British Journal of Pharmacology (2005) 146, 370–377
www.nature.com/bjp

Some structural determinants of the antiproliferative effect of heparin-like molecules on human airway smooth muscle

[1,2]Varsha Kanabar, [1]Stuart J. Hirst, [1]Brian J. O'Connor & *,[2]Clive P. Page

[1]Department of Asthma, Allergy and Respiratory Science, GKT School of Medicine, King's College London, Guy's Campus, London SE1 9RT and [2]Pharmacology and Therapeutics Division, Sackler Institute of Pulmonary Pharmacology, GKT School of Biomedical Sciences, King's College London, Guy's Campus, 5th Floor, Hodgkin Building, London SE1 9RT

1 Accumulation of airway smooth muscle (ASM) and its infiltration by mast cells are key pathological features of airway remodelling in asthma. Heparin, a major component of mast cell granules, inhibits ASM proliferation by an unknown mechanism. Here, unfractionated heparins and related glycosaminoglycans having structurally heterogeneous polysaccharide side chains that varied in molecular weight, sulphation and anionic charge were used to identify features of the heparin molecule that were required for its antiproliferative activity in cultured human ASM cells.

2 Proliferation induced by 10% fetal bovine serum (FBS) was abrogated by two unfractionated commercial heparin preparations (Sigma and Multiparin) and this effect was reproduced with each of three low-molecular weight heparin preparations (3, 5 and 6 kDa, respectively), demonstrating that antiproliferative activity resided in at least a 3 kDa heparin fraction.

3 *N*-desulphated 20% re-acetylated (*N*-de) heparin (anticoagulant) and *O*-desulphated heparin (*O*-de) (non-anticoagulant) fractions also inhibited FBS-dependent proliferation (rank potency: Sigma heparin > *O*-de > *N*-de) suggesting that the antiproliferative action of heparin involved *N*-sulphation but was independent of its anticoagulant activity.

4 Other sulphated molecules with variable anionic charge (dextran sulphate, fucoidan, chondroitin sulphates A or B, heparan sulphate) inhibited proliferation to varying degrees, as did the non-sulphated molecules hyaluronic acid and poly-L-glutamic acid. However, nonsulphated dextran had no effect.

5 In summary, attenuation of FBS-dependent proliferation of human ASM by heparin involves but does not depend upon sulphation, although loss of *N*-sulphation reduces antiproliferative activity. This antiproliferative effect is independent of anionic charge and the anticoagulant actions of heparin.
British Journal of Pharmacology (2005) **146,** 370–377. doi:10.1038/sj.bjp.0706333;
published online 18 July 2005

Keywords: Heparin; glycosaminoglycan; proliferation; airway; remodelling

Abbreviations: AHR, airways hyper-responsiveness; ASM, airway smooth muscle; D, dextran; DS, dextran sulphate; GAG, glycosaminoglycan; HS, heparan sulphate; LMW, low molecular-weight heparin; *O*-de, *O*-desulphated heparin; *N*-de, *N*-desulphated 20% reacetylated heparin; *N*-ac, *N*-desulphated 90% reacetylated heparin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PGA, poly-L-glutamic acid; VSM, vascular smooth muscle

Introduction

A component of tissue remodelling and excessive airway narrowing in asthmatic airways involves accumulation of airway smooth muscle (ASM), thought to result from aberrant hyperplastic and hypertrophic cell growth (Heard & Hossain, 1973; Ebina *et al*., 1993; Lambert *et al*., 1993). This single factor alone is suggested to be sufficient to cause airways hyper-responsiveness (AHR) (James *et al*., 1989). Possible mechanisms that underlie or amplify the remodelling in asthma and which may also drive increased ASM content include infiltration of inflammatory cells and leakage of plasma proteins, which may serve to increase the availability of profibrogenic and mitogenic factors within the vicinity of ASM (Shiels *et al*., 2000).

A recent study suggests a major difference between asthma and eosinophilic bronchitis (a condition characterised by cough, but not AHR or airflow obstruction) was infiltration of ASM bundles by mast cells (Brightling *et al*., 2002). Although mast cells produce a variety of lipid mediators, chemokines, cytokines and enzymes that may interact with ASM cells to cause AHR (Page *et al*., 2001; Robinson 2004), they are the only endogenous source of heparin in mammals, which may have a protective role by limiting imflammation and airway remodelling (Page, 1991). In support of this hypothesis, heparin is released upon mast cell degranulation (Green *et al*., 1993) and inhibits proliferation of ASM cultured from several species including bovine, canine, guinea-pig and man (Johnson *et al*., 1995; Kilfeather *et al*., 1995; Halayko *et al*., 1997), and is antiproliferative against ASM from asthmatic airways (Burgess *et al*., 2001).

Heparin and related glycosaminoglycans (GAGs) comprise alternating repeating disaccharide units of varying lengths with an amino sugar (either glucosamine or galactosamine) and uronic acid (either glucuronic or iduronic acid) residue (Tyrrell

*Author for correspondence; E-mail: clive.page@kcl.ac.uk

et al., 1999). These disaccharide units are variably *N*-sulphated, *O*-sulphated and *N*-acetylated. Furthermore, uronic acid residues are also carboxylated, which in combination with variable sulphate groups provide GAGs with a high net negative charge. Collectively, the above physical properties of heparin result in a molecule of varying sulphation, charge and size. Heterogeneity such as this likely underlies heparin's broad range of biological effects. In support of this possibility, heparin has anti-inflammatory and antimetastatic activities (Seeds *et al*., 1995; Fryer *et al*., 1997; Lever & Page, 2002), which are distinct from its anticoagulant property that resides within a defined pentasaccharide sequence (Tyrrell *et al*., 1999). Furthermore, sulphation, polymer size and anionic charge are reported to be important determinants of the antiproliferative activity of heparin in vascular smooth muscle (VSM) cultured from systemic or pulmonary vascular tissue (Karnovsky & Edelman, 1994; Joseph *et al*., 1997; Garg *et al*., 2000; 2002). However, the properties responsible for heparin's antiproliferative activity in ASM have been only partially characterised in bovine ASM (Kilfeather *et al*., 1995) and have not been examined at all in human ASM.

In the present study, we hypothesised that specific structural properties of the heparin molecule were responsible for its anti-proliferative activity and that identification of such properties would facilitate the design of polysaccharide sequences to target ASM proliferation specifically. Accordingly, we evaluated the contribution of sulphation, molecular size/weight and anionic charge as variables contributing to heparin's reported antiproliferative capacity in fetal bovine serum (FBS)-stimulated human ASM cells. Although identification of the molecular mechanism underlying heparin's antiproliferative activity in ASM was not the primary aim of our study, a nonanticoagulant heparin derivative was also examined to investigate whether the anticoagulant activity of heparin was required for its antiproliferative action on these cells.

Methods

Compounds

Unfractionated sodium heparin was obtained from two separate sources: Sigma (Cat #H3149, 17–19 kDa, 170–195 USP units mg^{-1}; Sigma-Aldrich Company Ltd, Poole, Dorset, U.K.) and CP Pharmaceuticals (30 kDa, 200 IU mg^{-1}; CP Pharmaceuticals Ltd, Wrexham, U.K.) and described hereafter as Sigma heparin and Multiparin, respectively. Low molecular-weight (LMW) heparins were from Sigma (3 and 6 kDa) and Leo Laboratories (5 kDa (Fragmin), Leo Laboratories, Princes Risborough, U.K.). *O*-Desulphated heparin (*O*-de) (<10 kDa) was a gift from Dr T. Kennedy (Carolinas Medical Health Care Foundation, Charlotte, NC, U.S.A.). Chondroitin sulphate A (5–50 kDa), chondroitin sulphate B (37.5 kDa), *N*-acetyl, *N*-desulphated (*N*-de) heparin (17–19 kDa), heparan sulphate (14.2 kDa), fucoidan (193 kDa), hyaluronic acid (3–5.8×10^3 kDa), dextran (9.5 kDa), dextran sulphate (DS) (10 kDa), poly-L-glutamic acid (3–15 and 50–100 kDa) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma. All cell culture reagents were from Invitrogen (Paisley, U.K.).

Isolation and culture of human ASM cells

Human ASM cells were obtained in accordance with procedures approved by the Guy's and St Thomas' Hospitals' Research Ethic's Committee from the lobar or main bronchus of 14 nonasthmatic subjects (12 smokers, two nonsmokers; mean age 64 ± 2 years, range 27–78 years; 11 male and three female) undergoing lung resection for carcinoma of the bronchus using methods described previously (Hirst *et al*, 2000). Fluorescent immunocytochemical and flow cytometric techniques confirmed that near-confluent, FBS-deprived early passage human ASM cells stained ($>95\%$) for smooth muscle-specific α-actin and calponin (Hirst *et al*, 2000). Cells at passages 3–8 were used in all experiments.

Cell stimulation and proliferation

Cells in multiwell plates were seeded at 10,000 cm^{-2} in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. After 24 h, cell growth was arrested by replacing the medium with FBS-free DMEM supplemented with 1 μM insulin, 5.5 $\mu g\,ml^{-1}$ transferrin, 6.7 $pg\,ml^{-1}$ selenium and bovine serum albumin (0.1%) for 72 h. Cells were then either left unstimulated or growth was initiated with DMEM containing 10% FBS in the presence or absence of each heparin or related GAG. Solutions were prepared fresh on the day of addition from a 100 $mg\,ml^{-1}$ stock solution. Fresh medium was replaced every 48–72 h. Proliferation was examined on days indicated either by direct cell counting using a haemacytometer or by the MTT reduction assay, as previously validated with human ASM cells (Hirst *et al*., 1992).

Data analysis

Data are mean$\pm$s.e.m. from cells cultured from *n* patients. Data were compared using one- or two-way repeated measures analysis of variance (ANOVA), where appropriate, followed by Bonferroni's *t*-test to evaluate differences between treatment groups (SigmaStat; SPSS Inc., Chicago, IL, U.S.A.). A probability value (*P*) of less than 0.05 was considered significant. Effective concentrations giving a 50% inhibition (IC_{50}) and extrapolated maximum responses were estimated for individual concentration–response curves using nonlinear least-squares regression (SigmaStat). IC_{50} values were converted to negative logarithmic values (pIC_{50}) for all statistical analysis, although for ease of comprehension IC_{50} values with the corresponding 95% confidence interval range are given in the text. The polymeric nature of heparin and related GAGs precluded use of molar concentrations.

Results

Unfractionated heparins

Unfractionated heparin from Sigma (comprising variable chain lengths) inhibited FBS-induced proliferation in a concentration and time-dependent manner ($P<0.001$, Figure 1a). Concentrations of at least 2.5–5 $mg\,ml^{-1}$ maintained cells in a nonproliferating state for all days examined. Direct comparison of the effect of Sigma heparin by MTT reduction with cell counts suggested cell counting was

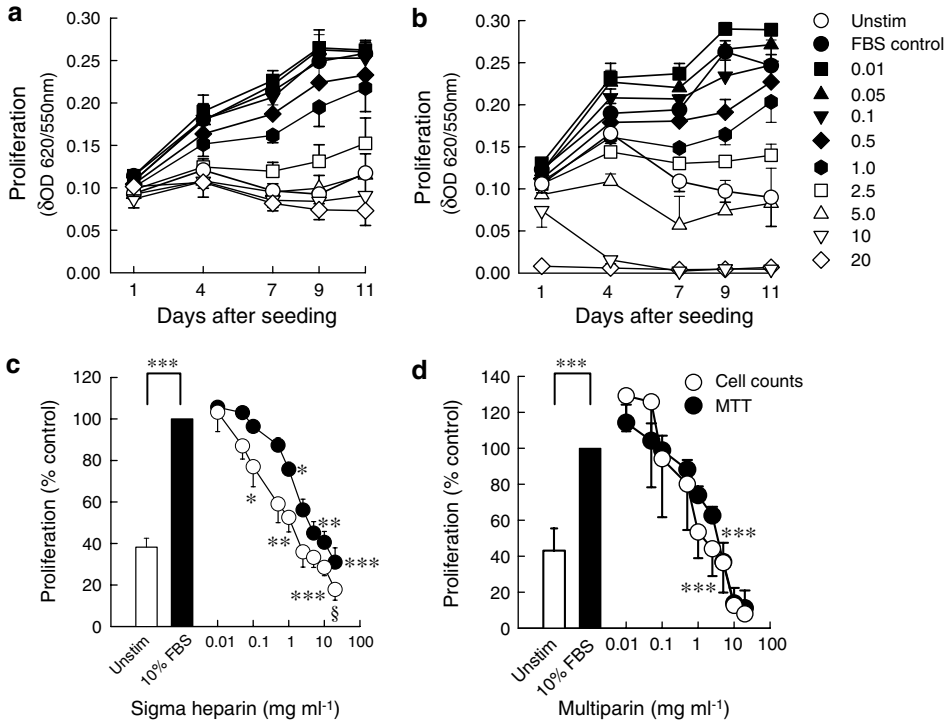

Figure 1 Attenuation of FBS-stimulated proliferation by unfractionated heparins. Human ASM cells were either unstimulated or stimulated with 10% FBS in the presence or absence of varying concentrations (mg ml^{-1}) of either Sigma heparin (a, c) or a clinically used commercial heparin preparation, Multiparin (b, d). Proliferation was assessed by MTT reduction (a, b) and by direct cell counting. Panels c and d compare proliferation at day 7 using both methods. Data are mean ± s.e.m. of six experiments using cells from individual donors. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with FBS alone; $^{\S}P<0.05$ compared with MTT reduction by two-way ANOVA.

approximately 1.8-fold more sensitive than MTT reduction for detecting the attenuating effects of heparin ($P=0.036$, Figure 1c). Likewise, another unfractionated heparin (Multiparin) that was standardised for anticoagulant activity and used clinically, prevented FBS-dependent proliferation in a concentration and time-dependent manner ($P<0.001$, Figure 1b) but also caused a variable, nonsignificant potentiation at the lowest concentrations examined (0.01–0.05 mg ml^{-1}). At day 7, however, there was no significant difference between Multiparin-dependent cell inhibition determined by MTT reduction or by direct cell counting ($P>0.05$, Figure 1d) and the MTT reduction assay was adopted for all subsequent studies. Concentrations at or exceeding 10 mg ml^{-1} Multiparin, but not below, caused cells to become rounded and detached when viewed under the microscope 24 h after treatment, also evidenced by optical density values well below those obtained for control unstimulated cells. Viability of detached cells, assessed by Trypan Blue exclusion, was >95% after treatment for 24 h with 10 mg ml^{-1} Multiparin, but fell to 42 ± 4% at day 4. The reduction in viability at late but not early time points of detached cells likely reflects loss of attachment-dependent survival by Multiparin rather than direct cytotoxicity. These effects were unrelated to the preservative in the commercial formulation (0.15% chlorocresol) as no effect was found on basal or FBS-stimulated proliferation (data not shown). In addition, neither Sigma heparin nor Multiparin (0.01–5 mg ml^{-1}) had any effects upon MTT reduction in the absence of mitogen ($P>0.05$, $n=4$) (data not shown), suggesting they had no direct mitogenic or cytotoxic action.

Role of sulphation

To examine a requirement for sulphation in the growth attenuating effects of heparin, a non-heparin-like compound, dextran sulphate (DS) was initially examined against FBS-stimulated proliferation and compared with its nonsulphated derivative, dextran (D, 0.01–20 mg ml^{-1}). DS significantly attenuated FBS-induced human ASM proliferation in a concentration-dependent manner (IC_{50} 2.0 [0.1–3.8] mg ml^{-1}; inhibition at 10 mg ml^{-1} 108 ± 10%), whereas D was without effect over the range of concentrations examined (Figure 2).

To investigate the role of sulphation within the heparin molecule in greater detail, heparan sulphate (HS) and chondroitin sulphates (CSA and CSB) were examined for antiproliferative activity and compared with Sigma heparin. FBS-dependent proliferation was prevented by HS in a concentration-dependent fashion (inhibition at 10 mg ml^{-1} 97 ± 9%, $P<0.001$, Figure 3a), which was not significantly different (IC_{50}: HS 5.7 [3.1–8.4] mg ml^{-1}, Sigma heparin 2.2 [1.1–3.2] mg ml^{-1}, $P>0.05$) from that observed with Sigma heparin (inhibition at 10 mg ml^{-1} 92 ± 8.0%, $P<0.001$). Both CSA and CSB also both inhibited FBS-stimulated proliferation (inhibition at 20 mg ml^{-1}: CSB 78 ± 13%, CSA 47 ± 9%, heparin 118 ± 3%) but were markedly less potent ($P<0.001$) compared with Sigma heparin (IC_{50}: CSB 11.9 [7.5–16.3] mg ml^{-1}, CSA >20 mg ml^{-1}, Sigma heparin 0.8 [0.3–1.2] mg ml^{-1}) such that the rank order of potency was Sigma heparin > CSB = CSA (Figure 3b).

Hyaluronic acid (HA), like heparin, is composed of glucosamine and glucuronic acid residues, but unlike heparin

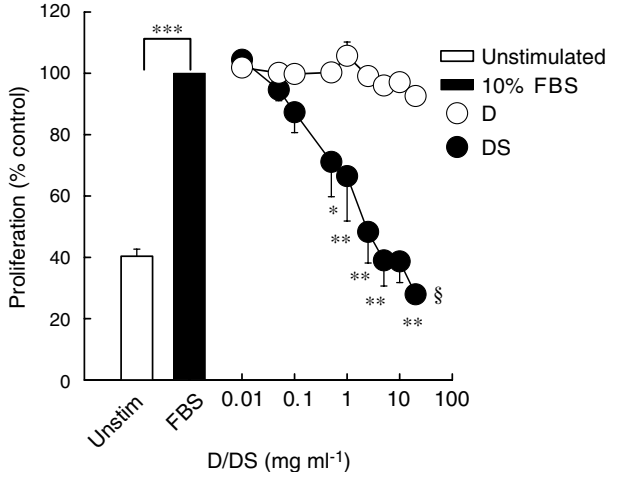

Figure 2 Attenuation of FBS-stimulated proliferation by sulphated but not nonsulphated nonheparin-like dextran (D) polymers. Proliferation at day 7 was determined by MTT reduction in the presence or absence of dextran sulphate (DS; 10 kDa) or D (9.5 kDa). Data are mean ± s.e.m. of five independent experiments using cells cultured from individual donors. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.01$ compared with FBS alone; $^{\S}P<0.001$ compared with D by two-way ANOVA.

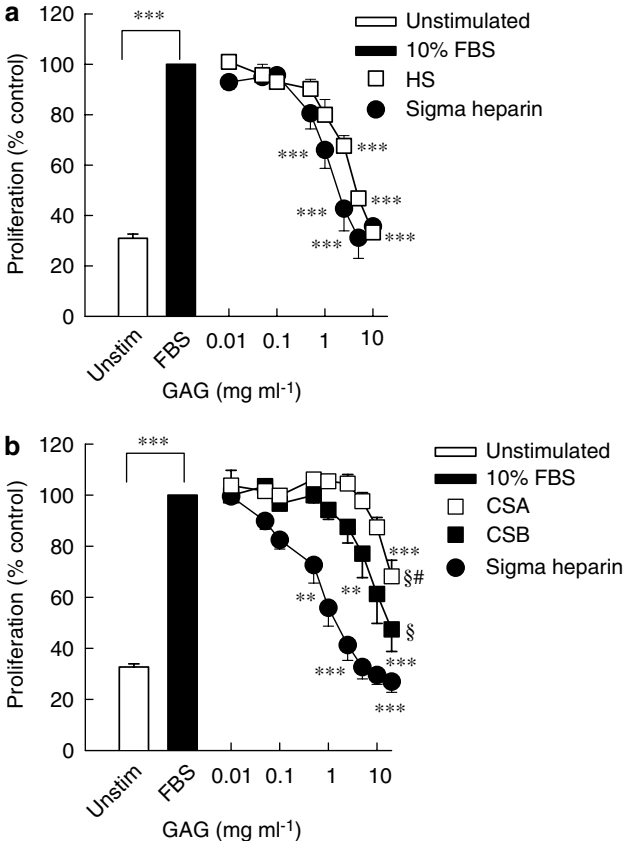

Figure 3 Attenuation of FBS-stimulated proliferation by (a) heparan sulphate (HS) and (b) by chondroitin sulphates (CS). Proliferation at day 7 was determined by MTT reduction in the presence or absence of HS, CSA or CSB and compared with attenuation by Sigma heparin. Data are mean ± s.e.m. of 2–5 independent experiments using cells cultured from 3–5 individual donors. $^{**}P<0.01$, $^{***}P<0.001$ compared with FBS alone, $^{\S}P<0.001$ compared with Sigma heparin, $^{\#}P<0.05$ compared with CSB by two-way ANOVA.

does not contain iduronic acid residues and is not sulphated (Varma & Varma, 1983). Treatment with HA caused a concentration-dependent inhibition of FBS-induced proliferation ($P<0.001$), which was markedly less than with Sigma heparin (IC_{50}: HA 22.0 [16.6–27.3] mg ml^{-1}, Sigma heparin 0.8 [0.3–1.2] mg ml^{-1}, $P<0.001$) (inhibition at 2.5 mg ml^{-1}: HA, 17 ± 3%, heparin, 93 ± 2%) (Figure 4).

Sulphation pattern and anticoagulant activity

We next examined if anticoagulant activity or positional sulphation were factors in the attenuated proliferation by heparin. We found FBS-stimulated proliferation was inhibited by *O*-de (non-anticoagulant), by *N*-de (anticoagulant), by *N*-desulphated 90% re-*N*-acetylated heparin (*N*-ac) and by Sigma heparin (Figure 5). Inhibition at 20 mg ml^{-1} was 93 ± 6, 89 ± 4, 103 ± 5 and 113 ± 8%, respectively). The antiproliferative effect of all three heparin derivatives was significantly less potent compared with Sigma heparin (IC_{50}: *O*-de 2.1 [1.3–2.8] mg ml^{-1}, *N*-de 14.6 [10.0–19.2] mg ml^{-1}, *N*-ac 2.9 [1.0–4.7] mg ml^{-1}, Sigma heparin 0.8 [0.3–1.2] mg ml^{-1}, $P<0.001$) with the rank order of potency against FBS being Sigma heparin > *O*-de ⩾ *N*-ac > *N*-de.

Role of molecular size

To establish if the antiproliferative effect of heparin was retained in smaller heparin fragments, three low molecular-weight (LMW) heparins (3, 5 and 6 kDa) were compared with the antiproliferative effects of Sigma heparin (17–19 kDa). FBS-dependent proliferation was prevented by each of the LMW heparins ($P<0.001$) (Figure 6). Similar extents of attenuation of FBS-stimulated proliferation were found with 3 and 5 kDa LMW heparin compared with Sigma heparin ($P>0.05$), although the 6 kDa fraction resulted in some cell loss above 5 mg ml^{-1}. Likewise, no differences in potency were

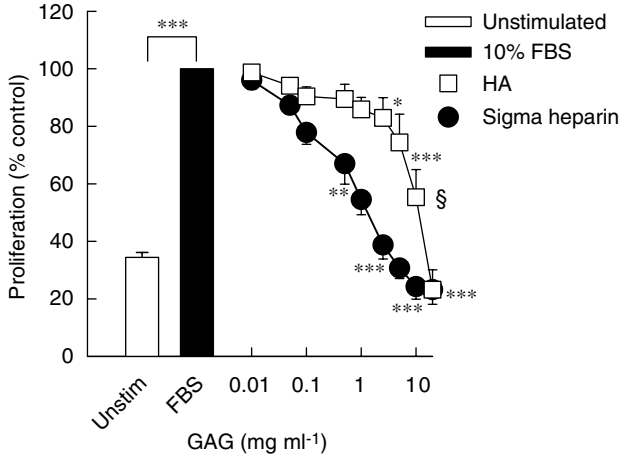

Figure 4 Effect of treatment with hyaluronic acid (HA) on FBS-dependent proliferation. Proliferation at day 7 was determined by MTT reduction in the presence or absence of HA and compared with native heparin (Sigma). Data are mean ± s.e.m. of five independent experiments using cells cultured from individual donors. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with FBS in the absence of GAG, $^{\S}P<0.01$ compared with Sigma heparin by two-way ANOVA.

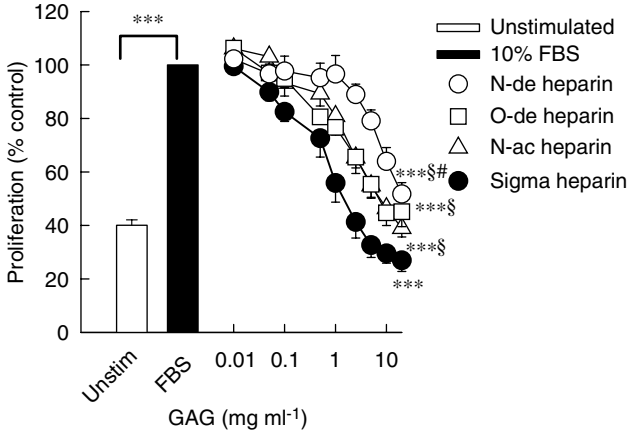

Figure 5 Attenuation of FBS-stimulated proliferation by *O*-desulphated heparin (*O*-de, non-anticoagulant), *N*-desulphated re-*N*-acetylated (*N*-de, anticoagulant) and *N*-acetylated heparin (*N*-ac, anticoagulant). Proliferation was determined by MTT reduction at day 7 in the presence or absence of *O*-de, *N*-de or *N*-ac and compared with attenuation by native heparin (Sigma). Data are mean ± s.e.m. of five independent experiments using cells cultured from individual donors. $^{**}P<0.01$, $^{***}P<0.001$ compared with FBS alone, $^{\S}P<0.001$ compared with Sigma heparin, $^{\#}P<0.05$ compared with *O*-de by two-way ANOVA.

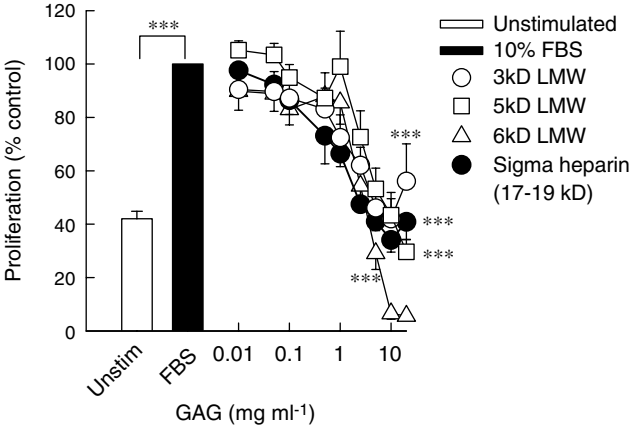

Figure 6 Attenuation of FBS-stimulated proliferation by LMW heparins. Proliferation was determined by MTT reduction at day 7 in the presence or absence of LMW heparin fractions of 3, 5 or 6 kDa and compared with attenuation by Sigma heparin (17–19 kDa). Data are mean ± s.e.m. of six independent experiments using cells cultured from individual donors. $^{***}P<0.001$ compared with FBS in the absence of LMW fractions by two-way ANOVA (see text for details of other statistical comparisons).

found (IC_{50}: 3 kDa 1.6 [0.3–3.0] mg ml^{-1}, 5 kDa 2.2 [0.8–3.6] mg ml^{-1}, 6 kDa 1.7 [0.1–3.5] mg ml^{-1}, Sigma heparin 1.1 [0.5–1.7] mg ml^{-1}, $P>0.05$).

Role of anionic charge

The contribution of anionic charge to the antiproliferative effects of heparin was explored using highly polyanionic peptides/polysaccharides with and without *N*- and *O*-sulphation. Poly-L-glutamic acid (PGA) was examined in two forms to control for differences in molecular size: PGA-1 (3–15 kDa) and PGA-2 (50–100 kDa), with PGA-1 being comparable in size to unfractionated Sigma heparin (17–19 kDa).

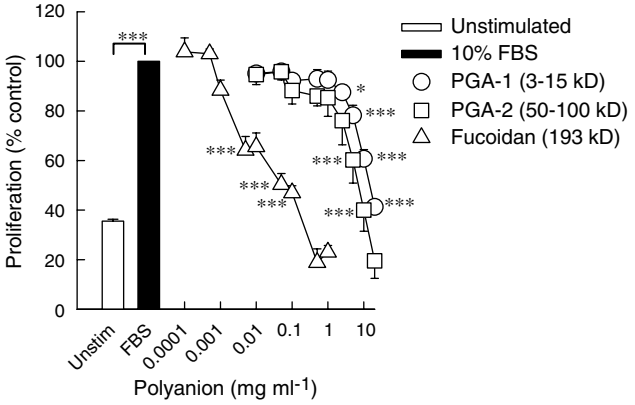

Figure 7 Attenuation of FBS-stimulated proliferation by polyanions. Proliferation was determined by MTT reduction at day 7 in the presence or absence of sulphated (fucoidan) or nonsulphated (PGA-1,-2) polyanions. Data are mean ± s.e.m. of five independent experiments using cells cultured from individual donors. $^{*}P<0.01$, $^{***}P<0.001$ compared with FBS in the absence of polyanions by two-way ANOVA.

All three polyanions inhibited FBS-induced cell proliferation ($P<0.001$). Maximal inhibition with fucoidan occurred at 1 mg ml^{-1} and was 124 ± 4%. Inhibition at 10 mg ml^{-1} PGA-1 and PGA-2 was 94 ± 6 and 62 ± 13%, respectively. Corresponding IC_{50} values were: fucoidan 0.03 [0.01–0.1] mg ml^{-1}, PGA-1 24.0 [10.8–37.0] mg ml^{-1} and PGA-2 18.4 [9.2–28.0] mg ml^{-1} (Figure 7). Overall, there were no differences in potency between the nonsulphated polysaccharides PGA-1 and PGA-2 (Figure 7) or Sigma heparin ($P>0.05$, not shown), supporting our findings with LMW heparins that molecular size (at least to 3 kDa) was not a determinant for heparin's antiproliferative activity in ASM. However, they were markedly less potent (approximately 500-fold) than the sulphated polysaccharide, fucoidan ($P<0.001$).

Discussion

Heparin's structural heterogeneity arises from polysaccharide side chains containing alternating residues of an amino sugar (glucosamine) and uronic acid (either glucuronic or iduronic acid), variably distributed with regions of sulphated domains (*O*-sulphate and *N*-sulphate) and less sulphated domains (*N*-acetylated). Additionally, heparin comprises irregular chain lengths and is highly negatively charged due to the sulphated and carboxylated groups present on the polysaccharide side chains (Tyrrell *et al*., 1999). These variables in chemical structure are thought to account for its diverse biological properties (Tyrrell *et al*., 1999; Lever & Page, 2002) and may explain why commercial preparations of heparin often differ widely in their antiproliferative activity against VSM (Jaques *et al*., 1967; Castellot *et al*., 1986; Grainger *et al*., 1993). Although, it is well-established that heparin is likewise antiproliferative for ASM cells from other species including man (Johnson *et al*., 1995; Kilfeather *et al*., 1995; Halayko *et al*., 1997), the key structural moieties on the heparin molecule required for this activity are unknown. Here, we demonstrate some of the structural properties of heparin necessary for inhibition of ASM cell proliferation. These include sulphation, particularly *N*-sulphation and to a much

lesser extent, 2-*O*- and 3-*O*-sulphation. We also show that the antiproliferative activity was independent of its anticoagulant activity, anionic charge and molecular size exceeding 3 kDa.

The importance of sulphation for the antiproliferative effect of heparin was initially identified with the nonheparin-like polymer DS, which unlike nonsulphated D inhibited FBS-dependent human ASM proliferation. Similar observations are reported elsewhere with bovine ASM (Kilfeather *et al*., 1995), rat VSM and rat fibroblasts (San Antonio *et al*., 1992). Although structurally unrelated to heparin, both heparin and DS are comparable in their high level of sulphation, with DS having 3.3 sulphate residues per disaccharide unit (Windholz *et al*., 1976) and heparin having 2.4 sulphate residues per disaccharide unit (Rabenstein, 2002). The compounding effect of DS polymers having varying molecular weights and hence differing overall sulphate content was not addressed directly in this study, but Kilfeather *et al*. (1995) demonstrated that DS polymers with molecular weights between 5 and 500 kDa were equally effective in preventing FBS-dependent proliferation of bovine ASM cells, suggesting that the absolute presence of sulphation is required. This is also supported by the finding that nonsulphated D lacked antiproliferative activity. However, the finding that the non-sulphated molecule HA also possessed antiproliferative activity was unexpected and contrasts with observations in VSM by Garg *et al*. (2000). However, the antiproliferative effect of HA occurred with higher concentrations (>5 mg ml^{-1}) and was markedly less effective compared with Sigma heparin. On balance, therefore, we suggest that sulphation is important but is not the sole property of the heparin molecule required for its antiproliferative activity.

We further examined the contribution of sulphation in experiments employing polysaccharides that exhibit less sulphation than heparin. Although structurally similar to heparin with side chains composed of the same monosaccharide building blocks, resulting in alternating glucosamine and hexuronic acid residues (either iduronic or glucuronic acid) of which most are glucuronic acid in HS chains, HS has a lower proportion of *N*- and *O*-sulphation and *N*-acetyl groups. The ratio of *N*-sulphated to *N*-acetylated glucosamine residues is 1 : 1 for HS but is 4 : 1 in heparin (Rabenstein, 2002). However, despite the reduction in overall sulphation within HS its antiproliferative activity was comparable with heparin itself. In similar studies with VSM heparin was found to be more effective (Garg *et al*., 2000), but in bovine ASM heparin was two-fold *less* antiproliferative than HS (Kilfeather *et al*., 1995). Although we did not examine over-sulphation of these molecules, Garg *et al*. (2000) found that over-sulphation of HS augmented its antiproliferative activity by 30%, whereas over-sulphation of heparin had no additional effect, which may indicate that heparin was already optimally sulphated. Consistent with the importance of sulphation, we found that the chondroitins, CSA and CSB, which are less sulphated than HS or heparin were poorly antiproliferative compared with heparin (average number of sulphate groups per repeating disaccharide unit for heparin is 2.5 compared with 1.0 for the chondroitins and 1.5 for HS (Varma & Varma, 1983). Moreover, the overall order of potency against FBS-stimulated proliferation in human ASM cells was heparin = HS $\gg$ CSB $\geqslant$ CSA $\geqslant$ HA. This is similar to reports elsewhere for inhibition of cell adhesion (San Antonio *et al*., 1992) and loosely correlates with the empirical sulphate content for each of the GAGs examined where heparin > HS > CSB = CSA > HA (Rabenstein, 2002).

Both heparin and HS are variably *N*-sulphated, *O*-sulphated and *N*-acetylated, whereas CSA and CSB though *N*-acetylated and *O*-sulphated are not *N*-sulphated (Varma & Varma, 1983). The finding that antiproliferative activity was lower for both chondroitins than for heparin or HS suggests that *N*-sulphation also contributes to the overall antiproliferative potency of heparin. That HS is less *N*- and *O*-sulphated compared with heparin but equally antiproliferative implies the level of *N*-sulphation present in HS side chains was sufficient and not below a threshold required for this activity. Consistent with this possibility, completely *N*-de, 20% re-*N*-acetylated heparin (*N*-de) retained antiproliferative activity but was less effective than heparin, indicating that removal of *N*-sulphation reduces but does not abrogate antiproliferative activity. In VSM studies, loss of *N*-sulphation results in complete loss of antiproliferative activity (Wright *et al*., 1989), which upon *N*-acetylation partially restores antiproliferative activity, perhaps due to neutralisation upon *N*-acetylation of the otherwise positively charged heparin molecule (Wright *et al*., 1989). The finding that 90% re-*N*-acetylation (*N*-ac) resulted in improved antiproliferative activity compared with 20% re-*N*-acetylation (*N*-de), but not to the same extent with heparin, is consistent with similar observations in VSM (Castellot *et al*., 1984; Tiozzo *et al*., 1993) and suggests that *N*-sulphation is required for heparin's antiproliferative activity. Although we did not examine a minimum level of *N*-sulphation required for antiproliferative activity, a recent study suggests heparin must retain 24% *N*-sulphate groups to retain comparable antiproliferative activity with native heparin (Longas *et al*., 2003). The antiproliferative activity of *O*-de (non-anticoagulant) and *N*-ac were similar but less effective than heparin suggesting that the pentasaccharide sequence required for anticoagulant activity does not play a role in attenuation of ASM proliferation, a finding supported in VSM *in vivo* (Guyton *et al*, 1980) and *in vitro* (Hoover *et al*., 1980). Collectively, since *O*-desulphated heparin retains *N*-sulphation and 6-*O* sulphation but not 2-*O* or 3-*O* sulphation (Fryer *et al*., 1997) our findings suggest attenuation of ASM cell proliferation by heparin involves *N*-sulphation and *N*-acetylation but not 2-*O* or 3-*O* sulphation.

We also investigated whether the overall size of the heparin polymer was a factor in the attenuation. All three LMW heparins examined prevented FBS-dependent proliferation, and were comparable in activity with Sigma heparin, suggesting that the antiproliferative activity of heparin resides in chains of 3–6 kDa and as little as 3 kDa. This agrees with previous findings in bovine ASM (Kilfeather *et al*., 1995) as well as a report by Tiozzo *et al*. (1991) who demonstrated a graduated loss of antiproliferative activity with decreasing molecular weight from 4.5–1 kDa LMW heparin fraction.

The importance of net charge within the heparin molecule to antiproliferative activity has not been widely investigated. The highly anionic, non-sulphated linear polysaccharides, PGA-1 and -2 though antiproliferative appeared less antiproliferative than heparin. That PGA-1 and -2 were equally effective, further suggests that increasing the overall negative charge by increasing the size of the molecule does not influence cell proliferation. However, this does not rule out a requirement for a minimum anionic charge. Previous studies report variable findings. For example, Joseph *et al*. (1997) showed that the

antiproliferative activity of a lower charge density (less negative) heparin fraction in VSM cells was similar to the parent heparin, but high charge density fractions were ineffective. This contrasts with the findings of Wright *et al.* (1989) who showed that increased negative charge was concomitant with antiproliferative activity. Our finding that the highly anionic non-sulphated linear polysaccharides, PGA-1 and -2 were equally effective but less effective than highly anionic and sulphated polysaccharide fucoidan suggest that both anionic charge and sulphation contribute to antiproliferative activity.

The underlying mechanism of heparin's antiproliferative action in ASM is unknown, although it has been shown to prolong cell cycle kinetics thereby increasing the interval between mitosis (Halayko *et al*., 1997). Much more is known in VSM where the binding of heparin to cell surface high-affinity sites results in selective modulation of mitogenic signalling pathways and altered expression of a specific subset of growth regulatory genes including immediate-early response genes, transcription factors, cell cycle machinery proteins and extracellular matrix components. Emerging data suggest that its antiproliferative effect requires upregulation of a novel growth arrest-specific gene *Cop-1* (also known as *CCN5, HICP, Wisp2* or *CTGF-L*), which is a member of the CCN (cysteine-rich 16/connective tissue growth factor/nephroblastoma overexpressed) family of secreted, cysteine-rich modular proteins implicated in growth regulation, migration and extracellular matrix production. Upregulation of this gene by heparin and secretion of the growth-suppressing protein COP-1/CCN5 is reported to be restricted to heparin-sensitive VSM cells and absent in heparin-resistant VSM or heparin-treated endothelial cells (Delmolino *et al*., 2001). Recent studies with siRNA against CCN5 have confirmed that it is required for a major component of the antiproliferative effect of heparin (Lake & Castellot, 2003). Other antiproliferative mechanisms are also under investigation. For example, extracellularly applied heparin suppresses elevated intercellular calcium levels *via* inhibition of store-operated calcium entry from the outside and its interference with calcium handling pathways may account for the diversity of its biological effects (Nemeth & Kurucz, 2005). Alternatively, suppression of VSM growth by heparin could involve sequestration of polypeptide growth factors present in FBS or its binding to integrins or other matrix elements required for proliferation. However, removal of heparin-binding proteins from FBS does not abrogate either FBS-dependent proliferation or the attenuating effects of heparin (Hoover *et al*., 1980). Which, if any, of these proposed mechanisms accounts for its antiproliferative effect in human ASM remains to be investigated.

In conclusion, our results demonstrate that specific structural properties of the heparin molecule are involved in its antiproliferative activity against FBS-dependent human ASM cells. These include sulphation, particularly *N*-sulphation and to a much lesser extent, 2-*O*- and 3-*O*-sulphation, which was independent of anticoagulant activity and molecular weight up to 3 kDa. Understanding the structural properties of the heparin molecule that underlie its antiproliferative activity offers opportunities for the design of 'tailor-made' sequences based on the heparin template to isolate specific therapeutic activities (Tyrrell *et al*., 1999; Lever & Page, 2002) including targeting the accumulation of ASM found in asthma.

We thank the thoracic surgeons, operating theatre staff and pathologists of Guy's and St Thomas' Hospitals, London, for supply of human lung tissue for culture of human airway smooth muscle cells.

References

BRIGHTLING, C.E., BRADDING, P., SYMON, F.A., HOLGATE, S.T., WARDLAW, A.J. & PAVORD, I.D. (2002). Mast-cell infiltration of airway smooth muscle in asthma. *N. Engl. J. Med.,* **346,** 1699–1705.

BURGESS, J.K., GE, Q., BLACK, J.L., KING, G. & JOHNSON, P.R.A. (2001). Heparin and PGE_2 inhibit proliferation of asthmatic and non-asthmatic airway smooth muscle cells in culture. *Am. J. Respir. Crit. Care Med.,* **158,** A503 (Abstract).

CASTELLOT, J.J., BEELER, D.L., ROSENBERG, R.D. & KARNOVSKY, M.J. (1984). Structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cells. *J. Cell Physiol.,* **120,** 315–320.

CASTELLOT, J.J., CHOAY, J., LORMEAU, J.C., PETITOU, M., SACHE, E. & KARNOVSKY, M.J. (1986). Structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cells. II. Evidence for a pentasaccharide sequence that contains a 3-*O*-sulfate group. *J. Cell Biol.,* **102,** 1979–1984.

DELMOLINO, L.M., STEARNS, N.A. & CASTELLOT, J.J. (2001). COP-1, a member of the CCN family, is a heparin-induced growth arrest specific gene in vascular smooth muscle cells. *J. Cell Physiol.,* **188,** 45–55.

EBINA, M., TAKAHASHI, T., CHIBA, T. & MOTOMIYA, M. (1993). Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. *Am. Rev. Respir. Dis.,* **148,** 720–726.

FRYER, A., HUANG, Y.C., RAO, G., JACOBY, D., MANCILLA, E., WHORTON, R., PIANTADOSI, C.A., KENNEDY, T. & HOIDAL, J. (1997). Selective *O*-desulfation produces nonanticoagulant heparin that retains pharmacological activity in the lung. *J. Pharmacol. Exp. Ther.,* **282,** 208–219.

GARG, H.G., CINDHUCHAO, N., QUINN, D.A., HALES, C.A., THANAWIROON, C., CAPILA, I. & LINHARDT, R.J. (2002). Heparin oligosaccharide sequence and size essential for inhibition of pulmonary artery smooth muscle cell proliferation. *Carbohydr. Res.,* **337,** 2359–2364.

GARG, H.G., THOMPSON, B.T. & HALES, C.A. (2000). Structural determinants of antiproliferative activity of heparin on pulmonary artery smooth muscle cells. *Am. J. Physiol. Lung Cell Mol. Physiol.,* **279,** L779–L789.

GRAINGER, D.J., WITCHELL, C.M., WATSON, J.V., METCALFE, J.C. & WEISSBERG, P.L. (1993). Heparin decreases the rate of proliferation of rat vascular smooth muscle cells by releasing transforming growth factor beta-like activity from serum. *Cardiovasc. Res.,* **27,** 2238–2247.

GREEN, W.F., KONNARIS, K. & WOOLCOCK, A.J. (1993). Effect of salbutamol, fenoterol, and sodium cromoglycate on the release of heparin from sensitized human lung fragments challenged with *Dermatophagoides pteronyssinus* allergen. *Am. J. Respir. Cell Mol. Biol.,* **8,** 518–521.

GUYTON, J.R., ROSENBERG, R.D., CLOWES, A.W. & KARNOVSKY, M.J. (1980). Inhibition of rat arterial smooth muscle cell proliferation by heparin. *In vivo* studies with anticoagulant and nonanticoagulant heparin. *Circ. Res.,* **46,** 625–634.

HALAYKO, A.J., RECTOR, E. & STEPHENS, N.L. (1997). Characterisation of molecular determinants of smooth muscle cell heterogeneity. *Can. J. Physiol. Pharmacol.,* **75,** 917–929.

HEARD, B.E. & HOSSAIN, S. (1973). Hyperplasia of bronchial muscle in asthma. *J. Pathol.,* **110,** 319–331.

HIRST, S.J., BARNES, P.J. & TWORT, C.H. (1992). Quantifying proliferation of cultured human and rabbit airway smooth muscle cells in response to serum and platelet-derived growth factor. *Am. J. Respir. Cell Mol. Biol.,* **7,** 574–581.

HIRST, S.J., TWORT, C.H. & LEE, T.L. (2000). Differential effects of extracellular matrix proteins on human airway smooth muscle cell proliferation and phenotype. *Am. J. Respir. Cell Mol. Biol.,* **23,** 335–344.

HOOVER, R.L., ROSENBERG, R., HAERING, W. & KARNOVSKY, M.J. (1980). Inhibition of rat arterial smooth muscle cell proliferation by heparin II. *In vitro* studies. *Circ. Res.,* **47,** 578–583.

JAMES, A.L., PARE, P.D. & HOGG, J.C. (1989). The mechanics of airway narrowing in asthma. *Am. Rev. Respir. Dis.,* **139,** 242–246.

JAQUES, L.B., KAVANAGH, L. & LAVALLEE, A. (1967). A comparison of biological activities and chemical analyses for various heparin preparations. *Arzneimittel-Forschung,* **17,** 774–778.

JOHNSON, P.R., ARMOUR, C.L., CAREY, D. & BLACK, J.L. (1995). Heparin and PGE_2 inhibit DNA synthesis in human airway smooth muscle cells in culture. *Am. J. Physiol. Lung Cell Mol. Physiol.,* **269,** L514–L519.

JOSEPH, P.A., GARG, H.G., THOMPSON, B.T., LIU, X. & HALES, C.A. (1997). Influence of molecular weight, protein core and charge of native heparin fractions on pulmonary artery smooth muscle cell proliferation. *Biochem. Biophys. Res. Commun.,* **241,** 18–23.

KARNOVSKY, M.J. & EDELMAN, E.R. (1994). Heparin/heparan sulphate regulation of vascular smooth muscle behaviour. In: *Airways and Vascular Remodelling in Asthma and Cardiovascular Disease: Implications for Therapeutic Intervention,* ed. Page, C.P. & Black, J.L. pp. 45–70. New York: Academic Press.

KILFEATHER, S.A., TAGOE, S., PEREZ, A.C., OKONA-MENSA, K., MATIN, R. & PAGE, C.P. (1995). Inhibition of serum-induced proliferation of bovine tracheal smooth muscle cells in culture by heparin and related glycosaminoglycans. *Br. J. Pharmacol.,* **114,** 1442–1446.

LAKE, A.C. & CASTELLOT, J.J. (2003). CCN5 modulates the antiproliferative effect of heparin and regulates cell motility in vascular smooth muscle. *Cell Commun. Signal.,* **1,** 5–18.

LAMBERT, R.K., WIGGS, B.R., KUWANO, K., HOGG, J.C. & PARE, P.D. (1993). Functional significance of increased airway smooth muscle in asthma and COPD. *J. Appl. Physiol.,* **74,** 2771–2781.

LEVER, R. & PAGE, C.P. (2002). Novel drug development opportunities for heparin. *Nat. Rev. Drug Discov.,* **1,** 140–148.

LONGAS, M.O., GARG, H.G., TRINKLE-PEREIRA, J.M. & HALES, C.A. (2003). Heparin antiproliferative activity on bovine pulmonary artery smooth muscle cells requires both *N*-acetylation and *N*-sulfonation. *Carbohydr. Res.,* **338,** 251–256.

NEMETH, K. & KURUCZ, I. (2005). Suppression of Ca^{2+} influx by unfractionated heparin in non-excitable intact cells *via* multiple mechanisms. *Biochem. Pharmacol.,* **69,** 929–940.

PAGE, C.P. (1991). One explanation of the asthma paradox: inhibition of natural antiinflammatory mechanism by beta 2-agonists. *Lancet,* **337,** 717–720.

PAGE, S., AMMIT, A.J., BLACK, J.L. & ARMOUR, C.L. (2001). Human mast cell and airway smooth muscle cell interactions: implications for asthma. *Am. J. Physiol. Lung Cell Mol. Physiol.,* **281,** L1313–L1323.

RABENSTEIN, D.L. (2002). Heparin and heparan sulfate: structure and function. *Nat. Prod. Rep.,* **19,** 312–331.

ROBINSON, D.S. (2004). The role of the mast cell in asthma: induction of airway hyperresponsiveness by interaction with smooth muscle? *J. Allergy Clin. Immunol.,* **114,** 58–65.

SAN ANTONIO, J.D., LANDER, A.D., WRIGHT, T.C. & KARNOVSKY, M.J. (1992). Heparin inhibits the attachment and growth of Balb/c-3T3 fibroblasts on collagen substrata. *J. Cell Physiol.,* **150,** 8–16.

SEEDS, E.A., HORNE, A.P., TYRRELL, D.J. & PAGE, C.P. (1995). The effect of inhaled heparin and related glycosaminoglycans on allergen-induced eosinophil infiltration in guinea-pigs. *Pulm. Pharmacol.,* **8,** 97–105.

SHIELS, I.A., TAYLOR, S.M. & FAIRLIE, D.P. (2000). Cell phenotype as a target of drug therapy in chronic inflammatory diseases. *Med. Hypotheses,* **54,** 193–197.

TIOZZO, R., CINGI, M.R., REGGIANI, D., ANDREOLI, T., CALANDRA, S., MILANI, M.R., PIANI, S., MARCHI, E. & BARBANTI, M. (1993). Effect of the desulfation of heparin on its anticoagulant and antiproliferative activity. *Thromb. Res.,* **70,** 99–106.

TIOZZO, R., REGGIANI, D., CINGI, M.R., BIANCHINI, P., OSIMA, B. & CALANDRA, S. (1991). Effect of heparin derived fractions on the proliferation and protein synthesis of cells in culture. *Thromb. Res.,* **62,** 177–188.

TYRRELL, D.J., HORNE, A.P., HOLME, K.R., PREUSS, J.M. & PAGE, C.P. (1999). Heparin in inflammation: potential therapeutic applications beyond anticoagulation. *Adv. Pharm.,* **46,** 151–208.

VARMA, R. & VARMA, R.S. (1983). Chemistry and metabolism of glycosaminoglycans and proteoglycans. In: *Mucopolysaccharides. Glycosaminoglycans of Body Fluids in Health and Disease,* pp. 3–48. Berlin: Walter de Gruyter.

WINDHOLZ, M., BUDAVERI, S., STROUNTSOS, L. & FERTIG, M. (1976). The Merck Index. 9th edn. pp. 387, New Jersey: Merck Rahway.

WRIGHT, T.C.J., CASTELLOT, J.J.J., PETITOU, M., LORMEAU, J.C., CHOAY, J. & KARNOVSKY, M.J. (1989). Structural determinants of heparin's growth inhibitory activity. Interdependence of oligosaccharide size and charge. *J. Biol. Chem.,* **264,** 1534–1542.

(Received April 12, 2005
Accepted June 9, 2005)
Published online 18 July 2005)