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Biochemical and functional characterization of glycosaminoglycans released from degranulating rat peritoneal mast cells: Insights into the physiological role of endogenous heparin

Rebecca Lever [a, *], Amir Smailbegovic [a, b], Yanira Riffo-Vasquez [b], Elaine Gray [b, c], John Hogwood [c], Stephen M. Francis [d], Neville V. Richardson [d], Clive P. Page [b], Barbara Mulloy [b, c]

[a] UCL School of Pharmacy, Brunswick Square, London, WC1N 1AX, UK
[b] Sackler Institute of Pulmonary Pharmacology, Institute of Pharmaceutical Science, King's College London, London, SE1 9NH, UK
[c] National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, EN6 3QG, UK
[d] School of Chemistry, University of St. Andrews, St. Andrews, KY16 9ST, UK

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ABSTRACT

The properties of commercially prepared heparin as an anticoagulant and antithrombotic agent in medicine are better understood than is the physiological role of heparin in its native form, where it is uniquely found in the secretory granules of mast cells. In the present study we have isolated and characterised the glycosaminoglycans (GAGs) released from degranulating rat peritoneal mast cells. Analysis of the GAGs by NMR spectroscopy showed the presence of both heparin and the galactosaminoglycan dermatan sulphate; heparinase digestion profiles and measurements of anticoagulant activity were consistent with this finding. The rat peritoneal mast cell GAGs significantly inhibited accumulation of leukocytes in the rat peritoneal cavity in response to IL-1β ($p < 0.05$, $n = 6$/group), and inhibited adhesion and diapedesis of leukocytes in the inflamed rat cremasteric microcirculation in response to LPS ($p < 0.001$, $n = 4$/group). FTIR spectra of human umbilical vein endothelial cells (HUVECs) were altered by treatment of the cells with heparin degrading enzymes, and restored by the addition of exogenous heparin. In conclusion, we have shown that rat peritoneal mast cells contain a mixture of GAGs that possess anticoagulant and anti-inflammatory properties.

1. Introduction

The glycosaminoglycan (GAG) heparin was discovered a century ago and, as an anticoagulant drug, ranks as one of the most commonly used agents in modern medicine [1]. Whilst much is now known about the nature of commercially prepared pharmaceutical heparin, both in its unfractionated and low-molecular weight forms, with respect to structure, biological activity and clinical effects [2–4], the physiological role of endogenous heparin is considerably less well understood. It has long been known, however, that heparin possesses additional effects that are both separate to, and separable from, its well-characterized effects on blood coagulation, many of which involve modulation of aspects of immune or inflammatory cell function [5,6]. In contrast to the closely related GAG heparan sulphate, the ubiquitous expression of which alone goes some way towards explaining its pivotal role in normal physiology [7,8], mammalian heparin is produced exclusively by mast cells. In this respect, heparin has been suggested to be primarily important for the storage of histamine and certain pro-inflammatory granule proteins within the mast cell [9,10]. However, it would seem unlikely that a potent anticoagulant molecule having a broad range of biological activities [11] should be biosynthesized solely for this purpose, and indeed solely within a cell type found outside the vasculature. The localization of mast cells close to vessels of the microcirculation though, as well as their more recent description in pathological tissue sites including tumors and atheromatous plaques [12,13], suggests that endogenous heparin may be important in regulation of pathophysiological

* Corresponding author.
E-mail addresses: rebecca.lever@ucl.ac.uk (R. Lever), clive.page@kcl.ac.uk (C.P. Page).

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responses, as well as in normal physiology. It has been suggested that heparin, released from activated mast cells, may be involved in physiological regulation of inflammation [14] through the binding and neutralization of cytotoxic and pro-inflammatory proteins, thus limiting the extent of the inflammatory response and potential tissue damage and remodelling as part of homeostasis.

Many of the non-anticoagulant actions of heparin are mediated through interactions with proteins such as chemokines and growth factors, which often depend upon the binding of heparan sulphate for full activity [15–17]. Whilst the structural basis of the anticoagulant activity of heparin is well understood (reviewed in 11), the exact structural requirements for the majority of the anti-inflammatory effects of heparin remain to be fully determined. The ability of heparin to interact with a wide variety of proteins can vary from strongly sequence specific, such as the binding of antithrombin, to relatively non-specific, in part due to the size and polyanionic nature of the molecule [18,19]. In this regard, it is important to consider that commercially-available heparin, which is usually extracted from porcine intestinal mucosa, is standardized only for its anticoagulant activity, which depends heavily on the presence of the high affinity antithrombin-binding pentasaccharide [18]. Therefore, any biological activity confined to other polysaccharide sequences contained within the heparin structure may not necessarily correlate with the total amount of material present in the resultant heterogenous mixture, and may even be fractionated out by the current techniques for preparing heparin commercially as an anticoagulant. A greater understanding of the nature of the GAGs present in mast cells may elucidate the physiological role(s) of endogenous heparin and potentially facilitate the design of drugs to mimic specific biological effects of heparin other than anticoagulant activities.

In the present study, therefore, we have sought to examine the nature of GAGs released from peritoneal mast cells of the rat as a product of their degranulation.

2. Experimental procedures

Animals- Male Sprague-Dawley rats (200–250 g; Harlan, UK) were housed in an animal unit on a 12:12 h light:dark cycle, with access to standard laboratory chow and water *ad libitum*, for at least seven days prior to experimentation. All experiments were performed in accordance with local Ethical and UK Home Office approval and guidelines.

Isolation of rat mast cell GAGs (*RMCG*)- Rats were euthanized by CO_2 exposure and their peritoneal cavities immediately lavaged with 20 mL normal saline containing 0.05 mM EDTA. Recovered cells were washed in modified HBSS (Ca^{2+}/Mg^{2+} free) at 250 × *g* for 2 min, followed by density-dependent centrifugation to separate mast cells from mononuclear cells. Mast cell pellets were re-suspended in 5 mL buffer (PBS containing 0.1 mg mL^{-1} HSA and 5.6 mM glucose) and incubated for 10 min at 37° C prior to addition of a further 5 mL buffer containing 5 μg mL^{-1} compound 48/80 and incubation for a further 20 min at 37° C. Gross cellular material was removed by centrifugation at 150*g* for 10 min and discarded. Supernatants were then transferred to 1 mL micro-centrifuge tubes and centrifuged at 10,000 rpm for 15 min to sediment intact granules. Supernatants (A) were collected and transferred to a refrigerator and pelleted granules were re-suspended in 1 mL 2 M NaCl by vortexing, then incubated at room temperature for 30 min to facilitate the release of granule contents. The suspension was again centrifuged at 10,000 rpm for 15 min, supernatants (B) were collected and dialysed overnight against five changes of 1.5 L dH_2O and pellets were discarded. Supernatants A and B were added to poly-L-lysine agarose (Sigma-Aldrich) packed into 5 ml columns (6 mL per columns, pre-washed with 3 × 3 mL 2 M NaCl), which were capped and placed on a roller for 60 min at room temperature. Unbound contents were washed with 3 × 3 mL dH_2O and bound contents eluted with 4 × 3 mL of 1.5 M NaCl. Eluents derived from supernatants A and B were combined, dialysed overnight against 5 changes of 1.5 L dH_2O and freeze-dried. Average RMCG yield was 0.26 mg per 10^6 cells, with 0.8–1.2 × 10^7 cells retrieved per cavity estimated by weight of material.

Heparinase digestion- 1 mg mL^{-1} solutions of the RMCG, unfractionated heparin (5th International Standard; NIBSC) and heparan sulphate (HS1 as previously described [20]) were prepared, respectively, and each solution treated with 10 μL heparinase I (approximately 0.02 IU) from *F. heparinum* (EC: 4.2.2.7) (a kind gift of Leo Pharma, Ballerup, Denmark). Absorbance at 234 nm was monitored for 60 min (heparin and heparan sulphate) or 120 min (mast cell material).

Molecular weight distribution- The molecular weight distributions for RMCG and the USP Heparin Sodium Identification Reference Standard (USP, Rockville, MD, USA) were determined by size exclusion chromatography/gel permeation chromatography (SEC/GPC) as described in Ref. [21]. Briefly, samples were taken up to a concentration of 5 mg mL^{-1} in 0.1 M ammonium acetate containing 2 mg mL^{-1} alpha-cyclodextrin as a flowrate marker. Duplicate chromatography runs were performed on a column system consisting of TSK SWXL guard column, TSK G4000 SWXL and TSK G3000 SWXL columns in series, with 0.1 M ammonium acetate as the mobile phase at 0.6 mL min^{-1} and refractive index detection. The peak molecular weight M_p, weight average molecular weight M_w, number average molecular weight M_n and polydispersity were calculated using Cirrus software (Agilent, Santa Clara, CA, USA).

Anticoagulant activity- Assessment was carried out using two plasma based assays (activated partial thromboplastin time, APTT), using sheep plasma (First Link, UK in accordance with the European Pharmacopoeia (01/2008:20705) or human plasma (NBTS, UK). Purified reagent assays were also carried out to investigate antithrombin dependent inhibition of factor Xa and factor IIa activity (USP34 NF26) and heparin cofactor II (HCII) dependent inhibition of thrombin. Two heparin preparations, bovine mucosa and porcine mucosa, from the NIBSC panel were included as comparators. All assays used the 6th International Standard for Unfractionated Heparin (07/328, NIBSC, UK) as the standard with data analysis carried out using the parallel line bioassay model (Combistats, EDQM).

NMR spectroscopy- RMCG (~5 mg) was dissolved in 99.8% D_2O and transferred to a 5 mm NMR tube. One dimensional ^1H and two dimensional TOCSY, and NOESY spectra, were recorded at 500 MHz, 60 °C, using a Varian Unity 500 NMR spectrometer, with pulse sequences supplied by the manufacturer. Chemical shifts are reported relative to deuterated trimethylsilylpropionic acid sodium salt (TSP-d_4) (Sigma-Aldrich Ltd. UK) at 0 ppm.

Effects in in vivo *models of inflammation*- For peritoneal inflammatory cell recruitment experiments, rats (as before) were injected i.p. with the RMCG, or an equal volume of vehicle (200 μL saline), 30 min prior to the administration of 20 ng rat recombinant interleukin-1β (Sigma-Aldrich Ltd, UK) or vehicle (200 μL saline). Animals were euthanized 2 h later and peritoneal cavities lavaged immediately with 20 mL saline. Total cells in lavage fluids were counted and differential cell counts were obtained from cytospin preparations, stained using the DiffQuick system (Gamidor, UK). For intravital microscopy of the cremaster muscle, rats were administered RMCG or saline i.v. immediately prior to s.c injection of 25 μg LPS to the scrotal sac. Four hours later, animals were anaesthetized with urethane (2 mg kg^{-1} i.p.). Cremaster muscles were exteriorized following midline incision and carefully exposed over a transparent viewing area of a heated microscope stage, maintained at 37° C, and constantly superfused with Tyrode-HEPES buffer.