Heparanase is an endo-β-glucuronidase that cleaves heparan sulfate (HS) chains of heparan sulfate proteoglycans on cell surfaces and in the extracellular matrix (ECM). Heparanase, overexpressed by most cancer cells, facilitates extravasation of blood-borne tumor cells and causes release of growth factors (such as FGF-2 and VEGF) sequestered by HS chains, thus accelerating tumor growth and metastasis. Inhibition of heparanase with HS mimics is a promising target for a novel strategy in cancer therapy. In this study, in vitro inhibition of recombinant heparanase was determined for heparin derivatives differing in degrees of 2-O- and 6-O-sulfation, N-acetylation, and glycol-splitting of nonsulfated uronic acid residues. The contemporaneous presence of sulfate groups at O-2 of IdoA and at O-6 of GlcN was found non-essential for effective inhibition of heparanase activity, provided that one of the two positions retains a high degree of sulfation. N-desulfation/N-acetylation involved a marked decrease in the inhibitory activity for degrees of N-acetylation higher than 50%, suggesting that at least one NSO3 group per a disaccharide unit is involved in interaction with the enzyme. On the other hand, glycol-splitting of preexisting or of both preexisting and chemically generated nonsulfated uronic acids dramatically increased the heparanase-inhibiting activity, irrespective of the degree of N-acetylation. Indeed, N-acetylated heparins in their glycol-split forms inhibit heparanase as effectively as the corresponding N-sulfated derivatives. Whereas heparin and N-acetyl heparins containing unmodified GlcA residues inhibit heparanase by acting, at least in part, as substrates, their glycol-split derivatives are no more susceptible to cleavage by heparanase. Glycol-split N-acetyl heparins do not release FGF-2 from ECM and fail to stimulate its mitogenic activity. The combination of high inhibition of heparanase and low release/potentiation of ECM-bound growth factor indicates N-acetylated, glycol-split heparins as potential antiangiogenic and antimitastatic agents, more effective than their counterparts with unmodified backbones.

Introduction

Heparanase is a mammalian endo-β-D-glucuronidase that cleaves heparan sulfate (HS) chains at a limited number of sites (1-3). Cloning of the heparanase cDNA by several groups (1-6), suggests that a single functional HS-degrading endoglycosidase is expressed in mammalian cells. The enzyme is synthesized as a latent 65 kDa precursor that undergoes proteolytic cleavage, yielding 8 kDa and 50 kDa subunits that heterodimerize to form a highly active enzyme (7, 8). Heparanase enzymatic activity participates in degradation and remodeling of the extracellular matrix (ECM), facilitating, among other activities, cell invasion associated with cancer metastasis, angiogenesis and inflammation (1-3, 9). Heparanase upregulation has been documented in a variety of human tumors correlating, in some cases, with increased vascular density and poor postoperative survival (10-13). Heparanase over expression has also been noted in several other
pathologies such as cirrhosis (14), nephrosis (15) and diabetes (16). In addition to its intimate involvement in the egress of cells from the bloodstream, heparanase activity releases from the ECM and tumor microenvironment a multitude of HS-bound growth factors, cytokines, chemokines and enzymes which affect cell and tissue function, most notably angiogenesis (17, 18). These observations, the anti-cancerous effect of heparanase gene silencing (ribozyme, siRNA) (19) and of heparanase-inhibiting molecules (non-anticoagulant species of heparin and other sulfated polysaccharides) (20, 21), as well as the unexpected identification of a predominant functional heparanase (1-3), suggest that the enzyme is a promising target for development of new anti-cancer drugs.

HS and the structurally-related heparin are present in most animal species. They are glycosaminoglycans constituted by repeating disaccharide units of a uronic acid (either D-gluconic, GlcA, or L-iduronic, IdoA) and D-glucosamine (either N-acetylated, GlcNAc, or N-sulfated, GlcNSO$_3$) and bear sulfate substituents in various positions (22-25). Though derived from the common biosynthetic precursor N-acetyl heparosan (=GlcA-GlcNAc)$_n$, HS and heparin have different structures, HS being less sulfated and more heterogeneous than heparin. The two glycosaminoglycans have also different locations in tissues: whereas HS is a component of the ECM and of the surface of most cells, heparin is stored in granules of mast cells and co-released with histamine into the circulation upon cellular degranulation, mainly in cases of allergic and inflammatory reactions and anaphylactic stress. On the other hand, exogenous heparin is widely used as an anticoagulant and antithrombotic drug and is of increasing interest for novel therapeutical applications (24, 25, 27).

As an analog of the natural substrate of heparanase, heparin is commonly considered as a potent inhibitor of heparanase (20, 21, 28-31). This activity is attributed, in part, to its high affinity interaction with the enzyme and limited degradation, serving as an alternative substrate. Early reports (20, 21, 30, 31) showed that heparin and some chemically modified species of heparin, as well as other sulfated polysaccharides (22, 32) which inhibit tumor cell heparanase, also inhibit experimental metastasis in animal models, while other related compounds that lack heparanase-inhibiting activity fail to exert an anti-metastatic effect (20-22, 30-32). Regardless of the mode of action, heparin and LMWH were reported to exert a beneficial effect in cancer patients (33), stimulating research on the potential use of modified, non-anticoagulant species of heparin and HS in cancer therapy.

Screening of heparin derivatives permitted to identify some of its structural features associated with inhibition of the enzyme. As a general trend, the heparanase inhibiting activity increases with increasing degrees of O-sulfation. However, N-sulfates seems to exert little effect, since they can be replaced by N-acyl (N-acetyl, N-succinyl, or N-hexanoyl) groups without substantial loss of inhibitory activity (20-22, 30-32). No significant differences were found between the currently used unfractionated heparins and low-molecular weight heparins, and a tetradecasaccharidic fragment (34). 2-O-desulfated derivatives were shown to retain the inhibitory activity, whereas N-desulfated, N-acetylated derivatives displayed a reduced activity (35). In the present study, relationships between structure and heparanase inhibiting activity of heparin were studied using a larger number of heparins and heparin derivatives, including some with various degrees of 6-O-sulfation of GlcN and 2-O-sulfation of IdoA residues, as well as “glycol-split” derivatives obtained by controlled periodate oxidation/borohydride reduction of natural (36) or partially 2-O-desulfated heparins (37, 38). Glycol-splitting of C2-C3 bonds of nonsulfated uronic acid residues was suggested to interfere with the biological interactions of heparin by providing flexible joints between protein binding sequences (37-39). When framing heparin sequences that bind FGF-2, glycol-split residues were shown not to impair the binding to FGF-2. However, they prevented activation of FGF-2 and FGF-2-induced angiogenic activity (37, 38). The present study shows that glycol-splitting enhances the heparanase-inhibiting activity of heparin. Based on the observation that N-acetyl groups do not prevent, and may even assist, recognition by heparanase (40, 41) and taking into account that N-acetyl heparin, as opposed to heparin, does not release angiogenic factors from ECM (34), we have prepared and tested heparins with various degrees of N-acetylation/N-sulfation, together with some of their glycol-split derivatives. N-acetylated, glycol-split heparins were shown to
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3 inhibit heparanase more efficiently than the corresponding nonglycol-split N-acetylated heparins.

MATERIALS AND METHODS

Materials - All chemicals were of reagent grade from Sigma Aldrich Chemicals and used as supplied. Heparins were commercial preparations from pig mucosa (H-1 to H-3 from LDO, Trino Vercelese, Italy, and H-6 from Hepar), from beef mucosa (H-4 and H-5, LDO), and from beef lung (H-7, Upjohn, Kalamazoo, USA). The corresponding contents of major sulfate groups, as evaluated by \(^{13}\)C NMR spectroscopy (42) and expressed as mole percent of IdoA\(^2\)SO\(_3\), GlcNSO\(_3\), and GlcN(SO\(_3\) or Ac)\(_6\)SO\(_3\) per disaccharide unit, were: H-1: 69, 89, 79; H-2: 68, 85, 82; H-3: 64, 85, 82; H-4: 62, 89, 60; H-5: 66, 92, 60; H-6: 65, 86, 82; and H-7: 86, 98, 95. The average molecular weights (Mw, in Da, by GPC-HPLC (43)) were: H-1: 14,200; H-2: 18,100; H-3: 19,600; H-4: 18,800; H-5: 18,200; H-6: 23,200; and H-7: 21,600. Sample desalting was carried out by dialysis against water with 1000 Da cut-off tubes or by fractionation on a 2.5 x 100 cm Sephadex G-25 column (Pharmacia), using 10% ethanol in water as eluent and UV detection at 210 nm. Molecular weight determinations were performed by GPC-HPLC on a Viscotex instrument equipped with VE1121 pump, Rheodyne valve 100 \(\mu\)l and TDA (Triple Detector Array) 302 equipped with IR, viscosimeter and 90° light scattering systems. Two 300 x 7.8 mm TSK GMPWXL Viscotek columns were used, with 0.1M NaNO\(_3\) as eluent (flow 0.6 ml/min). Samples were dissolved in the eluent solution at the concentration of 15 mg/ml (43).

NMR spectra were recorded at 500 MHz for \(^1\)H and 125 MHz for \(^{13}\)C with a Bruker AMX spectrometer, equipped with a 5-mm \(^1\)H/X inverse probe. The spectra were obtained at 45°C from D\(_2\)O solutions (15 mg/0.5 ml D\(_2\)O, 99.99% D). Chemical shifts, given in parts per million downfield from sodium-3-(trimethylsilyl)-propionate, were measured indirectly with reference to acetone in D\(_2\)O (\(\delta\) 2.235 for \(^1\)H and \(\delta\) 30.20 for \(^{13}\)C). The \(^{13}\)C NMR spectra were recorded at 300 or 400 MHz with a Bruker AC300 or AMX-400 spectrometer.

Recombinant human heparanase - Recombinant enzymatically active heparanase was purified from heparanase-transfected CHO cells (4). Briefly, CHO cells were harvested with trypsin, centrifuged and the cell pellet was suspended in 20 mM citrate phosphate buffer pH 5.4. The suspension was subjected to 4 cycles of freeze and thaw (-70°C/37°C, 5 min each), the cell extract was centrifuged (18,000 rpm, 15 min, 2-8°C) and the supernatant collected and filtered through a 0.45 \(\mu\)M filter. The filtrate was applied onto Source 15 S column (Pharmacia) equilibrated with 20 mM phosphate buffer, pH 6.8. The column was washed (20 mM phosphate buffer, pH 6.8, followed by 20 mM phosphate buffer, pH 8.0) and heparanase was eluted with a linear gradient (0 to 35%) of 8 column volumes of 1.5 M NaCl in 20 mM phosphate buffer, pH 8.0. Active fractions were pooled and applied onto Fractogel EMD SO\(_3\)- (Merck) column equilibrated with 20 mM citrate phosphate buffer, pH 5.4. Heparanase was eluted with a linear gradient (0 to 22%) of 1 column volume followed by 10 column volumes (22% to 25%) of 1.5 M NaCl in 20 mM phosphate buffer, pH 8.0. Finally, heparanase eluted from the Fractogel column was applied onto HiTrap heparin column (Pharmacia) equilibrated with 20 mM phosphate buffer, pH 8.0, and eluted with a linear gradient of 1 column volume (0 to 20%) and 15 column volumes (20% to 28%) of 1.5 M NaCl in 20 mM phosphate buffer pH 8.0. Eluted fractions were analyzed by gradient SDS-PAGE, stained with Gelcode® (Pierce) and pooled according to their purity. At least 90% pure, highly active heparanase preparation was obtained, containing the active 50 and 8 kDa heparanase subunits and, to a lower extent, the 65 kDa pro-heparanase (8). Active recombinant human heparanase was also produced in insect cells as described (7). The construct encoding the 8 and 50 kDa heparanase subunits was kindly provided by Dr. E. McKenzie (Oxford Glycoscience Ltd., UK) (7). Similar results were obtained with both preparations.

Preparation of heparin derivatives

6-O-desulfated heparin - Procedure A: an extensively 6-O-desulfated heparin also partially (~15%) 2-O-desulfated (\(^{16}\)OdeS-H\(_A\)), where the superscript denotes the degree of 6-O-desulfation), Mw 16,000, was prepared according to Nagasawa et al (44), starting from the pyridinium salt of heparin H-1, under solvolytic conditions (10 mg/ml in DMSO: water 9:1) at 100°C for 2.5 h followed by resulfation of free amino groups with sulfur trioxide/trimethylamine complex in alkaline...
aqueous medium (45). Procedure B: 6-O-desulfated-heparins (\(^{77}\)Odes-[H], Mw 19,000; \(^{73}\)Odes-[H] [B], Mw 17,700, and \(^{65}\)Odes-H[H], Mw 20,400) were prepared according to Matsuo et al (46) by O-desulfation followed by activation with N-methyl-N-(trimethylsilyl)trifluoroacetamide (MTSTFA) or N,O-bis (trimethylsilyl)acetamide (BTSA) without N-desulfation. Heparin H-1 (200 mg) was converted into its pyridinium salt and soaked in pyridine (20 ml). After addition of 4 ml MTSTFA, the solution was heated for 4 h at 80°C to yield \(^{77}\)Odes-H, or for 8 h at 60°C to yield \(^{73}\)Odes-H. Heparin (H-1) was converted into its pyridinium salt and soaked in pyridine (30 ml) then added of 6 ml BTSA. The solution was heated for 2 h at 60°C to yield \(^{65}\)Odes-S-H.

2-O-desulfated heparins - Procedure A: 2-O-desulfated heparin in the IdoA form [H, IdoA(A), Mw 17,700], was prepared according to Jaseja et al (47). Heparin (500 mg) was simply dissolved in 500 ml of 0.1 M NaOH and the solution was frozen and lyophilized. The residue dissolved in 500 ml distilled water was dialyzed and the product was isolated by evaporation under reduced pressure. Its \(^{13}\)C NMR spectrum closely corresponded of the one reported in the literature (48), indicating an essentially complete conversion of the original Ido2SO3 residues into IdoA residues. Procedure B: 2-O-desulfated heparin in the GalA form [H, GalA(B), Mw 12,600], was prepared by a modification of methods used by Perlin (47, 49), essentially as previously described (48). Heparin (500 mg) was dissolved in 10 ml of 1M NaOH, then heated at 85°C for 1 h times. After cooling below 30°C, the solution was brought to pH 7 with 0.1 M HCl and heated at 70°C for 48 h to give (after cooling, dialysis and freeze drying) GalA the derivative with typical \(^{13}\)C NMR spectrum (48).

\(^{N}\)Acetylated heparins – \(^{N}\)-acetylated heparins (\(^{6}\)AH, where the superscript x denotes the degree of \(^{N}\)-acetylation as referred to total GlcN) were prepared by time-controlled N-desulfation under solvolytic conditions (44). Briefly, the pyridinium salt of heparin was stirred at 20-25 °C in DMSO: water (9:1) for different times (30, 60, 90, 100, 120 min and 8 h) in order to obtain intermediates with different degrees of N-desulfation, which upon \(^{N}\)-acetylation with acetic anhydride in alkaline aqueous medium (NaHCO\(_3\), 4°C, 2 h) (50) gave \(^{29}\)NAH, Mw 22 kDa; \(^{30}\)NAH, Mw 21 kDa; \(^{30}\)NAH, Mw 21 kDa; \(^{70}\)NAH, Mw 22 kDa; and 8 h for \(^{92}\)NAH, Mw 13.7 kDa, and \(^{106}\)NAH, Mw 15.7 kDa.

Glycol-split heparins and glycol-split \(^{N}\)-acetylated heparins were prepared by exhaustive periodate-oxidation and borohydride reduction of heparin and \(^{N}\)-acetyl heparins, respectively, without (36) or with (37, 38) prior partial 2-O-desulfation. For the first series of glycol-split, \(^{N}\)-acetyl heparins, H-1 and \(^{29}\)NAH, \(^{39}\)NAH, \(^{50}\)NAH, \(^{70}\)NAH, and \(^{100}\)NAH, 250 mg samples were dissolved in 6 ml H\(_2\)O and the solutions were added of 6 ml 0.1 M NaIO\(_4\). The solutions were stirred at 4°C for 16 h in the dark. The reactions were stopped adding 1 ml ethyleneglycol and the solutions were dialyzed through a 1000 Da cut-off tubes for 16 h. Solid sodium borohydride (60 mg) was added to the retentate solutions in several portions under stirring. After 2-3 h the pH was adjusted to 4 with 0.1 M HCl, and the solutions were neutralized with 0.1 M NaOH. After desalting and dialysis, the final products were recovered by freeze-drying to yield RO.H, Mw 15.7 kDa, \(^{26}\)NA,RO.H, Mw 17 kDa, \(^{46}\)NA,RO.H, Mw 16 kDa, \(^{53}\)NA,RO.H, Mw 11.25 kDa, \(^{67}\)NA,RO.H, Mw 15 kDa, and \(^{100}\)NA,RO.H, Mw 20.2 Kd, respectively. For the second series of N-acetylated, glycol-split heparins (NAH, gs), 250 mg samples of H-1, \(^{29}\)NAH, \(^{39}\)NAH, \(^{50}\)NAH, \(^{70}\)NAH, and \(^{100}\)NAH, were dissolved in 5 ml of 1M NaOH, then heated at 60°C for 30 minutes. After cooling below 30°C, the solutions were brought to pH 7 with 0.1 M HCl and heated at 70°C for 48 h to give (after cooling, dialysis and freeze drying) partial conversion of Ido2SO3 to GalA. Products were treated as described above to yield the corresponding glycol-split derivatives H\(^{52}\)gs, Mw 11 kDa, \(^{29}\)NAH, \(^{46}\)gs, Mw 6 kDa, \(^{41}\)NAH, \(^{64}\)gs, Mw 8.5 kDa, \(^{53}\)NAH, \(^{64}\)gs, Mw 9.5 kDa, and \(^{70}\)NAH, \(^{59}\)gs, Mw 9.3 kDa. The glycol-splitting (gs) percentages were evaluated by integration of the anomeric \(^{13}\)C NMR signals at 106.5 ppm (A) and at 102 ppm (B), corresponding to the split uronic acid residues and 2-O-sulfated iduronic acid residues, respectively; gs = [A/(A+B)] x 100. Products obtained without generation of additional nonsulfated uronic acid residues had a content of glycol-split residues and 2-O-sulfated iduronic acid residues, respectively; gs = [A/(A+B)] x 100.

Products obtained by glycol-splitting of both the preexisting and the newly generated nonsulfated uronic acids (IdoA or GalA) were designated as H\(_g\),gs (or NAH,\(_g\),gs if derived from \(^{N}\)-acetyl
were determined by integration of the $^{13}$C NMR and the corresponding molecular weight values freeze-drying. The depolymerization degrees (DP) ethanol, were dissolved in water and recovered by cooled at 4°C then added of 75 mg of NaNO$_2$ and the pH was adjusted to 2 with 0.1 M HCl. The solution was stirred at 4°C for 20 min and then the pH was brought to 7. Solid NaBH$_4$ (1 g) was added in several portions under stirring. After 2-3 h, the pH was adjusted to 4 with 0.1 M HCl and the solution was neutralized with 0.1 M NaOH. The products (LMW H-1, 6.5 kDa; LMW-H, 49gs, 6.3 kDa; LMW-H, 49gs, 3.0 kDa; LMW-H, 49gs, 5.4 kDa) obtained by precipitation with 3 volumes of ethanol, were dissolved in water and recovered by freeze-drying. The depolymerization degrees (DP) and the corresponding molecular weight values were determined by integration of the $^{13}$C NMR signals at 98-107 ppm and 82, 85 and 87 ppm, corresponding to total C1 and C2, C3 and C5 of the anhydro-mannitol unit, respectively. The percentage of glycol-splitting, expressed as glycol split residues referred to total uronic acids, was evaluated by integration of the $^{13}$C NMR signals at 106.5 ppm and 102 ppm, corresponding to C1 of the split uronic residues and 2-O-sulfated iduronic acids, respectively.

**Cells** - Cultures of bovine corneal endothelial cells were established from steer eyes and maintained in DMEM (1 g glucose/liter) supplemented with 5% newborn calf serum, 10% FCS and 1 ng/ml FGF-2, as described (4, 52). Confluent cell cultures were dissociated with 0.05% trypsin and 0.02% EDTA in phosphate buffered saline (PBS) and sub-cultured at a split ratio of 1:8 (52).

**Preparation of dishes coated with ECM** - Bovine corneal endothelial cells were plated into 35-mm tissue culture dishes at an initial density of 2 x 10$^5$ cells/ml and cultured as described above, except that 4% dextran T-40 was included in the growth medium (4, 52). On day 12, the subendothelial ECM was exposed by dissolving the cell layer with PBS containing 0.5% Triton X-100 and 20 mM NH$_4$OH, followed by four washes with PBS (52). The ECM remained intact, free of cellular debris and firmly attached to the entire area of the tissue culture dish. To produce sulfate labeled ECM, Na$_2$SO$_4$ (Amersham, Buckinghamshire, UK) was added (25 µCi/ml) on days 2 and 5 after seeding and the cultures were incubated with the label without medium change and processed as described (4, 52). Nearly 80% of the ECM radioactivity was incorporated into HSPG.

**Heparanase inhibition activity** - Heparin species were tested for their ability to inhibit heparanase, using metabolically sulfate labeled ECM as a substrate (28, 29). Briefly, sulfate labeled ECM coating the surface of 35-mm culture dishes was incubated (4 h, 37°C, pH 6.0) with recombinant human heparanase (40 ng/ml) in the absence and presence of different concentrations (0.2-1.0-5.0 µg/ml) of each heparin species. The reaction mixture contained: 50 mM NaCl, 1 mM DDT, 1 mM CaCl$_2$, and 10 mM buffer Phosphate-Citrate, pH 6.0. To evaluate the occurrence of proteoglycan degradation, the incubation medium was collected and applied for gel filtration on Sepharose 6B columns (0.9 x 30 cm). Fractions (0.2 ml) were eluted with PBS at a flow rate of 5 ml/h and counted for radioactivity. The excluded volume ($V_v$) was marked by blue dextran and the total included volume ($V_i$) by phenol red. Nearly intact HSPG are eluted from Sepharose 6B just after the void volume ($V_v$) with PBS containing 0.02% gelatine (34). The ECM remained intact, free of cellular debris and firmly attached to the entire area of the tissue culture dish. To produce sulfate labeled ECM, Na$_2$SO$_4$ (Amersham, Buckinghamshire, UK) was added (25 µCi/ml) on days 2 and 5 after seeding and the cultures were incubated with the label without medium change and processed as described (4, 52). Nearly 80% of the ECM radioactivity was incorporated into HSPG.

**Release of ECM-bound FGF-2** - ECM-coated wells (four well plates) were incubated with iodinated FGF-2 (1-2 x 10$^5$ cpm/ng, 1.5-2.5 x 10$^6$ cpm per 0.25 ml/well, 3 h, 24°C), and the unbound FGF-2 was removed by four washes with PBS containing 0.02% gelatine (34). The ECM was then incubated (3 h, 24°C) with the various heparins and modified heparins, and aliquots of the 0.25 mL incubation medium were counted in a gamma counter to determine the amount of released material. The remaining ECM was washed twice with PBS, solubilized with 1 N NaOH, and the radioactivity was counted in a
gamma-counter (34). The percentage of released \(^{125}\text{I}-\text{FGF-2}\) was calculated from the total ECM-associated radioactivity. “Spontaneous” release of \(^{125}\text{I}-\text{FGF-2}\) in the presence of incubation medium alone was 7-12% of the total ECM-bound FGF-2 (34). Each experiment was performed 3-5 times, yielding similar results.

Stimulation of FGF-2 mitogenic activity. A cytokine-dependent, heparan sulfate deficient, lymphoid cell line (BaF3) engineered to express FGFR1 (53, 54) was applied to investigate the effect of heparin derivatives on FGF-2 mediated cell proliferation. These cells (clone F32) respond to FGF-2 only in the presence of exogenously added heparin, HS, or some modified species of heparin. Briefly, F32 cells (2 x 10\(^4\)/well) were plated into 96-well microtiter plates in the presence of 2.5 or 5.0 ng/ml FGF-2 and increasing concentrations of the test compound, in a total volume of 250 µl. Forty eight h later, 1 µCi of ³H-thymidine was added per well, the cells were incubated for additional 6 h and collected with a harvester. Incorporated thymidine was determined by liquid scintillation counting using a topCount microplate counter (53, 54).

**Results**

Preparation (and schematic presentation) of chemically modified species of heparin - The relationship between sulfation patterns and heparanase-inhibiting activity of heparin species with unmodified backbone was studied using O-desulfated heparin derivatives and N-desulfated, N-acetylated heparins of various degrees of substitution prepared starting from a well-characterized pig mucosal heparin (H-1), using established procedures with slight modifications. 6-O-Desulfation was accomplished using two different procedures, the first (A) involving solvolytic desulfation (44) and the second (B) through activated silyl acetamides (46). As reported (46), attempts to obtain extensively 6-O-desulfated heparins using procedure A involved also partial 2-O-desulfation (10-15%, by NMR analysis). Therefore, the extensively 6-O-desulfated heparin H,\(^{16}\)6Odes was obtained only via procedure B. 2-O-Desulfation of heparin was also performed using two different procedures, leading to different products. The first procedure (A), involving lyophilization of alkaline solutions, quantitatively removes the 2-OSO\(_3\) groups retaining the IdoA residues in their original configuration (47). The second one (B), which involves heating of alkaline solutions, is more easily controlled in order to generate also partially 2-O-desulfated heparins (37) and to convert the 2-O-sulfated L-IdoA residues into D-GalA residues (47-49). Glycol-split derivatives were prepared by periodate oxidation/borohydride reduction of both unmodified heparin and partially 2-O-desulfated heparins as previously described (37). The same procedure was applied to obtain glycol-split derivatives of N-acetyl heparins of various degrees of N-acetylation and 2-O-desulfation. Details are given in Experimental Procedures.

Figure 1 shows the general scheme for the preparation of derivatives of heparin, N-acetyl heparins and the corresponding glycol-split derivatives. The structure of a heparin chain is schematized as composed of N-acetylated (NA), N-sulfated (NS) and mixed NA/NS domains, the prevalent sequences being those of the trisulfated disaccharide (TSD) (Formula 1) (24). For simplicity, only chains containing the antithrombin-binding region (ATBR) are presented, although this region is contained in only about one third of the chains of heparin. The prevalent structure of 6-O-desulfated heparins is shown in formula 2 and that of 2-O-desulfated heparins retaining the L-IdoA configuration is shown in 3. Formula 4 shows the prevalent structure of heparins that underwent inversion of configuration to L-GalA during 2-O-desulfation of L-IdoA. Fully N-acetylated heparins are represented by the general formula 5. Partially N-acetylated heparins have different percentages of N-acetyl groups, the complement to 100% N-substitution being N-SO\(_3\) groups. Glycol-splitting is depicted in Fig. 1 in two different ways. Figure 1A refers to heparin (H) and fully N-acetylated heparin (NAH) glycol-split without any previous modification of their structures. The corresponding
glycol-split products are referred to as “reduced oxyheparins” (RO-H) (36) and “N-acetyl-reduced oxyheparins” (NA-ROH), respectively. Figure 1 B refers to heparin and NAH (in the example, 50% N-acetylated heparin 50NA-H) glycol-split after removal of 2-O-SO$_3$ groups to reach a total content of 50% nonsulfated uronic acid residues, the nonmodified residues being IdoA2SO$_3$ (37). De-O-sulfation of one out of two IdoA residues followed by glycol-splitting gave heparins with prevalent structure 6, corresponding to sequences of poly-pentasulfated trisaccharides (p-PST) separated by glycol-split uronic acid residues (sU) (37). Low-molecular weight species of heparin and representative heparin derivatives were obtained by controlled nitrous acid depolymerization (51) of heparin, 50% glycol-split heparin, and the RO-derivative of 50% N-acetylated heparin.

All compounds were analyzed by mono and two-dimensional, $^1$H and $^{13}$C NMR spectroscopy (37). Analytical data, expressed as relative molar content of 6-OSO$_3$, 2-OSO$_3$, and NSO$_3$ groups are summarized in Table 1. Superscripts on abbreviations for 6-O-desulfated (6DeS), 2-O-desulfated (2DeS), N-acetylated (NA) and glycol-split (gs) heparins, represent relative percentages of 6-O- and 2-O-desulfation, N-acetylation, and glycol splitting, respectively.

Heparanase inhibition by heparin derivatives - Typical heparanase-inhibition curves, showing the gel filtration profiles of sulfate labeled degradation fragments released by heparanase from metabolically labeled ECM in the absence (control) and presence of 1 µg/ml of unmodified heparin and fully N-acetylated heparin are presented in Fig. 2. Inhibition is reflected by the decreased amounts and Kav values of HS fragments released from ECM and eluted as peak II (fractions 20-35), in comparison with control incubation of the ECM with recombinant heparanase in the absence of inhibitors (28, 29). Heparanase activity is calculated as the total amount of cpm eluted in peak II multiplied by the Kav (i.e., elution position) of these fragments. The heparanase inhibitory activity (expressed as percent inhibition of heparanase) of almost all heparins at concentrations of 5, 1, and 0.2 µg/ml, is shown in Table 1. Most of the data represent the average of several separate experiments (numbers indicated). Standard deviations, indicated for each heparin, were usually lower than 5 for the most active compounds and did not exceed 20 as a mean for the less effective ones.

Data in Table 1 confirm that heparin is a strong inhibitor of heparanase (~70% inhibition at 1 µg/ml). No significant differences in inhibitory activity were found among H-1 and other heparin preparations from pig mucosa, beef mucosa, and beef lung (data not shown) in spite of significant differences in their sulfation patterns (detailed in the Experimental Section). Also, activity differences found between the parent heparin and its low-molecular weight species as well as between glycol-split 50NAH and its LMW species were not significant. On the other hand, well-defined significant differences in heparanase-inhibiting activity were associated with specific chemical modifications of heparin. As illustrated in Fig. 3, whereas either 6-O-desulfation or 2-O-desulfation with retention of L-IdoA configuration had little or no effect on the heparanase inhibitory activity of heparin, 2-O-desulfation with change of configuration of the L-IdoA residues to L-GalA markedly decreased the inhibitory activity of heparin.

Also, complete removal of N-sulfate groups followed by N-acetylation resulted in a substantial decrease of the inhibitory activity (Fig 3). However, as illustrated in Fig. 4, this effect was only noted for N-acetylation degrees higher than approximately 50%. On the other hand, glycol-splitting markedly increased the heparanase-inhibiting activity of both heparins and N-acetylated heparins and restored the inhibitory effect lost upon N-acetylation of heparin (Fig. 4 and Table 1). This effect is illustrated in Fig. 4 and Table 1 for N-acetylated heparins of the RO-type (i.e., 25% glycol-split), which almost completely inhibited the heparanase activity (to less than 10% of the control at 1 µg/ml and to 20-30% at 0.2 µg/ml), irrespective of their degree of N-acetylation. Glycol-splitting extended to newly-generated nonsulfated IdoA/GalA residues in heparin and N-acetylated heparins gave products showing high heparanase inhibitory activity. The dose-dependence of the heparanase inhibitory activity is illustrated in Figure 5 for heparin (H), fully N-acetylated heparin and its RO derivative. IC$_{50}$ values calculated from the corresponding curves are >5 µg/ml for NAH, ~0.4 µg/ml for H-1, and ~0.2 µg/ml for 100NA, RO.H.

Gel permeation chromatographic analysis of some products of heparanase digestion,
performed under conditions of the enzyme inhibition assay, indicated that whereas heparin (not shown) and N-acetyl heparin are cleaved by heparanase (as previously shown for heparin) (40), their glycol-split derivatives are not susceptible to cleavage, as illustrated for fully N-acetylated, RO-heparin in Fig. 6A and 52% glycol-split heparin (H,52gs) in Fig. 6B.

**Effect of modified heparins on release of ECM-bound FGF-2 and stimulation of FGF-2 mitogenic activity** - Some of the heparin derivatives were tested for their capacity to release FGF-2 from ECM (18, 34). As demonstrated in Fig. 7, dose-response curves of the FGF-2-releasing activity of glycol-split heparin (H,52gs) and its corresponding low-molecular weight derivative (LMW-H,49gs) were almost superimposable to those reported for heparin (34), indicating that glycol-splitting does not substantially modify the FGF-2-releasing properties of heparin. Also, the curves of the RO derivative and of heparin are superimposable (data not shown). Fig. 7 also shows that glycol-split, N-acetylated heparins behave similarly to non glycol-split NAH (34) in that they release ECM-bound FGF-2 consistently less than unmodified heparin. 100NAH (not shown) and 100NA,RO.H exhibited the lowest FGF-2 releasing activity among the tested compounds, yielding only about twice the spontaneous release observed in presence of the buffer (PBS) alone.

The ability of heparin, 100 NAH and 100NA,RO.H to promote the mitogenic activity of recombinant FGF-2 was investigated using a cytokine-dependent, heparan sulfate deficient, lymphoid cells (BaF3) engineered to express FGFR1 (53, 54). Unlike heparin, both fully N-acetylated heparin (100NAH) and its glycol-split counterpart molecule (100NA,RO.H) failed to stimulate the mitogenic activity of FGF-2, beyond the basal level obtained in the absence of added heparin (Fig. 8). Thus, while glycol splitting of NAH fully restored its heparanase-inhibiting activity, it failed to induce a similar restoration of the ability to displace ECM-bound FGF-2 and to stimulate the mitogenic activity of recombinant FGF-2.

**DISCUSSION**

The HS chains of HSPG in the ECM and on the surface of EC are the natural substrates for heparanase. HSPG, expressed by virtually all cells, are tought to play key roles in numerous biological settings, including embryogenesis, cytoskeleton organization, cell migration, wound healing, inflammation, cancer metastasis and angiogenesis (17, 26). These multiple functions, exerted via distinct mechanisms, are modulated by heparanase through endoglycosidic cleavage of HS (1-3). The site of cleavage is the β-glycosidic linkage of a GlcA residue which must be flanked by N-sulfated or N-acetylated α-linked GlcN residues. At least one O-SO₃ group is essential for efficient recognition by the enzyme (40, 41). The tri-dimensional structure of heparanase is not yet known in detail. Translation of the primary structure of heparanase over another endo β-glycosidase (β-xylanase) shows clusters of basic amino acid residues, at least one of which conceivably implicated in binding to sulfate groups of the substrate (55). Our preliminary studies, applying point mutations and deletions, as well as synthetic peptides, identified aminoacid residues 158-171 as the predominant HS binding domain of the heparanase molecule². HS/heparin and derived oligosaccharides must have a minimal octasaccharidic size to be good substrates for heparanase (40, 41). However, the enzyme can be efficiently inhibited also by shorter but more extensively sulfated oligosaccharides such as maltohexaose polysulfate (MHS) and phosphomannopentaose polysulfate (PI-88) (22). Heparin, though less sulfated than these persulfated oligosaccharides, is a good inhibitor of heparanase activity (28) and is active in experimental metastasis models, as well (20-22). The inhibitory activity of heparin is lost upon extensive O-desulfation and/or a decrease in chain length below a tetradsaccharidic size (20, 34).

Previous reports on the effects of selective O-desulfation on the heparanase-inhibiting activity of heparin (35) are essentially confirmed by the present study. In fact, removal of either the 6-O-sulfate group on the glucosamine residue or the 2-O-sulfate group on the iduronic acid residue only slightly reduced the inhibitory activity of heparin (Fig. 3).

The decrease in inhibitory activity observed in the present study for extensively 6-O-desulfated heparin prepared by the solvolytic method, appears associated with a concomitant partial 2-O-desulfation. On the other hand, the consistently lower heparanase-inhibiting activity of partially 2-O-desulfated derivative obtained...
with procedure B (involving change of configuration from L-IdoA to L-GalA) as compared with that of the 2-O-desulfated derivative obtained by procedure A (with retention of the L-IdoA configuration) is likely associated with different conformational properties of IdoA and GalA. IdoA residues have been demonstrated to be endowed with a unique conformational flexibility ("plasticity"). Such a plasticity, associated with different equienergetic conformations of IdoA residues, all co-existing in a rapid dynamic equilibrium, can currently explain the better protein binding capacity and associated biological properties of IdoA-containing sequences, as compared to the more rigid GlcA-containing ones (56). Based on simple conformational criteria, GalA is expected to have very much the same conformational rigidity as GlcA.

Replacement of N-sulfate groups with N-acetyl groups does not completely suppress the heparanase-inhibitory activity of heparin (34), its activity being reduced to about one third (35), as confirmed by the present study. However, it is noteworthy that a substantial decrease in heparanase-inhibiting activity was only observed for degrees of N-acetylation higher than about 50%. As shown in Table 1 and illustrated in Fig. 4, for degrees of N-acetylation lower than 40%, the inhibitory activity remained essentially the same as that of heparin. This is taken as an indication that only one half of the NSO₃ groups of heparin are essential for complete inhibition of heparanase. The accepted conformation of the N-sulphated (NS) domains of heparin is represented by helices where sets of three sulfate groups (NSO₃, 2SO₃ and 6SO₃) alternate on each side of the chain (57). The observation that only one out of two N-sulfate groups is required to inhibit heparanase, and the assumption that the N-desulfation/N-acetylation reaction occurs randomly along the NS domains, would accordingly suggest that heparin and its derivatives with unmodified backbone bind the enzyme only from one side of the chain.

The effect of glycol-splitting of heparin on heparanase inhibition is largely new. Lapierre et al. (35) reported that periodate oxidation/borohydride reduction of nonsulfated uronic acids of heparin, leading to a product corresponding to H₂⁵gs of the present study, did not impair the inhibitory activity of heparin, a finding taken by these authors as an indication that nonsulfated IdoA were not essential for the activity. The present results on heparins subjected to glycol-splitting only at the level of pre-existing nonsulfated uronic acids (RO.H) (36) and of both the preexisting and the newly generated ones (such as H₅₂gs) (37, 38) indicate that in fact glycol splitting resulted in a marked general increase in the inhibition of the heparanase activity by heparin species. A reasonable explanation of such an effect is that formation of glycol-split residues, involving elimination of conformational constrains with formation of three additional degrees of rotational freedom per each split residue, generates flexible joints which separate from each other heparin sequences containing IdoA₂SO₃ residues, thus facilitating the docking of these sequences to sites essential for heparanase activity. The proposed “extra flexibility” (39) induced by these joints reinforces the binding-driving influence of the already existing intrinsic conformational “plasticity” of iduronate residues. More notable, glycol-splitting also increased the heparanase-inhibiting activity of N-acetylated heparins, even that of fully N-acetylated heparin, whose heparanase binding capacity is very weak when their backbone is unmodified.

Glycol-splitting involves substantial loss of the anticoagulant activity of heparin (36). It is now clear that the main reason for such an effect is the cleavage of C(2)-C(3) bonds of the GlcA residue of the pentasaccharidic sequence (Fig. 1, formula 7). This residue in its unmodified form is essential for binding to antithrombin and whenever it is glycol-split (as in sequence 8), the heparin affinity for antithrombin is completely lost (24, and references therein).

The heparanase inhibiting properties of glycol-split, extensively N-acetylated heparins cannot be explained by the model discussed before where only sulfate groups (perhaps in addition to the uronate carboxyl groups) on the same side of the heparin helix are involved in binding to the enzyme. Such a model is compatible with heparanase binding of heparins that contain no more than 50% N-acetyl groups.

Retention of strong inhibitory activity upon removal all N-SO₃ groups and their substitution with nonpolar N-acetyl groups followed by glycol splitting of nonsulfated uronic acids, implies that efficient docking to heparanase, favored by the flexible joints generated by glycol-
splitting, occurs with a conformation different from that adopted by heparin with a degree of N-acetylation lower than 50%.

Conformational polymorphism is not uncommon in heparin sequences bound to proteins. Thus, X-ray studies showed that heparin sequences may bind FGF-1 in more than one, equally favored conformations (58). Increasing evidence is being accumulated that the plasticity of iduronate residues, combined with some rotational freedom of both uronic acid and amino sugar residues around the glycosydic linkages, favor several possibilities of binding to basic domains of proteins (59). As expected (37, 39), glycol splitting appears to further increase the molecular flexibility of heparin chains, as determined by small angle X-ray scattering (60) and NMR spectroscopy supported by molecular modeling studies (61).

It appears that heparin contains both recognition/cleavage and inhibition sites for heparanase and that its inhibition of the enzyme involves also competition (40). Size profiling of HS degradation products by heparanase in the presence of N-acetyl heparin (Fig. 6A) showed that the fully N-acetylated derivative NAH, is also cleaved by the enzyme. On the contrary, the gel filtration profile obtained in the presence of glycol-split heparin is practically superimposable to that obtained in the absence of the enzyme (Fig. 6B), indicating that modification of GlcA residues totally abolishes cleavage by the enzyme.

HS assembles ligands and receptors into ternary signaling complexes, best exemplified by the FGF/FGFR/heparin complex (62, 63). Following cleavage by heparanase, the multitude of polypeptides sequestered and regulated by HS (17) become bio-available (1, 18) and this requires a tight regulation of their activity, applying, among other approaches, modified species of heparin and HS. The present results on the effect of glycol-splitting on the ability of heparins to release FGF-2 from ECM, extend previous observations on the effect of O-sulfation and N-acetylation on this property (34). As illustrated in figure 7, glycol-split derivatives of both heparin (including LMWH) and NAH did not substantially modify the ability to displace FGF-2 from ECM. In other words, while both native and glycol-split heparins efficiently released FGF-2 from ECM, their N-acetylated counterparts exhibited a markedly reduced ability to displace FGF-2, reflecting the essential involvement of N-sulfate groups in this interaction. This observation supports the finding that glycol-split heparins bind FGF-2 (37) and VEGF (64) with very much the same affinity as unmodified heparins. However, since H50gs inhibits dimerization of FGF-2 (37), it is conceivable that the ECM-bound growth factor is released by glycol-split heparins in an inactive form. Moreover, we have demonstrated that unlike native heparin and LMWH, NAH and even more so glycol split NAH (100NAH,25gs, i.e., NA,RO,H) failed to stimulate FGF-2 mediated proliferation of HS deficient lymphoid cells (Fig. 8). The remarkable heparanase-inhibitory activity of N-acetylated, glycol-split heparins, together with the low levels of FGF-2 that they release from ECM and their inability to stimulate the mitogenic activity of FGF-2, indicate this class of chemically modified heparins as potential antiangiogenic and antimetastatic agents. These compounds also markedly inhibit wound angiogenesis in transgenic mice over-expressing the heparanase gene (65). Furthermore, our preliminary experiments show that some of these heparin derivatives effectively abolish experimental lung colonization of intravenously administered B16-BL6 mouse melanoma cells (66 and our unpublished results).

Retrospective analyses suggest that treatment of venous thromboembolism in cancer patients with low-molecular-weight heparins (LMWH) is associated with additional benefits in terms of their survival (33). The experiments presented in this study were undertaken to develop heparin-based molecules for efficient inhibition of heparanase activity. Polysulfated chains such as those of heparin are expected to envelope the basic clusters of heparanase and compete with its binding to HS. Confirming previous findings (34), we showed that such an activity is retained by a LMW heparin. Moreover, we have demonstrated such a retention also for three representative LMW glycol-split derivatives. Further studies are planned to determine, for each type of derivatives, the shortest chains as well as the shortest IdoA2SO3-containing sequences still showing significant inhibition of the enzyme, and to elucidate whether the carboxylate groups of glycol-split residues participate in binding and inactivation of heparanase.

In conclusion, we have applied desulfation strategies and controlled glycol-splitting in order to remove sulfate groups not necessarily involved
in heparanase recognition and inhibition and to improve the molecular flexibility and biological interactions of heparin. Generation of specific heparanase-inhibiting compounds such as those described in this study, is important not only as a proof of concept, but also as a promising approach to develop heparin-based anti-cancer lead compounds devoid of side effects.

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1The abbreviations used are: HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; ECM, extracellular matrix; FGF-2, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; IdoA, L-iduronic acid; GlcN, D-glucosamine; GlcA, D-glucuronic acid; GlcNAc, N-acetyl D-glucosamine; GlcNSO3, D-glucosamine N-sulfate; LMWH, low-molecular weight heparin; NMR, nuclear magnetic resonance; GPC-HPLC, gel permeation chromatography – high performance liquid chromatography; TDA, triple detector assembly; BTSA, N,O-bis (trimethylsilyl) acetamide; SDS-PAGE, sodium dodecylsulfate – polyacrylamide electrophoresis; MTSFA, N-methyl-N-(trimethylsilyl) trifluoroacetamid; DMSO, dimethyl sulfoxide; NA, N-acetylated; NAH, N-acetyl heparin; NS, N-sulfated; TSD, trisulfated disaccharide; NAD, N-acetylated disaccharide; ATBR, antithrombin binding region; p-PST, poly-pentasulfated trissaccharide; gs, glycol-split; sU, split uronic acid; RO.H, reduced oxyheparin; PBS, phosphate-buffered saline; FGFR1, fibroblast growth factor receptor – 1; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; EDTA, ethylene diamine tetracetic acid.

2Levi-Adam et al, in preparation.

*Key words: heparanase inhibition/heparin derivatives/glycol-splitting*
TABLE 1
Distribution of sulfate groups and heparanase inhibitory activity of heparin and derivatives

| SO₃ groups (mole) | Heparanase inhibitory activity, % |  |
|------------------|----------------------------------|--|
|                  | 5 µg/ml                          | 1 µg/ml | 0.2 µg/ml |
|                  | Mean [SD (N)]                    | Mean [SD (N)] | Mean [SD (N)] |
| NS               |                                   |         |           |
| A6OS             |                                   |         |           |
| 12OS             |                                   |         |           |

**HEPARIN**

| H-1          | 89 | 79 | 69 | 95.4 [3.7 (4)] | 73.5 [13.5 (7)] | 37.2 [20.2 (5)] |

**6-O-DESULFATED H-1**

| H, 716OdeS (A) | 81 | 29 | 55 | 78.9 [14.2 (4)] | 50.5 [25.6 (7)] | 31.3 [31.5 (6)] |
| H, 776Odes (B) | 87 | 23 | 67 | 93.4 [66.0 (2)] | 60.5 [9.4 (2)] | 16.6 [16.6 (2)] |
| H, 736OdeS (B) | 78 | 27 | 64 | 88.9 [79.9 (3)] | 70.9 [18.0 (3)] | 44.9 [36.0 (2)] |
| H, 466OdeS (B) | 82 | 56 | 68 | 79.9 [84.8 (3)] | 63.4 [17.0 (6)] | 35.9 [32.6 (5)] |

**2-O-DESULFATED H-1**

| H, IdoA (A)    | 83 | 85 | 0  | 98.6 [13.3 (3)] | 64.7 [17.8 (3)] | 35.6 [35.6 (3)] |
| H, GalA (B)    | 86 | 74 | 0  | 57.8 [18.9 (2)] | 11.5 [11.0 (2)] | 16.7 [16.7 (2)] |

**N-DESULFATED, N-ACETYLATED H-1**

| 29NAH          | 71 | 80 | 72 | 91.9 [8.3 (3)] | 88.4 [11.6 (4)] | < 15 [15.0 (4)] |
| 39NAH          | 61 | 80 | 71 | 78.9 [4.2 (2)] | 76.6 [7.4 (2)] | < 15 [7.4 (2)] |
| 50NAH          | 50 | 79 | 70 | 87.0 [13.0 (2)] | 52.0 [4.4 (2)] | < 15 [4.4 (2)] |
| 58NAH          | 41 | 79 | 68 | 68.4 [32.9] | 32.9 [15.3 (2)] | < 15 [15.3 (2)] |
| 70NAH          | 30 | 75 | 65 | 71.3 [6.1 (3)] | 37.9 [20.2 (5)] | < 15 [20.2 (5)] |
| 92NAH          | 8  | 71 | 73 | 46.2 [9.6 (2)] | 32.7 [18.0 (4)] | 0 [0.0 (2)] |
| 100NAH         | 0  | 78 | 66 | 68.4 [32.9] | 32.9 [15.3 (2)] | < 15 [15.3 (2)] |

**GLYCOL-SPLIT H-1**

| RO.H            | 89 | a  | 67 | 97.4 [5.8 (2)] | 91.3 [15.8 (2)] | 50.3 [50.3 (2)] |
| H, 52gs         | 89 | a  | 48 | 83.5 [7.3 (4)] | 79.1 [17.7 (6)] | 62.0 [1.6 (3)] |

**N-ACETYLATED, GLYCOL-SPLIT H-1**

| 26NA, RO.H      | 74 | 80 | 77 | 91.5 [12.6 (3)] | 91.1 [3.1 (4)] | 72.1 [6.0 (2)] |
| 40NA, RO.H      | 60 | 80 | 71 | 100.0 [10.0] | 72.3 [72.3] |
| 53NA, RO.H      | 47 | 79 | 71 | 98.2 [18.5] | 94.8 [31.1 (6)] | 85.3 [6.8 (3)] |
| 67NA, RO.H      | 33 | 79 | 79 | 98.5 [2.1 (2)] | 93.9 [2.2 (3)] | 84.0 [7.0 (2)] |
| 100NA, RO.H     | 0  | 71 | 75 | 93.0 [4.2 (2)] | 92.5 [5.0 (3)] | 93.8 [2.6 (2)] |
| 29NAH, 60gs     | 71 | a  | 40 | 88.6 [6.0 (2)] | 79.6 [15.0 (2)] | 62.4 [62.4] |
| 43NAH, 60gs     | 57 | a  | 40 | 94.5 [2.5 (2)] | 70.1 [17.1 (2)] | 72.2 [1.6 (2)] |
| 57NAH, 64gs     | 42 | a  | 36 | 95.1 [2.3 (2)] | 87.0 [10.5 (2)] | 73.8 [73.8] |
| 70NAH, 59gs     | 30 | a  | 41 | 92.9 [4.2 (2)] | 87.8 [12.5 (2)] | 89.2 [89.2] |

**LMW HEPARIN and DERIVATIVES**

| LMW-H-1        | 82 | 77 | 66 | 86 | 47.7 [35.0 (2)] | 43.1 [15.3 (2)] |
| LMW-H, 40gs    | 87 | a  | 51 | 95.7 [1.8 (2)] | 86.2 [2.8 (3)] | 65.8 [65.8] |
| LMW-H, 59gs    | 89 | a  | 51 | 84.4 [10.0 (2)] | 69.9 [10.0 (2)] | 73.8 [73.8] |
| LMW-50NA, gs   | 50 | 79 | 75 | 95.4 [0.6 (2)] | 90.4 [3.0 (3)] | 89.2 [89.2] |

SD : standard deviation = \(\sqrt{\frac{\sum(y_i-\bar{y})^2}{(N-1)}}\). N : number of experiments.; a = 75± 5% (see text)
FIGURE LEGENDS

**Figure 1.** A) Schematic representation of glycol-splitting of pre-existing nonsulfated uronic acid residues of heparin (H) and N-acetyl heparin (NAH). Heparin chains are represented as composed of N-sulfated (NS) domains prevalently made up of trisulfated disaccharide (TSD) sequences, N-acetylated (NA) domains with prevalent N-acetylated disaccharide (NAD) sequences, and mixed (NA/NS) domains. The location of antithrombin-binding region (ATBR) is arbitrary (24). For simplicity, only chains containing the ATBR are represented and the GlcA residue of this region is shown as the only nonsulfated uronic acid residue in the NS region. B) Schematic representation of partial 2-O-desulfation and glycol-splitting of both the pre-existing and the newly generated nonsulfated uronic acid residues of heparin (H) and 50% N-acetylated heparin (50NAH). The represented schematic structure of glycol-split heparin corresponds to derivative H50gs, with splitting of about 50% of the total uronic acid residues, prevalently represented by repeating sequences PST.sU and actually consisting of about 25% pre-existing and 25% newly generated uronic acid residues (38). The example for glycol-split N-acetylated heparin corresponds to the model derivative 50NAH,50gs.

**Figure 2.** Representative heparanase-inhibition curves for heparin and N-acetyl heparin. Sulfate labeled ECM was incubated (4 h, 37°C, pH 6.0) with recombinant human heparanase (40 ng/ml) in the absence (control) and presence of 1 µg/ml heparin (H-1) or N-acetyl heparin (NAH). Sulfate labeled degradation fragments released into the incubation medium were analyzed by gel filtration on Sepharose 6B. The figure shows Peak II (corresponding to fractions 20-35) used to calculate percent residual activity of the enzyme (see later).

**Figure 3.** Heparanase inhibitory activity of O-desulfated species of heparin. Sulfate labeled ECM was incubated (4 h, 37°C, pH 6.0) with recombinant heparanase (40 ng/ml) in the absence (contr) and presence of 1 (white bars) or 5 (dashed bars) µg/ml unmodified heparin (H-1), NAH, or each of the indicated specifically desulfated heparins (6OdeS-H; 2OdeS-H; H,GalA). Sulfate labeled degradation fragments released into the incubation medium were analyzed by gel filtration on Sepharose 6B. Kav of peak II (see Fig. 2), calculated for each compound, was multiplied by the total cpm eluted in peak II. Results are presented as % of control. Residual heparanase activity = Kav x total cpm in peak II (% of control).

**Figure 4.** Inhibition of heparanase by N-acetyl heparins and the corresponding 25 % glycol-split (RO) derivatives. Sulfate labeled ECM was incubated (4 h, 37°C, pH 6.0) with heparanase (40 ng/ml) in the presence of 1 µg/ml N-acetyl heparins (NAH) with increased percentage of N-acetylation (% NAc), or with the corresponding 25% glycol-split derivatives (NA,RO.H). Sulfate labeled material released into the incubation medium was analyzed by gel filtration and heparanase enzymatic activity (Kav x total cpm in peak II) is presented as % of the 100% activity obtained in the absence of inhibitor.

**Figure 5.** Dose-dependence of heparanase-inhibition by heparin, N-acetyl heparin, and 25% glycol-split (RO) N-acetyl heparin. Sulfate labeled ECM was incubated (4 h, 37°C, pH 6.0) with heparanase (40 ng/ml) in the absence or presence of increasing concentrations of heparin (H), 100% N-acetyl heparin (100NAH), or the corresponding 25% glycol-split (RO), 100% N-acetyl heparin (100NA,RO,H). Sulfate labeled material released into the incubation medium was analyzed by gel filtration. Heparanase enzymatic activity (total cpm in peak II x Kav) is presented as % of the activity (100%) obtained in the absence of heparin.

**Figure 6.** Glycol-splitting inhibits cleavage by heparanase. Gel filtration profiles of fully N-acetylated heparin (A) and 52% glycol split heparin (B) before (a) and after (b) incubation with heparanase. 2 mg of each compound were incubated for 48 h at 37°C in 40 mM phosphate-citrate buffer, pH 5.8, with or without 4 µg recombinant heparanase in a total volume of 50 µl. The samples were lyophilized, then
redisolved in 0.5 ml water and analyzed by GPC-HPLC using 300 x 7.8 mm TSK PW 2000 and PW 3000 columns and a refraction index detector. The sharp peak is from salts.

**Figure 7.** Effect of combined N-acetylation and glycol-splitting on release of ECM-bound FGF-2. ECM-coated wells were incubated (3 h, 24°C) with iodinated FGF-2 and the unbound FGF-2 was washed away, as described in “Experimental Procedures”. The ECM was then incubated (3h, 24°C) with the indicated species of N-acetyl and glycol-split heparins and aliquots of the incubation medium were counted in a gamma counter. The remaining ECM was solubilized and its radioactivity counted and used to calculate the percentage of ECM-bound $^{125}$I-FGF-2 released by each compound. Each data point is the average of triplicate wells and the variation did not exceed 10% of the mean.

**Figure 8.** N-acetyl heparin and its corresponding glycol-split derivative fail to stimulate the mitogenic activity of FGF-2. BaF3 (F32) lymphoid cells were plated into 96-well microtiter plates in the presence of 2.5 ng/ml FGF-2 and increasing concentrations of heparin (H-1), 100% N-acetylated heparin (100NAH), or the corresponding 25% glycol-split, 100% N-acetylated heparin (100NA,RO,H). After 48 h, $^3$H-thymidine was added (1 µCi/well) and the amount of incorporated thymidine was determined as described in “Experimental Procedures”. Each data point is the average of triplicate wells and the variation did not exceed 10%.
FIG. 1

A) NS-domain NA/NS-domain NA-domain

H

RO-H

NAH

NA-ROH

B)

H

H,50gs

50NA-H

50NA-H50gs

a) Periodate oxidation/borohydride reduction; b) controlled alkaline 2-O-desulfation

GlcNSO3, GlcNSO3,6SO3, GlcNAc6SO3, GlcNAc

IdoA2SO3, IdoA, GlcA, GalA, glycol-split GlcA, glycol-split IdoA/GalA
FIG. 2
FIG. 3

![Bar graph showing residual heparanase activity for different treatments.](image-url)

- **contr.**
- **Hep.**
- 6OdesH
- 2OdesH
- H,GalA
- NAH

**X-axis:** Treatments

**Y-axis:** Residual heparanase activity

- **5 µg/ml**
- **1 µg/ml**
FIG. 4

![Graph showing the relationship between % NAc and Total cpm in peak II x Kav, % of control, with data points for NAH and NA,RO,H.](http://www.jbc.org/Downloaded from)
FIG. 5

Total cpm in peak II x Kav, % of control vs. µg/ml.

- H
- 100% NAH
- 100% NAH.RO
FIG. 7

![Graph showing the release of ECM bound 125I-FGF in response to varying concentrations of different heparin-derived heparanase inhibitors.](http://www.jbc.org/Downloaded from)

- **LMWH<sup>49gs</sup>**
- **H<sub>52gs</sub>**
- **LMW<sub>50NA,RO,H</sub>**
- **53NA,RO,H**
- **43NAH<sup>60gs</sup>**
- **LMW<sub>50NA,RO,H</sub>**
- **100NA,RO,H**

**Y-axis:** Release of ECM bound 125I-FGF, %

**X-axis:** µg/ml
Modulation of the heparanase-inhibiting activity of heparin through selective desulfation, graded N-acetylation, and glycol-splitting
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