Inhibition of serum-induced proliferation of bovine tracheal smooth muscle cells in culture by heparin and related glycosaminoglycans

¹S.A. Kilfeather, S. Tagoe, A.C. Perez, K. Okona-Mensa, R. Matin & C.P. Page

Sackler Institute of Pulmonary Pharmacology, Department of Thoracic Medicine, Kings College School of Medicine and Dentistry, London SE5 9PJ

- 1 The effect of heparin and related glycosaminoglycans on bovine airway smooth muscle proliferation has been investigated.
- 2 Foetal bovine serum stimulated division of bovine trachealis smooth muscle cells in a concentration-dependent fashion at concentrations between 1 and 30%.
- 3 Heparin (0.1–100 $\mu\text{g ml}^{-1}$), heparan sulphate (0.1–100 $\mu\text{g ml}^{-1}$) and fragmin (0.1–100 $\mu\text{g ml}^{-1}$) inhibited smooth muscle division in a concentration-dependent fashion between 0.1–100 $\mu\text{g ml}^{-1}$. A heparin disaccharide did not exhibit inhibition of division at 100 $\mu\text{g ml}^{-1}$.
- 4 Dextran sulphate at molecular weights of 5×10^3 and 5×10^5 concentration-dependently inhibited division between 0.1–100 $\mu\text{g ml}^{-1}$. Dextran without sulphation did not exhibit inhibition of division at 100 $\mu\text{g ml}^{-1}$.
- 5 The magnitude of inhibition of proliferation did not reach 100% for any compounds examined at concentrations up to 100 $\mu\text{g ml}^{-1}$ during incubations for 5 and 14 days. IC_{50} values for inhibition of proliferation ranged between 1–5 $\mu\text{g ml}^{-1}$.
- 6 These findings suggest that heparin and related glycosaminoglycans inhibit bovine airway smooth muscle cell division.

Keywords: Heparin; glycosaminoglycans; airway; smooth muscle; division

Introduction

The smooth muscle component of airways has largely been investigated for its contribution to airway smooth muscle tone in several diseases such as asthma and bronchitis. Changes in structure of the airway wall including increases in airway smooth muscle mass have also been observed in asthmatics (Dunnill, 1969; Ebina *et al.*, 1990; 1993) and there is evidence supporting a relationship between changes in airway smooth muscle mass and the bronchial hyperresponsiveness characteristic of asthma (James *et al.*, 1989). However, the mechanisms controlling airway smooth muscle cell (ASMC) division and size are poorly defined. The range of potential mitogenic stimuli for ASMC is extensive, but the number of endogenous factors with an established antiproliferative action on ASMC is very limited.

The inhibitory action of glycosaminoglycans including heparin and heparan sulphate on vascular smooth muscle cell (VSMC) (Karnovsky & Edelman, 1994) and fibroblast division (Jackson *et al.*, 1991) is well established. Heparin containing mast cells are abundant in the airways and cell surface heparan sulphate glycosaminoglycans are ubiquitous. Furthermore, it has recently been demonstrated that human lung parenchyma can release heparin at antiproliferative concentrations following allergen exposure (Green *et al.*, 1993). Glycosaminoglycans therefore provide potential regulators of ASMC behaviour. The absence of a proliferative action of heparan sulphate on ASMC division has been demonstrated (Panettieri *et al.*, 1990), but the potential antiproliferative effects of glycosaminoglycans on ASMC division have not been examined.

Heparins are polymers composed of alternating glucuronic and iduronic saccharides with O- and N- sulphation and carboxylation. The antiproliferative action of heparin in VSMC is independent of anticoagulant activity, indicating

that the antithrombin III binding site is not a requirement (Guyton *et al.*, 1980). Certain characteristics of the glycosaminoglycans are required for antiproliferative activity, including a degree of charge density and minimum molecular size (Castellot *et al.*, 1984; Wright *et al.*, 1989). Thus, desulphated heparin or heparin fragments of less than hexasaccharide units are not antiproliferative in VSMC (Karnovsky & Edelman, 1994).

In the present investigation we have examined the possible influence of glycosaminoglycans on ASMC. In addition we have examined requirements of minimum molecular size and sulphation by examining antiproliferative activity of dextrans and low molecular weight heparin fragments on ASMC division. Preliminary findings of this work have been presented to the European Respiratory Society (Kilfeather *et al.*, 1993).

Methods

Smooth muscle cell culture

Airway smooth muscle cells were obtained from bovine trachealis muscle. Trachealis muscle was obtained from adult cattle killed by captive bolt and was dissected free of connective tissue under aseptic conditions under a dissection microscope. The muscle was washed in calcium- and magnesium-free phosphate buffered saline (PBS) and cut into 1–2 mm³ pieces for explanting. The explants were washed in medium 199 (M199) supplemented with gentamicin (50 $\mu\text{g ml}^{-1}$) and plated in 100 mm tissue culture-treated Petri dishes in M199 at pH 7.3 supplemented with foetal bovine serum (FBS, 10%), glutamine (2 mM), gentamicin (50 $\mu\text{g ml}^{-1}$) and amphotericin B (2.5 $\mu\text{g ml}^{-1}$), and maintained at 95% air/5% CO₂ in a humidified atmosphere at 37°C. Cells migrated from the explants after one week of plating. The explants

¹ Author for correspondence.

were removed when cells migrating from the explant had reached a density of 10 per cent. The cells were then allowed to reach confluence before replating. For replating, cells were washed three times in PBS and removed from the plate as previously described (San Antonio *et al.*, 1992) by incubation with PBS and EDTA (1 mM). The cells were replated for further population growth or directly onto a 96 well tissue culture plated at 10 or 20% confluence for examination of cell division at 5 days or plated at 100 cells/well for examination of division at 14 days. A lower cell density was adopted for plating in experiments conducted over 14 days to maintain exponential growth characteristics during the incubation period. Cells were refed every 3 days. Cells were examined up to passage 4. Cells from separate animals were used for each experiment.

Smooth muscle cell identification

Smooth muscle cells were identified by immunofluorescence using an antibody against alpha smooth muscle actin (anti- α -SM-1) as previously described (Skalli *et al.*, 1986).

Smooth muscle cell division

Following plating for 24 h in 10% FBS, cells were growth arrested by maintenance in M199 supplemented with 0.5% FBS for 72 h. This concentration of FBS was insufficient to stimulate division (Figure 1). Cell division was reinitiated by incubation with FBS (10%) or varying concentrations for examination of concentration-dependent stimulation of division by FBS. Compounds under examination were introduced with 10% FBS. Cell division was monitored after 5 or 14 days by cell counting following nuclear staining with propidium iodide and giemsa. Cells were washed in phosphate buffered saline with calcium and magnesium three times before fixing in methanol and air drying. Fixed cells were then stained with propidium iodide (0.05 mg ml⁻¹, 30 min) or giemsa (GS-500, Sigma 2 min). Stained cells were then washed in deionised water. Staining with propidium iodide and giemsa gave identical results, although giemsa staining was found to be preferable due to stability of the stain and lack of requirement for fluorescence microscopy. Cell location while counting was assisted by creation of a grid on the underside of the wells with a scalpel blade.

Compounds

All compounds including tissue culture components and antibodies for immunofluorescence were obtained from Sigma (Poole, UK). Fragmin was obtained from Pharmacia (Milton Keynes, UK). Tissue culture plastics were obtained from Bibby Sterilin (UK).

Data analysis

Comparison of cell densities within experiments was by Student's *t* test. Concentration-response curves were analysed from Hill coefficients. Statistical significance was accepted with $P < 0.05$.

Results

Smooth muscle cell culture

After four days following explant plating, cells were observed migrating from explants. These cells and cells at subsequent passages stained positive for α -smooth muscle actin. Maintenance of cells at confluence in the presence of FBS (10%) resulted in the formation of multilayered cells characteristic of smooth muscle cells and often referred to as 'Hill and valley'.

Effect of heparin and related compounds on smooth muscle cell division

FBS induced a concentration-related increase in cell division between 1 and 30% (Figure 1). A submaximal concentration of 10% serum was chosen in subsequent experiments to examine potential inhibitory effects of glycosaminoglycans. In initial experiments, effects of glycosaminoglycans were examined at 100 μ g ml⁻¹, since the findings of previous investigations have demonstrated maximal antiproliferative effect on VSMC at this concentration (Wright *et al.*, 1989). Heparin, heparan sulphate, fragmin and dextran sulphate all inhibited cell division at 5 days (50% at 100 μ g ml⁻¹, $P < 0.05$) (Figure 2). The antiproliferative action of heparin in VSMC is dependent upon a minimum molecular weight. We have examined molecular weight dependency in the antiproliferative action of heparin against ASMC. The disaccharide of heparin (α -DUA-[1-4]-GlcNS-6S, derived from heparinase I and II-treated heparin) did not influence cell division in experiments where heparin exhibited significant inhibition of division (Figure 2). Previous studies in VSMC and fibroblasts have revealed a requirement for sulphation in the antiproliferative action of heparin. We have investigated a requirement for sulphation in the antiproliferative action of dextran sulphate by examining a dextran devoid of sulphation, but with high molecular weight (5×10^5) to avoid

Figure 1 The effect of foetal bovine serum (FBS) on bovine trachealis smooth muscle division. Cells were incubated with FBS for 5 days and cell density measured as described in methods. The data points are mean with s.e.mean derived from one of two experiments conducted with eight replicates for each mean.

Figure 2 The effect of heparin, and related glycosaminoglycans (100 μ g ml⁻¹) on FBS (10%)-induced ASMC division. Cells were incubated with FBS for 5 days and cell density measured as described in methods: (H) heparin, (HS) heparan sulphate, (F) fragmin, (DS) dextran sulphate, (D) dextran, (DiH) heparin disaccharide. Columns are means with s.e.mean derived from one of three experiments conducted with eight replicates for each mean. * $P < 0.05$ compared to FBS alone.

Figure 3 The concentration-dependency of inhibition of ASMC division by glycosaminoglycans. Cells were incubated with and without FBS and glycosaminoglycans (GAG) for 5 days and cell density measured as described in methods. Cells incubated without glycosaminoglycan ($\square$), with heparin ($\bullet$), heparan sulphate ($\circ$) and fragmin ($\blacksquare$). The data points are mean with s.e.mean derived from one of three experiments conducted with eight replicates for each mean.

Figure 4 The concentration-dependent inhibition of ASMC division by dextran sulphate. Cells were incubated with and without FBS for 5 days and cell density measured as described in methods. Cells incubated without dextran sulphate ($\blacksquare$), with dextran sulphate (molecular weight 5,000, $\circ$; molecular weight 500,000 $\square$) for 5 days and cell density measured as described in methods. The effect of heparin ($100 \mu\text{g ml}^{-1}$, $\bullet$) on cell division is also included. The data points are mean with s.e.mean derived from one of two experiments conducted with eight replicates for each mean.

potential limitation of molecular size. Division in response to 10% serum was inhibited in a concentration-dependent fashion by heparin ($0.1\text{--}100 \mu\text{g ml}^{-1}$) (Figure 3). Heparan sulphate and fragmin, a low molecular weight heparin also induced concentration-dependent inhibition of cell division ($0.1\text{--}100 \mu\text{g ml}^{-1}$) (Figure 3). Dextran sulphate at molecular weights of 5×10^3 , and 5×10^5 ($0.1\text{--}100 \mu\text{g ml}^{-1}$) also exhibited concentration-dependent inhibition of cell division (Figure 4). Heparin included as a positive control inhibited division to a similar extent as observed in other experiments. An intermediate size dextran sulphate (molecular weight 4.5×10^4) also inhibited division at $100 \mu\text{g ml}^{-1}$ by 50% ($54.4 \pm 3.3\%$). We have not observed significant differences in potency between heparin-related compounds and dextran sulphates exhibiting inhibition of ASMC division.

The possibility that proliferation could be restored in the presence of heparin over a period of 14 days was also examined. In these experiments cells were incubated with heparin for 14 days before monitoring effects of cell division. Heparin inhibited division over 14 days by approximately 75% ($75.1 \pm 2.3\%$) (Figure 5), providing support for sustained inhibition of ASMC division by heparin.

Heparin inhibits attachment of both fibroblasts and vascular smooth muscle cells to collagen substrata (LeBaron *et al.*, 1989; San Antonio *et al.*, 1992). We examined the possibility that heparin at concentrations between 0.1 and $100 \mu\text{g ml}^{-1}$ could interfere with cell attachment to tissue culture plastic by examining attachment over 24 h; no effect of heparin on cell attachment was observed.

Discussion

This paper provides evidence that heparin and related glycosaminoglycans can inhibit cell division in bovine ASMC. This finding extends the established antiproliferative action of heparin observed in aortic VSMC and recently observed in pulmonary vascular smooth muscle (Benitz *et al.*, 1986; 1990). The present findings in ASMC with respect to potency and efficacy of heparin for antiproliferative effects also reflect findings in VSMC (Castellot *et al.*, 1984).

The antiproliferative properties of heparin were first recognised following investigations of the interdependence of anticoagulant and antiproliferative activity in VSMC *in vivo*

Figure 5 Inhibition of ASMC division by heparin during a 14 day incubation. Cells were maintained for 14 days in the presence and absence of FBS. Cell density was measured as described in Methods. The data points are means with s.e.mean derived from one of three experiments conducted with 12 replicates for each mean. * $P < 0.01$; ** $P < 0.001$ compared to FBS without heparin.

(Guyton *et al.*, 1980). The findings of these and subsequent investigations *in vitro* demonstrated a direct inhibitory effect on smooth muscle division independent of anticoagulant activity (Hoover *et al.*, 1980). Further examination of antiproliferative actions of heparin on VSMC *in vitro* has revealed a direct relationship between antiproliferative effect and both molecular size and charge density (Castellot *et al.*, 1984). Fragmin, used in the present investigation, contains fragments of molecular weight in the region of 4,000, while the heparin disaccharide has a molecular weight of 665. The difference in inhibitory activity on ASMC between these compounds is indicative of a minimum molecular size requirement in the inhibitory action of glycosaminoglycans on ASMC division. These findings suggest similarity in molecular size requirements of glycosaminoglycan actions in ASMC and VSMC, since disaccharides of heparin are not antiproliferative in VSMC (Wright *et al.*, 1989).

The range of dextran sulphate molecular weights examined in this investigation (5,000–500,000) are above the molecular weight threshold observed between fragmin and the heparin disaccharide and this could account for the absence of a relationship between molecular size and antiproliferative activity in this range. Although dextran sulphate inhibits VSMC and Swiss 3T3 fibroblast division (San Antonio *et al.*, 1992), a relationship between molecular size and antiproliferative effect has not been demonstrated for dextran sulphates.

The present findings demonstrate an antiproliferative effect of dextran sulphate against ASMC that appears to be dependent on sulphation, since dextran without sulphation at the highest molecular weight (500,000) was without effect on ASMC division. This finding also reflects inhibition by dextran sulphate and absence of inhibition by dextran in fibroblasts (San Antonio *et al.*, 1992). The nature of sulphation could be important, since O-sulphation is required in antiproliferative activity in VSMC (Wright *et al.*, 1989) and antiproliferative activity is not observed in dermatan sulphate and the chondroitins 4 and 6 sulphate in either VSMC or 3T3 fibroblasts (Castellot *et al.*, 1981; San Antonio *et al.*, 1992).

Interruption of ASMC attachment by heparin to tissue culture-treated plastic was not observed under conditions in which significant inhibition of division occurred. This finding does not exclude the possibility that interference by glycosaminoglycans in ASMC attachment and migration could be a contributory factor to potential antiproliferative activity *in vivo* or a collagen substratum *in vitro*. Interference with cell attachment through competition with glycosaminoglycan binding sites in attachment-dependent cells remains a potential mode of inhibition of division or migration.

Taken together our findings demonstrate that certain classes of glycosaminoglycans exhibit antiproliferative activity in ASMC. The requirements for this action that have been identified by the present investigation include a certain molecular size within heparins and sulphation within dextrans. In these aspects and in terms of efficacy and potency, the profile of glycosaminoglycan activity is consistent with the actions of glycosaminoglycans in VSMC. Further investigation is required to establish whether sulphation alone or the nature of the sulphation is important in defining antiproliferative activity in ASMC.

Airways are embryologically derived from the intestine and we cannot assume that the actions and underlying mechanisms of glycosaminoglycans will be the same in ASMC and VSMC. The mechanism of action of glycosaminoglycans in VSMC has not been completely defined and speculation concerning the mechanism of antiproliferative action of glycosaminoglycans in ASMC is not possible without extensive biochemical examination. It is improbable, however, that the incomplete inhibition of ASMC division by glycosaminoglycans reflects development of a glycosaminoglycan-resistant cell population during the course of these experiments. Heparin also inhibits thymidine labelling conducted over 1 h, 24 h after mitogenic stimulation by approximately 50%. It is more probable therefore that incomplete inhibition reflects

the glycosaminoglycan insensitivity of certain intracellular mitogenic signalling pathways. Heparin-resistant cell populations can be developed following prolonged division in the presence of heparin (Pukac *et al.*, 1990), but this process is not achieved over the time courses used in the present investigation. Experiments conducted over 14 days in the present investigation demonstrate that a significant antiproliferative action is maintained over this period as opposed to a possible resumption of normal division rate after inhibition for 5 days. Division occurred in the presence of heparin during this period, indicated by incomplete inhibition of division by heparin. This implies that over this time period adaption to the inhibition by heparin is not observed. Division is not, therefore, delayed by heparin, but subject to prolonged inhibition for at least 14 days. The extent of inhibition is apparently greater at 14 days than at 5 days. This is a cumulative effect reflecting divergence in population size of two cell populations exhibiting different exponential growth rates.

These findings have relevance to broader issues of inflammation, structure and hyperresponsiveness of airways. Several changes in structural cell behaviour occur in asthma including increased smooth muscle mass that appear to be related to airway hyperresponsiveness (James *et al.*, 1989). Heparin is released from human parenchyma upon exposure to allergen or calcium ionophores at concentrations that would influence ASMC division *in vitro* (Green *et al.*, 1993). Glycosaminoglycans modulate vascular smooth muscle phenotypic expression towards a nondividing contractile phenotype (Chamley-Campbell *et al.*, 1981; Campbell *et al.*, 1992); an action that could contribute to antiproliferative effects on ASMC division observed in the present investigation. Inflammatory cells are major sources of heparanase, which disrupt glycosaminoglycan linkages and thereby release fragments from cellular heparan sulphate. Released glycosaminoglycans could influence lymphocyte (Sy *et al.*, 1983; Brenan & Parish, 1986; Lider *et al.*, 1990) and eosinophil trafficking (Seeds *et al.*, 1993), while disrupted cell surface heparan sulphate could affect glycosaminoglycan antiproliferative activity in the airway, as previously suggested for the vasculature (Campbell *et al.*, 1992). Persistent or recurring inflammation in the airways could thereby influence structural changes observed in airways of asthmatics. Glycosaminoglycans, either free or incorporated as proteoglycans could be involved in this process.

In conclusion, these findings suggest a role for endogenous glycosaminoglycans in regulation of airway smooth muscle division and airway structure. Manipulation of airway glycosaminoglycan levels would therefore be of relevance to airway remodelling and consequent influence on airway responsiveness.

This work was supported by grants from Rhone Poulenc Rorer and the British Council.

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(Received May 24, 1994
Revised November 21, 1994
Accepted December 1, 1994)