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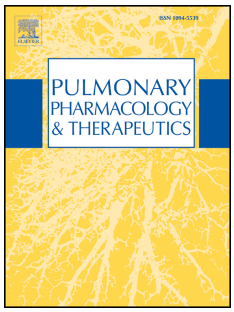

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Rebecca Lever, Amir Smailbegovic, Yanira Riffo-Vasquez, Elaine Gray, John Hogwood, Stephen M. Francis, Neville V. Richardson, Clive P. Page, Barbara Mulloy

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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

[1]Rebecca Lever, [1,2]Amir Smailbegovic, [2]Yanira Riffo-Vasquez, [2,3]Elaine Gray, [3]John Hogwood, [4]Stephen M. Francis, [4]Neville V. Richardson, [2]Clive P. Page, and [2,3]Barbara Mulloy

[1]UCL School of Pharmacy, Brunswick Square, London, WC1N 1AX, UK

[2]Sackler Institute of Pulmonary Pharmacology, Institute of Pharmaceutical Science, King's College London, London, SE1 9NH, UK

[3]National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, UK

[4]School of Chemistry, University of St. Andrews, St. Andrews, KY16 9ST, UK

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To whom correspondence should be addressed: Rebecca Lever, UCL School of Pharmacy

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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.

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 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.

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 x *g* for 2 minutes, 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 minutes at 37^{o} C prior to addition of a further 5 mL buffer containing 5 µg mL^{-1} compound 48/80 and incubation for a further 20 minutes at 37^{o} C. Gross cellular material was removed by centrifugation at 150 x *g* for 10 minutes and discarded. Supernatants were then transferred to 1 mL micro-centrifuge tubes and centrifuged at 10,000 rpm for 15 minutes 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 minutes to facilitate the release of granule contents. The suspension was again centrifuged at 10,000 rpm for 15 minutes, 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 x 3 mL 2 M NaCl), which were capped and placed on a roller for 60 minutes at room temperature. Unbound contents were washed with 3 x 3 mL dH_2O and bound contents eluted with 4 x 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 x 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 minutes (heparin and heparan sulphate) or 120 minutes (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 (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 TSL 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 (~5mg) was dissolved in 99.8% D_2O and transferred to a 5mm NMR tube. One dimensional ^1H and two dimensional TOCSY, and NOESY spectra, were recorded at 500 MHz, 60 ^oC, 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^o C, and constantly superfused with Tyrode-

HEPES buffer. Unbranched, post-capillary venules of 30-50 μm diameter (≥ 5 per animal) were viewed under a Zeiss Axioskop 2 FS microscope, fitted with a x 40 water-immersion lens and a x 10 eye piece. Digital images were captured using an ORCA flash digital camera (Hamamatsu, Japan) attached to an Axio-Workstation computer and images were viewed and recorded for subsequent off-line analysis using IHC acquisition software (Hamamatsu, Japan). Leukocyte rolling flux was quantified as the number of rolling cells passing a fixed point on the venular wall per 30 s and adherent leukocytes were considered those cells that were stationary for at least 30 seconds within a given 100 μm vessel wall segment. Migrated cells were classed as all cells present within a 100 μm^2 area of the surrounding extravascular tissue. *FTIR spectroscopy of the endothelial glycocalyx*- Human umbilical vein endothelial cells (HUVECs; TCS Cellworks Ltd., U.K.) were cultured to confluency in 6-well tissue culture plates (Corning Costar Ltd., U.K.) at 37 ^oC, 5% CO_2, in medium (MCDB 131) supplemented with fetal bovine serum (2% v/v), hydrocortisone (1 ng mL^{-1}), gentamicin (50 μg mL^{-1}), amphotericin-B (50 ng mL^{-1}) and human epidermal growth factor (10 ng mL^{-1}). Cultures were washed three times with phosphate buffered saline, to remove culture medium, and some wells were incubated with a combination of the enzymes heparinases I, II and III (Sigma-Aldrich Ltd., U.K.; 60 minutes at room temperature, each at 0.5 IU mL^{-1}). Following heparinase treatment, monolayers were washed and some of these wells subsequently received unfractionated heparin (500 IU mL^{-1} Multiparin®, CP Pharmaceuticals Ltd., Wrexham, U.K. – Multiparin is a porcine intestinal mucosal heparin; 20 minutes at room temperature) and were washed again. Cells were removed from the plates using a rubber policeman, blotted onto the FTIR crystal and gently dried under nitrogen to remove excess buffer. ATR-FTIR spectra were taken using a 6021 Galaxy Series spectrometer (Mattson Instruments Ltd., U.K.), set at 50 scans per run.

RESULTS

Molecular weight distribution- RMCG was found to be of lower average molecular weight (peak, number and weight averages) than typical porcine unfractionated heparin, but with greater dispersity (Table 1). Moreover, the chromatogram for RMCG exhibits a non-symmetrical peak, which may indicate the presence of more than one distinct population of molecules within the material, in contrast to the symmetrical peak for the heparin standard (Figure 1).

Susceptibility to heparinase digestion- Figure 2 shows reaction progress curves for the digestion of equal concentrations of heparin standard, RMCG and heparan sulphate, respectively, by a fixed concentration of heparinase I, as measured by the change in absorbance at 234 nm. RMCG was less susceptible to the actions of this enzyme than standard heparin, both in terms of initial rate and plateau, but more susceptible than heparan sulphate, suggesting the material to be comprised substantially, but not

exclusively, of heparin chains. After 60 minutes exposure to the enzyme, unsaturated uronic acid generation from the RMCG was approximately half of that from the heparin standard at the same time point, and 2.5 times that from heparan sulphate. However, when the reaction time was extended to two hours, allowing it to plateau, total generation from the RMCG was increased to approximately 60% of that obtained from the heparin standard and more than three times that from heparan sulphate.

Anticoagulant activity- The RMCG was found to give a valid potency estimation against the porcine unfractionated heparin standard (07/328), although the specific activity of RMCG is lower than porcine and bovine heparin (Table 1) . The APTT assay using sheep plasma was found to give the highest specific activity of 78 IU mg^{-1} (Table 1). The APTT assay using human plasma and the HCII based assay gave similar specific activity, 61 IU mg^{-1}, whilst the two antithrombin dependent assays gave lower activity, anti-Xa 55 IU mg^{-1} and anti-IIa 46 IU mg^{-1}. The ratio of anti-Xa to anti-IIa activity was 1.2.

^{1}H-NMR spectra of the RMCG- display signals (Figure 3) are consistent with the RMCG material being predominantly heparin (22,23), but interestingly containing a substantial proportion, possibly as much as 40-50%, of dermatan sulphate (24). Specifically, in spite of the rather broad resonances from this sample, the TOCSY spectrum of RMCG contains spin systems consistent with the presence of the IdoA residue (2-O-sulfo-α-L-iduronic acid; I2S) and glucosamine residues (α-D-N-sulfoglucosamine-6-O-sulphate; GlcNS,6S) that together make up the major trisulphated disaccharide repeat unit of heparin (Table 2). In addition, resonances were apparent in the ^{1}H spectrum that correspond to the presence of the galactosamine (β-D-N-acetylgalactosamine-4-O-sulphate; GalNac4S) and non-sulphated IdoA residue (α-L-iduronic acid; I) in the major disaccharide repeat unit of dermatan sulphate (Table 2). Comparisons with literature values shown in Table 2 confirm that the signals attributable to GalNAc4S are characteristic of those in dermatan sulphate (24) rather than chondroitin-4-sulphate (28), and the signals attributable to IdoA are characteristic of those in dermatan sulphate rather than in heparin.

Anti-inflammatory activity- was measured *in vivo* in the rat in two models of inflammation. Pre-treatment with RMCG inhibited accumulation of leukocytes in the peritoneal cavity in response to the cytokine IL-1β, when both agents were administered i.p. (Figure 4A). In addition, systemic pre-treatment with RMCG inhibited the firm adhesion and diapedesis of leukocytes in the cremasteric microcirculation, without significantly affecting the number of rolling cells (Figure 4B-D).

FTIR spectroscopy of the endothelial glycocalyx- FTIR spectroscopy of HUVECs yielded spectra indicating the presence of sulphated functional groups on the cell surface. Differences were observed in the spectra of untreated endothelial cells when compared with those from enzymatically-treated cells (Figure 5), in that peaks in the window associated with the presence of sulphate groups were abolished following treatment with heparinase enzymes, suggesting degradation of heparan sulphate on the endothelial surface. Interestingly, addition of exogenous heparin to enzymatically treated HUVECs led to

restoration of the sulphate peak in the spectra of these cells, with introduction of additional peaks found in the spectrum of heparin itself, suggesting the binding of heparin to the cell surface of the endothelium.

DISCUSSION

A specific physiological role for heparin, over and above that associated with its general characteristics as a GAG, has yet to be defined. Heparin is sometimes considered to be a specialized form of heparan sulphate and, indeed, many of the important protein-binding characteristics of heparan sulphate are known to be shared, and often exceeded, by heparin. In the case of the anticoagulant actions of heparin, this increased potency is known to be due in large part to the relatively frequent occurrence of the antithrombin-binding pentasaccharide sequence that is expressed more rarely in heparan sulphate chains, whereas for other examples of GAG-protein interactions, that are less specific in terms of the GAG structure involved, the significantly greater sulphation density of heparin appears to be the key feature.

In the present study, we have isolated GAGs from degranulating rat peritoneal mast cells in good yield using affinity chromatography on poly-L-lysine agarose. This technique has the advantage of reducing to a minimum any co-purification of mast cell granule proteins with the GAGs. We have found that endogenous heparin released by degranulating rat peritoneal mast cells is characterised by a relatively low molecular weight profile by comparison with a porcine mucosal heparin reference standard. Free GAGs released from mast cells on degranulation have been depolymerised from their original macromolecular form by mast cell heparanase (25). The molecular weight distribution of the resulting GAG mixture is characteristic of the source tissue and species; for example, porcine mucosal and bovine lung heparins have consistently different molecular weight profiles (26). Using NMR spectroscopy we have also observed the presence of dermatan sulphate; signals from both IdoA and GalNAc are present, with chemical shift values characteristic of dermatan sulphate (Table 2), clearly distinguishable from those of unsulphated IdoA in heparin sequences (27) and GalNAc in chondroitin sulphate A (28). The presence of dermatan sulphate in rat peritoneal mast cells has been suggested by comparison of the disaccharide products of digestion with chondroitinase ABC (to which DS is sensitive) with chondroitinase AC (to which DS is not sensitive)(29). Here we confirm the presence of DS by direct spectroscopic observation of the GAG mixture, without degradation or separation of its components. Minor proportions of chondroitin-4-sulphate (CSA), and traces of chondroitin-4,6-sulphate (CSE) found by Akiyama et al. may be present, but are not visible in our NMR spectra (29). The presence of both

heparin and dermatan sulphate chains presumably gives rise to the skewed molecular weight distribution of the rat GAGs (Fig. 1); the mode of depolymerisation of mast cell DS is not currently known to us.

There have been a few previous attempts to look at the release of endogenous rat heparin (30,31), the first of which (30) characterized the whole serglycin proteoglycan rather than the GAG. Wang and Kovanen have also previously reported the release of endogenous heparin from rat mast cells, and studied the ability of this material to inhibit the proliferation of aortic vascular smooth muscle cells, suggesting that the endogenous heparin may be more potent in this regard than an exogenous heparin preparation (31).

We have demonstrated in this study that rat peritoneal mast cell GAGs, consisting of a mixture of heparin and DS, possess significant anti-inflammatory activity in a number of assays in addition to a clear ability to act as an anti-coagulant. The GAG content of mast cells and of other granule-containing cells such as basophils has not been accurately determined thus far, although it has been proposed that in rodents there are two types of mast cell referred to as connective tissue (containing heparin) and mucosal (containing chondroitin sulphate), but only based on histochemical staining (32,33). It has been previously suggested that heparin is found in mast cell granules whereas chondroitin sulphate is found in basophils (34). However, in the present study, the mast cell granule contents we have examined contain both heparin and dermatan sulphate (chondroitin sulphate B), suggesting that both GAGs may occur in the same granule (though it is possible that our peritoneal mast cell preparation includes two separate cell populations).

The time course profile of heparinase I digestion of the RMCG indicates that roughly 60% of its constituent material is susceptible to the enzyme, which is specific for the highly sulphated sequences typical of heparin rather than the low-sulphated HS sample. The anticoagulant profile for the RMCG is also consistent with the presence of both heparin and DS. DS exclusively potentiates heparin cofactor II inhibition of thrombin, whereas heparin potentiates both antithrombin and HCII dependent inhibition of thrombin. Since measurable activity was found using antithrombin dependent assays, it can be concluded that RMCG contains the prerequisite pentasaccharide sequence for potentiation of antithrombin. A ratio between the antithrombin anti-Xa and anti-IIa of 1.2 highlights the overall lower molecular weight of RMCG in comparison to heparin. Since expression of anti-IIa activity requires a longer chain length than anti-Xa activity, a higher ratio suggests the presence of a higher proportion of lower molecular weight material. This observation fits in with the relatively lower molecular weight of the RMCG by comparison with porcine heparin. Overall the anticoagulant potency of the RMCG is lower than both bovine and porcine heparins, which may be due to the presence of a substantial proportion of DS, though relatively low molecular weight rat heparin may also have inherently lower activity.

Nonetheless as has been reported for exogenous heparin (for example 35,36), the endogenous heparin we have isolated and characterised was able to inhibit the infiltration of neutrophils into the rat peritoneal cavity suggestive of anti-inflammatory activity. Furthermore, using intra-vital microscopy we have also

shown that endogenous heparin was able to inhibit the adhesion and transmigration of leukocytes across the vascular endothelium. These findings extend previous observations that exogenous heparin can inhibit leukocyte infiltration into a number of tissues, both experimentally (35,37) and clinically (38). Our results support the hypothesis (14) that the release of endogenous heparin in an inflammatory response may serve to act as a homeostatic braking mechanism to limit cell infiltration into tissues and by virtue of the well known ability of heparin to neutralise the actions of various cationic pro-inflammatory mediators, may also serve to limit the effects of the mediators released from infiltrating inflammatory cells.

It has recently been demonstrated that the loss of the glycocalyx from the endothelium lining blood vessels following exposure to heparanase released by inflammatory cells may play an important role in the subsequent trafficking of inflammatory cells into tissues (39), and indeed we have recently reported that recombinant human heparanase is pro-inflammatory and can elicit leukocyte infiltration into tissues (33). Furthermore, we have also recently reported that heparanase inhibitors are able to inhibit neutrophil recruitment into lung tissue following certain types of inflammatory insult (40). It is therefore of interest that by use of FTIR spectroscopy, we have observed the loss of sulphation on the surface of vascular endothelial cells exposed to heparinase and that we could restore this barrier by adding back exogenous heparin. Given the proximity of mast cells to blood vessels it thus remains plausible that the release of endogenous heparin serves as a "top up" mechanism to help preserve the integrity of the glycocalyx during normal defence mechanisms further supporting the suggestion that endogenous heparin may play an important physiological role in regulating the innate inflammatory response (14). It is of interest therefore that patients with allergy, where there is regular mast cell degranulation in response to sensitising allergens, have been reported to have increased levels of "circulating heparin like material" (41) which may account for the observations that atopic patients can have a mild haemostatic defect (42), and even have evidence of less calcification of their arteries (43). However, more recent observations have suggested that there may a loss of heparin in the circulation of patients with asthma (44) and that some patients can have a hyper-coaguable state (45).

It is clear from heparin preparations used in medicine that heparin composition varies between species and tissue of origin (46). Heparin from bovine lung differs markedly in the degree of sulphation from porcine intestinal mucosal heparin (22), and both are different from bovine intestinal mucosal heparin (47). We can speculate that these differences in structure and in relative content of heparin *vs,* chondroitin/dermatan sulphate may have functional significance in the context of each particular tissue and species. Clearly, further research is needed to clarify these structure activity relationships of endogenous GAGs.

In conclusion, we have isolated and partially characterised the GAGs released from degranulating rat peritoneal mast cells and demonstrated the presence of both heparin and DS. Whilst it remains to be

determined what the physiological role of this endogenous material is, we have provided evidence that this material exhibits anti-coagulant activity comparable to heparin obtained from other species and shows anti-inflammatory activity.

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TABLE 1

A: Peak molecular weight M_p, number average molecular weight M_n, weight average molecular weight M_w and polydispersity M_w/M_n for rat peritoneal mast cell GAGs (RMCG) and for a heparin identity standard, the USP Heparin Sodium Identity Reference Standard (USP ID RS). Results are the means of duplicate determinations.

B: Anticoagulant potencies of RMCG, a bovine and porcine heparin sample estimated against 6th International Standard for Unfractionated Heparin using a parallel line analysis model. Values are calculated using multiple concentrations of unknown samples against the standard to give IU/mg and 95% confidence limits range for the estimated value.

A	*Mp*	*Mn*	*Mw*	Polydispersity (*Mw/Mn*)	
RMCG	7333	7471	12027	1.611	
USP ID RS	15346	12718	15856	1.247	

B	Potency in IU/mg (95% Confidence Limits)				
	Sheep Plasma APTT Assay	Human Plasma APTT Assay	anti-Xa assay	anti-IIa assay	HCII assay
RMCG	78 76 – 81	61 57 – 64	55 54 – 56	46 44 – 47	61 54 – 68
Bovine Heparin	108 (103 – 113)	89 (85 – 93)	94 (91 – 97)	87 (80 – 94)	120 (112 – 129)
Porcine Heparin	200 (194 – 206)	185 (175 – 195)	197 (186 – 208)	192 (175 – 211)	199 (186 – 214)

TABLE 2

Assignment of the ^{1}H NMR spectrum of RMCG: chemical shifts in ppm at 500 MHz, 60 ºC, in D_2O relative to TSP at 0 ppm.

	Heparin					Dermatan sulphate				
	GlcNS,6S		IdoA2S		GalNAc4S			IdoA		
	This study	Ref. 22	This study	Ref. 22	This study	Ref. 24	Ref 28	This study	Ref. 24	Ref 27
H1	5.39	5.39	5.21	5.24	4.65-4.7	4.7	4.60	4.90	4.90	5.04

H2	3.27	3.31	4.30	4.37	4.04	4.05	4.03	3.54	3.54	3.78
H3	3.69	3.70	4.20	4.23	4.02	3.95	4.01	3.92	3.92	4.12
H4	3.76	3.78	4.11	4.13	4.65	4.65-4.7	4.74	4.10	4.11	4.08
H5	4.02	4.06	4.80	4.81	3.85	3.8	3.81	4.72	4.72	4.84
H6	n.d.	4.37			3.80	3.75-3.8	3.82			
H6’	n.d.	4.30					3.77			
	GlcNAc									
CH_3	2.05	2.05			2.08	2.08	2.02			

1. In chondroitin 4 sulphate at 60 deg. C
2. In the heparin-derived sequence IdoA-GlcNS,6S at 40 deg. C.

FIGURE LEGENDS

Figure 1: A**.** Size exclusion chromatograms of rat mast cell GAGs (RMCG; peak A) and a sample of typical unfractionated heparin (the USP Heparin Sodium Identification RS; peak B). Peak C is the flow-rate marker alpha-cyclodextrin, and peak D is a salt peak

Figure 2: Heparinase I digestion profiles of RMCG (A), unfractionated heparin standard (B) and heparan sulphate (C). In (A) the spectrophotometer was reprogrammed at 60 minutes to extend the data collection period, leading to a gap in the readings at that time.

Figure 3: A. ^{1}H NMR spectrum (500 MHz, 60 ºC in D_2O) of rat mast cell GAGs. Some of the characteristic resonances of heparin and dermatan sulphate are annotated.

Figure 4: A. IL-1β-induced (20 ng) accumulation of cells in the peritoneal cavity of the rat and the effect of 10 µg kg^{-1}RMCG administered locally. Open bars indicate total cell counts and filled bars neutrophil counts *(*$p \leq 0.05$ *vs*. IL-1β, n= 6/group). B-D. Rolling (B), firmly adherent (*$p<0.05$ vs LPS) (C) and transmigrated (*$p<0.001$ vs LPS) (D) cells in the cremasteric microcirculation of the rat inresponse to 25 µg of LPS and the effect of RMCG administered systemically (1 and 10 µg Kg^{-1},n= 4/group).

Figure 5: FTIR spectra of HUVECs treated with vehicle (top), heparinases I, II and III (0.5 IU mL^{-1}; second from top) and heparinases I, II and III followed by unfractionated heparin (0.5 IU mL^{-1} and 500 IU mL^{-1}, respectively, third from top). The spectrum of unfractionated heparin itself is also shown (bottom).

Figure 1

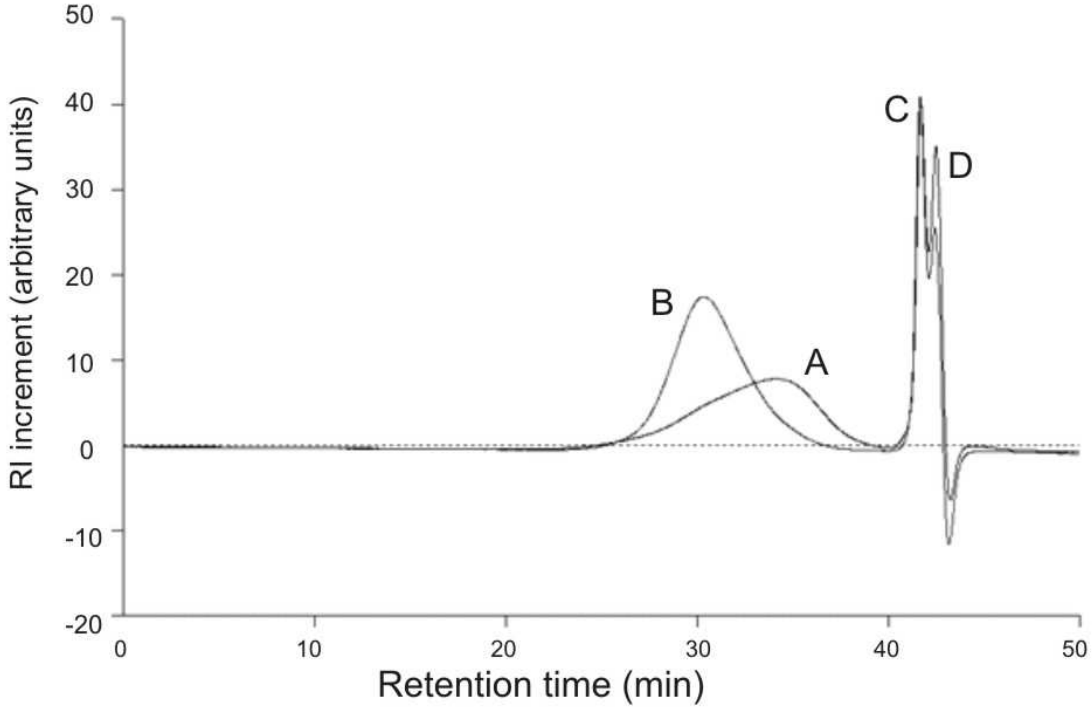

Figure 2

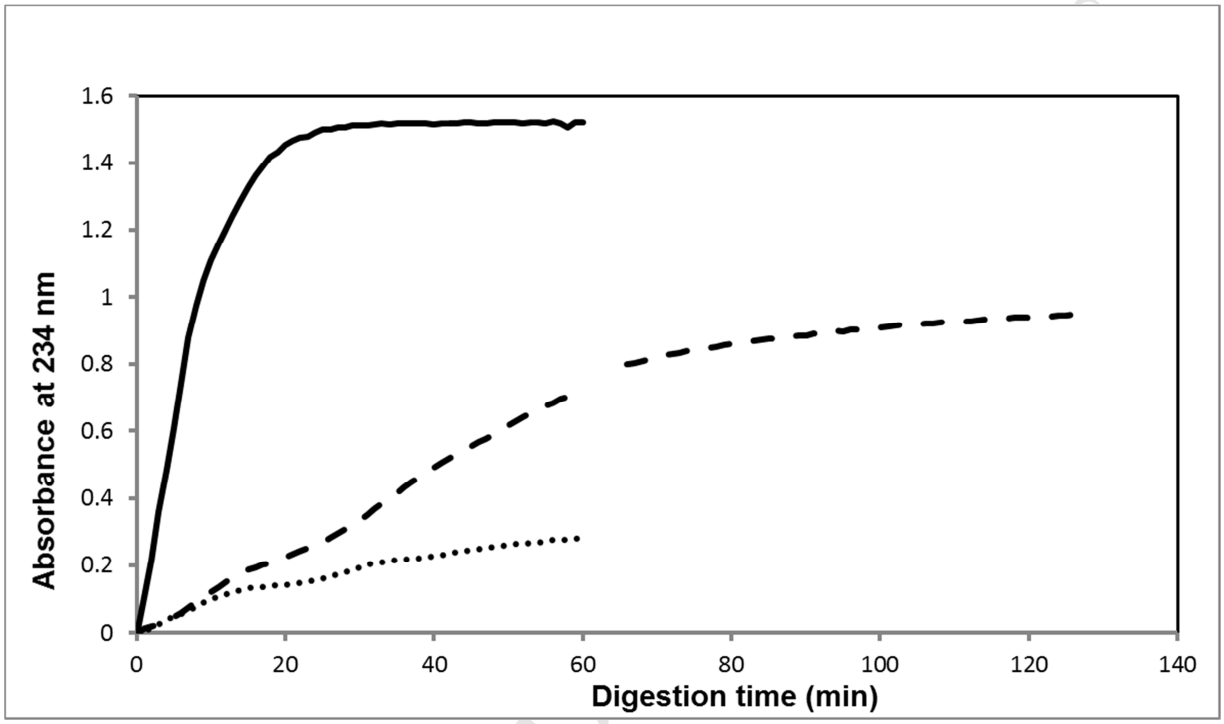

Figure 3

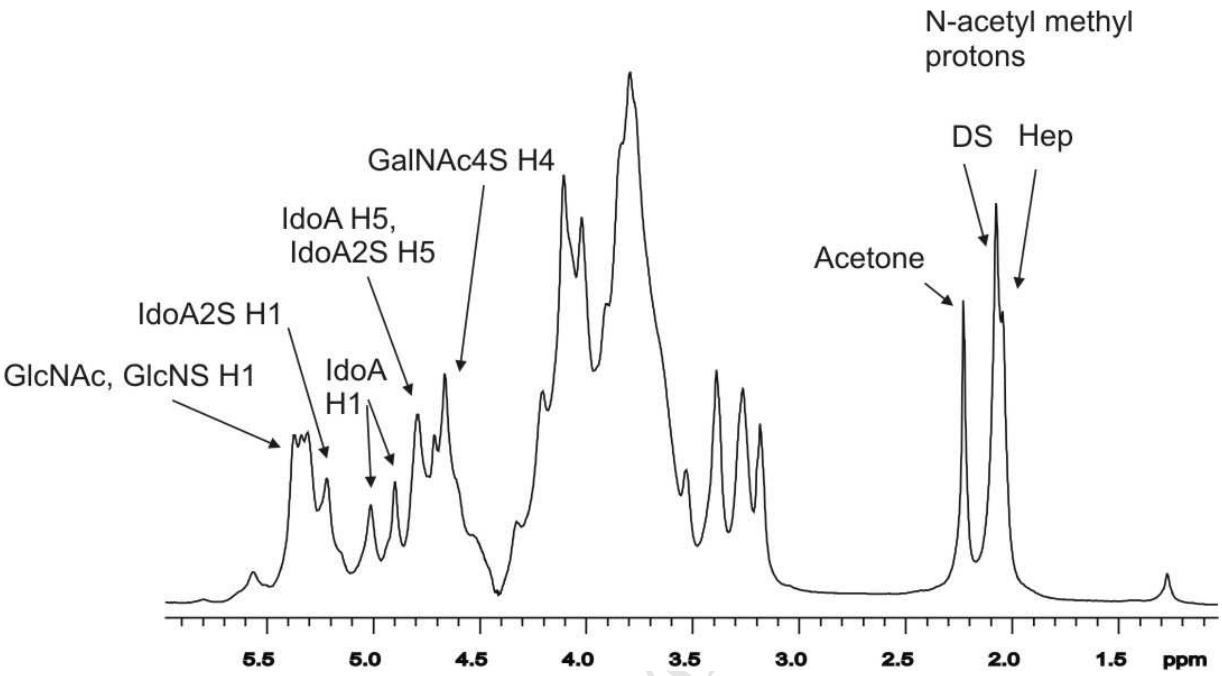

Figure 4

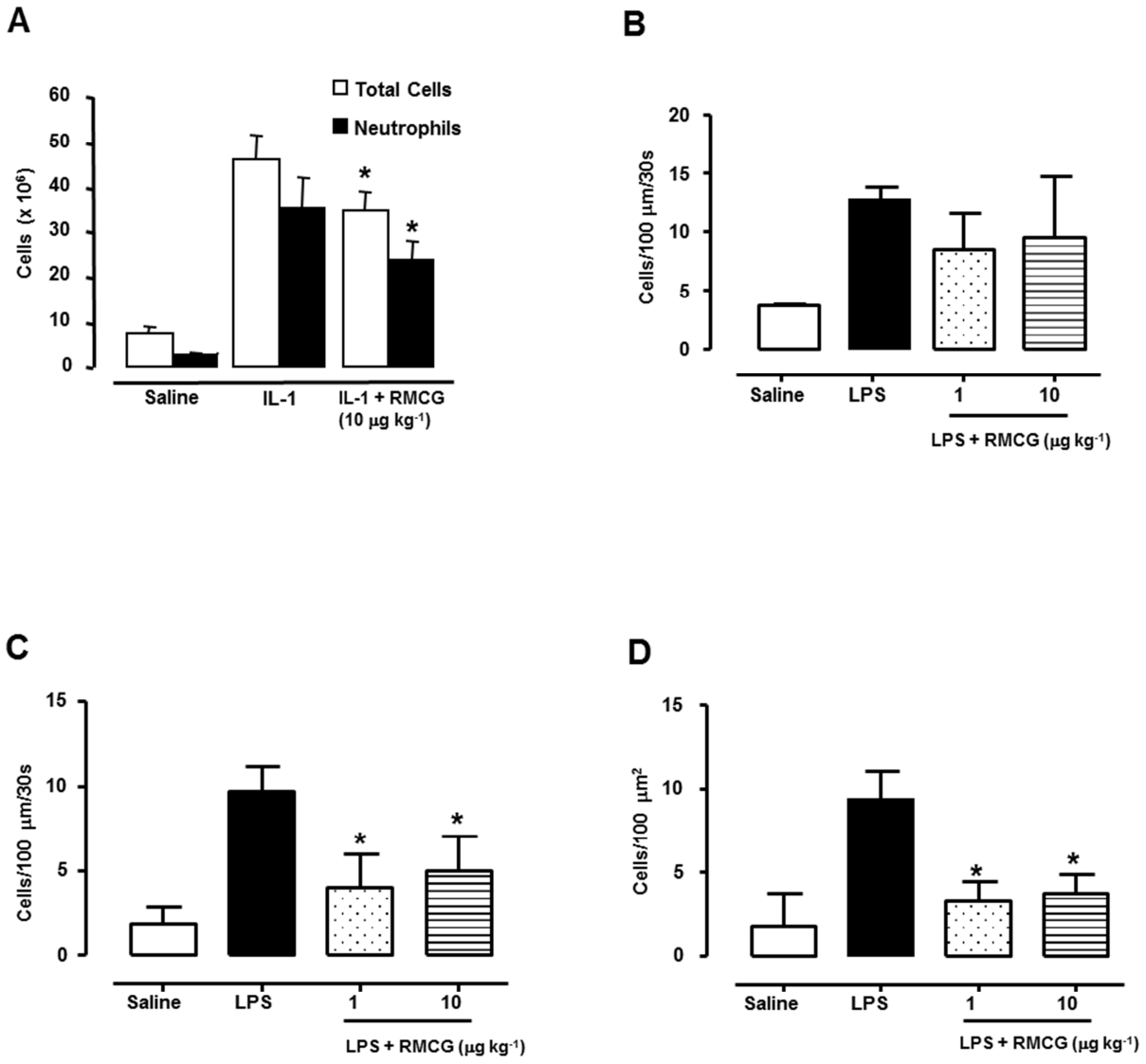
A
Total Cells
Neutrophils
Cells (x 10⁶)
60
50
40
30
20
10
0
Saline
IL-1
IL-1 + RMCG
(10 µg kg⁻¹)
B
Cells/100 µm/30s
20
15
10
5
0
Saline
LPS
1
10
LPS + RMCG (µg kg⁻¹)
C
Cells/100 µm/30s
15
10
5
0
Saline
LPS
1
10
LPS + RMCG (µg kg⁻¹)
D
Cells/100 µm²
15
10
5
0
Saline
LPS
1
10
LPS + RMCG (µg kg⁻¹)

Figure 5