing a major pattern of signals superimposed on minor signals mostly associated with GlcNAc-containing sequences. Also evident in the spectrum are weak signals due to minor reducing end-groups, expected from the somewhat reduced Mw of the product (9800) vs 17 000 for the parent heparin. Independent proof of structure 4 was provided through mild hydrolysis of p-PST-sU, which generated the trisaccharide PST-R (where R is the remnant of a glycol split uronic acid) as a major fragment (unpublished data).

The 1H and 13C chemical shifts of prominent signals of the final product are reported in Table 1 together with the corresponding assignments obtained by analysis of 2D COSY and TOCSY spectra (not shown). Signal patterns were clearly associated to each of the four residues chiefly constituting the novel polysaccharide. The area ratios for the four anomeric signals (1H for two N,6-desulfated GlcN residues at 5.39 and 5.32 ppm, and at 5.33 and 4.98 for the two other residues, with corresponding 13C signals at 99.8, 98.9, 102.2, and 106.9) are very close to 1:1:1:1. The 1H anomeric signal of the second residue corresponds to that of IdoA2SO₃. The H-2 signal of the fourth residue correlates with protons at 3.78 and 3.68 ppm, respectively, associated with a primary alcohol function, with the corresponding 13C signal at 64.9 ppm. Another set of signals of this latter residue (1H at 3.91 and 13C at 62.4 ppm) correlates with two protons in the TOCSY spectrum, at 4.25 and 4.64 ppm, respectively, its overall signal pattern being the one expected (47) for a glycol-split residue sU, with C(1) and C(4) bearing CH₂OH substituents.

The presence of four prevalent residues is clearly shown in the HSQC spectrum of the pPST-sU product (Figure 3a). To confirm that the two glucosamine and the two uronic acids are arranged as in 4, the position of the interglycosidic linkages was determined by HMBC experiments. The correlations shown in the HMBC spectrum (Figure 3b) indicate connections between H1 of A1 and A2 with C4 of I and sU, and between H4 of A1 and A2 with C1 of sU and I, respectively. The same correlations across the glycosidic linkages (H1 and H4 of contiguous residues) are also indicated by the 2D NOESY experiments (data in Table 2), thus confirming the predicted sequence. It should be noted that the NMR spectra of the partially 2-O-desulfated and exhaustively glycol-split heparin of the present study are deceptively simple and do not fully reflect the structural heterogeneity associated with the presence of different types of glycol-split uronic acid residues. In fact, whereas splitting of the C(2)−C(3) bonds eliminates the stereocchemical differences between the ido and galacto configuration of the precursors (i.e., between the IdoA residues preexisting in unmodified heparin and the GalA residues generated by opening of the epoxide rings), the gluco configuration of the original GlcA residues is not modified throughout the epoxidation-epoxide hydrolysis-periodate oxidation—reduction reactions, and NMR signals of glycol-split GlcA and IdoA/GalA are superimposed in the present spectra.

Table 1: 1H and 13C Chemical Shifts of 4 (prevalent p-PST-sU sequences)

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Both experimental NOE effects and carbon spin−spin relaxation times (T₂) obtained for sequence 4 are compared in Tables 2 and 3 with those for heparin (sequence 1). T₂ values for most of the overlapping signals, not measurable by classical 1D experiments, were obtained using a modified double INEPT 2D sequence (35). The average T₂ values for 4 are twice larger than those calculated for 1. Although the comparison is not strictly homologous since the Mw of the heparin derivative is somewhat lower than that of the heparin used for relaxation measurements (9800 vs 12 000 Da), the relaxation data strongly suggest that chains made up of sequences 4 are much more flexible than those of the parent polysaccharide. Preliminary molecular modeling calculations indicated that the H1−H5 distance in the sU residue in the favored conformers of 4 is short enough to generate an NOE effect, as actually observed for p-PST-sU (Table 2). By contrast, the most stable conformation of heparin (background model in Figure 4) has measurable NOE effects only for H1 and H4 protons of adjacent residues. The conformer shown in Figure 4, obtained by modeling calculations, is one of the major contributors to the conformation of sequences 4 compatible with the additional NOE effect of 3% observed between protons H1 and H5. Such a conformation clearly involves a drastic distortion of the chain conformation from that of heparin alone (21) as well as of heparin oligosaccharides co-crystallized with FGF2 (19) and with FGF2/FGFR1 (23) (see also refs 8 and 20).

**FGF2/p-PST-sU Interaction.** Previous observations had shown that heparin protects FGF2 from proteolytic cleavage in a dose-dependent fashion and that the protective effect depends on the interaction of the glycosaminoglycan (GAG) with the growth factor and not with the proteolytic enzyme.
(37). On this basis, p-PST-sU was compared to unmodified heparin for the capacity to prevent FGF2 digestion by trypsin. As shown in Figure 5a, the two molecules exert a similar protective effect, with an ED50 equal to approximately 0.5 µg/mL. In agreement with its poor FGF2-binding capacity (37), N-acetyl heparin taken as a negative control was five times less effective.

In a second set of experiments, to evaluate further their ability to interact with FGF2, heparin, and p-PST-sU were assessed for the capacity to bind FGF2 and to prevent formation of the HSPG/FGF2/FGFR ternary complex (48). For this purpose, we utilized an experimental model in which disruption of the complex abolishes FGF2-mediated cell–cell attachment of HSPG-deficient CHO mutants transfected with FGFR1 (A745 CHO flg-1A cells) to wild-type CHO–K1 cells expressing HSPGs but not FGFR (37). In this model, a significant number of FGFR1-transfectants adhere to the HSPG-bearing monolayer in the presence of 30 ng/mL FGF2 (140 (20 cells/field) but not in the absence of the growth factor (20 (4 cells/field). Specificity of the cell–cell interaction was demonstrated by the inability of CHO mutants nontransfected with FGFR1 to adhere to the monolayer also in the presence of FGF2 (15 (3 cells/field). Again, both GAGs exerted a similar inhibitory activity on FGF2-mediated cell–cell adhesion whereas N-desulfated/N-acetylated heparin was ineffective (ID50 equal to 0.08 µg/mL, 0.05 µg/mL, and 50 µg/mL for unmodified heparin, p-PST-sU, and N-acetyl heparin, respectively) (Figure 5b).
FGF2 Antagonist Heparin Sequences

Biochemistry, Vol. 41, No. 33, 2002 �10525

FIGURE 5: FGF2/p-PST-sU interaction. (a) Effect of p-PST-sU on FGF2 tryptic cleavage. FGF2 was incubated at 37 °C with trypsin in the presence of the GAG and its integrity was quantified by SDS–PAGE followed by computerized image analysis of the gel. (b) Effect of p-PST-sU on FGF2-mediated cell–cell adhesion. HSPG-deficient FGFR1 transfectants were added to wild-type CHO monolayers in the presence of FGF2 and the GAG and the bound cells were counted. GAGs: p-PST-sU (●), heparin (○), and N-acetyl heparin (▲).

FIGURE 6: Effect of p-PST-sU on FGF2 oligomerization. (a) Chemically cross-linked FGF2 dimers were quantified after incubation of [125I]FGF2 with the GAG. (b) Binding of [125I]FGF2 to FGFR1, evaluated in A745 CHO flg-1A cells in the presence of the GAG. GAGs: p-PST-sU (●) or heparin (○). Each point is the mean of three determinations in duplicate.

Next, we compared the ability of the two GAGs to induce FGF2 oligomerization (49). As shown in Figure 6a, unmethylated heparin stimulates the formation of the FGF2 dimer. The effect is dose-dependent and described by a bell-shaped curve. Indeed, heparin causes an increase in the amount of FGF2 dimer only at low doses, that is in the presence of a molar excess of FGF2 that favors the interaction of more than one molecule of FGF2 with a single GAG chain. No significant increase in the formation of FGF2 dimer is observed instead at a FGF2:heparin molar ratio equal or higher than 1:1, when only a single FGF2 molecule will interact with the GAG chain. As anticipated, the modifications of the structure of the heparin chain introduced in p-PST-sU caused a significant decrease (p < 0.001, paired two-population t-test) of the capacity of the GAG to induce FGF2 dimerization in the dose range between 0.001 and 1 ng/mL (Figure 6a). No induction of FGF2 dimerization was exerted by N-acetyl heparin (data not shown).

Previous observations had shown that heparin-induced oligomerization of FGF2 facilitate its interaction with FGFR and FGFR dimerization in cells lacking endogenous heparan sulfate (50). On this basis, we have compared p-PST-sU and unmodified heparin for the capacity to favor the interaction of [125I]FGF2 with FGFR1 in HSPG-deficient A745 CHO flg-1A transfectants. In agreement with its reduced capacity to facilitate FGF2 dimerization, p-PST-sU is approximately 100 times less potent than unmodified heparin in favoring the binding of [125I]FGF2 to FGFR1 in A745 CHO flg-1A cells (Figure 6b).

Taken together, the above experiments indicate that p-PST-sU retains an FGF2-binding capacity similar to that exerted by unmodified heparin. As anticipated, however, p-PST-sU has lost, at least in part, the capacity to induce FGF2 oligomerization.

Effect of p-PST-sU on the Biological Activity Exerted by FGF2 on Endothelial Cells. To assess the effect of p-PST-sU on the biological activity exerted by FGF2 on endothelial cells in culture, fetal bovine aortic endothelial GM 7373 cells were incubated with FGF2 in the absence of increasing concentrations of unmodified heparin, N-acetyl heparin, or p-PST-sU and cell proliferation was evaluated 24 h thereafter. As shown in Figure 7a, p-PST-sU inhibits the mitogenic activity exerted by FGF2 with a potency significantly higher than the other GAGs under test (ID50 equal to 0.1 g/mL, 100 µg/mL, and > 100 µg/mL for p-PST-sU, unmodified heparin, and N-acetyl heparin, respectively). Also, p-PST-sU was a more potent inhibitor for FGF2-induced [3H]-thymidine incorporation in BAE cells (ID50 equal to 1 µg/mL, 100 µg/mL, and > 100 µg/mL for p-PST-sU, unmodified heparin, and N-acetyl heparin, respectively) (Figure 7b). Differences in HSPG composition of the cell surface may explain the different potency shown by each GAG in the two endothelial cell types.

These data prompted us to assess the capacity of p-PST-sU to exert an anti-angiogenic activity in vivo. The chick embryo chorioallantoic membrane (CAM) expresses FGF2 mRNA and protein (51, 52) and endogenous FGF2 plays a limiting role in the development of the vascular system of this embryonic membrane (51). On this basis, the CAM represents an in vivo system suitable to assess the impact of putative anti-angiogenic FGF2 antagonists on blood vessel formation during development. As shown in Table 4, p-PST-sU exerts a significant inhibitory activity on blood vessel formation when applied at day 8 on the top of the CAM at...
50 or 100 μg/embryo via a gelatin sponge implant (43). At
day 12 of development this results in a 60% decrease in the
average number of macroscopic blood vessels surrounding
the implant (7 ± 2 vs 3 ± 1 total macroscopic blood vessels
in control and p-PST-sU-treated sponges, respectively; n =
10). Histologic analysis of the CAMs followed by planimetric
counting of microscopic blood vessels at the sponge-CAM
boundary confirmed the inhibitory effect of p-PST-sU on
angiogenesis (7 ± 3 vs 0 ± 0 microvessels per 0.125 mm²
for control and p-PST-sU-treated CAMs, respectively, n =
10). Interestingly, p-PST-U (i.e., p-PST-sU before glycol-
splitting) and a preparation of heparin that underwent partial
glycol-splitting without previous partial 2-O-desulfation
(RO-heparin), showed some antiangiogenic activity even
though much lower than that exerted by the p-PST-sU
derivative (Table 4). No macroscopic and microscopic
inhibition of angiogenesis was observed in CAMs treated
with unmodified or N-acetyl heparin (Table 4).

**DISCUSSION**

Biochemical studies with natural and synthetic glycosami-
 noglycan fragments have provided evidence that heparin and
HS “activate” FGF2 by binding to the growth factor and by
favoring oligomerization and formation of ternary complexes
with its cellular receptors FGFRs (2). Oligosaccharides as
small as tetrasaccharide are able to bind FGF2, provided they
contain at least one GlcNSO₄ and one IdoA2SO₃ residue (5,
16, 19). However, only GAG chains longer than octa-
decasaccharide are able to activate FGFRs and to trigger
mitogenic signals. Molecular modeling and X-ray diffraction
studies have clearly shown that such a minimum size is
required for building up assemblies of FGF and FGFR
molecules (8, 20). Indeed, in crystalline FGF2/FGFR1
assembles the growth factor and its receptor generate a
“basic canyon” which can ideally accommodate a relatively
long heparin molecule in its extended helical conformation
(7).

Although not all the sulfate groups of heparin/HS are
essential for formation of 1:1 complexes with FGF2 (12–
15), most of the complexes investigated thus far involve the
trisulfated disaccharide sequences (TSD) constituted by
alternating IdoA2SO₃ and GlcNSO₄SO₃ residues. These
sequences, which are the chief product of biosynthesis of
mammalian heparins and are much less represented in most
HS species (12), are involved in binding of a large number of
proteins (10, 20, 53). In pig mucosal heparins currently
used in therapy, segments constituted by TSD sequences are
on the average 8 disaccharide units long (46), i.e., long
enough to bind to FGF2 and induce its dimerization and
complexation with FGFRs. In the present work we showed
that binding and induced activation of FGF2 can be
modulated by generating sulfation gaps along the glycosami-
noglycan chain, more specifically by removing sulfate groups
from IdoA2SO₃ residues. We also showed that splitting
the C(2)–C(3) bonds of the resulting nonsulfated IdoA residues
(12) together with those of preexisting nonsulfated uronic acids
(GlcA and IdoA) generates flexible joints between the
unmodified heparin sequences that confer FGF2 antagonist
activity and anti-angiogenic capacity to the p-PST-sU heparin
derivative. Relevant to this point is the observation that both
partial 2-O desulfation and glycol-splitting are required for
a potent anti-angiogenic activity of the heparin derivative.
Indeed, RO-heparin (that underwent glycol-splitting in the
absence of a partial 2-O desulfation) and the p-PST-U
derivative (that underwent partial 2-O desulfation in the
absence of glycol-splitting) exert only a very modest anti-
angiogenic effect in the CAM assay when compared to
p-PST-sU.

The role of local rigidity and flexibility along the heparin
chains has been widely discussed in the context of the
anticoagulant properties mediated by antithrombin III (AT)
(12). The peculiar characteristics of IdoA residues to adopt
more than one of two or three equienergetic conformations
confers to IdoA-containing GAGs a “plasticity” that greatly
enhances the binding ability of these polysaccharides (12,
53). In fact, whereas FGF2 in its crystalline state with heparin
tetra/hexasaccharides selects the chair 1 C 4 conformation of
the GlcA residue of the pentasaccharide sequence constituting
the active site for AT (53). As expected from both partial
2-O desulfation and exhaustive glycol-splitting (the first
modification impairing the HC1-mediated contribution, and
the second one eliminating the AT-mediated contribution to
the anticoagulant activity), the novel heparin derivative
described in this paper has an anticoagulant activity even
lower than reduced oxyheparin (RO–H, prevalently p-PST-
U, data not shown).

Unfractionated, unmodified heparin modulates cell growth
in general and angiogenesis in particular, sometimes with
opposite effects depending on the cellular system and the
type of assay (57). Since proliferation of endothelial cells is
triggered by angiogenic signals, inhibition of growth of these
cells is expected whenever FGF2 is not activated through
oligomerization and formation of ternary complexes with
FGFRs. However, the growth of endothelial cells is a

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (μg/embryo)</th>
<th>Angiogenesis inhibition (inhibited CAMs/total CAMs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>100</td>
<td>0/10</td>
</tr>
<tr>
<td>Heparin</td>
<td>100</td>
<td>0/10</td>
</tr>
<tr>
<td>N-acetyl heparin</td>
<td>100</td>
<td>0/10</td>
</tr>
<tr>
<td>RO-heparin</td>
<td>100</td>
<td>3/10</td>
</tr>
<tr>
<td>p-PST-U</td>
<td>100</td>
<td>3/10</td>
</tr>
<tr>
<td>p-PST-sU</td>
<td>50</td>
<td>5/10</td>
</tr>
<tr>
<td>p-PST-tU</td>
<td>100</td>
<td>8/10</td>
</tr>
</tbody>
</table>

* Gelatin sponges containing the sample under test were applied on
the CAM at day 8 of development. CAMs were scored macroscopically
for inhibition of neovascularization at day 12 as described in Materials
multifactorial event, and inhibition of FGFs (and/or their cellular receptors) does not always correspond to inhibition of cell growth. However, the effects of angiogenesis-inducing (or -inhibiting) factors can be different in different animal species (58). Whereas unmodified heparin inhibits growth of GM 7373 cells (Figure 6a), its effect on BAE cells is quite similar to the one of its N-acetylated derivative taken as negative control. In both cases p-PST-sU has a clear inhibiting effect, suggesting a prevailing mechanism of inhibition of FGF2.

The reported effects of unmodified heparin on angiogenesis and related events such as metastasis are paradoxical. In the presence of corticosteroids, heparin exerts a still inhibiting effect, suggesting a prevailing mechanism of inhibition of FGF2.

ACKNOWLEDGMENT

The authors thank Dr. Sara Guglieri for valuable assistance in molecular modeling and Prof. R. A. Lane for critical reading of the manuscript.

REFERENCES


BI020118N