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Note

Heparin oligosaccharide sequence and size essential for inhibition of pulmonary artery smooth muscle cell proliferation

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Dedicated to Professor Derek Horton on the occasion of his 70th birthday

Abstract

Heparin has a wide range of important biological activities including inhibition of pulmonary artery smooth muscle cell proliferation. To determine the minimum size of the heparin glycosaminoglycan chain essential for antiproliferative activity, porcine intestinal mucosal heparin was partially depolymerized with heparinase and fractionated to give oligosaccharides of different sizes. The structure of these oligosaccharides was fully characterized by 1D and 2D ^{1}H NMR spectroscopy. These oligosaccharides were assayed for antiproliferative effects on cultured bovine pulmonary artery smooth muscle cells (PASMCs). The tetrasaccharide (4-mer) exhibited no heparin-like activity. Decasaccharides (10-mers) and dodecasaccharides (12-mers) displayed a reduced level of activity when compared to full-length heparin. Little effect on activity was observed in deca- and dodecasaccharides with one less 2-*O*-sulfo group. The 14-, 16-, and 18-mers showed comparable growth-inhibition effects on PAMSC as porcine intestinal mucosal heparin. These data suggest that a 14-mer is the minimum size of oligosaccharide that is essential for full heparin-like antiproliferative activity. Since the 14- to 18-mers have no 3-*O*-sulfo groups in their glucosamine residues, their full activity confirms that these 3-*O*-sulfonated glucosamine residues, which are required for heparin's anticoagulant activity, are not an essential requirement for antiproliferative activity. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

In the normal physiological state, smooth muscle cells (SMCs) are in a quiescent growth state in pulmonary arterial walls. This is regulated by a balance between inhibitory and mitogenic factors.[1–3] Heparin is essentially a highly sulfonated subclass of heparan sulfate that consists primarily of S-domains, having a high content of sulfo groups and a relatively low level of structural diversity.[4–6] Heparin and heparan sulfate have been reported to inhibit PASMCs growth in vitro,[7–13] PASMCs Na^+/H^+ exchange,[14] and PASMCs hypertrophy.[15] Heparin is also found to reduce vascular remodeling in response to hypoxia in mice, rat and guinea pigs in vivo.[16–19]

Structural determinants of antiproliferative activity of heparin on bovine PASMCs have been recently reviewed.[20] However, precise structural determinants of the antiproliferative properties of heparin have not been well understood as yet. Earlier studies on the size requirements of heparin for vascular smooth muscle cell antiproliferative activity have shown that for full activity, the smallest active heparin oligosaccharide is a dodecasaccharide. These oligosaccharides were prepared by chemical cleavage of heparin.[21,22] The reported importance of 2-*O*-sulfo groups in glucuronic acid residues of the heparin glycosaminoglycan chain is

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controversial. Wright and co-workers have presented evidence that the 2-*O*-sulfo group containing glucuronic acid is not required for antiproliferative activity of heparin.[23] In contrast, Tiozzo and co-workers have found that the 2-*O*-sulfo group containing glucuronic acid residue is required for antiproliferative activity.[24] In addition to this, Castellot and co-workers have found that the presence of 3-*O*-sulfo groups on the internal glucosamine residue of a synthetic pentasaccharide is critical for growth inhibitory capacity.[22] In a recent study, we found that 3-*O*-sulfo groups in glucosamine residues are not critical for full length heparin's antiproliferative activity.[10]

In our studies aimed at determining the structural requirements within heparin essential for native heparin's antiproliferative properties,[11] we found that: (a) high-molecular-weight and low-molecular-weight heparin fractions have identical antiproliferative properties; (b) the antiproliferative properties of heparin reside in the glycosaminoglycan chains; and (c) heparin fractions with lower overall charge density, obtained by fractionation on a DEAE-cellulose column, have antiproliferative activity not seen in fractions with higher charge density. Recently, we have reported that complete sulfonation of native heparin did not enhance its bovine PASMC antiproliferative activity in comparison to the starting heparin preparation.[25]

To understand the structural requirements, particularly the heparin glycosaminoglycan chain size necessary for full antiproliferative activity, different size oligsaccharides from heparin were prepared under mild conditions by enzymatic treatment with heparin lyase I (heparinase). Heparinase cleaves heparin at the linkage between *N*-sulfo-α-D-glucosamine (a 6-*O*-sulfo group can be present) and 2-*O*-sulfo-α-L-iduronic acid, affording an unsaturated 2-*O*-sulfo uronic acid at the non-reducing end of the oligosaccharide product.[26] The resulting heparin oligosaccharides were then fractionated and characterized by 1D and 2D ^{1}H NMR spectroscopy and assayed for antiproliferative activity on bovine pulmonary artery smooth muscle cells (PASMCs). The content of 2-*O*-sulfo groups in the hexuronic acid residues and 3-*O*-sulfo groups in the glucosamine residues and their effect on the antiproliferative properties of these oligomers have also been investigated.

2. Results and discussion

In an earlier publication, we showed that different commercially available heparin preparations from different sources vary in their inhibitory effects on bovine PASMCs.[14] Moreover, we found that the percent inhibition of PASMC growth by heparin decreases with decreased dose. In addition to this, we have also recently found that: (a) high- and low-molecular-weight heparin preparations have no appreciable difference in their antiproliferative activity on bovine PASMCs; (b) the antiproliferative activity of heparin resides in the glycosaminoglycan chains and not in the peptide core; and (c) heparin fractions by DEAE cellulose column chromatography, with low overall charge density, have antiproliferative properties not seen in fractions with high charge density fraction.[11]

To evaluate the minimum oligosaccharide size necessary to retain full antiproliferative activity, we cleaved a commercial heparin under mild conditions to give oligosaccharides of different sizes. This starting heparin was first examined by cellulose acetate electrophoresis (data not given). The analysis data showed that this heparin preparation also contained two bands as reported in other heparin preparations earlier.[10] This heparin preparation was depolymerized to prepare oligosaccharides for bioassay as described below.

Heparin was partially depolymerized with heparinase[26] and fractionated by GPC and semipreparative SAX-HPLC. Using this approach it was possible to prepare heparin-derived oligosaccharides that were $>90\%$ pure as assessed using analytical SAX-HPLC, gradient PAGE and normal- and reverse-polarity CE.[27,28] The structure of these oligosaccharides, which was deduced using 2-dimensional correlation spectroscopy and nuclear Overhauser effect NMR spectroscopy,[29] is presented in Fig. 1. Tetrasaccharide (Fig. 1(a)) (**1**), decasaccharide (**3**) and dodecasaccharide (**5**) each contain repeating sequences of heparin's major trisulfated disaccharide unit, →4)-*N*-sulfo-α-D-glucosamine 6-*O*-sulfate (1→4)-α-L-iduronic acid-2-*O*-sulfate(1→. Decasaccharide (**2**) and dodecasaccharide (**4**) each have reduced sulfation. These oligosaccharides contain a disaccharide with one less 2-*O*-sulfo group and have a sequence in which a glucuronic acid residue replaces the 2-*O*-sulfo iduronic acid residue at the position immediately adjacent to the reducing-end (Fig. 1(b)).

Purification of larger oligosaccharides (>12-mers) possesses several challenges. First, while GPC and SAX-HPLC give good resolution between a tetrasaccharide and a hexasaccharide, differing in molecular weight and net charge by 50%, it gives poor resolution between a tetradecasaccharide and hexadecasaccharide differing in molecular weight and net charge by 14%. Second, a hexadecasaccharide with reduced sulfation can have a molecular weight and net charge that is nearly identical to a fully sulfated tetradecasaccharide. Third, the number of structural and sequence permutations increase with oligosaccharide size, resulting in a large number of possible tetradecasaccharide structures, thus, confounding their separation. While the first problem is inherent to the separation methods available, the last two problems are because of the complex-

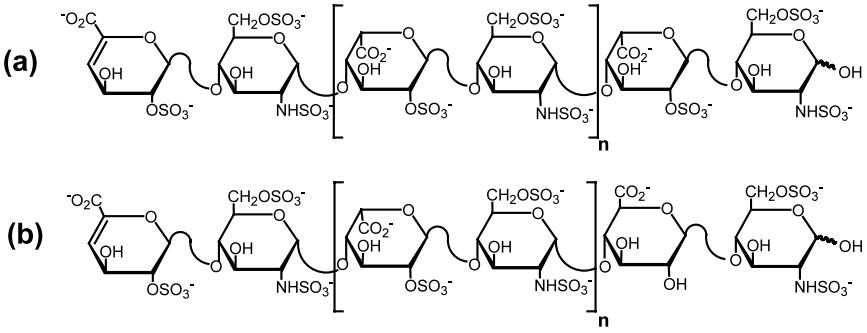

Fig. 1. Structure of heparin-derived oligosaccharides. Shown is a fully sulfated structure (a) corresponding to tetrasaccharide (**1**), decasaccharide (**3**), dodecasaccharide (**5**) and the major component in the purified tetradecasaccharide (**6**), hexadecasaccharide (**7**) and octadecasaccharide (**8**) fractions, where $n = 0$, 3, 4, 5, 6 and 7, respectively. Also shown is an undersulfated structure (b) corresponding to decasaccharide (**2**) and dodecasaccharide (**4**), where $n = 3$ and 4, respectively. All the oligosaccharides were used as their sodium salts.

ity of the mixture being separated. To decrease the similarity of components in the higher oligosaccharide fractions (14–18-mer) these mixtures were chemically treated to degrade oligosaccharides having unsulfated uronic acid residues. Refractionation of this treated oligosaccharide mixture provided fractions containing primarily a tetradecasaccharide (**6**), hexadecasaccharide (**7**) and octadecasaccharide (**8**). Gradient PAGE and analytical SAX-HPLC confirmed that each sample was size and charge uniform. CE analysis suggested that each sample was $\sim 60\%$ pure. Based on the known selectivity of periodate oxidation for vicinal diols, the structure of the previous heparin-derived oligosaccharide isolated in our laboratory[29] and the known composition and structural variability of heparin,[4,5] the most likely structure for the major component in each is the fully sulfated oligosaccharide.

Gradient PAGE analysis of each of the heparin-oligosaccharides is presented in Fig. 2. This gel confirms the size purity of each sample and suggests that sound conclusions on the relationship between oligosaccharide size and biological activity can be made based on these samples. The tetradecasaccharide (**6**) (14-mer) was subjected to further analysis by reverse-polarity capillary electrophoresis (CE) and high-field ^{1}H NMR spectroscopy (Fig. 3). CE showed it to be 94% pure and NMR confirmed its structure (Fig. 1, legend).

The above oligosaccharides (Fig. 1) were assayed for their antiproliferative activities (Fig. 4). Due to the difficulties (discussed above) in preparation of these oligomers in large amounts, low doses (5 μg/mL) of the oligosacccharides were used in the assay. Therefore, the inhibition effect on growth of PASMCs observed with the parent heparin and the oligosaccharides is less than 50%. Our results indicate that the 14-mer (Fig. 1(a), **6**) and above, 16- and 18-mers (Fig. 1(a), **7** and **8**), have full antiproliferative activity on PASMCs. This suggests that 14-mer is the smallest oligomer necessary for heparin-like PASMC antiproliferative activity. The sequence of the 14-mer as determined by NMR is as given in Fig. 1(a), $n = 5$. Castellot et al. have shown that a 12-mer is essential for maximal antiproliferative activity of aortic vascular SMCs.[21] This difference in the size of oligomer required for antiproliferative activity suggests the origin of vascular smooth muscle cells (i.e., pulmonary or aortic) is important to determine the size of oligomer of heparin for antiproliferative activity.

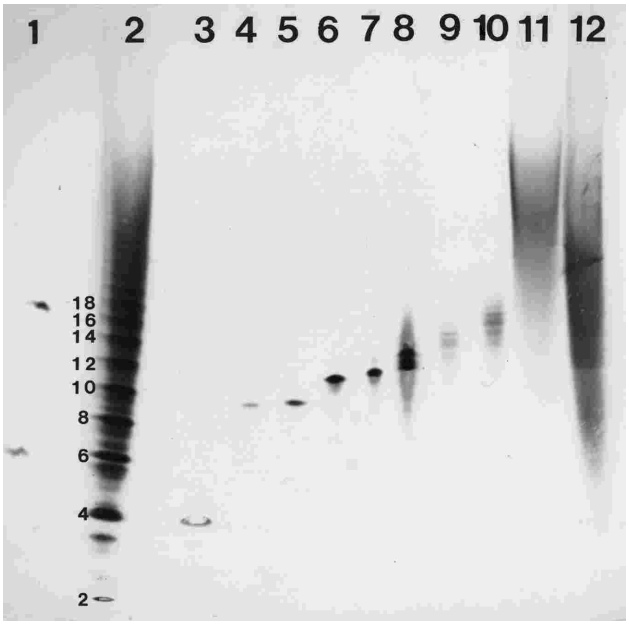

Fig. 2. Gradient PAGE analysis of heparin-derived oligosaccharides. The twelve lanes of this gel contain: (1) bromophenol blue marker; (2) a mixture of heparin-derived oligosaccharides, the major bands in which correspond to fully sulfated oligosaccharides (the numbering to the left corresponds to the degree of polymerization of each major band);[27] (3) tetrasaccharide **1**; (4) decasaccharide **2**; (5) decasaccharide **3**, (6) dodecasaccharide **4**; (7) dodecasaccharide **5**; (8) purified tetradecasaccharide (14-mer) fraction **6**; (9) purified hexadecasaccharide (16-mer) fraction **7**; (10) purified octadecasaccharide (18-mer) fraction **8**; (11) heparan sulfate; and (12) heparin.

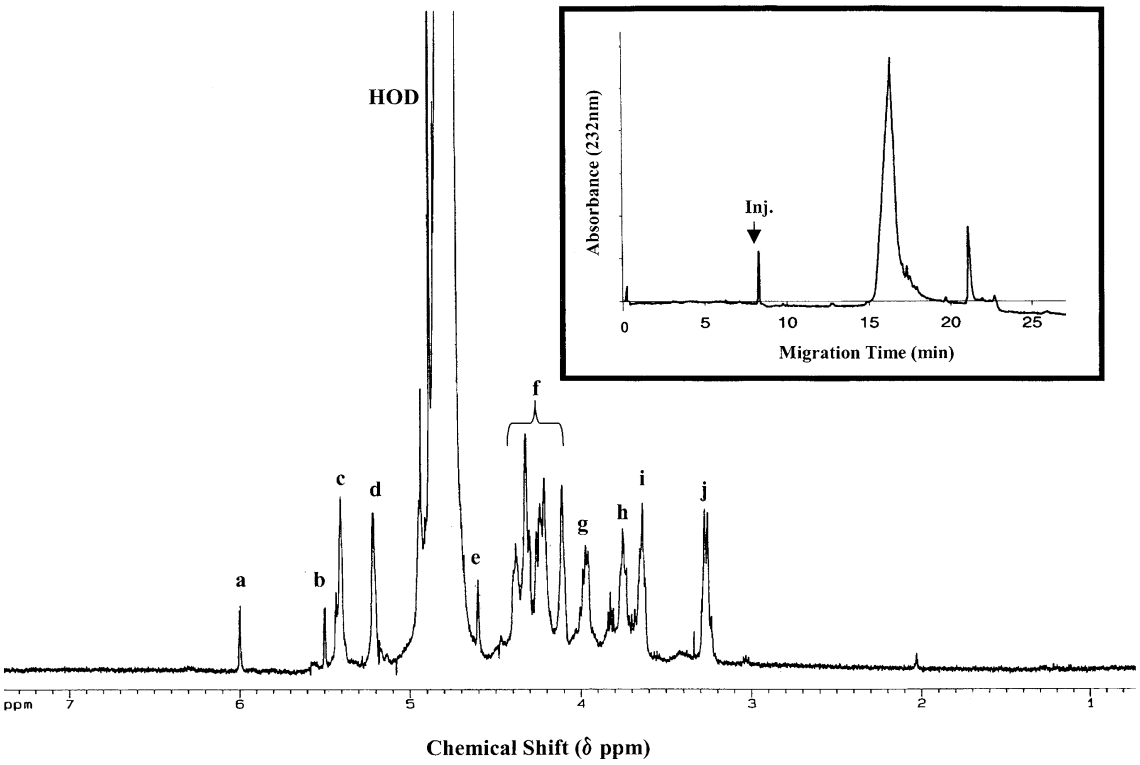

Fig. 3. ^{1}H NMR spectrum (600 MHz) of tetradecasaccharide (**6**) in D_2O and purity analysis by reverse-polarity capillary electrophoresis (CE) (29) (inset). Residues A–N in the tetradecasaccharide (**6**) correspond to its 14 saccharide units with A at the non-reducing end and N at the reducing end. Major signals in the NMR spectrum assigned by 2D ^{1}H NMR correspond to: (a) A4 (proton at the 4-position of non-reducing terminal residue A); (b) A1; (c) B1, D1, F1, H1, J1, L1, N1; (d) C1, E1, G1, I1, K1, M1; (e) A2; (f) C2, E2, G2, I2, K2, M2 and A3, C3, E3, G3, I3, K3, M3 and C4, E4, G4, I4, K4, M4 and B6, D6, F6, H6, J6, L6, N6; (g) B5, D5, F5, H5, J5, L5, N5; (h) B4, D4, F4, H4, J4, L4, N4; (i) B3, D3, F3, H3, J3, L3, N3; and (j) B2, D2, F2, H2, J2, L2, N2. CE (inset) shows a major peak at 16.5 min corresponding to tetradecasaccharide (**6**) that is 94% pure.

These results support the earlier finding that the growth effect of heparin is cell-type dependant.[30]

The heparin-derived tetrasaccharide (Fig. 1(a), $n = 0$) is inactive against pulmonary artery smooth muscle cells. The decasaccharide and dodecasaccharides (10- and 12-mers) (Fig. 1(a), $n = 3$ and 4) have weak antiproliferative activities (Fig. 4, column d and f). The 2-*O*-sulfo hexuronic acid next to reducing end glucosamine residue of 10- and 12-mers (Fig. 1(b), $n = 3$ and 4) has little effect on their biological activities. Our results on the requirements of 2-*O*-sulfo groups are similar to the results of Wright et al.[23] Our work and that of Wright et al.[23] was carried out with more structurally characterized heparin oligosaccharides. Other studies that indicate otherwise involve desulfation of unfractionated heparin, which is structurally more heterogeneous, thus making the analysis of results complicated. The structure of the oligomers in this study has no 3-*O*-sulfo groups on glucosamine residues (Fig. 1). The 3-*O*-sulfo on the internal glucosamine residue has been reported critical for the growth-inhibitory capacity of a synthetic pentasaccharide.[22] A report published from our laboratory has presented evidence that in unfractionated heparin, the 3-*O*-sulfo group of glucosamine is not critical for it's antiproliferative activity.[10] The growth inhibitory activity of the 14–18-mer heparin fragments, containing no 3-*O*-sulfo

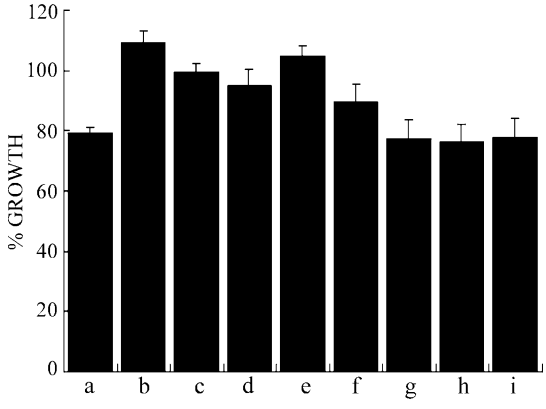

Fig. 4. Percent growth of bovine pulmonary artery smooth muscle cells grown in media containing 10% FBS plus: Column a, heparin; Column b, 4-mer [Fig. 1(a), $n = 0$]; Column c, 10-mer (undersulfated) [Fig. 1(b), $n = 3$]; Column d, 10-mer [Fig. 1(a), $n = 3$]; Column e, 12-mer (undersulfated) [Fig. 1(b), $n = 4$]; Column f, 12-mer [Fig. 1(a), $n = 4$]; Column g, 14-mer [Fig. 1(a) $n = 5$]; Column h, 16-mer [Fig. 1(a), $n = 6$] and; Column i, 18-mer [Fig. 1(a), $n = 7$].

groups on glucosamine residues, confirm our previous results.

In conclusion, these results on native heparin oligomers obtained by cleavage of depolymerized heparin under very mild conditions, i.e., by enzymatic cleavage, suggest that: (a) the 14-mer is the minimal size of native heparin fragment required for full heparin-like antiproliferative activity on PASMCs; (b) the number of 2-*O*-sulfo groups on the hexuronic acid residues has little influence on the growth inhibitory effects of heparin fragments; and (c) the data further confirms that a 3-*O*-sulfo group on glucosamine residues is not a critical requirement for oligomers for their heparin-like activity.

3. Experimental

Materials.—Porcine mucosal heparin, sodium salt (145 U/mg) was from Hepar Industries, Franklin, OH. Heparin lyase I (heparinase, E.C. 4.2.2.7) used in the large-scale depolymerization was from IBEX, Montreal, Canada. Sephadex G-50 (superfine) from Pharmacia Biochemicals, Piscataway, NJ and Bio Gel P-2 (fine), was from BioRad, Richmond, CA. SAX-HPLC was performed on 5-μm Spherisorb columns from Phase Separation, Norwalk, CT of dimensions 0.64 × 25 cm (analytical) and 2.5 × 25 cm (semipreparative).

Gradient polyacrylamide gel electrophoresis (PAGE) was performed on a 32-cm vertical slab gel unit SE620, from Hoefer Scientific Instruments, San Francisco, CA. CE was performed using a fused silica capillary, 75 μm i.d. × 375 μm o.d., 78 cm long on a Dionex Capillary Electrophoresis System.

Preparation of heparin oligosaccharides.—The detailed method for preparation and characterization of heparin oligosaccharides from the size of tetrasaccharide (4-mer) to dodecasaccharide (12-mer) has been previously published.[29]

The higher oligosaccharide fraction, eluting from Sephadex G50 after the void but before the dodecasaccharide, was refractionated on Sephadex G50 to obtain fractions having sizes corresponding to tetradecasaccharides (14-mers), hexadecasaccharides (16-mers) and octadecasaccharides (18-mers). Each higher oligosaccharide fraction (1 g in 10 mL of water) was treated with sodium metaperiodate (10 mL of 0.5 M solution) for 18 h at 25 °C to oxidize any vicinal diols present in these oligosaccharides.[31] The newly formed aldehydes present in the ring-opened sugars were reduced by the addition of 1 g of sodium borohydride over 1 h, all the while maintaining the pH at 7.0 through the addition of acetic acid. Finally, the glycosidic linkages to the ring-opened sugars were broken, and the excess sodium borohydride was decomposed by adjusting the pH of the solution to 3.0 with HCl and heating the solution to 100 °C. The solution containing each oligosaccharide fraction was cooled to rt, the pH adjusted to 7.0, the volume reduced by rotary evaporation, the sample desalted on a Bio-Gel P2 column and lyophilized to afford a yellow powder.

Each periodate treated sample was applied repeatedly to a semipreparative SAX-HPLC column. The major peak in each fraction was collected, desalted on a Bio-Gel P2 column and lyophilized to afford a purified oligosaccharide as a white powder. Gradient PAGE with Alcian blue staining was used to confirm the size of each purified oligosaccharide.[27] To assess the purity of each oligosaccharide, high-sensitivity silver staining was used.[32] Analytical SAX-HPLC with detection at 232 nm was used to assess the charge purity of each oligosaccharide. Finally, both normal and reverse polarity CE was used to confirm oligosaccharide purity.[28]

Cellulose acetate plate electrophoresis of native heparin.—Heparin was analyzed on a cellulose acetate plate (6 × 7.5 cm) by electrophoresis as described by Cappelletti et al.[33] After electrophoresis the heparin and standard glycosaminoglycans were made visible with Alcian Blue.

Cultured pulmonary artery smooth cells proliferation assay.—Smooth muscle cell proliferation assays were preformed as previously described.[10] Briefly, isolated bovine pulmonary artery smooth muscle cells were seeded at 1.5×10^4 cells/well into a six-well tissue culture plate, grown for 2 days, then growth arrested for 48 h by reducing the serum concentration of the medium from 10 to 0.1%. Media was then changed to experimental samples containing either standard medium [RPMI-1640 with 10% fetal bovine serum (FBS)], growth arrest media (0.1% FBS) or standard media containing oligomers/heparin (5 μg/mL). All media contained streptomycin (100 μg/mL), penicillin (100 U/mL) and amphotericin B (1.25 μg/mL). After 4–5 days growth, cells were lifted with trypsin/EDTA and then counted using a Coulter counter. The percent growth was calculated as:

$$\%\ \text{growth} = \frac{\text{net cell growth in oligomer/heparin}}{\text{net cell growth in standard medium}} \times 100$$

Statistical analysis.—Results are presented as mean ± standard error of the mean. Comparisons among groups were made with a factorial analysis of variance (ANOVA), using the STATEVIEW software package (SAS Institute Inc., Cary, NC) for Macintosh computers. If ANOVA were significant, multiple comparisons were made using Fisher protected least-significant difference (PSLD) test. In all cases, significance was set up as $P < 0.05$.

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