The glycosaminoglycan heparin inhibits the growth of several cell types in vitro including smooth muscle cells and rat cervical epithelial cells. The commercially available heparin which has antiproliferative activity is a structurally heterogeneous polymer that undergoes extensive modifications during maturation. In this report we have performed structure-function studies on heparin's antiproliferative activity using three different cell types: both rat and calf vascular smooth muscle cells and rat cervical epithelial cells. The minimal oligosaccharide size requirements for antiproliferative activity were determined for the three cell types using oligosaccharide fragments of defined length prepared by nitrous acid cleavage and gel filtration and a synthetic pentasaccharide. The size requirements are similar but not identical for the different cell types. Hexasaccharide fragments are antiproliferative for all three cell types but the synthetic pentasaccharide inhibits the growth of only the rat and calf vascular smooth muscle cells. The interdependence between size and charge for antiproliferative activity was investigated using chemically modified oligosaccharides as well as oligosaccharides prepared from heparin and separated into fractions of differing charge by ion-exchange chromatography. There is a strong interdependence between size and charge for antiproliferative activity. For example, increasing the charge of inactive tetrasaccharide fragments by O-oversulfation makes them antiproliferative whereas reducing the charge of active larger fragments causes them to lose their antiproliferative activity. Finally the importance of 2-O-sulfate glucuronic acid moieties for antiproliferative activity was investigated using heparin preparations that lack 2-O-sulfate glucuronic acid. These compounds possess antiproliferative activity indicating that 2-O-sulfate glucuronic acid is not required for antiproliferative activity.

The glycosaminoglycan heparin is a highly charged anionic molecule that exerts a variety of biochemical and biological effects. These effects include binding to antithrombin III and heparin cofactor II which result in the inactivation of serine proteases involved in blood coagulation (1-3), interactions with acidic and basic fibroblast growth factors (4-6), potentiation of angiogenesis (7, 8), altering the activity of various enzymes (9-12), effects on immune responses (13), and modulation of cell growth (14-20). The effects of heparin on cell growth appear to be complex since they depend both on the cell type studied as well as on the total set of growth factors present at the time the cells are studied. For example, although heparin inhibits the growth of vascular smooth muscle cells (VSMC) (14-16) and rat cervical epithelial cells (RCEC) in the absence of epidermal growth factor (EGF) (17), heparin stimulates the growth of human vascular endothelial cells in the presence of acidic fibroblast growth factors (18).

The commercially available heparin that expresses these varied effects is derived from mast cells and is a structurally heterogeneous polymer composed of repeating uronic acid-N-acetyl-D-glucosamine disaccharide units that have undergone a variety of chemical modifications during maturation (reviewed in Ref. 21). These modifications include C5 epimerization of D-glucuronic acid to form L-iduronic acid and extensive sulfation. D-Glucosamine residues can undergo O-sulfation at C6 and C3 together with N-sulfation, whereas L-iduronic acid and rarely glucuronic residues can be O-sulfated at C2. As a result of these modifications, mature heparin polymers contain disaccharide units that are unsulfated mixed with disaccharide units that have varying degrees of sulfation (22-24). These structural variations may be important in determining the biological and biochemical properties of a particular preparation of heparin.

In previous studies we have investigated the structural requirements for heparin's antiproliferative effect for vascular smooth muscle cells (25, 26). Those studies indicated that the size of the oligosaccharide component is important since a synthetic pentasaccharide and larger fragments retained activity whereas disaccharide and tetrasaccharides were inactive. Moreover both the N- and O-sulfates of native heparin were important for antiproliferative activity and a 3-O-sulfate on the internal glucosamine of the synthetic pentasaccharide fragment was critical for the activity of this particular oligosaccharide. In this paper we have extended our analysis of the structural determinants of the antiproliferative effect of heparin in several ways. Since the growth effects of heparin are dependent on the cell type studied (27), we have compared the saccharide chain length requirements for heparin's growth inhibitory effect in the following three cell types: calf and rat VSMC and rat cervical epithelial cells. Second, we have extended our previous studies on the role of sulfation in the
antiproliferative activity of whole heparin by studying the interdependence of oligosaccharide size and degree of sulfation in antiproliferative activity. For these studies we have utilized oligosaccharide fragments of defined length that were either separated by ion-exchange chromatography into fractions of charge or were chemically modified to alter their charge. Third, since studies utilizing hepatocytes have suggested that heparin molecules containing 2-O-sulfate glucuronic acid localize in the nucleus in a growth state-dependent manner (28), we have investigated whether the presence of 2-O-sulfate glucuronic acid residues are critical for antiproliferative activity. Finally, we have investigated whether small oligosaccharide fragments that lack antiproliferative activity themselves are capable of blocking the antiproliferative activity of larger active oligosaccharide fragments.

**MATERIALS AND METHODS**

**Cells**—Rat aortic SMC from Sprague-Dawley rats (CD strain, Charles River Breeding Laboratories Inc., Wilmington, MA) were isolated, cultured, and characterized as previously described (16). In brief, the abdominal segment of the aorta was removed and the fascia cleaned away using a dissecting microscope. The aorta was cut longitudinally, and small pieces of media were dissected from the vessel wall. The dissected media was placed in culture dishes in RPMI-1640 medium that contained 20% fetal calf serum, 4 mm glutamine, 100 mg/ml penicillin, and 100 µg/ml streptomycin. By 1-2 weeks SMC appeared by indirect immunofluorescence (30) and characteristic appearance of confluent cultures. All cultures were maintained at 37°C in a 5% CO2 atmosphere of the N-desulfated, N-acetylated oligosaccharides was performed as described above. The product was recovered by alcoholic precipitation (1.5 volumes of ethanol) after addition of mineral salt (NaC1, 20 g/liter). The precipitate was washed with water and cleaving with periodate oxidation at pH 5 (concentration 10-3 M) cholera toxin. The collagen gels used for culture were prepared by mixing acid-soluble collagen prepared from rat-tails (1.1 with 10 × RPMI-1640 and 0.1 N NaOH) and allowing the gel to form overnight (32). After cultures become confluent, they are subsequently passaged at 1:5 split ratios by incubating the gels in 0.1% collagenase for 1 h at 37°C followed by treatment with trypsin (0.025%/EDTA (0.9 mm) for 10 min at 37°C. Cultures between the 4th and 15th passage that had been maintained in CEGM were used in these studies.

**Growth Studies**—To assay for the antiproliferative activity of heparin in SMC, 5-10 × 10^5 SMC were plated in 16-mm multwell plates in the appropriate growth medium. After 24 h, the cultures were growth arrested by washing with Hank’s balanced salt solution containing 15 mM HEPES, pH 7.4, and placed in RPMI with 0.1% fetal calf serum for 72 h. Growth-arrested cultures of rat SMC were released from Go by feeding with fresh RPMI with 20% fetal calf serum, and growth-arrested cultures of calf SMC were released by feeding with fresh Dulbecco’s modified Eagle’s medium with 10% calf serum. Control cultures were grown in the appropriate medium in the absence of heparin, and experimental cultures were fed medium containing the designated concentrations of specific oligosaccharide fragments. SMC cultures were not refed during the experiments. To assay for the antiproliferative activity of heparin for RCEC, cultures maintained on collagen gels were plated at 1.5 × 10^5 cells/16-mm multwell dish using CEGM. Prior to plating, the dishes were precoated with a film of collagen by incubating the multwells with collagen for 15 min and then aspirating the multwells. The remaining film of collagen was allowed to dry, 24 h after plating the RCEC were rinsed with preformed salt solution. The plates were fed with 7.5% porcine serum containing the appropriate concentrations of oligosaccharides. It should be pointed out that RCEC cultures cannot be reversibly growth arrested by low serum treatment, and the effects of heparin were therefore studied on exponentially growing cells. RCEC cultures were refed at 48-h intervals.

Upon reaching confluence, cell number was determined by counting the total number of cells after detachment with trypsin/EDTA in triplicate cultures using a Coulter Counter (model 2B1). Trypsinized cultures were stained directly with hemacytometer modified in the interdependence of oligosaccharide size and degree of sulfation on cellular inhibition. The percent inhibition is determined from the ratio of net growth in the presence of heparin to the net growth in the absence of heparin.

**Preparation, Characterization, and Chemical Modification of Heparin Oligosaccharide Fragments**—Heparin was cleaved by nitric acid under controlled conditions (33). For this reaction 2 g of the sodium salt of pig mucosal heparin (Chyce, injectable grade) were dissolved in 20 ml of water. 76 mg of sodium nitrite were added and the pH of the solution was lowered to 2.5 with concentrated hydrochloric acid. The desaminative cleavage was allowed to proceed during 60 min. The pH of the solution was then raised to 10 with concentrated sodium hydroxide, and 20 mg of sodium borohydride were added. After stirring overnight and adjusting the pH at 7, the resulting oligosaccharide mixture (1.6 g) was recovered by ethanol precipitation and fractionated by gel-filtration on a Ultrogel ACA-202 column (100 × 5-cm) eluted with 0.5 M sodium chloride. The use of such a column allowed separation of heparin oligosaccharide fragments by size. After solvent evaporation in vacuo and pooling the desialylated oligosaccharides were lyophilized. The oligosaccharide fragments were analyzed by HPLC molecular sieving on a Waters 1-60 column (0.78 × 30-cm) previously calibrated with a series of heparin oligosaccharides of known molecular weights. This analysis indicated that the oligosaccharides were predominantly monodisperse and their molecular weights corresponded to the ones expected for di-, tetra-, hexa-, octa-, deca-, dodeca-, tetradeca-, hexadeca-, and octadecasaccharides.

**O-Sulfuration** of the oligosaccharides was performed using the sodium salts of the oligosaccharides prepared as described above. These were dissolved in water and passed through a Dowex 50-H⁺ cation exchange column (20 ml). The solution was then neutralized with tetrabutylammonium hydroxide and lyophilized. The resulting tetrabutylammonium salt Was thoroughly dried at 50°C under high vacuum. The salt was dissolved in dry dimethylformamide and sulfur trioxide-trimethylamine complex added. After 24 h at 50°C the solution was cooled, a saturated solution of sodium acetate in ethanol was added, and the solution was poured into cold ethanol. After centrifugation the white precipitate was desalted on a column of Sephadex G-15. GPC-HPLC analysis of the compounds confirmed the presence of the desired oligosaccharide fragments. The analysis indicates that the extra sulfate groups are preferentially introduced at primary alcohols of glucosamine and 2-5 anhydromannitol residues.

**N-Acetylated oligosaccharides were prepared by selectively N-desulfating oligosaccharides using the technique of Nagasawa and Inoue (34) and N-acetylation by repeated addition of acetic anhydride at pH 7-9. After gel filtration the product was lyophilized. O-Sulfation of the N-desulfated, N-acetylated oligosaccharides was performed as described for N-sulfated derivatives. Carboxyl-reduced heparin was prepared from the sodium salt of pig mucosal heparin utilizing the method of Shively and Conrad (35). The oversulfated carboxyl-reduced heparin was obtained by dissolving the tetrabutylammonium salt of carboxyl-reduced heparin (90 mg) in dimethylformamide (3 ml) and sulfur trioxide-pyridine complex added (190 mg) after prior cooling at 0°C. After 2 h, water (3 ml) was added, followed by sodium hydroxide allowed to proceed up to pH 11. After gel filtration (Bioexclusion G-25) the product was passed through a Dowex 50 Na⁺ column and lyophilized (41 mg). Conductimetric determination revealed the presence of 4.75 eq sulfate group/mg of compound.

**Periodate oxidation of the sodium salt of pig mucosal heparin (Chyce, injectable grade) utilizing the heparin desulfatase method** and water and cleaving with periodate oxidation at pH 5 (concentration of heparin and sodium periodate, 2%). The solution was then dialyzed and β elimination conducted in basic conditions (sodium hydroxide, final molarity 0.2 N) and fragments reduced by sodium borohydride. The product was recovered by alcohol precipitation (1.5 volumes of ethanol) after addition of mineral salt (NaCl, 20 g/liter). The precipi-
proliferative Effect in Different Cell Types—Previous studies have demonstrated that the smallest active oligosaccharide size and antiproliferative activity is obtained with dodecasaccharide fragments of defined sizes were prepared by nitrous acid cleavage followed by gel filtration. This procedure produces oligosaccharides with even numbers of sugar residues which when analyzed by HPLC molecular sieving were found to be practically monodisperse and have molecular weights that correspond to the ones expected for di-, tetra-, hexa-, octa-, deca-, dodeca-, tetradeca-, hexadeca-, and octadecasaccharides. Fig. 1 compares the growth inhibitory activity of these oligosaccharides on RCEC and rat VSMC. It should be pointed out that we routinely find variations in the sensitivity of cells to the growth inhibitory effects of heparin from experiment to experiment. For example, the degree of inhibition seen at 100 μg/ml of native heparin varied between 55 and 95% in a number of experiments performed with different isolations and different passage numbers of RCEC over a 1.5-year period. Therefore to make comparisons of the antiproliferative activity of different oligosaccharides, the different preparations were always tested in the same experiment.

Disaccharides produced by nitrous acid cleavage have very low antiproliferative activity for both cell types. Tetrascarabides have very low antiproliferative activity for both RCEC and rat VSMC. Hexasaccharides show some growth inhibitory activity for both cell types with 100 μg/ml inhibiting RCEC growth 39% and rat VSMC growth 38%. Octasaccharides were found to be growth inhibitory as native heparin for RCEC with 100 μg/ml of native heparin inhibiting growth 59% and 100 μg/ml of the octasaccharide inhibiting growth 50%. In other experiments decasaccharides were also found to have the same antiproliferative activity as native heparin. Octasaccharides have relatively less growth inhibitory activity for VSMC than does native heparin. When a chemically synthesized pentasaccharide that represents the unique saccharide sequence of the heparin-binding site to antithrombin III was tested, differences were also found between RCEC and rat VSMC. This pentasaccharide has almost as much growth inhibitory activity for rat VSMC as do hexasaccharide fragments and is also growth inhibitory for calf VSMC. However, this pentasaccharide has only minimal growth inhibitory activity when tested on RCEC with 100 μg/ml resulting in only 9% inhibition of growth (Fig. 1). These results indicate that although the size requirements for antiproliferative activity in smooth muscle cells and epithelial cells are similar they are not identical.

Influence of Sulfation on Antiproliferative Activity—Utiliz-

![Fig. 1. Relationships between oligosaccharide size and antiproliferative effect.](image-url)
Characterization of chemically modified oligosaccharides

Oligosaccharides were prepared from native heparin by controlled nitrous acid depolymerization and separated into fractions of discrete sizes by gel filtration. The chemical modifications of the oligosaccharides and charge ratios were performed as described under "Materials and Methods."

| Gel-filtered oligosaccharides | O-Over sulfated | N-Desulfated, N-acetylated | N-Desulfated, N-acetylated, O-oversulfated |
|------------------------------|----------------|---------------------------|------------------------------------------|
| Tetrasaccharide              | 1.71           | 2.62                      | 1.40                                     | 2.17                                     |
| Hexasaccharide               | 1.81           | 2.79                      | 1.27                                     | 2.11                                     |
| Octasaccharide               | 1.88           | 2.67                      | 1.61                                     | 1.88                                     |

Fig. 2. Effects of chemical modification on antiproliferative activity of oligosaccharides. Cell number was determined after treatment for 5-7 days with the various concentrations of chemically modified octasaccharides, hexasaccharides, and tetrasaccharides. RCEC were exponentially growing at the start of the experiments whereas both rat VSMC and calf VSMC were released from G_0 block on day 0. Data are given as mean values for two separate experiments: ● unmodified oligosaccharides; ○ O-oversulfated oligosaccharides; □ N-desulfated, N-acetylated oligosaccharides; ■ N-desulfated, N-acetylated, and subsequently O-oversulfated oligosaccharides.

Noncovalent modification of heparin by nitrous acid involves depolymerization into oligosaccharides and chemical modification by desulfation, acetylation, 0-oversulfation, or 0-Oversulfation. Table I demonstrates that negative charge contributed by carboxyl groups is important in determining antiproliferative activity. In contrast to the results obtained with increasing the degree of sulfation, reduction of the degree of sulfation of the oligosaccharides by removal of N-sulfates followed by reacetylation of the N-positions results in a marked reduction in antiproliferative activity of all of the fragments for all of the cell lines. When the sulfation of the N-desulfated, N-acetylated oligosaccharides is increased by subsequently O-oversulfating the fragments, they regain most of their antiproliferative activity. The degree of sulfation of the N-desulfated, N-acetylated, O-oversulfated fragments is similar to that of the unmodified oligosaccharide fragments (Table I) and the antiproliferative activities of the two compounds are similar. In previous studies utilizing RCEC it has been shown that native heparin is not directly cytotoxic since EGF blocks the antiproliferative effects of heparin (17). To insure that the growth inhibitory effects observed with the modified oligosaccharides are not due to direct cytotoxicity, the oligosaccharides were added to RCEC in the presence of 15 ng/ml EGF. In the presence of EGF, the growth inhibitory effects of both the unmodified and modified oligosaccharides were greatly reduced. For example, the percentage growth inhibition seen at 100 μg/ml of O-oversulfated hexasaccharide or the O-oversulfated, N-desulfated, N-acetylated hexasaccharide was reduced to 6 and 3%, respectively, in the presence of EGF.

Fig. 2 demonstrates that negative charge as contributed by N-sulfation and O-sulfation is important in determining antiproliferative activity. To investigate the contribution of negative charge contributed by carboxyl groups to antiproliferative activity, the effects of both carboxyl-reduced heparin and oversulfated carboxyl-reduced heparin on rat VSMC were determined (Table II). Reduction of the carboxyl groups of native heparin results in a 31–36% decrease in growth inhibitory activity compared to native heparin. When the

Role of carboxyl groups in antiproliferative activity of heparin

Serum arrested rat VSMC were fed fresh media containing 20% fetal calf serum and the designated concentrations of the heparin preparations. Percent inhibition of growth was determined from direct cell counts 5 days later. Values represent mean ± S.E. for three separate experiments.

| Concentration | Native heparin | Carboxyl-reduced | Oversulfated carboxyl-reduced |
|---------------|---------------|------------------|------------------------------|
| μg/ml         |               |                  |                              |
| 1             | 16 ± 8        | 10 ± 4           | 22 ± 3                       |
| 10            | 47 ± 6        | 30 ± 6           | 57 ± 7                       |
| 100           | 69 ± 5        | 44 ± 9           | 76 ± 6                       |
| 200           | 83 ± 4        | 57 ± 7           | 89 ± 2                       |
carboxyl-reduced heparin is subsequently oversulfated, it regains full antiproliferative activity. This indicates that negative charge contributed by both sulfate groups as well as carboxyl groups is important in determining the antiproliferative activity of heparin.

When the data given in Fig. 2 are replotted to look at size relationships for oligosaccharides within a given chemical modification, a size dependence for antiproliferative activity is found for each of the modifications that have antiproliferative activity using each of the cell types. An example of this relationship obtained with RCEC is given in Fig. 3. Although O-oversulfation causes a marked increase in antiproliferative activity, the O-oversulfated tetrasaccharide is less growth inhibitory than the O-oversulfated hexasaccharides and octasaccharides. Similar results are seen with N-desulfated, N-acetylated oligosaccharides that were subsequently O-oversulfated. These oligosaccharides show a size relationship of antiproliferative activity that is identical to that of unmodified oligosaccharides. These results indicate that both the size of the oligosaccharides and the degree of sulfation are important in determining antiproliferative activity.

It should be noted that the loss of antiproliferative activity that occurs after N-desulfation, N-acetylation of the oligosaccharides contrasts with previous results showing that N-desulfated, N-acetylated native heparin retains antiproliferative activity for rat VSMC (25). To insure consistency of the present experiments with our previous work, the desulfated, N-acetylated native heparin was included as a control in several experiments and was found to have good antiproliferative activity (63% inhibition at 100 μg/ml) for rat VSMC. Using RCEC we have also found that N-desulfated, N-acetylated oligosaccharides lack antiproliferative activity whereas native heparin (approximately 15,000 M, and 40–50 residues) that was N-desulfated, N-acetylated has significant antiproliferative activity. This difference between native heparin and the oligosaccharides underscores the interdependence of size and charge in determining antiproliferative activity.

An additional approach to analyzing the interdependence of size and charge on antiproliferative activity was to use tetrasaccharides and hexasaccharides prepared from native heparin by nitrous acid cleavage and subsequently separated into fractions of differing charge densities on the basis of ion-exchange chromatography, as described above under “Materials and Methods.” HPLC analysis of these individual hexasaccharide fractions is given in Fig. 4. These anionic series allowed us to test a wide range of naturally occurring charge densities within a given size oligosaccharide. The antiprolif-

![Fig. 3. Relationships between size and antiproliferative activity of chemically modified oligosaccharides.](image)

![Fig. 4. Characterization of hexasaccharide fractions separated on the basis of charge.](image)
ferative activities of the variously charged fractions were assayed using both RCEC and rat VSMC. The results obtained with 100 µg/ml of the fractionated hexasaccharide molecules are shown in Fig. 5. Both RCEC and VSMC responded similarly to the different fractions and show increasing growth inhibition as the charge of the hexasaccharides increases. For example, the most charged hexasaccharide fractions inhibited RCEC growth 57% in these experiments whereas the least charged fraction showed absolutely no growth inhibitory activity. In this particular series of experiments, nonfractionated hexasaccharides at the same concentration gave 52% inhibition of RCEC and 49% inhibition of VSMC. This is the same amount of growth inhibition as was obtained with the most charged fraction obtained by ion-exchange chromatography. Similar studies were performed with tetrasaccharide fractions separated on the basis of charge density. The most charged fraction of tetrasaccharides was slightly more growth inhibitory for VSMC than the least charged fraction. No clear hierarchy of activity in the tetrasaccharide series could be demonstrated with RCEC (data not shown).

Role of 2-0-Sulfate Glucuronic Acid and Antithrombin III-binding Sequence in Growth Inhibitory Activity—Recently it has been demonstrated that heparan sulfate is present in the culture medium, the pericellular matrix, cytoplasm, and nucleus of cultured rat hepatocytes (28). This heparan sulfate included an unusual form containing a high content of β-D-2-0-sulfate glucuronic acid → d-glucosamine-N-O-(SO₄)₂; disaccharide units which was found in the nuclear pool of nonproliferating cells. The presence of this unusual disaccharide in the nuclear heparan sulfate pool led us to investigate whether or not the presence of 2-0-sulfate glucuronic acid is important for antiproliferative activity. For these studies two different compounds prepared by controlled periodic acid depolymerization of native heparin were used (Table III). Both compounds were analyzed by 13C NMR analysis to verify that neither compound contained detectable levels of 2-0-sulfate glucuronic acid moieties. In order to determine the influence of 2-0-sulfation on the chemical shift of 13C NMR signals of glucuronic acid, we synthesized two derivatives: (methyl 4-0-methyl-β-D-glucopyranoside) uronic acid and its 2-0-sulfated counterpart.3 13C NMR analysis of these derivatives indicated that heparin compounds containing 2-0-sulfated glucuronic acid would have signals at 103 ppm. A spectrum of the anomeric region (90-110 ppm) of one of the periodate-derived products is given in Fig. 6. Only two signals are seen that correspond to C-1 of 2-0-sulfated iduronic acid (101.7 ppm) and C-1 of N-sulfated glucosamine (99.4 ppm). No signal corresponding to 2-0-sulfated glucuronic acid is detectable at 103 ppm indicating that 2-0-sulfated glucuronic acid represents less than 5% of total uronic acids in these compounds. In addition no signal corresponding to C-1 of unsulfated glucuronic acid is detected at 104.5 ppm. Growth studies utilizing these periodate-derived products demonstrated that both had significant antiproliferative activity for RCEC and rat and calf VSMC (Table III). In addition we have compared the antiproliferative activities of decasaccharide fragments prepared either by controlled nitrous acid depolymerization or by controlled periodic acid depolymerization. The latter preparation contains no detectable glucuronic acid or 2-0-sulfated glucuronic acid by 13C NMR. Both of these decasaccharides have significant growth inhibitory activity for RCEC and rat and calf VSMC (Table III). These results indicate that 2-0-sulfate glucuronic acid residues are not essential for heparin's antiproliferative activity.

A number of studies both in vivo and in vitro have clearly demonstrated that heparin’s antiproliferative activity is independent of heparin’s whole blood anticoagulant activity (reviewed in 42). However, previously we have found that a synthetic pentasaccharide is antiproliferative for VSMC and that the 2-0-sulfate of the internal glucosamine of this pentasaccharide is critical for antiproliferative activity (26). These results are somewhat paradoxical since the synthetic pentasaccharide actually represents the unique saccharide sequence for antithrombin III binding (43-46), and the 3-0-sulfate is critical for the binding of this pentasaccharide to antithrombin III and anticoagulant activity (47-49). Therefore, we wanted to further analyze the relationships between the antithrombin III-binding sequence and antiproliferative activity. The pentasaccharide sequence contains an unsulfated glucuronic acid and is therefore lost during depolymerization of heparin with periodic acid. Our findings that preparations of heparin depolymerized with periodate and which lack glucuronic acid by 13C NMR have significant antiproliferative activity supports the conclusion that the antithrombin III-binding sequence is not required for antiproliferative activity (Table III).

Lack of Competition between Small Inactive Fragments and Larger Antiproliferative Fragments—To determine whether small nonantiproliferative oligosaccharides are capable of blocking the growth inhibitory effects of larger, active heparin fragments, an excess of inactive di- and tetrasaccharide fragments were mixed with 18 saccharide and 10 saccharide fragments (Table IV). The small nonantiproliferative oligosaccharides do not block the antiproliferative effects of the larger heparin fragments. For example, addition of 10 µg/ml of the decasaccharide fragment alone results in 88% inhibition of RCEC growth in these particular experiments. When a 200-fold molar excess (40-fold excess on a mass basis) of the inactive disaccharide is added together with the decasaccharide fragment the cells still show an 86% inhibition of growth. Similar results were found utilizing rat VSMC and using the inactive tetrasaccharide instead of the disaccharide and with an 18-saccharide fragment instead of the decasaccharide. These results demonstrate that small inactive oligosaccha-

3 M. Petitou and P. Duchaussoy, unpublished data.
Heparin's Structure-Function Relationships

The various heparin fragments were produced as described under "Materials and Methods." The % growth inhibition caused by 100 µg/ml of each of the fractions was determined after 5-7 days exposure of exponentially growing RCEC or rat VSMC released from G0 block on day 0.

| Description | Molecular weight | USP anticoagulant activity | Presence of 2-O-sulfate glucuronic acid | Presence of pentasaccharides ATIII-binding sequence | % Growth inhibition at 100 µg/ml |
|-------------|------------------|----------------------------|----------------------------------------|--------------------------------------------------|--------------------------------|
| Controlled periodic acid depolymerization and alcohol fractionation | 5000-8000 | >5 | No | No | 51  |
| Controlled periodic acid depolymerization | 2000-5000 | 0 | No | No | 54  |
| Decasaccharide produced by nitrous acid depolymerization and fractionation | ~3000 | 8 | No | Yes | 45  |
| Decasaccharide produced by periodic acid depolymerization and fractionation | ~3000 | 0 | No | No | 50  |

Effects of small oligosaccharides on the antiproliferative activity of larger heparin fragments

Exponentially growing RCEC were fed fresh media containing the designated concentrations and combinations of the oligosaccharides. Percent inhibition was determined from direct cell counts 5 days later. Values represent mean ± S.E. for three experiments.

| Antiproliferative species | Blocking oligosaccharide | Inhibition | % |
|--------------------------|--------------------------|------------|---|
| Decasaccharide (10)      | None                     | 88 ± 2     |   |
| Decasaccharide (10)      | Disaccharide (400)       | 86 ± 2     |   |
| Decasaccharide (10)      | Disaccharide (100)       | 82 ± 1     |   |
| Decasaccharide (10)      | Tetrasaccharide (400)    | 80 ± 2     |   |
| Decasaccharide (10)      | Tetrasaccharide (100)    | 80 ± 1     |   |
| Octadecasaccharide (10)  | None                     | 92 ± 2     |   |
| Octadecasaccharide (10)  | Disaccharide (400)       | 85 ± 10    |   |
| Octadecasaccharide (10)  | Disaccharide (100)       | 92 ± 4     |   |
| Octadecasaccharide (10)  | Tetrasaccharide (400)    | 88 ± 3     |   |
| Octadecasaccharide (10)  | Tetrasaccharide (100)    | 89 ± 1     |   |
| Disaccharide (100)       | None                     | 9 ± 9      |   |
| Disaccharide (400)       | None                     | 18 ± 16    |   |
| Tetrasaccharide (100)    | None                     | 0          |   |
| Tetrasaccharide (400)    | None                     | 0          |   |

Fig. 6. 13C NMR spectrum of the anomeric region (90-110 ppm) showing the absence of a signal corresponding to 2-O-sulfated glucuronic acid in oligosaccharides obtained after periodate oxidation of heparin. Only two signals are evident in the anomeric region, at 101.7 ppm (C-1 of N-sulfated glucosamine). Studies on model compounds indicate that a 2-O-sulfated glucuronic acid would give a signal at 103 ppm while a glucuronic acid usually yields a signal at 104.5 ppm. Neither of these signals are detected in the oligosaccharide obtained after periodate oxidation.

The anticoagulant activity of heparin is a function of its ability to facilitate the formation of a molecular complex between antithrombin III and serine proteases of the coagulation system. The inhibition of factor IIa by antithrombin III in the presence of heparin is dependent on the size, charge, and specific chemical composition of heparin (reviewed in 50). Recently, we have been characterizing the structural requirements for another of heparin's activities, its antiproliferative activity. Both size and charge are also important in determining whether a particular heparin species is growth inhibitory for VSMC. Using oligosaccharides produced by controlled depolymerization of heparin we previously demonstrated that hexamers but not tetramers had antiproliferative activity for rat VSMC (25). In addition, a completely synthetic pentasaccharide sequence has antiproliferative activity for those cells (26).

Studies comparing the growth inhibitory effects of heparin on different cell types have shown that variations occur in both their sensitivity to heparin as well as the specific effects of heparin (40). For example, heparin blocks rat VSMC late in the G1 phase of the cell cycle and causes a down-regulation of EGF receptors (41, 51), whereas heparin blocks RCEC earlier in the G1 phase and causes an increase in the number of EGF receptors (39). Because of these differences it was of interest to compare the saccharide chain length requirements for heparin's growth inhibitory effect in different cell types. The current results indicate that the oligosaccharide size requirements for antiproliferative activity are similar in the two different cell types. Hexasaccharides inhibit both RCEC as well as rat and calf VSMC and although the synthetic pentasaccharide inhibits rat and calf VSMC but not RCEC,
O-oversulfated tetrasaccharide fragments are capable of inhibiting all three cell types. Sulfation of the native heparin molecule is important in determining antiproliferative activity for rat VSMC. Utilizing chemically modified native heparin both N- and O-sulfates were found to be important for antiproliferative activity (25). The current results indicate that carboxyl groups also contribute to antiproliferative activity. To investigate whether there is an interdependence between size and sulfation of oligosaccharides for antiproliferative activity, we have studied a series of chemically modified oligosaccharides. O-Sulfation was also found to be important for antiproliferative activity of the oligosaccharide fragments. O-Oversulfation increases the activity of moderately active fragments and results in measurable activity in an otherwise inactive tetrasaccharide fragment. N-Desulfation followed by N-acetylation of octasaccharides and hexasaccharides results in a loss of antiproliferative activity. This loss of activity can be overcome by subsequent O-oversulfation of these fragments which restores the sulfation ratio back to that seen with the nonmodified fragments. However, for each of the chemical modifications, the octasaccharide is more active than hexasaccharides and tetrasaccharides. Therefore, both the sulfation ratio as well as size are important in determining the antiproliferative activity of oligosaccharide fragments. This relationship between size and charge was also demonstrated by the findings that although N-desulfation followed by N-acetylation of octasaccharides causes the oligosaccharides to lose antiproliferative activity; this same modification of native heparin does not affect antiproliferative activity. To analyze whether a similar interdependence of size and charge on antiproliferative activity exists for nonchemically modified oligosaccharides, we have produced oligosaccharide fragments from heparin by controlled nitrous acid depolymerization and separated them into fractions of differing charge by ion-exchange chromatography. Hexasaccharide fractions prepared in this way show an excellent correlation between charge and antiproliferative activity with the most charged fraction exhibiting the greatest antiproliferative activity.

The synthetic pentasaccharide sequence that has growth inhibitory activity for rat VSMC but not RCEC represents the unique saccharide sequence of the heparin-binding site to antithrombin III and is a heparin type glycosaminoglycan since it has alternating uronic acids of various degrees of sulfation and glucosamines (44–46). An internal N-sulfated 3-6-di-O-sulfated-d-glucosamine is essential for the antithrombin III binding of this compound and previously we have demonstrated that the 3-O-sulfate is also essential for its antiproliferative activity on VSMC. Since a number of different studies have demonstrated that heparin preparations devoid of whole blood anticoagulant activity exhibit antiproliferative activity (reviewed in 42), it seems somewhat paradoxical that this oligosaccharide exhibited antiproliferative activity. Our current findings that this compound is not antiproliferative for RCEC suggests that the antithrombin III-binding sequence is not uniquely responsible for the antiproliferative activity of native heparin. This interpretation is further supported by the finding that two separate larger heparin fragments that lack the pentasaccharide sequence have antiproliferative activity for both VSMC and RCEC. In addition, in preliminary studies we have determined the antiproliferative activity for VSMC of a slightly modified pentasaccharide that lacks a 2-O-sulfate on the internal iduronic acid. This particular pentasaccharide has greatly diminished antithrombin III binding when compared to the unmodified pentasaccharide but has similar antiproliferative activity.

Conrad and co-workers (28) have found that unusual heparan sulfates containing a high content of β-d-glucuronosyl (2-SO₄) → d-glucosamine N- O-SO₄₃ disaccharide units are present in the nucleus of hepatocytes and that both the rate of heparan sulfate synthesis as well as the structural features of cell-associated heparan sulfate are altered by the growth state of the cells. This suggests that β-d-2-O-sulfate glucuronic acid may be important for antiproliferative activity. To test this possibility we have assayed heparin fractions that lack 2-O-sulfate glucuronic acid and found that they have significant antiproliferative activity. Therefore, the presence of 2-O-sulfate glucuronic acid is not required for antiproliferative activity in our assay systems. However, these results do not rule out the possibility that endogenous 2-O-sulfate glucuronate-rich heparin species may play a growth regulatory role in cells.

Inhibition of the serine protease thrombin by antithrombin III in the presence of heparin appears to require the formation of a trimolecular complex between the three molecules (52–54). Although the antithrombin III-binding site for heparin recognizes a unique pentasaccharide sequence, a heparin molecule of at least 16–20 saccharides is actually required for the formation of the complex and thrombin inhibition. To investigate whether an oligosaccharide too small to have antiproliferative activity itself was capable of binding to either an intracellular or extracellular recognition site and inhibit antiproliferative activity, large excesses of inactive di- and tetrasaccharides were added together with active octadesaccharides and decasaccharide fragments. The smaller fragments lacked the ability to inhibit the activity of the larger fragments suggesting that binding to the oligosaccharide recognition site is sufficient for antiproliferative activity.

The above structure-function experiments have characterized a number of important structural requirements of heparin's antiproliferative activity and in particular demonstrate that there is an interdependence of oligosaccharide size and charge. These results suggest that neither a single precise arrangement of sulfates nor a specific sulfate are essential for antiproliferative activity. In addition, the data suggest that a specific saccharide composition is also not required for antiproliferative activity. Therefore, the antiproliferative effects of heparin appear to be regulated by different sorts of structural determinants than are the binding of heparin to antithrombin III or the localization of heparan sulfate to the nucleus of cells. The understanding of these structural requirements should prove useful in determining the mechanisms responsible for heparin's antiproliferative activity.

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