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Effect of carboxyl-reduced heparin on the growth inhibition of bovine pulmonary artery smooth muscle cells

Hari G. Garg [a,*], Hicham Mrabat [a], Lunyin Yu [a], Craig Freeman [b], Boyangzi Li [c], Fuming Zhang [c], Robert J. Linhardt [c], Charles A. Hales [a]

[a] Department of Medicine, Pulmonary/Critical Care Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA
[b] Immunology Program, The John Curtin School of Medical Research, The Australian National University, Canberra, ACT, Australia
[c] Departments of Chemistry and Chemical Biology, Biology and Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

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ABSTRACT

Heparin (HP) inhibits the proliferation of bovine pulmonary artery smooth muscle cells (BPASMC's), among other cell types in vitro. In order to develop a potential therapeutic agent to reverse vascular remodeling, we are involved in deciphering the relationship between the native HP structure and its antiproliferative potency. We have previously reported the influence of the molecular size and the effects of various *O*-sulfo and *N*-acetyl groups of HP on growth-inhibitory activity. In this study, to understand the influence of carboxyl groups in the HP structure required for endogenous activity, a chemically modified derivative of native HP was prepared by converting the carboxyl groups of hexuronic acid residues in HP to primary hydroxyl groups. This modification procedure involves the treatment of HP with *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide followed by reduction with $NaBH_4$ to yield carboxyl-reduced heparin (CR-HP). When compared to the antiproliferative potency of native HP on cultured BPASMC's at three dose levels (1, 10, and 100 μg/mL), the CR-HP showed significantly less potency at all the doses. These results suggest that hexuronic acid residues in both major and variable sequences in HP are essential for the antiproliferative properties of native HP.

1. Introduction

Smooth muscle cells (SMCs) play an important role in pulmonary hypertension associated with chronic hypoxia. The proliferation of SMCs is proposed to be a key process in increasing pulmonary hypertension.[1–4] Many pharmaceutical interventions have been tried in order to prevent inappropriate proliferation of smooth muscle cells. Among these candidates, heparin (HP, Fig. 1) was found to be an effective inhibitor of the proliferation of SMCs.[5]

HP is a structurally heterogenous complex molecule that possesses numerous biological properties in vivo and in vitro.[6] Commercially available HPs derived from mast cells contain repeating disaccharide units of uronic acid (UA) and glucosamine (GlcN) residues (Fig. 1). The biosynthesis of HP involves the following steps in sequence: (a) N-deacetylation and N-sulfonation, (b) hexuronosyl C-5 epimerization and (c) O-sulfonation.[7] Incomplete modifications are responsible for the HP structural heterogeneity and complexity. Besides HP's ionic character, the other structural features responsible for its antiproliferative activity are very poorly understood.

For the past several years our laboratory has been engaged in an in vitro study to determine the structure–function relationships among HP-type compounds in the inhibition of the proliferation of bovine pulmonary artery smooth muscle cells (BPASMCs). The results of these studies, so far lead us to understand that (a) HP's antiproliferative properties reside in the glycosaminoglycan (GAG) chain;[8] (b) both *N*-sulfo groups and *N*-acetyl groups are required for the antiproliferative effect;[9] (c) fully O-sulfonated HP did not exhibit enhanced antiproliferative activity over that of native HP;[10] (d) the GlcN residues in HP are not critical as GAGs containing galactosamine also show activity;[10] (e) loss of *N*-sulfo and 6-*O*-sulfo groups in GlcN residues of HP reduces its antiproliferative potency,[11] (f) substitution of the free hydroxyl groups of HP with bulky alkyl groups increases its antiproliferative activity,[12] (g) the size of HP does not affect the growth-inhibitory properties,[13] and (h) 2-O-desulfonation of HP uronic acids decreases its antiproliferative activity.[13]

The purpose of this investigation is to determine if the carboxyl groups of HP's hexuronic acid (UA) residues are critical for its antiproliferative effect on BPASMCs. In this study we converted HP's carboxyl groups to primary hydroxyl groups through treatment with *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide, followed by sodium borohydride reduction (Fig. 1).[14] The growth-inhibitory effect in BPASMCs was then evaluated for both carboxyl-reduced CR-HP and HP.

* Corresponding author. Tel.: +1 617 726 5632; fax: +1 617 724 9650.
E-mail address: Hgarg@partners.org (H.G. Garg).

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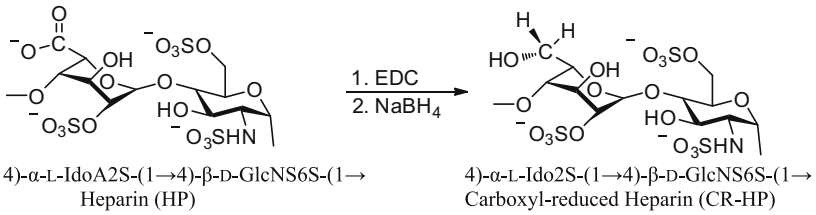

Figure 1. A representative structure of the major repeating disaccharide in HP and its conversion into CR-HP.

2. Experimental

2.1. Materials

Porcine mucosal HP was obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). Fetal bovine serum was obtained from Bio Whittaker (Walkersville, MD). Cell culture medium, RPMI-1640 (Mediatech, Washington, DC), contained streptomycin (Lilly, Indianapolis, IN), penicillin (Pfizer, New York, NY), and amphotericin B (GIBCO, Grand Island, NY), which were purchased commercially. Dialysis membranes of 3500 D molecular weigh cut-off (MWCO) were from Spectrum Laboratories (Rancho Dominguez, CA). Chemicals used in polyacrylamide gel electrophoresis (PAGE) and the apparatus were obtained from Bio-Rad (Hercules, CA). All chemicals for modifying HP were obtained from Sigma–Aldrich Chemical CO.

2.2. Methods

2.2.1. Preparation of CR-HP

An adaptation of a previously described micro-scale procedure was used.[15] HP (250 mg in 50 mL of water) was carboxyl reduced by first forming an active ester by adding *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC, 1 mg) at room temperature, followed by acidification with 10 mL of 0.04 M hydrochloric acid and stirring for 1 h. Reduction of the active ester was accomplished with freshly prepared 2 M $NaBH_4$ (200 mL in 2 portions) at 50 °C for 2 h. Excess $NaBH_4$ was decomposed with HOAc. Finally, CR-HP was isolated by either precipitation with abs EtOH followed by centrifugation or by dialysis using 3500 MWCO membrane.

2.2.2. Characterization of CR-HP[16]

The molecular weight of CR-HP was determined using polyacrylamide gel electrophoresis (PAGE),[17] and its chemical structures were confirmed by ^{1}H NMR spectroscopy.[18] CR-HP was analyzed by PAGE, and the molecular weight was calculated based on a previous literature method.[17] An equal amount of each sample (5 μL at 2 mg/mL) was combined with one volume of 50% (w/v) sucrose, and the mixture was loaded into a stacking gel of 5% (total acrylamide) and fractionated with a 22% resolving gel. Electrophoresis was performed at 200 V for 80 min. The gel was stained and fixed with Alcian Blue in 2% (w/v) HOAc. The average molecular weight of CR-HP was determined using a standard curve based on heparin oligosaccharide standards. CR-HP (10 mg) was dissolved in 0.5 mL of D_2O (99.996%, Sigma–Aldrich) and freeze-dried three times to remove the exchangeable protons. The samples were re-dissolved in 0.5 mL of D_2O. Spectra were recorded at 300 K on either a 500- or 600-MHz spectrometer.

2.2.3. Cultured bovine pulmonary artery smooth cell (BPASMC's) proliferation assay

BPASMC proliferation assays were preformed as previously described.[19] Briefly, isolated BPASMC's in passages 4–6 were seeded at 1.5×10^4 cells/well into 6-well tissue culture plates, grown for two days, then growth-arrested for 48 h by reducing the serum concentration of the medium from 10% to 0.1%. The medium was then changed for experimental samples to contain either standard medium [RPMI-1640 with 10% fetal bovine serum (FBS)], growth-arrest media (0.1% FBS) or standard media containing HP's and their chemically modified derivatives. All media contained streptomycin (10 μg/mL), penicillin (100 U/mL) and amphotericin B (1.25 μg/mL). After 4 days, BPASMC's present in the cell culture wells were rinsed with Hank's balanced salt solution to remove the remaining cell culture medium. No dead cells (Trypan Blue exclusion) were observed in the control preparations and in the treated culture medium containing heparin derivatives. After detachment of BPASMC's with trypsin/EDTA, the cell numbers were determined by a direct cell count.

The HP derivative was dissolved (1 mg/mL) in distilled sterile water, then 14 μL, 140 μL, or 1.4 mL of this solution was added to the culture medium (13.99, 13.86, and 12.6 mL, respectively), and 2 mL of the resulting media was added to each well. The chemically modified HP derivative and parent HP sample was completely soluble, as the culture medium was clear (no turbidity) after the addition of the HP derivatives. In the present study 1 μg/mL, 10 μg/mL, and 100 μg/mL of HP derivatives were used.

The percent growth inhibition was calculated as:

$$\left(1 - \frac{\text{net cell growth in treated medium}}{\text{net cell growth in standard medium}}\right) \times 100$$

where the net cell growth corresponds to cell growth in standard or treated medium minus cell growth in growth-arrest media. Differences in growth reflected in the differences in thymidine incorporation were not measured because the secretion of high amounts of endogenous thymidine in cell culture prevents the assessment of DNA synthesis with labeled thymidine.[20]

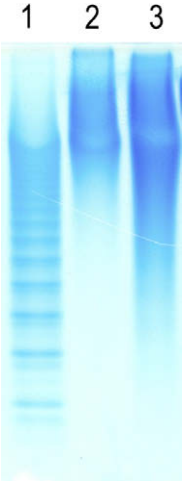

Figure 2. PAGE analysis of HP and CR-HP. Lane 1 shows a ladder of heparin oligosaccharide standards,[17] lane 2 is HP and lane 3 is CR-HP.

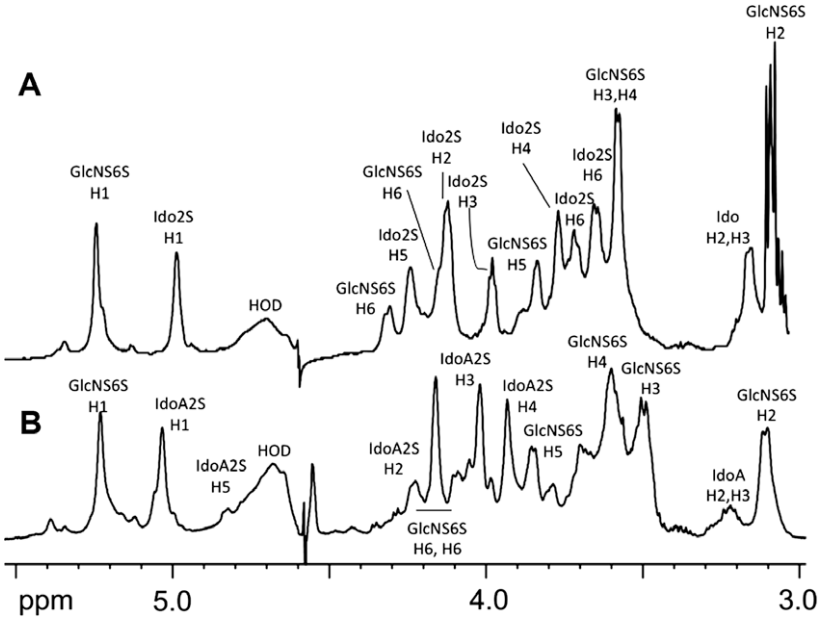

Figure 3. Expanded ^{1}H NMR spectra from 3 to 5.5 ppm of (A) CR-HP and (B) HP at 500 MHz and 300 K. Signals are labeled based on assignments made using 2D NMR spectroscopy.

2.2.4. Cell-growth statistics

The results are reported as mean ± standard error of the mean.

Comparisons among groups were made with a factorial analysis of variance (ANOVA), using the Statview software package (SAS Institute, Cary, NC 27513) for Macintosh computers. If ANOVA were significant, multiple comparisons were made using the Fisher protected least significant difference (PSLD) test. Significance was set as p <0.01.

3. Results and discussion

The CR-HP derivative was prepared by chemically modifying HP (Fig. 1) to evaluate the significance of CO_2H groups of the uronic acid residues present in the major and variable sequences of heparin, for the growth-inhibition properties of BPASMCs. This HP derivative was obtained by treatment of HP with EDC, followed by $NaBH_4$ to give the CR-HP derivative (Fig. 1). The average molecular weight of the HP and CR-HP derivatives was examined by PAGE (Fig. 2). Analysis of this gel showed that the average molecular weight of CR-HP (MW_{avg} 10,600) was slightly lower than that of the original HP (MW_{avg} 13,700). This was anticipated as the $NaBH_4$ reduction of the ester takes place under basic conditions that can result in some β-elimination on the uronic acid residues. Thus, care must be taken to use conditions that are as mild as possible in this reaction to afford complete or nearly complete reduction without concomitant chain depolymerization.

HP and CR-HP derivatives were next examined by ^{1}H NMR spectroscopy to confirm their chemical structures. The ^{1}H NMR spectra of the HP sample and its CR-HP derivative are shown in Figure 3. Assignments were made using correlation spectroscopy (COSY) and totally correlated spectroscopy (TOCSY) (data not shown). The starting HP was consistent with a pure porcine intestinal heparin allowing the convenient assignment of the six prominent signals associated with 2-amino-2-deoxy-*N*-sulfo-6-*O*-sulfo-D-glucopyranose and the six prominent signals associated with 2-*O*-sulfo-L-idopyranosyluronic acid. The CR-HP shows the same signals assignable to *N*-sulfo-6-*O*-sulfo-D-glucopyranose, but with 6 new signals associated with 2-amino-2-deoxy-2-*O*-sulfo-L-idopyranose. Of particular note are the two signals at 3.67 and 3.72 ppm corresponding to the methylene group of the newly formed primary hydroxyl group of 2-*O*-sulfo-L-idopyranose. The differences in the ^{1}H NMR spectra suggest complete or nearly complete reduction of the hexuronic acid.

Both HP and CR-HP were assayed for their growth-inhibition properties against BPASMCs. Figure 4 shows the effects of HP and CR-HP at three concentrations (1 μg, 10 μg, and 100 μg/mL). Cells grown in HP derivative-free medium were defined as 0% inhibition, bar 1, and cells grown in 0.1% fetal bovine serum as 100% inhibition, bar 2. Treatment of cultures of BPASMCs at three dose levels of HP produced the following growth inhibitions: 23 ± 3%, bar 3; 42 ± 4%, bar 4; 68 ± 3%, bar 5; respectively. The addition of the CR-HP derivative to the cell culture resulted in decreases in growth inhibition by 26 ± 2%, bar 6; 50 ± 2%, bar 7; 81 ± 1%, bar 8, respec-

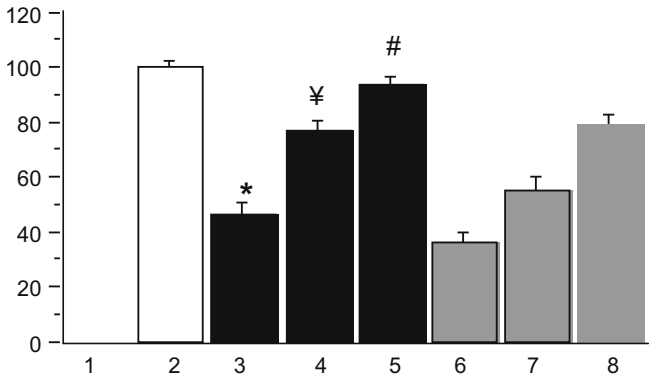

Figure 4. Growth inhibition of BPASMC by HP and a CR-HP derivative. Percent growth inhibition of bovine pulmonary artery smooth muscle cell grown in media containing: bar 1, 10% FBS without HP as negative control (−); bar 2, 0.1% FBS without HP as positive control (+); bar 3, 10% FBS plus 1 μg of HP; bar 4, 10% FBS plus 10 μg of HP; bar 5, 10% FBS plus 100 μg of HP; bar 6, 10% FBS plus 1 μg of CR-HP; bar 7, 10% FBS plus 10 μg of CR-HP; bar 8, 10% FBS plus 100 μg of CR-HP. (*) Significant inhibition of growth of PASMCs by 1 μg of HP compared to 1 μg of CR-HP; (ø) significant inhibition of growth of PASMCs by 10 μg of HP compared to 10 μg of CR-HP; (#) significant inhibition of growth of PASMCs by 100 μg of HP compared to 100 μg of CR-HP. p <0.001.

tively. At all dose levels there was a decrease in the inhibition of growth of CR-HP compared to the native HP preparation. These results show that the growth-inhibition properties of CR-HP are not completely abolished as in human umbilical cord vein (HUCV) SMCs as reported by Wessel et al.[21] These differences may result from a different response of CR-HP to BPASMC and HUCVSMC, respectively.[22]

Early reports[23–25] on the structural determinants of HP inhibitory activity on different types of cells have shown that structural variations are important in determining the HP biological properties of HP. These reports also suggested a strong interdependence between size and charge for antiproliferative activity.

In conclusion, we have clearly demonstrated that the carboxyl group of the iduronic acid and glucuronic acid residues in the major sequence and variable sequences of HP are important for antiproliferative potency of BPASMCs. These results are critical in the design of effective candidates for treatment of pulmonary hypertension, which is initiated by the increase in the growth of BPASMCs.

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