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RESEARCH PAPER

Heparin and structurally related polymers attenuate eotaxin-1 (CCL11) release from human airway smooth muscle

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Background and purpose: The glycosaminoglycan heparin has anti-inflammatory activity and is exclusively found in mast cells, which are localized within airway smooth muscle (ASM) bundles of asthmatic airways. Interleukin (IL)-13 induces the production of multiple inflammatory mediators from ASM including the eosinophil chemoattractant chemokine, eotaxin-1. Heparin and related glycosaminoglycan polymers having structurally heterogeneous polysaccharide side chains that varied in molecular weight, sulphation and anionic charge were used to identify features of the heparin molecule linked to anti-inflammatory activity.
Experimental approach: Cultured human ASM cells were stimulated with interleukin (IL)-13 in the absence or presence of heparin and related polymers. Eotaxin-1 was quantified using chemokine antibody arrays and ELISA.
Key results: Unfractionated heparin attenuated IL-13-dependent eotaxin-1 production and this effect was reproduced with low molecular weight heparins (3 and 6 kDa), demonstrating a minimum activity fragment of at least 3 kDa. N-desulphated, 20% re-N-acetylated heparin (anticoagulant) was ineffective against IL-13-dependent eotaxin-1 production compared with 90% re-N-acetylated (anticoagulant) or O-desulphated (non-anticoagulant) heparin, suggesting a requirement for N-sulphation independent of anticoagulant activity. Other sulphated molecules with variable anionic charge and molecular weight exceeding 3 kDa (dextran sulphate, fucoidan, chondroitin sulphate B) inhibited IL-13-stimulated eotaxin-1 release to varying degrees. However, non-sulphated dextran had no effect.
Conclusions: Inhibition of IL-13-dependent eotaxin-1 release by heparin involved but did not depend upon sulphation, though loss of N-sulphation reduced the attenuating activity, which could be restored by N-acetylation. This anti-inflammatory effect was also partially dependent on anionic charge, but independent of molecular size above 3 kDa and the anticoagulant action of heparin.
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Keywords: airway smooth muscle; asthma; eotaxin-1; glycosaminoglycan; heparin; inflammation

Abbreviations: ASM, airway smooth muscle; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; CSA, chondroitin sulphate A; CSB, chondroitin sulphate B; D, dextran; DS, dextran sulphate; GAG, glycosaminoglycan; IL, interleukin; LMW heparin, low molecular weight heparin; O-de heparin, O-desulphated; N-de 20%ac heparin, N-desulphated, 20% re-N-acetylated heparin; N-de 90%ac heparin, N-desulphated, 90% re-acetylated heparin; PGA, poly-L-glutamic acid

Introduction

Airway smooth muscle (ASM) myositis involves the production of multiple pro-inflammatory cytokines and chemokines including eotaxin-1 (CC chemokine ligand 11; CCL11), polypeptide growth factors and extracellular matrix components, as well as expression of cell surface receptor molecules involved in lymphocyte adhesion. This supports the possibility that ASM participates directly, through recruitment and activation of eosinophils and other infiltrating inflammatory cells, to perpetuate asthmatic airway inflammation and airway hyperresponsiveness (reviewed by Panettieri, 2002; Hirst, 2003).

The chemokine eotaxin-1 is a potent and specific eosinophil chemoattractant (Jose *et al*., 1994) whose expression is

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markedly increased by human ASM cells obtained from subjects with asthma both constitutively and upon cytokine stimulation (Ghaffar *et al*., 1999; Chan *et al*., 2006). A key stimulus eliciting eotaxin-1 release from ASM *in vitro* is the Th2 (T helper-2) cytokine, interleukin (IL)-13 (Hirst *et al*., 2002; Moore *et al*., 2002). Excessive IL-13 production in atopic and non-atopic asthma is well documented (Huang *et al*., 1995; Kotsimbos *et al*., 1996). Transgene pulmonary overexpression of IL-13 in mice is associated with several key pathological features of airways inflammation and remodelling also observed in patients with chronic severe asthma, including lymphocyte and eosinophil accumulation, mucus cell metaplasia, subepithelial fibrosis and airway hyperresponsiveness (Zhu *et al*., 1999). In addition, IL-13 is released by primed sensitized human mast cells upon exposure to eotaxin-1 (Grunig *et al*., 1998).

Airway wall mast cells are increased in number in patients with atopy and asthma and are specifically localized within the ASM bundles in asthma (Ammit *et al*., 1997; Brightling *et al*., 2002). Emerging evidence suggests that a complex bidirectional interplay exists between ASM and mast cells involving the production of lipid mediators, chemokines, cytokines and enzymes that may lead to airway hyperresponsiveness (Page *et al*., 2001; Robinson, 2004; Kaur *et al*., 2006). Mast cells are also the only endogenous source of heparin in mammals. Heparin has been shown to inhibit growth responses of ASM cultured from several species including bovine, canine, guinea-pig and man (Johnson *et al*., 1995; Kilfeather *et al*., 1995; Kanabar *et al*., 2005) and has been found to exert anti-inflammatory effects in a wide range of *in vitro* assays, animal models and in human disease (Seeds *et al*., 1995; Rose and Page, 2004). The release of heparin upon mast cell degranulation (Green *et al*., 1993) may play a protective role by limiting inflammation and airway remodelling (Rose and Page, 2004).

Heparin and related glycosaminoglycans (GAGs) comprise a class of carbohydrates with alternating repeating disaccharide units with an amino sugar (either glucosamine or galactosamine) and uronic acid (either glucuronic or iduronic acid) residue (Tyrrell *et al*., 1999). These disaccharide units are of irregular chain length and variably N-sulphated, O-sulphated (regions of high sulphation) and N-acetylated (regions of low sulphation). Furthermore, uronic acid residues are also carboxylated, which in combination with variable sulphate groups provide GAGs with a high net negative charge. Collectively, these physical properties of heparin result in a molecule of varying sulphation, charge and size. Heterogeneity such as this is likely to underlie heparin's broad range of biological effects (Lever and Page, 2002). Previously, we and others have shown that heparin inhibits human ASM proliferation (Johnson *et al*., 1995; Kilfeather *et al*., 1995) and have identified some of the structural requirements for this effect including N-sulphation (Kanabar *et al*., 2005). However, the critical physicochemical properties of the heparin molecule underlying its anti-inflammatory effect in the lung have not been investigated in detail.

The purpose of this study was to explore if specific structural properties within the heparin molecule could target the secretory phenotype of ASM by suppressing production of pro-inflammatory cytokines and chemokines. On the basis of its previously characterized antiproliferative action on human ASM (Kanabar *et al*., 2005), we hypothesized that the degree and position of sulphation of heparin would be important determinants of anti-inflammatory activity on ASM and that identification of such properties would facilitate development of polysaccharide sequences that could target ASM specifically. Accordingly, we evaluated, along with heparin, the ability of heparin analogues and polysaccharides, varying in the degree and position of sulphation, molecular weight, charge density and anticoagulant activity, to regulate the release of eotaxin-1 induced by IL-13.

Methods

Isolation and culture of human airway smooth muscle cells

Human ASM cells were obtained in accordance with procedures approved by the Guy's and St Thomas' Hospitals' Research Ethics Committee from the lobar or main bronchus of 13 non-asthmatic subjects (mean age 62 ± 5 years, range 35–78 years; nine male and four female) undergoing lung resection for carcinoma of the bronchus using methods described previously (Hirst *et al*., 2000, 2002). Fluorescent immunocytochemical and flow cytometric techniques confirmed that near-confluent, serum-starved early passage human ASM cells stained (>95%) for smooth muscle-specific α-actin and calponin (Hirst *et al*., 2000). Cell passages 3–6 were used in all experiments.

Cell stimulation

Cells in multiwell plates were seeded at a density of $10\,000\,cm^{-2}$ in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Near-confluent cells were growth-arrested for 72 h in fetal bovine serum-free RPMI-1640 supplemented with $10\,\mu g\,mL^{-1}$ insulin, $5.5\,\mu g\,mL^{-1}$ transferrin and $6.7\,\mu g\,mL^{-1}$ sodium selenite and BSA (0.1%). Cells were pretreated with or without varying quantities ($mg\,mL^{-1}$) of unfractionated heparin or related polymers for 1 h and then incubated in the continued presence of these compounds in the presence or absence of stimulation for 24 h with maximally effective concentrations of IL-13 ($10\,ng\,mL^{-1}$) or IL-4 ($1\,ng\,mL^{-1}$) (Hirst *et al*., 2002). Cell-free, cell-conditioned media were collected and stored at −80 °C until detection and quantification of eotaxin-1 levels by antibody arrays or by ELISA.

Chemokine antibody array and eotaxin-1 ELISA

The RayBio Human Chemokine Antibody Array 1 (RayBiotech, Norcross, GA, USA) was employed to assay multiple chemokines in cell-conditioned media from unstimulated and IL-13-stimulated ASM cells treated as above with and without heparin ($5\,mg\,mL^{-1}$). Thirty-eight different chemokines including eotaxin-1 were evaluated according to the manufacturer's instructions. A list and map of chemokines detected can be found at http://www.raybiotech.com/map/human_chemokine_map.pdf. To exclude interference by

heparin with the detection of chemokines (including eotaxin-1), cell-conditioned media collected from IL-13-stimulated cells were 'spiked' with heparin (5 mg mL^{-1}) immediately after collection. Semiquantitative chemokine levels were visualized by enhanced chemiluminescence (Amersham-Pharmacia, Amersham, UK) and quantified (ImageQuant; Molecular Dynamics, Sunnyvale, CA, USA) on autoradiographs that depicted spots within a linear range of exposure. The variation from membrane to membrane between duplicate positive control spots ranged from 0 to 8%. Chemokine levels were quantified against internal controls within each array and compared with other samples as fold increases of values assigned to the same chemokine under unstimulated conditions on a separate array.

Levels of eotaxin-1 in conditioned medium were also determined in duplicate by specific sandwich ELISA as described previously (Chan *et al*., 2006). To exclude interference by heparin with the eotaxin-1 ELISA, 1000 pg mL^{-1} of recombinant human (rh)-eotaxin-1 was prepared in varying concentrations of unfractionated heparin (0.1, 1, 5 and 10 mg mL^{-1}) in RPMI containing 0.1% BSA. In three experiments, incubation of heparin (5 and 10 mg mL^{-1}) for 24 h with 1000 pg mL^{-1} rh-eotaxin-1 inhibited the detection of rh-eotaxin-1 by 23 ± 3 and 34 ± 2%, respectively, but had no effect on rh-eotaxin-1 recovery at concentrations below 5 mg mL^{-1} heparin (data not shown). All subsequent samples were routinely diluted at least 20-fold to ensure that the maximum concentration of heparin in the ELISA did not exceed 0.5 mg mL^{-1}. Levels of eotaxin-1 were calculated initially as ng per mL per 10^6 cells and then where appropriate expressed as a percentage of the response to IL-13 or IL-4 alone. The minimum ELISA detection limit for eotaxin-1 was 20 pg mL^{-1}.

Data and statistical analysis

Effective concentrations giving a 50% inhibition (IC_{50}) and extrapolated maximum responses were estimated for individual concentration–response curves using nonlinear least-squares regression (r^2 values were >0.9 and the curve fit described by the equation $y = y_0 + (ax/b + x)$, SigmaPlot 10; SPSS Inc., Chicago, IL, USA). IC_{50} values were converted to negative logarithmic values for all statistical analysis, although for ease of comprehension IC_{50} values (±95% confidence interval range) are given in the text. All other values are given as mean ± s.e.mean from ASM cells cultured from *n* patients. Raw data values were compared using Student's *t*-test or one-way ANOVA with a Bonferroni's *post hoc* test as appropriate (SigmaStat 3.5; SPSS Inc.). A probability (*P*) value of <0.05 was considered significant. The polymeric nature of heparin and related molecules precluded use of molar concentrations, and, thus, IC_{50} values could not be compared between molecules. Differences in concentration–response relationships were therefore compared by two-way ANOVA.

Compounds

All oligosaccharides were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) with the exception of O-desulphated heparin (O-de heparin, <10 kDa), which was a gift from Dr T Kennedy (Carolinas Medical Health Care Foundation, Charlotte, NC, USA). Oligosaccharides investigated were as follows: unfractionated heparin (17–19 kDa, 170–195 USP units mg^{-1}), low molecular weight (LMW) heparins (3 and 6 kDa), chondroitin sulphate A (CSA, 5–50 kDa), chondroitin sulphate B (CSB, 37.5 kDa), N-desulphated, N-acetyl heparin (17–19 kDa), fucoidan (193 kDa), dextran (D, 9.5 kDa), dextran sulphate (DS, 10 kDa) and poly-L-glutamic acid (PGA, 3–15 kDa and 50–100 kDa). rh-IL-4, rh-IL-13 and rh-eotaxin-1 (CCL11) were purchased from R&D Systems (Abingdon, UK). All cell culture reagents were from Invitrogen (Paisley, UK).

Results

Effect of unfractionated heparin upon constitutive eotaxin-1 release

Unfractionated heparin (comprising variable chain lengths, 17–19 kDa), at the lowest concentration examined (0.01 mg mL^{-1}) significantly potentiated constitutive eotaxin-1 release (maximum increase of 61 ± 8% at 0.01 mg mL^{-1}, $n = 5$, $P < 0.001$, Figure 1a). Higher concentrations of unfractionated heparin resulted in concentration-dependent inhibition of constitutive eotaxin-1 release (IC_{50} heparin 1.8 (0.8–2.7) mg mL^{-1}, maximum inhibition of 74 ± 5% at 10 mg mL^{-1}, $P < 0.001$).

Effect of unfractionated heparin upon IL-13- and IL-4-induced eotaxin-1 release

Given the finding that eotaxin-1 release by IL-13 is dependent on the IL-4 receptor α-chain (Hirst *et al*., 2002), the effect of unfractionated heparin upon both IL-13-stimulated (10 ng mL^{-1}) and IL-4-stimulated (1 ng mL^{-1}) eotaxin-1 release was investigated. The release of eotaxin-1 by IL-13 (119 ± 24 ng per mL per 10^6 cells) and IL-4 (109 ± 27 ng per mL per 10^6 cells) under these conditions was similar ($P > 0.05$, $n = 6$).

Unfractionated heparin did not alter (maximum of 48 ± 20% at 0.05 mg mL^{-1}, $n = 4$, $P > 0.05$) IL-13-dependent eotaxin-1 release at low concentrations (<0.1 mg mL^{-1}) (Figure 1b). In contrast, marked inhibition of eotaxin-1 release was observed at higher heparin concentrations (maximum inhibition of 77 ± 8% at 10 mg mL^{-1}, $n = 6$, $P < 0.001$, Figure 1b). As with IL-13, eotaxin-1 release induced by IL-4 was inhibited by unfractionated heparin (maximum inhibition at 10 mg mL^{-1} of 95 ± 4%, $n = 6$, $P < 0.05$, Figure 1c). No differences were detected in the potency of the attenuating effect of heparin on either IL-13-dependent (IC_{50} heparin 2.0 (0.7–3.3) mg mL^{-1}) or IL-4-dependent (IC_{50} heparin 1.3 (1.0–1.7) mg mL^{-1}) eotaxin-1 production ($P > 0.05$, $n = 6$), suggesting a common mechanism of attenuation by heparin.

RayBio Human Chemokine Antibody Arrays were used to examine if heparin suppressed the release of multiple chemokines present in cell-conditioned media from IL-13-stimulated human ASM cells. The array identified upregulation of six chemokines by IL-13 (at least $P < 0.05$, $n = 3$).

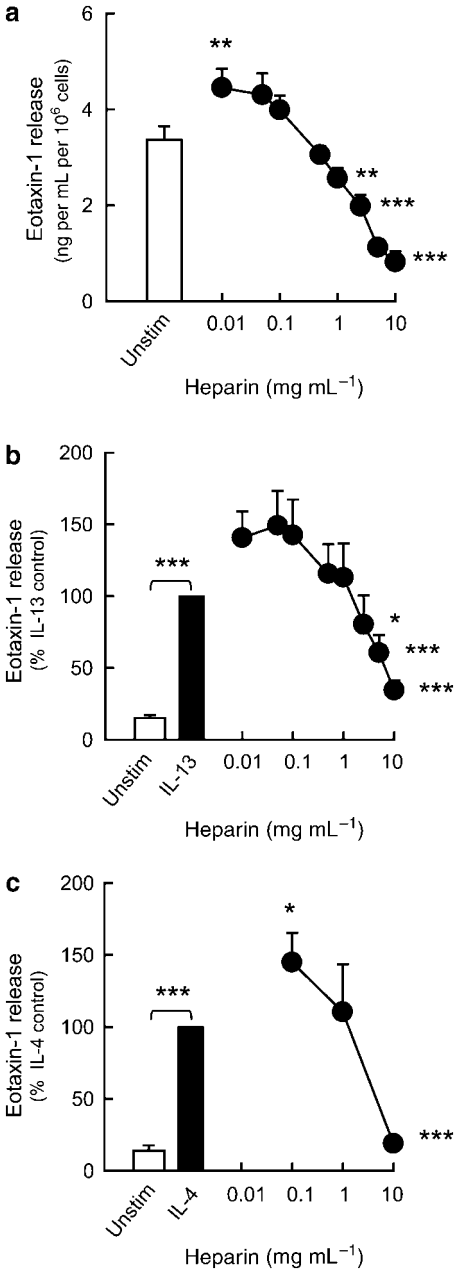


Figure 1 Attenuation by unfractionated heparin of constitutive eotaxin-1 release and release induced by the Th2-like cytokines. Subconfluent growth-arrested human airway smooth muscle (ASM) cells in the absence or presence of unfractionated heparin were left either (**a**) unstimulated (Unstim) for 24 h or treated with (**b**) 10 ng mL^{-1} interleukin (IL)-13 or (**c**) 1 ng mL^{-1} IL-4. Points are mean ± s.e.mean of duplicate values from independent experiments using cells at passages 4–6 from five to six donors. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with (**a**) Unstim or (**b** and **c**) cytokine-stimulated cells by one-way ANOVA followed by a Bonferroni's *t*-test.

These were eotaxin-1 (CCL11, 1.62-fold), growth-related oncogene-α (GROα (CXC chemokine ligand 1; CXCL1), 1.87-fold) and monocyte chemotactic peptide-3 (MCP-3 (CCL7), 2.38-fold) (Figure 2) as well as I-309 (CCL1, 1.50-fold), interferon-inducible I-TAC (T-cell-α chemoattractant (CXCL11), 1.41-fold), and MCP-2 (CCL8, 2.89-fold) (not shown). Heparin (5 mg mL^{-1}) suppressed the induction of these chemokines by IL-13 ($P<0.05$–0.01, $n=3$), but did not prevent IL-13-dependent MCP-3 production (Figure 2b). Failure to suppress MCP-3 production while preventing upregulation of multiple chemokines in the same samples suggested that attenuating effects of heparin on susceptible chemokines such as eotaxin-1 did not involve sequestration of the stimulus, IL-13. To exclude any interference by heparin with the detection of chemokines (including eotaxin-1), cell-conditioned media collected from IL-13-stimulated cells were 'spiked' with heparin (5 mg mL^{-1}). Under these conditions, the attenuation by heparin of IL-13-induced upregulation of eotaxin-1, GROα or MCP-3 did not occur (Figure 2b).

Role of sulphation

To examine any requirement for sulphation, the attenuating effect of unfractionated heparin on IL-13-dependent eotaxin-1 release was compared with heparin-like (CSA and CSB) or non-heparin-like (D and DS) sulphated compounds that varied either in the degree of sulphation (DS > heparin > CSA = CSB ≫ D) or in the position of sulphation (N-sulphated or O-sulphated) (Tyrrell *et al*., 1999; Rabenstein, 2002). As heparin is variably N-acetylated, the effect of N-desulphated and re-N-acetylated heparins was also examined.

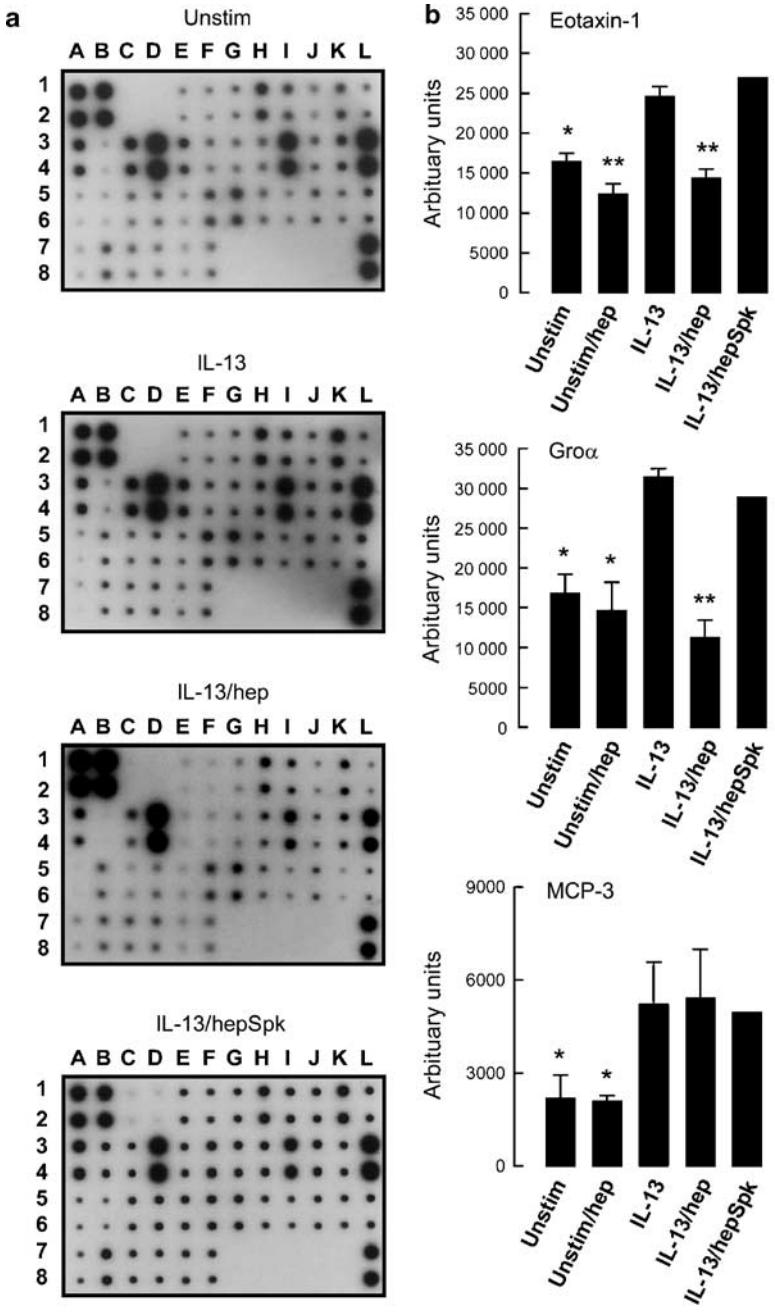
Dextran sulphate was examined and compared with its non-sulphated derivative, D. DS (0.1–10 mg mL^{-1}) prevented IL-13-induced eotaxin-1 release in a concentration-dependent manner (maximum inhibition of 100 ± 5% at 10 mg mL^{-1}, $n=6$, $P<0.001$), whereas D was without effect over the concentration range investigated (Figure 3a). CSA and CSB were also examined and IL-13-dependent eotaxin-1 release was increased by CSA (maximal increase of 57 ± 19% at 10 mg mL^{-1}, $n=6$, $P<0.001$, Figure 3b), but was attenuated by CSB (maximum inhibition of 39 ± 17% at 10 mg mL^{-1}, $n=6$, $P<0.05$).

Sulphation pattern and anticoagulant activity

We next examined if positional sulphation or anticoagulant activity was factors in the attenuated secretory responses by heparin. A molecule that was completely N-desulphated with 20% re-N-acetylation (N-de 20%ac, anticoagulant) did not prevent IL-13-dependent eotaxin-1 release. Increased acetylation to 90% within this N-desulphated molecule

Figure 2 (**a**) Human Chemokine Antibody Array for detection of proteins in conditioned medium from unstimulated (Unstim) or interleukin (IL)-13-stimulated human airway smooth muscle (ASM) cells treated with or without heparin (hep, 5 mg mL^{-1}). In some experiments, conditioned medium from IL-13-stimulated cells was immediately spiked with heparin (hepSpk, 5 mg mL^{-1}) to examine any potential direct interference by heparin on chemokine detection. (**b**) Examples of chemokines upregulated by IL-13 are shown for eotaxin-1, growth-related oncogene-α (GROα) and monocyte chemotactic peptide-3 (MCP-3). Chemokine levels were quantified against an internal control on the array and shown as mean ± s.e.mean of duplicate values from independent experiments from three separate donors. (**c**) Chemokine array spot map. See http://www.raybiotech.com/map/human_chemokine_map.pdf for key to abbreviations for chemokines. Pos = positive control; Neg = negative control. *$P<0.05$, **$P<0.01$ compared with IL-13-stimulated cells by one-way ANOVA followed by a Bonferroni's *t*-test.

(N-de 90%ac, anticoagulant) fully restored the attenuating activity (maximal inhibition 94 ± 3% at 10 mg mL^{-1}, $n = 6$, $P < 0.001$), which was similar to unfractionated heparin ($P > 0.05$ by two-way ANOVA, $n = 6$) (Figure 4a). O-de heparin unlike unfractionated heparin is non-anticoagulant and lacks 2-*O*- and 3-*O*-sulphates but retains 6-*O*-sulphates (Fryer



c

	A	B	C	D	E	F	G	H	I	J	K	L
1	Pos	Pos	Neg	Neg	BLC	CCL28	Ckβ8-1	CTACK	CXCL-16	ENA-78	Eotaxin-1	Eotaxin-2
2	Pos	Pos	Neg	Neg	BLC	CCL28	Ckβ8-1	CTACK	CXCL-16	ENA-78	Eotaxin-1	Eotaxin-2
3	Eotaxin-3	Fractalkine	GCP-2	GRO	GROα	HCC-4	I-309	I-TAC	IL-8	IP-10	Lymphotactin	MCP-1
4	Eotaxin-3	Fractalkine	GCP-2	GRO	GROα	HCC-4	I-309	I-TAC	IL-8	IP-10	Lymphotactin	MCP-1
5	MCP-2	MCP-3	MCP-4	MDC	MIG	MIP-1α	MIP-1β	MIP-1δ	MIP-3α	MIP-3β	MPIF-1	NAP2
6	MCP-2	MCP-3	MCP-4	MDC	MIG	MIP-1α	MIP-1β	MIP-1δ	MIP-3α	MIP-3β	MPIF-1	NAP2
7	PARC	RANTES	SDF-1α	SDF-1β	TARC	TECK	Blank	Blank	Blank	Blank	Blank	Pos
8	PARC	RANTES	SDF-1α	SDF-1β	TARC	TECK	Blank	Blank	Blank	Blank	Blank	Pos

et al., 1997). Attenuation of IL-13-dependent eotaxin-1 release by O-de heparin (maximal inhibition was 53 ± 8% at 10 mg mL^{-1}, $n=6$, $P<0.001$) was similar to that found with unfractionated heparin ($P>0.05$, by two-way ANOVA) (Figure 4b).

Role of molecular size

The effects of two LMW heparins (3 and 6 kDa) were examined upon IL-13-dependent eotaxin-1 release, and were compared with unfractionated heparin (17–19 kDa) to determine if the attenuating effect of heparin was retained in smaller heparin fragments. The 3-kDa LMW heparin caused a small potentiation at lower concentrations ($P<0.05$ by ANOVA), but significantly inhibited IL-13-stimulated eotaxin-1 release at higher concentrations (maximum inhibition of 87 ± 6% at 10 mg mL^{-1}, $P<0.001$). Inhibition was also found with 6 kDa LMW heparin (maximum inhibition of 90 ± 10% at 10 mg mL^{-1}, $n=6$, $P<0.001$). No differences were found in the attenuating effects of LMW heparins and unfractionated heparin ($P>0.05$, by two-way ANOVA) (Figure 5).

Role of anionic charge

The contribution of anionic charge to the secretion-attenuating effects of heparin was explored using non-sulphated highly anionic peptides/polysaccharides with and without N- and O-sulphation. PGA is non-sulphated and was examined in two forms that varied in molecular weight: PGA-1 (3–15 kDa) and PGA-2 (50–100 kDa), with PGA-1 being comparable in size to the unfractionated heparin (17–19 kDa). Additionally, the highly sulphated anion, fucoidan (N- and O-sulphated, 193 kDa) was compared with unfractionated heparin. Eotaxin-1 release induced by IL-13 was not prevented by the PGA-1. However, PGA-2, which possesses increased anionic charge, caused an attenuation (maximum inhibition of 70 ± 13% at 10 mg mL^{-1}, $P<0.05$ by ANOVA) similar to that found with heparin ($P>0.05$, by two-way ANOVA) (Figure 6). Likewise, fucoidan attenuated IL-13-stimulated eotaxin-1 release (maximum inhibition of 95 ± 11% at 1 mg mL^{-1}, $P<0.001$) and was more effective than heparin ($P<0.05$, by two-way ANOVA).

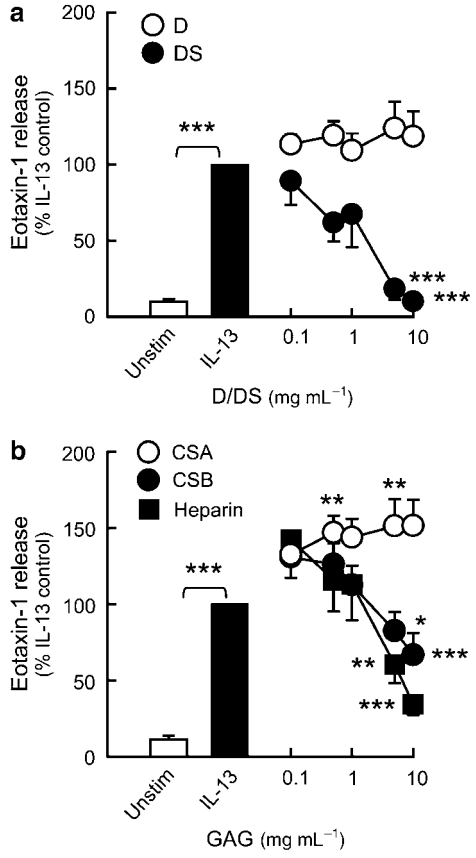


Figure 3 Attenuation of interleukin (IL)-13-induced eotaxin-1 release by variably sulphated heparin-like polymers. (**a**) Compares dextran sulphate (DS) with dextran (D) and (**b**) compares chrondroitin sulphates A and B (CSA/CSB) with unfractionated heparin. Points represent mean ± s.e.mean of duplicate values from independent experiments using cells from six human donors at passages 4–6. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with IL-13-stimulated cells by one-way ANOVA followed by a Bonferroni's *t*-test.

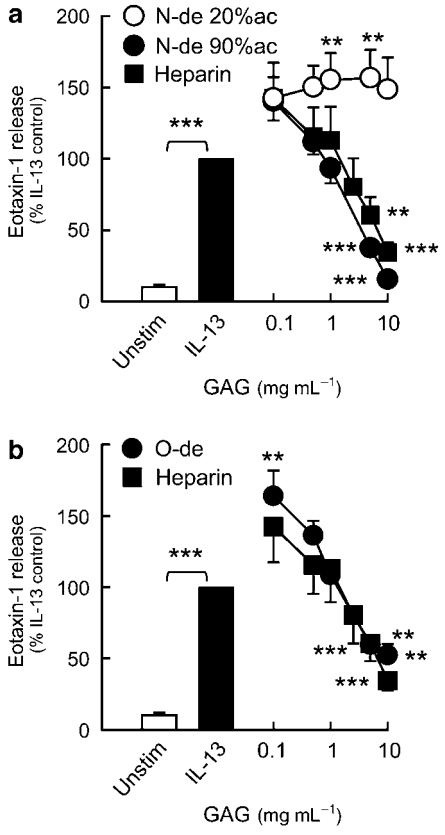


Figure 4 Attenuation of interleukin (IL)-13-induced eotaxin-1 release by variably sulphated and acetylated heparin-like polymers. (**a**) Compares unfractionated heparin with N-desulphated, 20% re-N-acetylated heparin (N-de 20%ac, anticoagulant), N-desulphated, 90% re-N-acetylated heparin (N-de 90%ac, anticoagulant) and (**b**) compares heparin with O-desulphated heparin (O-de heparin, non-anticoagulant). Points represent mean ± s.e.mean of duplicate values from independent experiments using cells from six human donors at passages 4–6. **$P<0.01$, ***$P<0.001$ compared with IL-13-stimulated cells by one-way ANOVA followed by a Bonferroni's *t*-test.

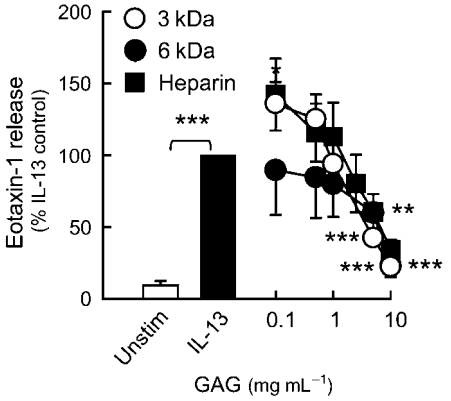


Figure 5 Attenuation of interleukin (IL)-13-stimulated eotaxin-1 production by low molecular weight (LMW) heparins. Human airway smooth muscle (ASM) cells were stimulated with IL-13 for 24 h in the presence or absence of LMW heparin fractions of 3 or 6 kDa and compared with attenuation by unfractionated heparin. Data are mean ± s.e.mean of six independent experiments using cells at passages 4–6 cultured from individual donors. $^{**}P<0.01$, $^{***}P<0.001$ compared with IL-13 in the absence of LMW fractions by ANOVA followed by a Bonferroni's *t*-test.

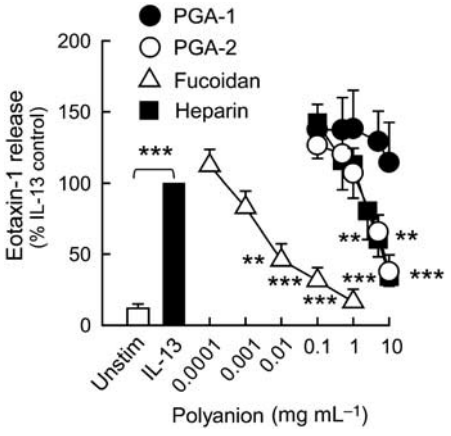


Figure 6 Attenuation of interleukin (IL)-13-stimulated eotaxin-1 production by polyanions. Human airway smooth muscle (ASM) cells were stimulated with IL-13 for 24 h in the presence or absence of sulphated (fucoidan) or non-sulphated (poly-L-glutamic acid, PGA) polyanions. Data are mean ± s.e.mean of six independent experiments using cells at passages 4–6 cultured from individual donors. $^{**}P<0.01$, $^{***}P<0.001$ compared with IL-13 in the absence of polyanions by ANOVA followed by a Bonferroni's *t*-test.

Discussion

Heparin's structural heterogeneity arises from polysaccharide side chains comprising alternating residues of an amino sugar (glucosamine) and uronic acid (either glucuronic or iduronic acid), which result in regions of sulphated domains (O-sulphated and N-sulphated) and less sulphated domains (N-acetylated). Additionally, heparin comprises irregular chain lengths and is highly anionic 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 and Page, 2002). Although, it is well-established that heparin is antiproliferative for ASM (Johnson *et al*., 1995; Kilfeather *et al*., 1995; Kanabar *et al*., 2005), its effects on ASM-derived chemokine production is unknown. Here, we demonstrate that heparin prevents IL-13-dependent eotaxin-1 production and identify some of the key structural moieties on the heparin molecule required for this activity. These include sulphation, particularly N-sulphation and to a much lesser extent, 2-O- and 3-O-sulphation. Furthermore, substitution of N-sulphation to 90% acetylation by re-N-acetylation restored activity comparable with unfractionated heparin. We also show that heparin's attenuating effect on eotaxin-1 secretion was associated with its non-anticoagulant activity, and was partially dependent on net anionic charge, but independent of molecular size above 3 kDa.

Owing to the highly anionic nature of GAGs, electrostatic interactions can form with other chemokines. Thus, a confounder at the outset was that heparin could bind eotaxin-1 and interfere with its detection or reduce the bioavailability of IL-13 and thus limit eotaxin-1 production. Alternatively, heparin could bind other factors in the culture media that could in turn modulate the release of eotaxin. In support of these possibilities, heparin is known to bind multiple chemokines including IL-8 (CXCL8), RANTES (regulated on activation, normal T-cell expressed and secreted; CCL5), MCP-1 (CCL2) and MIP (macrophage inflammatory peptide)-1β (CCL4) (Frevert *et al*., 2003; Johnson *et al*., 2004; de Paz *et al*., 2007; Ellyard *et al*., 2007), as well as several cytokines such as IL-1β, IL-2, IL-6, IL-10, TNF (tumour-necrosis factor)-α and IFN (interferon)-γ (Fernandez-Botran *et al*., 1999; Kuschert *et al*., 1999; Mummery and Rider, 2000; Salek-Ardakani *et al*., 2000; Bode *et al*., 2006). Heparin's capacity to bind cytokines and chemokines has been postulated to enhance the biological effects of chemokines by increasing their binding affinities for their respective receptors, although speculation exists as to whether this occurs *in vivo* over physiological pH ranges (Fernandez-Botran *et al*., 1999).

A recent report by Ellyard *et al*. (2007) demonstrates that heparin binds eotaxin-1 to increase its chemotactic activity *in vivo*. To establish if eotaxin-1 binding or IL-13 sequestration by heparin were important factors in explaining the attenuating effects of heparin on eotaxin-1 production from ASM, several strategies were employed. In the first, we ensured that the concentration of heparin in the ELISA detection step was below the level that could reduce the recovery of a known amount of rh-eotaxin-1. Although this limited direct interference with the assay, it did not exclude a possible reduction in detection of eotaxin-1 already bound to heparin. Control experiments indicated that recovery of rh-eotaxin-1 was reduced by no more than 35% at $10\,mg\,mL^{-1}$ heparin, which could not account for overall attenuating effect of heparin on released eotaxin-1, especially at the lower concentrations of heparin. Additionally, we examined the attenuating effect of heparin on multiple chemokines induced by IL-13 using an antibody array. In this, we confirmed that heparin prevented IL-13-dependent upregulation of eotaxin-1 and also other chemokines including GROα, I-309, I-TAC and MCP-2, but had no effect on upregulation of MCP-3. Reasons for the lack of effect of

heparin on MCP-3 release are unclear, but in the context of the suppression of other chemokines by heparin, it suggests that heparin was not acting via IL-13 sequestration. Likewise, addition (at the time of collection) of heparin to cell-conditioned media samples from IL-13-stimulated cells did not reduce the detection of eotaxin-1 (or GROα) by the array, further suggesting that heparin did not act to mask eotaxin-1 detection. Significantly, whereas Ellyard *et al*. (2007) reported that heparin can bind rh-eotaxin-1, other GAGs including CSB were found not to bind rh-eotaxin-1. In the present study, we found that both heparin and CSB were equivalent in preventing IL-13-dependent eotaxin-1 release. Thus, on balance, the attenuating effects of heparin on IL-13-stimulated eotaxin-1 release from ASM are not explained by sequestration of IL-13, the stimulus for eotaxin-1 production, or interference with the direct detection of eotaxin-1 by either the ELISA or the chemokine array.

The importance of sulphation for the attenuating effects of heparin on IL-13-dependent eotaxin-1 production was initially identified with the non-heparin-like polymer DS, which unlike non-sulphated D inhibited IL-13-dependent eotaxin-1 production by human ASM. Though structurally unrelated, 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 2.7 sulphate residues per disaccharide unit (Rabenstein, 2002; de Paz *et al*., 2007). The compounding effect of DS polymers having varying molecular weights and hence a differing overall sulphate content was not addressed directly in this study. We have previously shown, however, that several DS polymers with molecular weights between 5 and 500 kDa were equally effective in preventing fetal bovine serum-dependent proliferation of bovine ASM cells (Kilfeather *et al*., 1995), suggesting that the absolute presence of sulphation above a threshold is required. This is also supported by the finding that non-sulphated D had no effect on IL-13-dependent eotaxin-1 production.

Reports suggest that the number of sulphate groups in polysaccharides directly correlates with the level of bioactivity (Koyanagi *et al*., 2003). We further examined the contribution of sulphation in experiments employing polysaccharides that exhibit less sulphation than heparin. Consistent with the importance of sulphation, we found that CSA was poorly effective against IL-13-dependent eotaxin-1 production compared with heparin (average number of sulphate groups per repeating disaccharide unit for heparin is 2.7 compared with 1.0 for the chondroitins; Varma and Varma, 1983). In contrast, CSB, though less sulphated than heparin, was found to be as effective as heparin in attenuating eotaxin-1 release. A key difference between CSA and CSB is in the uronic acid content of the chrondroitin backbone, which in the case of CSA is glucuronate-based and iduronate in CSB (Casu *et al*., 1988). The greater flexibility of the iduronate residue in the CSB polysaccharide (compared with the glucuronate residue in CSA) is commonly used to explain the propensity of iduronate GAGs to interact with proteins and display a large number of different biological activities (Kawashima *et al*., 2002). This may have relevance in the context of large chondroitins such as versican that are elevated in the airways of patients with asthma (Huang *et al*., 1999). Moreover, heparin comprises both glucuronate and iduronate residues in its backbone, but it is unclear whether the iduronate content explains either the attenuating effect of heparin or CSB over CSA.

Heparin is variably N-sulphated, O-sulphated and N-acetylated (Varma and Varma, 1983). Salek-Ardakani *et al*. (2000) demonstrated that the ability of heparin to inhibit IL-10-induced CD14 expression upon monocytes/macrophages was dependent upon specific sulphation patterns. They demonstrated that loss of *N*-sulphates resulted in diminished inhibitory activity. Consistent with this, we found N-desulphated, 20% re-N-acetylated heparin (N-de 20%ac) was devoid of activity against IL-13-dependent eotaxin-1 production. In studies of vascular smooth muscle, loss of N-sulphation results in complete loss of antiproliferative activity (Wright *et al*., 1989). The finding that 90% re-N-acetylation (N-de 90%ac) in the molecule restored attenuating activity similar to unfractionated heparin against IL-13-stimulated eotaxin-1 release also agrees with studies in vascular smooth muscle where N-acetylation restores antiproliferative activity (Castellot *et al*., 1985; Tiozzo *et al*., 1993) and suggests that N-sulphation is required for heparin's eotaxin-1-attenuating activity. Although we did not investigate a minimum level of N-sulphation required for suppression of IL-13-dependent eotaxin-1 production, a recent study suggests heparin must retain 24% *N*-sulphate groups to retain comparable antiproliferative activity with native heparin (Longas *et al*., 2003). Suppression of eotaxin-1 release occurred also with the O-de heparin fraction (non-anticoagulant), suggesting that the pentasaccharide sequence required for anticoagulant activity did not play a role in the attenuation of IL-13-dependent eotaxin-1 production, a finding supported in vascular smooth muscle proliferation *in vivo* (Guyton *et al*., 1980) and *in vitro* (Hoover *et al*., 1980). Collectively, as O-de 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 eotaxin-1 production 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. Both the LMW heparins (3 and 6 kDa) examined prevented IL-13-dependent eotaxin-1 release, and were comparable in activity with unfractionated heparin, suggesting that the efficacy of heparin against IL-13-dependent eotaxin-1 release resides in chains of 3–6 kDa but may be retained in fractions <3 kDa. This agrees with previous findings for antiproliferative activity in human ASM (Kanabar *et al*., 2005), bovine ASM (Kilfeather *et al*., 1995) and a report by Tiozzo *et al*. (1991), who demonstrated a graded loss of antiproliferative activity with decreasing molecular weight from 4.5 to 1 kDa LMW heparin.

The importance of net anionic charge within the heparin molecule for antisecretory activity has not been investigated. PGA, a highly anionic, non-sulphated linear polysaccharide was examined in two forms, PGA-1 and PGA-2. PGA-1 (3–

15 kDa), although comparable in size to unfractionated heparin (17–19 kDa) did not prevent IL-13-stimulated eotaxin-1 release. However, when the larger polymer (PGA-2, 50–100 kDa) was examined, attenuating activity was recovered to a level similar to that with heparin, suggesting that an overall net negative charge was required for this activity. Previous studies examining the antiproliferative effect of heparin report variable findings. For example, Joseph *et al*. (1997) showed that the antiproliferative activity of a lower charge density (less negative) heparin fraction in vascular smooth muscle 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 polysaccharide PGA-1 was less effective than the highly anionic and sulphated polysaccharide fucoidan further suggests that sulphation contributes to suppression of eotaxin-1 release.

We observed that low concentrations ($0.1\,mg\,mL^{-1}$) of unfractionated heparin potentiated both constitutive and cytokine-induced eotaxin-1 release. This has not been reported previously and is a potentially undesirable side effect of any heparin-based therapeutic compound (de Paz *et al*., 2007). Potentiation was also observed with both LMW heparins and with N-de 20%ac, N-de 90%ac and O-de heparin, suggesting that neither the position of sulphation nor size of the heparin molecule were determinants for this effect. Furthermore, this profile contrasts with the observed pattern of activity for the attenuating effects of heparin and suggests that independent properties of the heparin molecule account for the potentiating role of heparin on ASM cells. Although GAGs are required for many chemokines to function and are reported to enhance chemokine/cytokine receptor binding affinity (Frevert *et al*., 2003; Johnson *et al*., 2004; de Paz *et al*., 2007; Ellyard *et al*., 2007), this would seem an unlikely explanation given that the potentiating effect of heparin occurred with both constitutive and cytokine-induced eotaxin-1 release. Further investigation is required to ascertain the nature and significance of the potentiation.

In conclusion, we provide new information for the anti-inflammatory activity of unfractionated heparin upon either constitutive release of eotaxin-1 from ASM or release induced by the Th2-like cytokines, IL-13 and IL-4. Additionally, we show that specific structural properties of the heparin molecule are involved in its secretion-attenuating activity against IL-13-dependent eotaxin-1 production. These include sulphation, particularly N-sulphation and to a much lesser extent, 2-O- and 3-O-sulphation and anionic charge. Attenuation was independent of anticoagulant activity and molecular weight above 3 kDa. Understanding the structural properties of the heparin molecule that underlie its anti-inflammatory activity offers opportunities for the design of 'tailor-made' sequences based on the heparin template for specific therapeutic uses (Tyrrell *et al*., 1999; Lever and Page, 2002) including suppression of eosinophilic mediators produced by ASM in allergic airways diseases such as asthma.

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Conflict of interest

The authors state no conflict of interest.

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