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Influence of Molecular Weight, Protein Core and Charge of Native Heparin Fractions on Pulmonary Artery Smooth Muscle Cell Proliferation[1]

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Heparin macromolecules have been shown to inhibit cultured pulmonary artery smooth muscle cell proliferation in vitro and prevent hypoxic vascular remodeling in vivo. In an attempt to understand the structural determinants of heparin's antiproliferative properties, we have fractionated an antiproliferative preparation of commercial heparin into low and high molecular weight fractions. Then the high molecular weight heparin fraction was further fractionated on a DEAE-cellulose column by charge density eluting with 0 - 1 M NaCl linear gradient. The heparin protein peptides were both removed and isolated. These heparin fractions were assayed for antiproliferative effects on cultured bovine pulmonary artery smooth muscle cells. No appreciable differences were found among high and low molecular weight heparin fractions The core peptides showed no antiproliferative activity. However, higher charge density fraction was less antiproliferative.

Under chronic hypoxic conditions, the pulmonary vascular bed undergoes remodeling characterized by hypertrophy and hyperplasia of smooth muscle cells (SMC) in the vascular media. The resultant narrowing of the vascular lumen and increasing pulmonary vascular resistance cause pulmonary hypertension (1,2). Heparins (HP) have been shown to inhibit cell growth *in vitro* in a variety of cell types, including pulmonary (3,4) and systemic vascular SMC (5-8). Heparin reduces vascular remodeling after hypoxia in mice, rat, and guinea pigs (3, 9-12). A correlation between the in vivo ability of heparin to inhibit remodeling and *in vitro* ability to inhibit SMC proliferation (3), SMC Na^+/H^+ exchange (4), and SMC hypertrophy (13) has been found.

Commercially available heparin preparations vary widely in their antiproliferative properties (3). These preparations are heterogeneous mixtures in terms of size and ionic charge. Heparin (HP) is a linear polysaccharide, a highly sulfated macromolecule, containing alternating residues of glucosamine and either glucuronic or iduronic acid with various O-sulfate, N-sulfate, and N-acetyl groups which are usually non-homogeously distributed along the glycosaminoglycan (GAG) chain (14). Heparin GAG chains range in molecular size from 3,000 to 30,000 Da and also differ in degree of sulfation.

Recent investigations have focused on correlation of structure with antiproliferative function of the HP molecule, with particular interest in the role of negative charge. Structure-function studies using chemically modified heparins, cleaved oligosaccharides or synthetic polysaccharides show variable results. Studies of synthetic pentasaccharides suggested a role for 3-O-sulfation on internal glucosamine, but this may not be critical in macromolecular heparins (15, 16). Tozzio and co-workers, using partially or totally 2-O-desulfated heparin derivatives showed decreasing antiproliferative properties as 2-O-sulfates were chemically deleted. They interpret their results to indicate that 2-O-sulfation was required for antiproliferative activity (17). Wright and co-workers, using oligosaccahrides with and without 2-O-sulfate glucuronic acid report that this 2-O-sulfation was not essential for antiproliferative activity (18). Other structural-function studies carried out by preparing discrete sizes of antiproliferative hep-

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arin fragments by chemically modifying heparin show that dodecasaccharide and larger fragments had antiproliferative activity (7, 18-20). The antiproliferative role of low molecular weight heparins remains unclear. Geary showed antiproliferative effects *in vitro*, but not *in vivo* (19), while Tozzio demonstrated progressive loss of antiproliferative activity with decreasing molecular weight (20). In summary, the above studies were based on chemically modified heparin or cleaved oligosaccharides, in which conformational molecular structure changes may occur. This may, in part, explain the variance in the above studies.

To extend our understanding of structural requirements necessary for native HP's antiproliferative properties, we compared the properties of unfractionated Upjohn heparin lot #1274G, which we have found to be a potent antiproliferative native HP preparation, with several fractions of the whole heparin. The heparin was fractionated without chemical modification, i.e. under mild conditions: (a) preparation of low and high molecular weight (MW) fractions, (b) isolation of protein core peptides by heparitinase treatment and (c) separation of the high molecular weight native heparin by anion exchange chromatography to give fractions differing in negative charge. Negative charge of heparin corresponds to the concentration of sodium chloride needed to elute the fraction from ion-exchange column (i.e. higher charge HP elutes with higher sodium chloride concentration). These fractions were then tested for antiproliferative effect on pulmonary artery smooth muscle cells in comparison to the whole Upjohn #1274G heparin.

MATERIALS AND METHODS

Reagents. Heparin lot #1274G (sodium salt) was the product of Upjohn Company, MI 49002, U.S.A. Glycosaminoglycans standards were supplied by Drs. M. Mathews and J. A. Cifonelli, University Chicago (Contract No. 1-AM-5-2205 from NIH, Bethesda, MD). DEAE-Cellulose ion-exchange resin was from Whatman International Inc. Fetal bovine serum and other reagents were obtained from Sigma, St. Louis, MO. Cell culture media, RPMI-1640 (Mediatech, Washington, DC), contained streptomycin (Lilly, Indianapolis, IN), penicillin (Pfizer, New York, NY), and amphotericin B (GIBCO, Grand Island, NY).

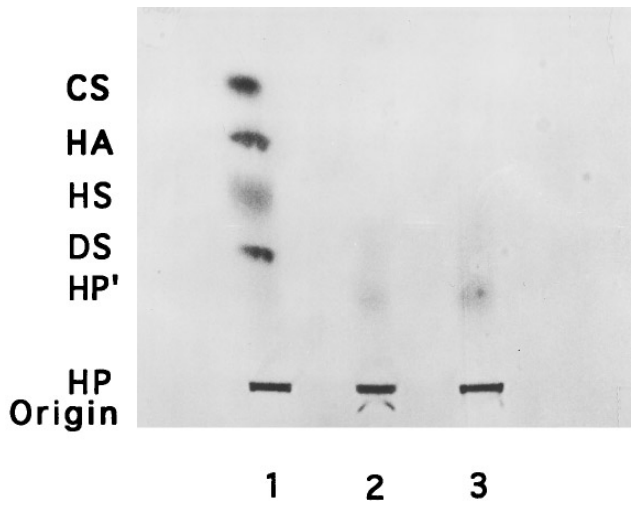

FIG. 1. Cellulose acetate electrophoresis of low and high density heparin fractions. Lane 1, a mixture of reference glycosaminoglycans: CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; HP and HP′, heparin; HA, hyaluronan. Lane 2, high molecular weight heparin. Lane 3, low molecular weight heparin.

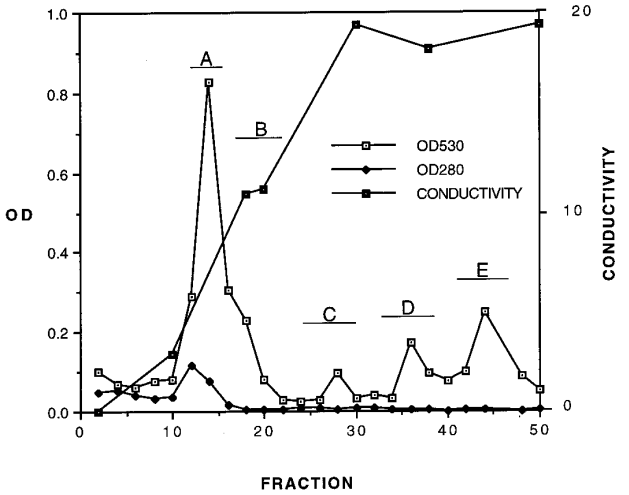

FIG. 2. DE-52 chromatography of high molecular weight heparin fraction from Upjohn heparin, lot #1274G. The column fractions were analyzed to determine the protein content (absorbance at 280 nm, ♦), the uronic acid content (absorbance at 530nm,□). The conductivity (■) relates to NaCl concentration in the elution buffer. The column fractions indicated were pooled to give different ionic strength fractions: A,B, C, D, and E.

Preparation of low and high molecular weight heparin. Upjohn heparin (lot #1274G, 20g) was dissolved in water (20ml) and transferred to a dialyzing bag (cut point about 3.5 kDa) and dialyzed against water (200 ml) three times. The dialyzate and high molecular weight native HP retained in the bag were lyophilized to give low and high molecular weight heparin fractions, respectively, without any chemical treatment. The yields of the two fractions so obtained and the composition of heparins are discussed in the results section. Both the heparin fractions were analyzed by cellulose acetate plate electrophoresis (Figure 1).

Fractionation of high molecular weight heparin preparation. Heparin (Upjohn lot #1274G, 20 mg) was dissolved in 7 M urea, 0.05 M Tris, pH 6.5-6.7 (buffer N, 5 ml) and applied to a DEAE-cellulose column (1.5 × 18 cms) packed with Whatman DE-52 and equilibrated with buffer N. The column was eluted with buffer N (60 ml) followed by a linear gradient of 0-1 M NaCl in buffer N (total volume 200 ml). The fractions were analyzed for uronic acid and protein contents. The fractions were pooled as shown in Figure 2, dialyzed against water, lyophilized. The yields of different fractions are summarized in Table 1. These fractions were analyzed for proteoglycan distribution by cellulose acetate electrolysis (Figure 3).

Analytical procedures. The protein contents of the column fractions were determined by measuring the absorbance at 280 nm. Uronic acid contents of the column fractions were determined using a carbazole reaction (21).

Cellulose acetate plate electrophoresis of heparin fractions. All the fractions from DEAE-cellulose column and low and high molecular weight heparins were analyzed on cellulose acetate plate (6 × 7.5 cms) by electrophoresis as described by Cappelletti et al. (22). After electro-

TABLE 1

Relative Quantities of Heparins Obtained from Different Fractions Eluted from DE-52 Column, and Proteoglycan Present in Different Fractions as Analyzed by Cellulose Acetate Electrophoresis

Fractions from Upjohn heparin lot #1274G	Amount in mg			Proteoglycan component**					
	I	II	III	HP	HP′	DS	HS	HA	CS
A	15.1	16.2	14.7	+	(+)				
B	0.9	0.5	2.5	+					
C	0.7	1.9	3.2	+					
D	0.7								
E	0.6		5.0	+					

* DEAE separation was performed 3 times, data is shown for each fractionations; I (20mg), II (20mg) and III (25mg).

** Indicates the presence of an Alcian Blue staining band with similar mobility to reference glycosaminoglycan samples, while (+) indicates the presence of minor band: CS, chondroitin sulfate; DS, dermatan sulfate; HA, hyaluronan; HP and HP′ heparin (HP′ is not digested with chondroitinase ABC); HS, heparan sulfate.

phoresis the heparin fractions and standard glycosaminoglycans were made visible by staining with Alcian Blue (Figures 1 and 3).

Removal of heparin core peptides. Upjohn heparin (lot 1274G) was treated 0.05M NaOH and 4 M $NaBH_4$ at 37°C for 24 hours, then neutralized with glacial acetic acid. Samples were then dialyzed against water and lyophilized. This procedure releases the HP GAG chains from any residual core peptides. Protein fragments were removed during dialysis. The remaining free GAG chains have the linkage region attached (23).

Preparation of the heparin core peptides. Upjohn heparin (lot 1274G) core peptides were prepared by digesting the heparin with Heparitinases I and II as described earlier (24). Briefly, heparin (2mg) was dissolved in 1 ml of 0.02M sodium acetate buffer (ph 7.0) containing 0.05% BSA and 0.002 M Ca $(OAc)_2$. To this solution was added 1 unit each of Heparitinase I and II and the reaction mixture was incubated at 37°C for 6 hr. The enzymatic reaction was terminated by heating in boiling water for 5 minutes. The reaction mixture was dialyzed against water (5 × 200ml) and lyophilized to yield core peptides.

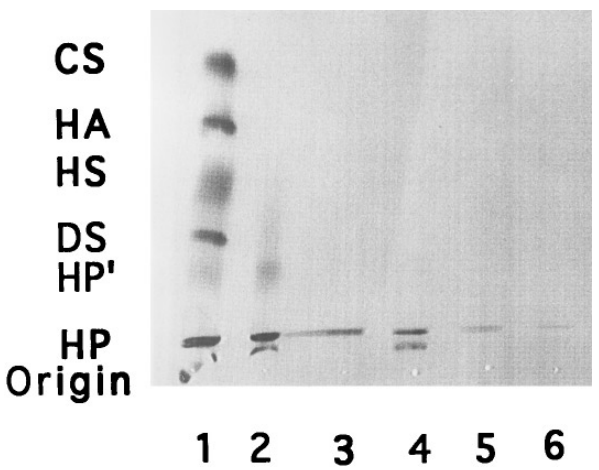

FIG. 3. Cellulose acetate electrophoresis of Fractions, A, B, C, D, and E. Lane 1, a mixture of reference glycosaminoglycans: CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; HP and HP′, heparin; HA, hyaluronan. Lanes 2, 3, 4, 5, and 6, heparin fractions A, B, C, D, and E respectively.

Chondroitinase ABC digestion. The heparin fraction (1 mg) was dissolved in 1 ml of 0.3 M sodium acetate, 0.1M tris (pH8.0) buffer and digested with chondriotinase ABC (0.25 unit) at 37° for 1 h (25). The enzymatic reaction was stopped by heating in boiling water for 1 min. The mixture was dialyzed against water (5 × 100 ml), lyophilized and analyzed by cellulose acetate plate electrophoresis as described by Cappelleti (22).

Cultured pulmonary artery smooth muscle cells proliferation assay. Smooth muscle cell proliferation assays were performed as previously described (26). Briefly, isolated bovine pulmonary artery smooth muscle cells were seeded at 1.5×10^4 cells/well into 6 well tissue culture plates, grown for 2 days, then growth arrested for 48 hours by reducing the serum concentration of the medium from 10% to 0.1%. Media was then changed to experimental samples containing either standard media (RPMI-1640 with 10% FBS), growth arrest media (0.1% FBS) or standard media containing heparin (10 mg/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 counted by Counter counter. The degree of growth inhibition was calculated as:

$$\% \text{ inhibition} = \frac{1 - \text{net growth in heparin}}{\text{net growth in standard media}} \times 100,$$

where net growth = growth in samples − growth in growth arrested media.

Statistics. Results are presented as mean ± standard error of the mean. Comparisons among groups were made with a factorial analysis of variance (ANOVA), using the statview software package (BrainPower, Inc., Calabassas, CA.) for Macintosh computers. If ANOVA were significant, multiple comparisons were made using the Fisher protected least significant difference (PSLD) test. In all cases, significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

We have previously shown different commercially available heparin preparations from Upjohn, Elkin-Sinn, and Choay to vary in their inhibitory effects on pulmonary artery smooth muscle cell growth, Na^+/H^+ exchange, and pulmonary artery smooth muscle cells hypertrophy in the order Upjohn > Elkin-Sinn > Choay (4, 13). In order to determine structure - function

relationship of native heparin we used the most potent antiproliferative heparin, Upjohn lot #1274G, for the present studies. This HP was fractionated into low and high molecular weight fractions, sequentially by dissolving in water, dialyzing against water without chemical depolymerization, then lyophilizing both the dialyzate and heparin retained in the dialysis bag. From 2 gm of the whole heparin, we obtained 0.433 gm (21.5%) of high MW and 1.5 gm (75%) low MW native HP fractions. In both the high and low MW fractions, there were approximately equal amounts of heparin and heparin like (HP′) proteoglycans. A "heparin like" proteoglycan is a heparin with electrophoretic properties, by cellulose electrophoresis, more in the range of heparan sulfate. Analysis of these fractions on cellulose acetate electrophoresis (Figure 1) showed two bands, one with the same mobility as standard heparin, and a second band with a slightly greater mobility than heparin and less mobility than dermatan sulfate, i.e. HP′. Chondroitin ABCase treatment did not destroy the HP′ band confirming that this band was not due to dermatan sulfate (data not shown).

The high MW heparin fraction was further fractionated by chromatography on a DEAE-Cellulose column. Figure 2 shows a typical elution pattern at different ionic strengths. The uronic acid positive fractions were pooled, dialyzed against water, and lyophilized. The amounts of heparin in different fractions are given in Table 1. The fractions that were eluted with higher ionic strength i.e. B to E when analyzed by cellulose acetate electrophoresis (Figure 3) showed a single band with the same mobility as heparin, whereas A fraction showed two bands, one with the same mobility as HP and a second band of HP′.

All these fractions were assessed for antiproliferative effect on bovine pulmonary artery smooth muscle cells. The PASMC growth in heparin free media was 100%. Growth of bovine pulmonary artery smooth cells in low and high molecular weight heparin fractions was not different, at 40.5 ± 4.3% and 41.9 ± 5.3% respectively, as compared to the growth with whole heparin (45.7 ± 5.6%, Figure 4). These data suggest that both the heparin fractions have carbohydrate sequences necessary for antiproliferative activity. Geary demonstrated similar antiproliferative effects in a low MW heparin in systemic SMC, but not in vivo (19). Contrary to our results, Tozzio reported a progressive reduction in antiproliferative activity with reduction in molecular weight obtained by chemical depolymerization, particularly below 2 kDa (20). The disparity in these results may reflect the method of low MW heparin preparation. Our low MW heparin, produced by dialysis with cut point 3.5 kDa, is a native heparin not subjected to chemical fragmentation. Tozzio's group prepared the 1 and 2 kDa derivatives by controlled depolymerization with H_2O_2 and cupric acid (20). It is possible that the chemical methods used to produce their HP derivatives have altered the structural determinants necessary for the antiproliferative properties of HP.

DEAE-cellulose chromatographic fractionation of the high molecular weight heparin fraction showed significant differences in antiproliferative activity. Figure 5 summarizes the results of studies of the pooled heparin fractions A - E. Sample A showed 53.7 ± 3.2% growth of PASMC as compared to 49.7 ± 1.7% for the parent heparin (lot #1274G) and 100% for heparin free media. The remaining samples, B,C,D, and E, showed no antiproliferative effect at 102.8 ± 3.5%, 108.7 ± 2.3%, 110.6 ± 3.2%, and 102.0 ± 1.7%, respectively. Therefore, the heparin fraction eluted from the DEAE

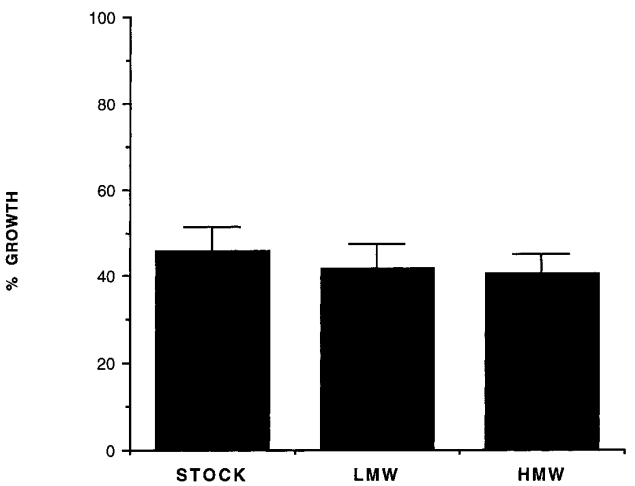

FIG. 4. % inhibition of growth in bovine pulmonary artery smooth muscle cells grown in media containing 10% FBS plus low and high molecular weight heparin fractions from Upjohn heparin lot #1274G.

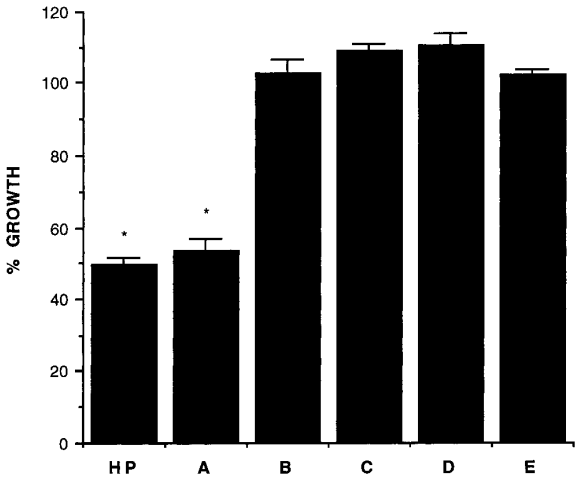

FIG. 5. % inhibition of growth in bovine pulmonary artery smooth muscle grown in media containing 10% FBS plus fractions A, B, C, D, and E (10 mg/ml) of heparin eluted from DE-52 column. *$p \leq 0.05$ as compared to fractions B,C,D and E.

column at lower charge density had antiproliferative properties similar to the parent product, whereas higher charge density fractions were not antiproliferative.

Our findings are in contrast to Wright and co-workers, who have also studied the role of charge in cell proliferation (18). They demonstrated an increase in antiproliferative activity with increasing negative charge. However, their studies were performed on chemically modified hexa and tetrasaccharides, compared with our studies on native heparin.

Wright and co-workers, using oligosaccharides with and without 2-O-sulfate glucuronic acid, also report that the 2-O-sulfate glucuronic acid was not essential for antiproliferative activity (18). In contrast, Tozzio and co-workers, using epoxidation to produce partially (20%) or totally desulfated heparin derivatives (2-O-desulfated heparins), showed decreasing antiproliferative properties as 2-O-sulfation was lost. Tozzio's results with N-desulfation were unclear. Both 25% and 55% N-desulfated heparins were markedly less antiproliferative to SMC than the original heparin, while total N-desulfation was not particularly different from the parent heparin. However, 40% desulfation was more antiproliferative. Nonetheless, Tozzio interprets these results to indicate that 2-O-sulfation and negative charge at the N-position were required for antiproliferative activity (17). These discrepancies may result from the conformational molecular structure changes in heparin as a result of chemical modification. Our data in native heparin suggest that charge density alone was not responsible for heparin's antiproliferative properties, but some other structural constraints might also be important. Further studies of heparin without chemical modification are needed to identify these structural constraints.

Commercial heparins retain a small peptide residual of the protein core. To firmly establish if there were any role for this core peptide in heparin's antiproliferative activity, we used two approaches. First we dissolved the core peptides using alkaline borohydrate. This procedure cleaved the carbohydrate-protein linkage of HP GAG chains by converting the xylose residue linked with Ser/Thr into xylitol and by cleaving the protein core/peptide. These peptides were removed during dialysis, leaving the GAG chains with accompanying linkage region oligosaccharide. The GAG chains released by this process have similar antiproliferative activity (45.5 ± 3.0%) to that of the parent heparin (44.6 ± 2.7%, Fig. 6A). To further understand the role of core peptides on antiproliferative activity of heparin, the glycosaminoglycan chains were removed by treating heparin with Heparitinases I and II. This process removes the GAG chains, leaving the protein core with linkage regions intact. The released protein core lost almost all the antiproliferative activity in comparison to whole heparin (88.3 ± 2.8 vs 48.8 ± 1.9, respectively,

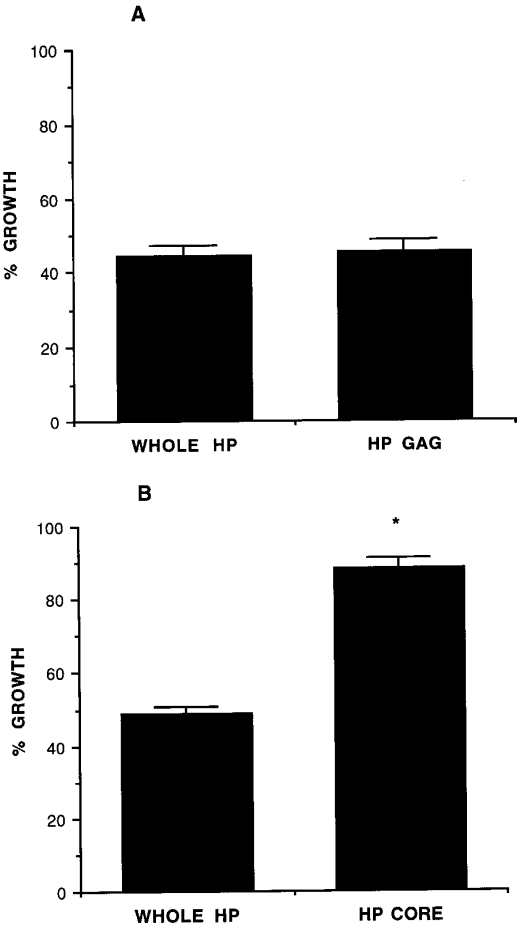

FIG. 6. (A) % inhibition of growth in bovine pulmonary artery SMC in media containing 10% FBS plus GAG chains (10 mg/ml) after $NaBH_4$ digestion of protein core. (B) % inhibition of growth in bovine pulmonary artery SMC in media containing 10% FBS plus protein core (10 mg/ml) obtained by treatment of Upjohn heparin # 1274G with heparitinases I and II. *$p \leq 0.05$ as compared to the parent heparin.

Fig. 6B). Heparitinases I and II alone had no antiproliferative effect (data not shown). These studies confirm that the antiproliferative properties of this heparin reside in the GAG chains.

In conclusion, we have demonstrated that: (1) High molecular weight and low molecular weight native heparin fractions have substituted carbohydrate moieties necessary for antiproliferative effect. (2) The antiproliferative properties of heparin reside in the GAG chains, not the protein core, and (3) Native heparin fractions with lower overall charge density have antiproliferative properties not seen in fractions with higher charge densities. These data suggest that other structural determinants such as charge distribution or conformation of GAG chains, rather than total charge, may determine the antiproliferative activity of heparins. This work in native HP fractions on structure-function determinants has not been investigated so far.

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