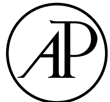
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Heparin inhibits SMC growth in the presence of human and fetal bovine serum

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Abstract

Heparin (HP) has antiproliferative as well as anticoagulant properties, but not all HP preparations are equally antiproliferative. A recent report found that HP lost its total antiproliferative activity when fetal bovine serum (FBS) was replaced with human serum (HS) in culture media. This observation led to the investigation of our most potent antiproliferative Upjohn HP preparation effects on bovine pulmonary artery smooth muscle cells (PASMC) and systemic SMC growth stimulated in the presence of either FBS or HS. Bovine PASMC, human PASMC, and bovine aortic SMC were treated with 10 μg/ml Upjohn HP in either 15% FBS or 15% HS and the cell number was determined by a Coulter counter. We found that Upjohn HP significantly inhibited bovine PASMC and systemic SMC proliferation in both HS and FBS. The antiproliferative activity of the above HP preparation in HS may lead to an effective treatment of pulmonary vascular and systemic remodeling.
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Keywords: Heparin; Pulmonary artery smooth muscle cell; Fetal bovine serum; Human serum

Under chronic hypoxic conditions, the pulmonary vascular bed undergoes remodeling characterized by hypertrophy and hyperplasia of smooth muscle cells (SMC). The resultant narrowing of the vascular lumen leads to an increase in pulmonary vascular resistance causing pulmonary hypertension [1,2]. Pulmonary hypertension contributes to the morbidity and mortality of a number of diseases associated with chronic hypoxia, including chronic obstructive pulmonary disease, cystic fibrosis, and hypoventilation syndromes [3]. At present, few options are available for treatment of pulmonary vascular remodeling.

Several strategies have been investigated to counter vascular SMC proliferation, one of which involves the use of heparin (HP) as an antiproliferative agent. HP has both anti-coagulant and anti-proliferative properties, which may be used as a possible therapeutic agent for the treatment of pulmonary vascular remodeling. HP is a highly sulfated linear polysaccharide composed of alternating residues of glucosamine (GlcN) linked by 1,4-glycosidic linkages with either β-D-glucuronic acid (GlcUA) or α-L-iduronic acid (IdoA). These substituted carbohydrate residues are usually heterogeneously distributed along the glycosaminoglycan (GAG) chain. The molecular size of the HP GAG chain varies from 3 to 30 kDa [4]. Heparin has been shown to reverse vascular remodeling after hypoxia in mice, rats, and guinea pigs [3,5–7] and also to inhibit cell growth in vitro in various cell types, including pulmonary artery SMC [8,9] and systemic vascular SMC [10–13]. In our laboratory, however, we have found that different commercial HP preparations vary in their antiproliferative potency against vascular SMC [9].

Most of the in vitro studies on the effects of HP upon vascular SMC, where HP was reported to inhibit cell proliferation, were conducted with the use of fetal bovine serum (FBS) as a growth supplement in culture medium. Underwood et al. [14] have recently demonstrated that while HP inhibited proliferation of vascular SMC in FBS, it was ineffective in the presence of human serum (HS). This prompted us to examine the growth inhibitory effect of our most potent antiproliferative HP preparation from Upjohn in the presence of HS. We hypothesized that the antiproliferative activity of Upjohn HP would be similar in the presence of either FBS or HS. To examine this hypothesis, bovine

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pulmonary artery smooth muscle cells (BPASMC), human pulmonary artery smooth muscle cells (HPASMC), and bovine aortic smooth muscle cells (BAOSMC) were cultured and treated with our anti-proliferative Upjohn HP containing media supplemented with either FBS or HS. The data demonstrated that the most potent antiproliferative Upjohn HP had similar growth inhibitory effect on pulmonary and systemic SMC in both types of sera, i.e., FBS and HS.

Materials and methods

In vitro isolation and culture of cells. Bovine pulmonary arteries were obtained from a local slaughterhouse. The method of Ross [15] with modifications by Yu et al. [16] was used for the isolation and culture of SMC, as previously described [17]. BPASMC were harvested and stored in liquid nitrogen. Cells previously frozen at passage two were thawed and grown to confluence in RPMI 1640 containing L-glutamine standard media (BioWhittaker, Walkersville, MD), with 10% FBS (BioWhittaker, Walkersville, MD) supplemented with 10,000 U/ml penicillin and 10,000 μg/ml streptomycin per ml (BioWhittaker, Walkersville, MD), and 250 μg/ml amphotericin B (Gibco, Grand Island, NY). The cells were incubated at 37 °C in air and 5% CO_2. BPASMC identification was completed by the immunofluorescence technique, which involved the use of a monoclonal antibody specific for smooth muscle α-actin (Sigma, St. Louis, MO) and a monoclonal anti-actin antibody (Sigma, St. Louis, MO). The polyclonal antifactor VIII antibody (Calbiochem, La Jolla, CA) was used as a negative control. Cells from passages four to six were used in these experiments.

In addition, HPASMC at passage two were obtained from Clonetics (Trademark of BioWhittaker, Walkersville, MD) and grown in SmGM-2 medium (Clonetics, Walkersville, MD) containing nutrients from SmGM-2 Bullet kit (Clonetics, Walkersville, MD). These cells were also incubated at 37 °C in air and 5% CO_2. Passages seven and eight cells were utilized in these experiments.

We also tested the antiproliferative effects of Upjohn HP on systemic vascular SMC in the presence of FBS and HS. BAOSMC were used to determine the anti-proliferative activity of heparin in the presence of both FBS and HS. BAOSMC were harvested from explants using the same protocol and media described previously for BPASMC. The cells were frozen in liquid nitrogen at passage two and passages four through six were used in these experiments.

Cultured smooth muscle cell proliferation assay. Smooth muscle cell proliferation assays were performed as previously described [17]. Briefly, the isolated BPASMC and BAOSMC were seeded at 1.25×10^4 cells/well into six-well tissue culture plates (Costar 3516; Corning Incorporated, Corning, NY) containing 2 ml of RPMI-1640 standard media with the antibiotic supplements and 10% FBS per well. The HPASMC were seeded under the same conditions but SmGM-2 media containing nutrients from SmGM-2 bullet kit were used instead of RPMI-1640. After 48 h, the cells were growth-arrested by decreasing the serum concentration of the cultured media to 0.1%. Following 48 h of the growth-arrested phase, the PASMC were treated with test media. The treatment groups were as follows: standard medium (RPMI-1640 supplemented with antibiotics) + 15% FBS, standard medium + 15% HS (BioWhittaker, Walkersville, MD), standard medium + 0.1% FBS, standard medium + 0.1% HS, standard medium containing HP + 15% FBS, and standard medium containing HP + 15% HS. The anticoagulant HP tested was obtained from Upjohn (Lot 1274G, The Upjohn, Kalamazoo, MI). HP was added to the standard media + 15% FBS or HS at 10 μg/ml dose and filtered with a 0.2 μm filter (Gelman Sciences, Ann Arbor, MI). This is a dose which we have previously shown to be highly effective in 10% FBS [9]. We used 15% FBS or HS in this study to duplicate the conditions used by Underwood et al. [14]. HPASMC were treated with only the standard medium + 15% HS, standard medium + 0.1% HS, and standard medium containing HP + 15% HS. Five wells per plate were harvested after four days of treatment for cell-number determination using a Coulter counter (Model ZM; Hialiah, FL). The experiments were repeated three times for each standard media containing 15% FBS or HS, 0.1% FBS or HS, and 15% FBS or HS with HP to give us a total of 15 samples ($n = 15$) per condition for all cell types. Using the cell number, the net growth of cells in control and heparin treated samples was determined by subtracting the average cell number in 0.1% FBS or HS (an estimate of the starting cell number) from the cell number at the end of the experiment (standard medium + 15% FBS or HS with or without heparin). The degree of growth was calculated as:

$$\text{Percent growth} = \left(\frac{\text{Net growth in heparin}}{\text{Average net growth in 15\% standard media}} \right) \times 100.$$

The average percent growth of the cell types in each condition was determined and presented in this study. The percent growth of BPASMC, HPASMC, and BAOSMC in standard media containing 15% FBS or HS after calculation was 100% while no growth and 0% growth were observed in cells treated with standard media + 0.1% FBS or HS.

Statistical analysis. All values were expressed as means ± SEM. Statistics were done using the computer program Statview (SAS Institute Inc., Cary, NC) with factorial ANOVA. If ANOVA were significant, multiple comparisons were made using Fisher's protected least significant difference test.

Results and discussion

Effect of HP on BPASMC proliferation in the presence of FBS or HS

In order to determine the effects of replacing FBS with human serum on cell growth inhibition, we used a potent lot of antiproliferative HP from Upjohn. The percent growth of cells in heparin free standard media containing 15% FBS was 100 ± 5% while no growth was observed (0 ± 1% growth) when the serum content was reduced to 0.1% (Fig. 1). Treatment of bovine PASMC with 10 μg/ml Upjohn HP in standard media with 15% FBS significantly inhibited growth as compared to cells grown in standard media containing 15% FBS without HP ($p < 0.0001$). Similarly, the addition of 10 μg/ml Upjohn HP to standard media containing 15% human serum also significantly inhibited cell growth as compared to standard media containing 15% human serum without HP ($p < 0.0001$). Heparin's antiproliferative effect was slightly more potent in FBS as compared to HS ($p < 0.05$). Thus, our antiproliferative HP at 10 μg/ml significantly inhibited BPASMC growth in the presence of both FBS and human serum.

Effect of HP on HPASMC growth in the presence of HS

In order to determine whether or not HPASMC would have a different response to HP than BPASMC,

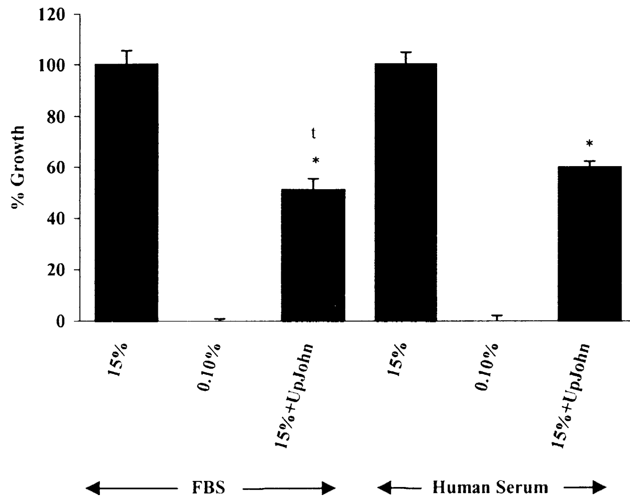

Fig. 1. Bovine pulmonary artery smooth muscle cells (BPASMC) treated with Upjohn heparin in FBS and human serum. BPASMC were treated with standard medium (RPMI-1640 supplemented with antibiotics) + 15% FBS, standard medium + 15% human serum (HS), standard medium + 0.1% FBS, standard medium + 0.1% HS, standard medium containing 10 μg/ml Upjohn heparin (Upjohn) + 15% FBS, and standard medium containing 10 μg/ml Upjohn heparin + 15% HS (Upjohn). After four days of treatment, cells were harvested and the cell number was determined. The cell percent growth was ascertained using previously described formula; $^{*}p < 0.0001$ vs. 15%; $^{t}p < 0.05$ vs. 15% HS + HP. Values are means ± SE; $n = 15$ in each group.

HPASMC were treated with RPMI-1640 standard media containing 15% HS, 0.1% HS, and 15% HS with Upjohn heparin, respectively (Fig. 2). Addition of 10 μg/ml heparin to standard media with 15% HS resulted in the inhibition of cell growth, which was statistically significant compared to standard media containing 15% HS without HP ($p < 0.0001$). These data demonstrated that Upjohn HP was also effective against HPASMC proliferation in the presence of HS.

Effect of HP on BAOSMC growth in the presence of FBS and HS

Akin to bovine and HPASMC, proliferation of BAOSMC was affected by the addition of antiproliferative HP into the media (Fig. 3). The treatment of BAOSMC with 10 μg/ml Upjohn heparin resulted in the decrease of cell growth in both FBS and HS ($p < 0.0001$ vs. 15%). Furthermore, HP's antiproliferative activity was more effective against BAOSMC growth in the presence of HS in comparison to FBS ($p < 0.05$). The results from BPASMC, HPASMC, and BAOSMC demonstrated that the activity of a potent antiproliferative lot of heparin from Upjohn was not compromised when FBS was replaced with HS.

In above studies, we determined the effects of replacing FBS with HS on cell growth inhibition. We have previously shown that different commercially available HP preparations from Upjohn, Elkin-Sinns, and Choay vary in their inhibitory effects on PASMC growth, and PASMC hypertrophy in the order of Upjohn > Elkin-Sinns > Choay [9,18]. Furthermore, HP preparations from the same company varied in their antiproliferative

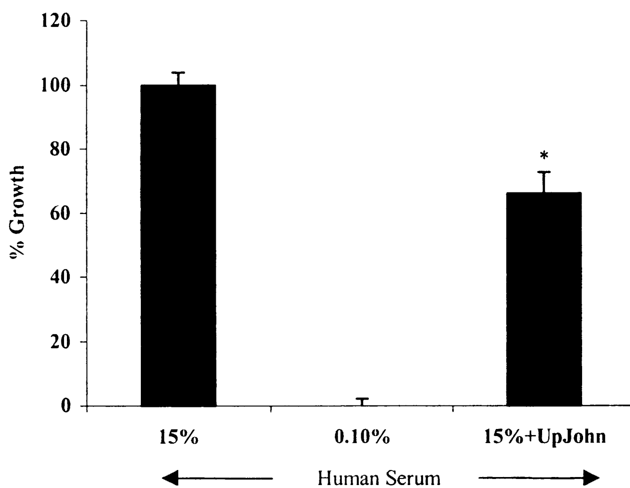

Fig. 2. Human pulmonary artery smooth muscle cells (HPASMC) treated with Upjohn heparin in human serum. HPASMC were treated with standard medium (RPMI-1640 supplemented with antibiotics) + 15% HS, standard medium + 0.1% HS, and standard medium containing 10 μg/ml Upjohn heparin + 15% HS (Upjohn). After four days of treatment, cells were harvested and the cell number was determined. The cell percent growth was ascertained using previously described formula; $^{*}p < 0.0001$ vs. 15%. Values are means ± SE; $n = 15$ in each group.

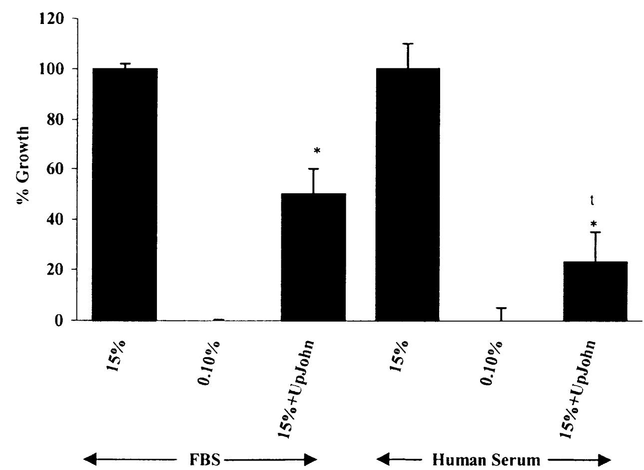

Fig. 3. Bovine aortic smooth muscle cells (BAOSMC) treated with Upjohn heparin in FBS and human serum. BAOSMC were treated with standard medium (RPMI-1640 supplemented with antibiotics) + 15% FBS, standard medium + 15% human serum (HS), standard medium + 0.1% FBS, standard medium + 0.1% HS, standard medium containing 10 μg/ml Upjohn heparin + 15% FBS (Upjohn), and standard medium containing 10 μg/ml Upjohn heparin + 15% HS (Upjohn). After four days of treatment, cells were harvested and the cell number was determined. The cell percent growth was ascertained using previously described formula; $^{*}p < 0.0001$ vs. 15%; $^{t}p < 0.05$ vs. 15% FBS+HP. Values are means ± SE; $n = 15$ in each group.

potency. We selected a potent antiproliferative HP lot from Upjohn for use in these experiments. Bovine PASMC, human PASMC, and bovine AOSMC were treated with standard media containing 10 μg/ml HP and supplemented with either 15% FBS or HS. A mild but significantly ($p < 0.05$) greater decrease in BPASMC proliferation was observed when the cells were treated with Upjohn HP in the presence of FBS in comparison to HS (Fig. 1). Upjohn HP treatment of human PASMC in HS supplemented standard media also was effective and resulted in the decrease of cell growth (Fig. 2). In addition, similar inhibition of bovine AOSMC growth due to HP treatment in FBS and HS was observed (Fig. 3). The results demonstrate that Upjohn HP's antiproliferative activity remains effective against bovine and human SMC proliferation in the presence of both FBS and HS. These data therefore suggest that the antiproliferative activity of Upjohn HP was not altered by the replacement of FBS with human serum.

Our results are opposite to an earlier study which demonstrated that HP failed to inhibit the proliferation of human vascular SMC in the presence of HS [14]. The degree of HP's antiproliferative potency is dependent upon the substitution of hydroxyl and amino groups of the HP glycosaminoglycan (GAG) chains. We have previously demonstrated that the antiproliferative activity of HP resides in the glycosaminoglycan chain and not in the protein core [19]. Furthermore, we have also shown that: (1) the basic sugar residue glucosamine can be replaced with another basic sugar residue, galactosamine; (2) O-sulfation of sugar residues of GAG chains is important for the antiproliferative activity; (3) *N*-sulfonate groups on basic sugar residue are not essential for growth inhibition of bovine PASMC [20]; (4) 3-*O*-sulfonate substitution of glucosamine residues is not essential in whole HP for antiproliferative activity [21]; (5) high molecular weight and low molecular weight of a given HP batch do not affect the potency [19]; (6) basic sugar residues of glucosamine are replaceable with galactosamine residues [20]; (7) anomeric linkage of acidic and basic sugar residues is not critical for antiproliferative activity; and (8) antiproliferative and anticoagulant activities reside in different domains of HP [22].

In conclusion, the above data show, in contrast to a previous report, that cultured human pulmonary artery, bovine pulmonary artery, and bovine aortic SMC are sensitive to inhibition by HP when cultured in HS. The variation in resistance to heparin in HS reported earlier may be due to the potency of the HP preparation. These results suggest a potential for the development of one of the potent antiproliferative HP preparations as a therapeutic agent for treatment of human vascular remodeling and serve a warning that not all heparin preparations are alike in their antiproliferative potency.

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