Published in final edited form as:

Carbohydr Res. 2010 June 16; 345(9): 1084–1087. doi:10.1016/j.carres.2010.03.026.

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

Hari G. Garg^{1,*}, Hicham Mrabat¹, Lunyin Yu¹, Craig Freeman², Boyangzi Li³, Fuming Zhang³, Robert J. Linhardt³, and Charles A. Hales¹

¹Department of Medicine, Pulmonary/Critical Care Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

²Immunology Program, The John Curtin School of Medical Research, The Australian National University, Canberra, ACT, Australia

³Departments of Chemistry and Chemical Biology, Biology and Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180

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 NaBH₄ 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, Figure 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 (Figure 1).

Tel: +1- 617-726-5632; Fax + 1-617-724-9650, Hgarg@partners.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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 (Figure 1).¹⁴ The growth-inhibitory effect in BPASMCs was then evaluated for both carboxyl reduced (CR)-HP and 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 3,500 D molecular weight 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.

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 and 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 NaBH₄ (200 mL in 2 portions) at 50 °C for 2 h. Excess NaBH₄ was decomposed with HOAc. Finally, CR-HP was isolated by either precipitation with abs EtOH followed by centrifugation or by dialysis using 3,500 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 ¹H 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 μ L 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 D₂O (99.996%, Sigma–Aldrich) and freeze-dried three-times to remove the exchangeable protons. The samples were re-dissolved in 0.5 mL of D₂O. Spectra were recorded at 300 K on either a 500- or 600-MHz spectrometer.

2.2.5. Cultured bovine pulmonary artery smooth cell (BPASMC's) proliferation assay—BPASMC proliferation assays were performed as previously described.¹⁹ Briefly, isolated BPASMC's in passages 4 to 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 μ g/mL), penicillin (100 U/mL) and amphotericin B (1.25 μ g/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 μ L, 140 μ L 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 addition of the HP derivatives. In the present study 1 μ g/mL, 10 μ g/mL, and 100 μ g/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

2.2.6 Cell-growth statistics—The results are reported as mean $\pm$ 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 (Figure 1) to evaluate the significance of CO₂H 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 NaBH₄ to give the CR-HP derivative (Figure 1). The average molecular weight of the HP and CRHP derivatives were examined by

PAGE (Figure 2). Analysis of this gel showed the average molecular weight of CRHP (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 1H NMR spectroscopy to confirm their chemical structures. The 1H 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- β -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- β -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 1H 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 μ g, 10 μ g, and 100 μ g/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, respectively. 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 reports²³⁻²⁵ 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.

Acknowledgments

This work was supported by National Institute of Health Grant HL039150 to Charles A. Hales and HL62244 and GM38060 to Robert J. Linhardt.

References

1. Geggel RL, Reid LM. Clin Perinatol 1985;11:525–549. [PubMed: 6386268]
2. Hales CA, Kradin RL, Branstetter RD, Zhu Y. J Am Rev Respir Dis 1983;128:747–751.
3. Meyrick B, Reid L. Exp Lung Res 1981;2:257–271. [PubMed: 7318779]

4. Hislop A, Reid L. *Br J Exp Pathol* 1976;57:542–554. [PubMed: 136978]
5. Clowes AW, Karnovsky MJ. *Nature* 1980;265:625–626. [PubMed: 859561]
6. Garg, HG.; Linhardt, RJ.; Hales, CA. *Chemistry and Biology of Heparin and Heparan Sulfate*. Garg, HG.; Linhardt, RJ.; Hales, CA., editors. Elsevier; Amsterdam: 2006. p. 513–532.
7. Mizumoto, S.; Kitagawa, H.; Sugahara, K. *Chemistry and Biology of Heparin and Heparan Sulfate*. Garg, HG.; Linhardt, RJ.; Hales, CA., editors. Elsevier; Amsterdam: 2006. p. 203–243.
8. Joseph PAM, Garg HG, Thompson T, Liu X, Hales CA. *Biochem Biophys Res Commun* 1997;241:18–23. [PubMed: 9405227]
9. Longas MO, Garg HG, Trinkle-Pereira JM, Hales CA. *Carbohydr Res* 2003;338:251–256. [PubMed: 12543557]
10. Garg HG, Yu L, Hales CA, Toida T, Toshi T, Capila IT, Linhardt RJ. *Arch Biochem Biophys* 1999;371:228–233. [PubMed: 10545209]
11. Garg HG, Yu L, Hales CA, Toida T, Toida T, Islam T, Linhardt RJ. *Biochim Biophys Acta* 2003;1639:225–231. [PubMed: 14636954]
12. Garg HG, Hales CA, Yu L, Butler M, Islam T, Xie J, Linhardt RJ. *Carbohydr Res* 2006;341:2607–2612. [PubMed: 16920092]
13. Garg HG, Mrabat H, Yu L, Freeman C, Li B, Zhang F, Linhardt RJ, Hales CA. *Carbohydr Res* 2008;343:2406–2410. [PubMed: 18656855]
14. Hoare DG, Koshland DE Jr. *J Biol Chem* 1967;242:2447–2453. [PubMed: 6026234]
15. Karamanos NK, Hjerpe A, Tsegenidis T, Engfeldt B, Antonopoulos CA. *Anal Biochem* 1988;172:410–419. [PubMed: 3189786]
16. Casu B, Lindahl U. *Adv Carbohydr Chem Biochem* 2001;57:159–206. [PubMed: 11836942]
17. Edens RE, Al-Hakim A, Weiler JM, Rethwisch DG, Fareed J, Linhardt RJ. *J Pharm Sci* 1992;81:823–827. [PubMed: 1328601]
18. Griffin CC, Linhardt RJ, van Gorp CL, Toida T, Hilenan RE, Schubert RL, Brown SE. *Carbohydr Res* 1995;276:183–197. [PubMed: 8536254]
19. Dahlberg CGW, Thompson BT, Joseph PM, Garg HG, Spence CR, Quinn DA, Bonventre JV, Hales CA. *Am J Physiol Lung Cell Mol Physiol* 1996;270:L260–L265.
20. Gehring H, Schroder A. *Biochem Biophys Res Commun* 1991;177:259–264. [PubMed: 2043111]
21. Wessel HP, Hosang M, Tschoop TB, Weimann BJ. *Carbohydr Res* 1990;204:131–139. [PubMed: 2279243]
22. Cindhuchao N, Quinn DA, Garg HG, Hales CA. *Carbohydr Res* 2003;302:184–188.
23. Hoover RL, Rosenberg RD, Haering W, Karnovsky M. *Circ Res* 1980;47:578–583. [PubMed: 6157501]
24. Castellot JJ, Addonizio ML, Rosenberg RD, Karnovsky M. *J Cell Biol* 1981;90:372–379. [PubMed: 7287812]
25. Wright TC, Johnstone TV, Castellot JJ, Karnovsky MJ. *J Cell Physiol* 1985;125:499–506. [PubMed: 3877732]

Figure 1.

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

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 ^1H NMR spectra from 3–5.5 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.

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 PSMCs by 1 µg of HP compared to 1 µg of CR-HP; (¥) significant inhibition of growth of PSMCs by 10 µg of HP compared to 10 µg of CR-HP; (#) significant inhibition of growth of PSMCs by 100µg of HP compared to 100 µg of CR-HP. $p < 0.001$