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Inhibition of serum and transforming growth factor beta (TGF-β1)-induced DNA synthesis in confluent airway smooth muscle by heparin

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1 Airway remodelling occurs in asthma and involves an increase in airway smooth muscle mass through cell proliferation and hypertrophy. Increased eosinophil density in the airways is a feature of asthma. Eosinophils exhibiting activation in the airways of asthmatics also exhibit increased expression of transforming growth factor beta (TGF-β1). We have examined the capacity of TGF-β1 and epidermal growth factor (EGF) to influence airway smooth muscle division and the effect of heparin on TGF-β1, EGF and serum-induced smooth muscle DNA synthesis in confluent airway smooth muscle cells (ASMC) as an indication of entry into S phase preceding mitogenesis.

2 ASMC were obtained from cell populations growing out from explanted bovine trachealis muscle sections. Cell division was monitored in sparse plated cells by direct cell counting following nuclear staining. Cell DNA synthesis in confluent cells was monitored by uptake of [^{3}H]-thymidine.

3 TGF-β1 (100 pM) inhibited FBS (10%)-induced smooth muscle division in sparsely plated cells (40%). TGF-β1 (100 pM) increased cell DNA synthesis (200%) in confluent cells in the presence of bovine serum albumin (BSA, 0.25%). EGF (0.7 nM) also increased airway smooth muscle DNA synthesis (69%) in the presence of BSA (0.25%). The facilitatory effect of TGF-β1 was observed between 1–100 pM, while that of EGF was observed between 20–200 pM.

4 Heparin inhibited serum and TGF-β1-induced DNA synthesis in confluent ASMC (55%), consistent with our previous observation of inhibition of division in sparsely populated ASMC (Kilfeather *et al*., 1995a). This action of heparin was observed between concentrations of 1–100 μg ml^{-1}. Heparin did not inhibit DNA synthesis in response to EGF. An anti-mitogenic effect of heparin was also observed against responses to combined exposure to TGF-β1 and EGF.

5 There was a clear inhibitory effect of heparin in absolute terms against serum-induced division in cells plated at 10, 20 and 45×10^3 cells cm^{-2}. The inhibitory effect of heparin was also observed at a plating density of 45,000 cells cm^{-2} when responses to serum were expressed as fold-stimulation of basal DNA synthesis.

6 These findings demonstrate a potential role of TGF-β1, EGF and heparin-related molecules in regulation of airway smooth muscle division.

Keywords: Airway; smooth muscle; heparin; transforming growth factor beta; epidermal growth factor; mitogenesis; glycosaminoglycans; DNA synthesis; tissue remodelling

Introduction

Changes in structure of the airway wall including increases in airway smooth muscle mass have been observed in asthmatics (Dunnil, 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). The range of potential mitogenic stimuli for airway smooth muscle cells (ASMC) is extensive, as is the range of potential cellular sources. Eosinophils, which exhibit increased density in airways of asthmatics, could contribute to airway remodelling through release of oxygen radicals, basic proteins, proteases and growth factors. Both transforming growth factor beta (TGF-β1) and alpha (TGF-α) are produced by eosinophils (Elovic *et al*., 1994) and airway eosinophil TGF-β1 expression is increased in asthma (Ohno *et al*., 1992). TGF-β1 is associated with tissue repair (Boarder & Ruoslahti, 1992) and is expressed by smooth muscle cells (Hamet *et al*., 1991; Fukuda, 1993).

TGF-β1 is a stable polypeptide with wide ranging effects mediated through receptors that are ubiquitous on mammalian cells (Bassing *et al*., 1994). The TGF-β polypeptides are highly conserved between species, demonstrating a fundamental role of TGF-β (Sporn & Roberts, 1990). TGF-β has been implicated in the development of lung fibrosis (Laurent *et al*., 1993; Giri *et al*., 1993) and TGF-β expression has been observed in human airways, but without generalized increased expression in airways of asthmatics (Aubert *et al*., 1994).

TGF-α is released from eosinophils and is analogous to epidermal growth factor (EGF). A mitogenic action of EGF on guinea-pig airway smooth muscle cells has been demonstrated (Stewart *et al*., 1994), raising the possibility that EGF-like factors could also contribute to airway remodelling. Receptors to TGF-β and EGF are coupled to differing signal transduction pathways, but can exert synergistic activity on cellular mechanisms involved in mitogenesis (Ranganathan & Getz, 1990).

Heparin and heparan sulphate are related glycosaminoglycans that exert inhibitory activity against vascular smooth muscle (Karnovsky & Edelman, 1994) and sparse and

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densely populated bovine (Kilfeather *et al*., 1995a), guinea-pig (Halayko *et al*., 1994) and human (Johnson *et al*., 1995) airway smooth muscle. Heparin is released by connective tissue mast cells in the lung and blood vessels, while heparan sulphate, TGF-β and EGF are released from smooth muscle and thereby have potential direct influence over smooth muscle development in respiratory and cardiovascular conditions (Karnovsky & Edelman, 1994; Hamet *et al*., 1991; Fukuda, 1993; Gibbon & Dzau, 1994). EGF-induced vascular smooth muscle division is not sensitive to inhibition by heparin and related glycosaminoglycans (Karnovsky & Edelman, 1994). There is also evidence to suggest that the insensitivity of EGF-induced responses to heparin is attributable to the independence of EGF-induced mitogenesis on protein kinase C-mediated pathways (Wright *et al*., 1989). The heparin sensitivity of EGF-induced mitogenic responses in airway smooth muscle cells has not been examined. Glycosaminoglycans could, however, influence TGF-β-induced cell proliferation through inhibition of underlying processes of cell division, but also through the involvement of glycosaminoglycans in TGF-β-cell surface receptor interaction. There are three known receptors for TGF-β (I, II and III) (Sporn & Roberts, 1990; Miyazono *et al*., 1994). Types I and II are involved in signal transduction, while type III is a chondroitin sulphate/heparan sulphate proteoglycan (betaglycan). The proximity of heparan sulphate proteoglycans to the cell surface receptors I and II and direct involvement of betaglycan in the type III receptor raises the possibility that heparin and other glycosaminoglycans could influence TGF-β-receptor interaction.

We have previously demonstrated inhibition of serum-induced proliferation of sparsely populated ASMC by heparin-related glycosaminoglycans (Kilfeather *et al*., 1995a). In the present investigation we have examined DNA synthesis as an indication of S phase entry in response to TGF-β1 in sparse and confluent cell populations. We have examined the effect of heparin on the TGF-β1, EGF and serum-induced DNA synthesis of densely populated ASMC. In addition we have examined the effect of variation in confluent cell density on serum-induced and heparin inhibition of DNA synthesis.

Methods

ASMC were obtained from bovine trachealis muscle. Trachealis muscle was obtained from adult cattle following sacrifice with a captive bolt and dissected free of connective tissue under aseptic conditions using a dissection microscope. The muscle was washed in calcium- and magnesium-free phosphate buffered saline (PBS) and cut into $1-2$ mm^3 pieces for explanting. The explants were washed in medium 199 (M199) supplemented with gentamicin (50 mg 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 mg ml^{-1}) and amphotericin B (2.5 mg ml^{-1}), and maintained at 95% air/5% CO_2 in a humidified atmosphere at 37°C. Cells migrated from the explants after 1 week of plating. The explants were removed when cells migrating from the explant had reached a density of approximately 10%. For replating, cells were washed three times in PBS and removed from the plate by incubation with PBS and EDTA (1 mM) as previously described (San Antonio *et al*., 1992). The cells were replated for further population growth or directly onto a 96 well tissue culture plate at either 3×10^2 cells cm^{-2} for examination of sparse plated cells, or 10, 20 or 45×10^3 cells cm^{-2} for examination of densely plated (confluent) cells. Cells were examined up to passage four.

Smooth muscle cell identification

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

Smooth muscle cell division in sparse plated cells

Following plating for 24 h in 10% FBS at 300 cells cm^{-2}, cells were growth arrested by maintenance in M199 supplemented with BSA (0.25%) for 24 h. Cell division was reinitiated by incubation with FBS, TGF-β or EGF for examination of concentration-dependent stimulation of division. Responses in terms of change in cell number were assessed at day 5 after introduction of stimuli. For cell counting, cells were washed in phosphate buffered saline (PBS) with calcium and magnesium three times before fixing in methanol and air drying. Fixed cells were then stained with giemsa (GS-500, Sigma, 2 min). Stained cells were then washed in deionized water. All cells in each well were counted. Cell location while counting was assisted by creation of a grid on the underside of the wells with a scalpel blade.

Smooth muscle cell DNA synthesis in densely plated cells

Cells plated in M199 and FBS (10%) at 10, 20 or 45×10^3 cells cm^{-2} in a 96 well plate were incubated with 0.25% BSA for 24 h prior to incubation with compounds under investigation. Twenty-four hours following addition of compounds 0.1 μci of [^{3}H]-thymidine was added to each well. After incubation for a further 24 h, each well was washed three times with PBS containing Ca^{2+} and Mg^{2+}, followed by incubation in trichloroacetic acid (10%, 4°C) for 30 min. The wells were then washed twice with ethanol and incubated overnight with Ca_2CO_3 containing PBS. Uptake of [^{3}H]-thymidine into nuclear material was then measured by beta-scintillation counting.

Materials

All compounds including tissue culture components and antibodies for immunofluorescence were obtained from Sigma (Poole, U.K.). Tissue culture plastics were obtained from Bibby Sterilin (U.K.).

Data analysis

Comparison of [^{3}H]-thymidine uptake was conducted by ANOVA and Student's *t*-test. Statistical significance was accepted with $P<0.05$.

Results

Cells plated at low density (300 cells cm^{-2}) contained cells with and without contact with others, but confluent colonies were uncommon at this cell plating density. Cells plated at high density (10,000–45,000 cells cm^{-2}) were always in contact with others in at least a monolayer. Cells at 45,000 cell cm^{-2} formed areas of both multilayered and monolayered cells, previously referred to as 'hill and valley' formations. The possibility that stimulated DNA synthesis of confluent ASMC could differ between differing confluent cell densities was investigated. ASMC exhibited a marked difference in both basal and serum-stimulated DNA synthesis between plating densities of 10,000–45,000 cells cm^{-1} (Figure 1).

DNA synthesis in response to FBS, TGF-β and EGF

Smooth muscle cells plated under sparse and confluent conditions exhibited mitogenic responses to FBS (Figure 2A, B). The potential for differing actions of TGF-β1 on sparse and confluent ASMC populations was investigated. TGF-β1 (100 pM) enhanced smooth muscle DNA synthesis in confluent smooth muscle cells in the presence of FBS (0.5%) (Figure 2A). The stimulatory effect on DNA synthesis in confluent cells appears to be masked in the presence of maximally stimulating concentrations of FBS (10%), (Figure 2A). TGF-β1 (100 pM) inhibited FBS (10%)-induced smooth muscle division in sparse-plated smooth muscle cells (Figure 2B). The inhibitory effect of TGF-β1 on sparse-plated smooth muscle cells was not evident at low concentrations of FBS (0.5%) exhibiting minimal mitogenic stimulation (Figure 2B). The potential for TGF-β1-induced DNA synthesis in confluent ASMC in the absence of serum was also examined. TGF-β1 alone increased DNA synthesis in a concentration-dependent fashion between 1–100 pM (Figure 3). As previously observed in guinea-pig ASMC (Stewart *et al*., 1994), EGF increased ASMC DNA synthesis in confluent cells. This action was also concentration-dependent between 10–200 pM (Figure 3).

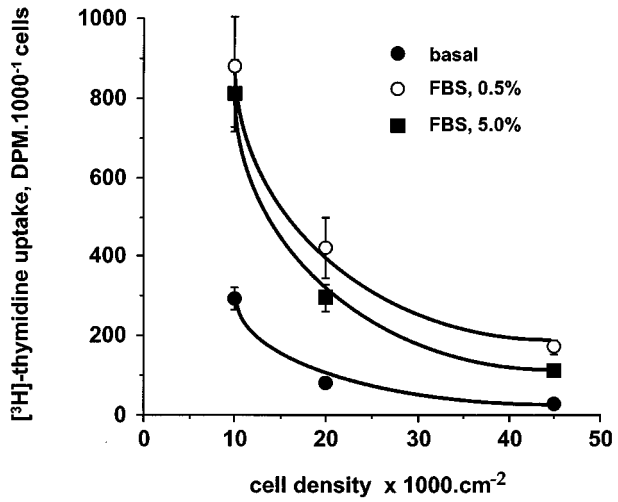

Figure 1 Effect of variation in confluent cell plating density on ASMC DNA synthesis. Cells were plated at 10,000 20,000 or 45,000 cells cm^{-2}. Following 24 h in serum-free conditions, incubation with FBS (0.5%, ■; or 5.0%, ○) or without FBS (control, ●) was for 24 h prior to measurement of [^{3}H]-thymidine uptake over 24 h. Points are means ± s.e.mean derived from six experiments conducted with ten replicates for each condition.

Effect of heparin on FBS, TGFβ and EGF-induced smooth muscle DNA synthesis

We have previously demonstrated heparin-induced inhibition of serum-stimulated ASMC division in sparsely populated cells (Kilfeather *et al*., 1995a). In the present investigation heparin inhibited DNA synthesis in the presence of 0.25% BSA and in response to FBS (0.5–5%) in confluent cells plated at 10,000–45,000 cells cm^{-2} (Table 1). However, in terms of fold-stimulation of basal DNA synthesis obtained in the presence of heparin, heparin only exhibited inhibition of DNA synthesis in response to serum at a plating density of 45,000 cells cm^{-2} (Figure 4). At 10,000 and 20,000 cells cm^{-2} heparin produced a consistent, but statistically insignificant increase in fold-stimulation of basal DNA synthesis obtained in the presence of heparin (Figure 4).

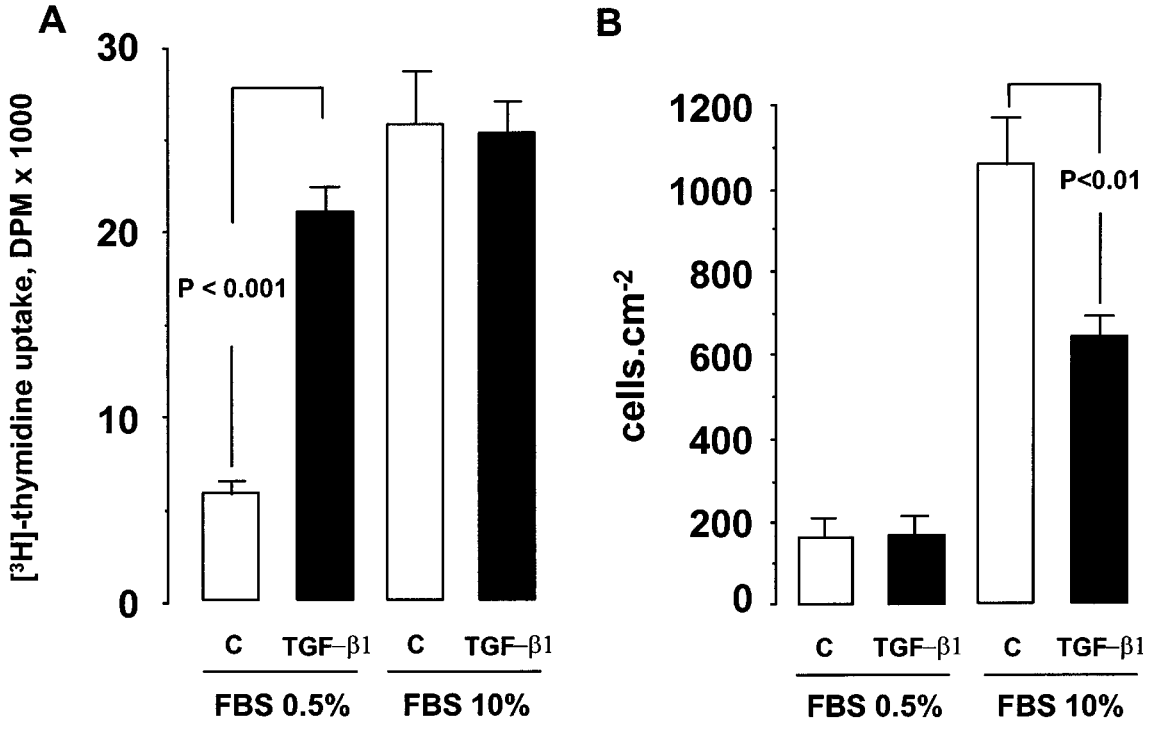

Figure 2 (A) Effect of TGF-β1 on [^{3}H]-thymidine uptake in confluent ASMC cells. Cells plated at 20,000 cell cm^{-2} were exposed to FBS (0.5 or 10%) in the presence or absence of TGF-β1 (100 pM) for 48 h and [^{3}H]-thymidine uptake measured over the final 24 h. Basal [^{3}H]-thymidine uptake was 1,820 ± 65. (B) Effect of TGF-β1 on proliferation of ASMC plated at low density. Cells (300 cm^{-2}) were incubated with FBS (0.5% or 10%) in the presence and absence of TGF-β1 (100 pM) for 5 days. Cells were counted by direct counting following Giemsa staining. Bars are mean ± s.e.mean. The values are derived from ten replicates in a representative experiment from three experiments.

Heparin inhibited DNA synthesis in response to TGF-β1 (100 pM) in a concentration-dependent fashion (Figure 5). Heparin did not influence ASMC DNA synthesis in response to EGF (70 pM) (Figure 6).

Effect of heparin on combined TGF-β1 and EGF-induced DNA synthesis

The effect of combined exposure to TGF-β1 and EGF on cell DNA synthesis was investigated together with the effect of heparin on the response to the combination. Co-stimulation with TGF-β1 and EGF produced a greater DNA synthesis than either of the two mitogens alone, suggesting at least an additive effect of TGF-β1 and EGF (Figure 6). The response to combined TGF-β1 and EGF exposure was significantly inhibited by heparin (Figure 6).

Discussion

We have demonstrated DNA synthesis in response to TGF-β1 on confluent ASMC. The concentration range in which TGF-β1-induced DNA synthesis was observed on confluent ASMC is consistent with the binding characteristics of TGF-β1 to cell surface TGF-β1 receptors (Ross *et al*., 1993a,b). The anti-mitogenic effect of TGF-β1 on sparsely plated ASMC is also consistent with effects on sparsely plated vascular smooth muscle cells (VSMC) (Assoian & Sporn, 1986). The differential effect of TGF-β1 on sparse and confluent VSMC has been attributed to differences in expression of cell surface receptors to TGF-β1 at these differing cell densities (Goodman & Majack, 1989). It is noteworthy that in densely populated ASMC we observed DNA synthesis in response to TGF-β1 at concentrations in the region of the reported Kd (88 pM) of the receptor population expressed in the densely populated vascular smooth muscle cells by Goodman & Majack (1989). This finding also underlines the importance of examining effects of factors influencing cell division in confluent in addition to sparse cell populations and at known cell densities.

TGF-β1 is a heparin binding growth factor and certain TGF-β1 binding sites are heparan sulphate-containing proteoglycans (Miyazono *et al*., 1994). In the context of growth factor-receptor interaction it is possible that heparin

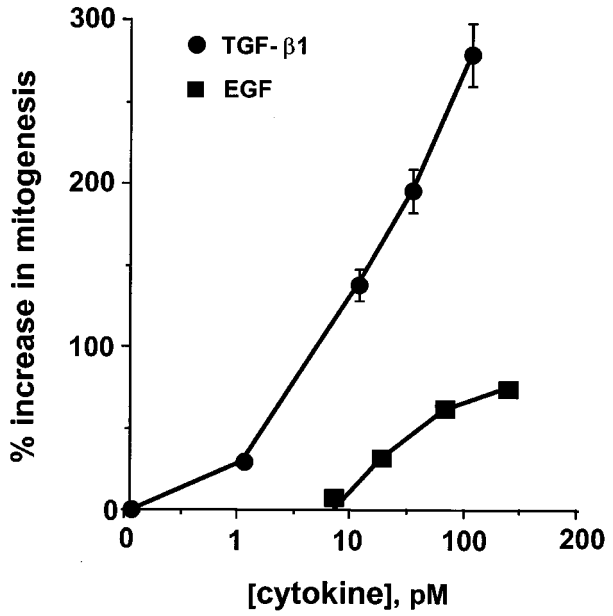

Figure 3 Concentration-effect relationship between TGF-β1 and EGF on ASMC DNA synthesis in the absence of FBS. Following 24 h in serum-free conditions confluent cells plated at 20,000 cells cm^{-2} were incubated with 0.25% BSA and increasing concentrations of TGF-β1 or EGF for 24 h prior to measurement of [^{3}H]-thymidine uptake over 24 h. Points represent means ± s.e.mean of data derived from three experiments using 10 replicates for each condition.

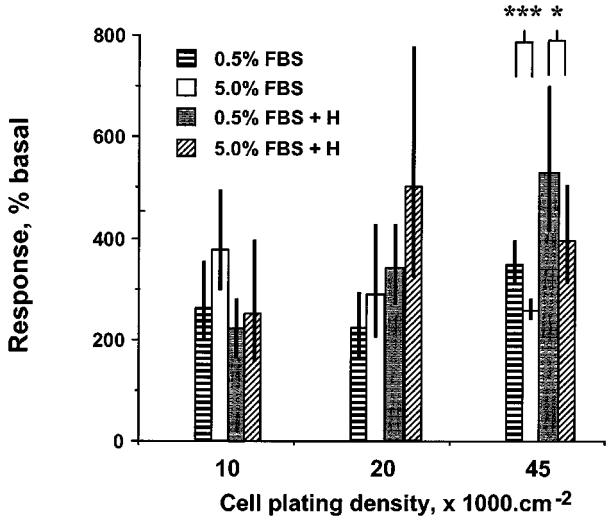

Figure 4 FBS-induced ASMC DNA synthesis in the presence and absence of heparin. Cells were plated at 10,000, 20,000 or 45,000 cells cm^{-2}. Following 24 h in serum-free conditions, cells were exposed to 0.25% BSA (basal) or FBS for 24 h in the presence or absence of heparin (100 μg ml^{-1}) prior to measurement of [^{3}H]-thymidine uptake over 24 h. Responses to FBS in the absence or presence of heparin are expressed as percent of corresponding basal obtained in the absence or presence of heparin respectively (0.5% FBS, ▤; 5% FBS, ▭; 0.5% FBS + heparin, ▭; 5% FBS + heparin, ▭). The effect of heparin on the basal responses under these conditions is shown in Table 1. Bars are geometric means with 95% confidence intervals. *, $P<0.05$; ***, $P<0.001$ compared to fold-stimulation in the absence of heparin.

Table 1 Effect of heparin (100 μg ml^{-1}) on basal and FBS-induced [^{3}H]-thymidine uptake

	% inhibition by heparin of [^{3}H]-thymidine uptake					
Cell plating density, × 1000 cm^{-2}	*Basal (BSA, 0.25% w/v)*	*n*	*FBS (0.25% v/v)*	*n*	*FBS (5.0% v/v)*	*n*
10	55.0 [45.0–67.8]	10	28.7 [17.9–46.0]	8	54.4 [38.5–76.7]	6
20	50.8 [44.6–57.7]	8	29.1 [19.0–44.7]	8	38.3 [29.3–50.2]	6
45	28.7 [18.7–42.0]	12	29.0 [20.3–41.8]	12	16.1 [9.4–27.5]	11

Effect of heparin (100 μg ml^{-1}) on DNA synthesis obtained in the presence and absence of FBS (0.5 or 5%) in cells plated at 10, 20 and 45,000 cells cm^{-2}. Values are geometric means with 95% confidence intervals. *n* denotes the number of experiments in which 10 replicates were used for each condition.

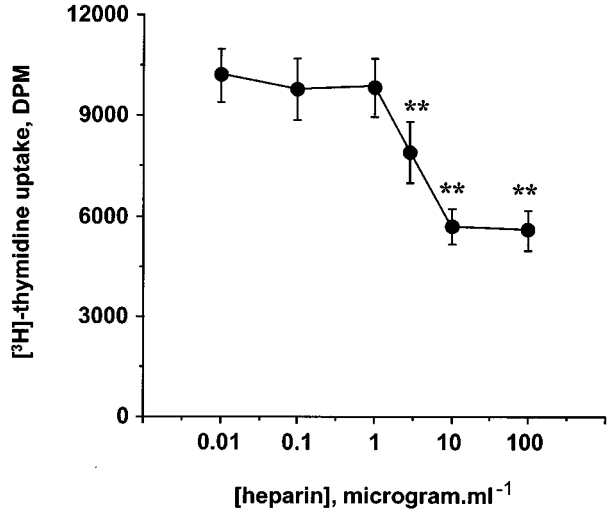

Figure 5 Concentration-effect relationship between heparin and TGF-β1-induced ASMC DNA synthesis. Following 24 h in serum-free conditions confluent cells plated at 20,000 cells cm^{-2} were incubated with TGF-β1 (100 pM) and increasing concentrations of heparin for 24 h prior to measurement of [^{3}H]-thymidine uptake over 24 h. Points represent means ± s.e.mean of ten replicates derived from five experiments. **, $P<0.01$ compared to TGF-β1 alone.

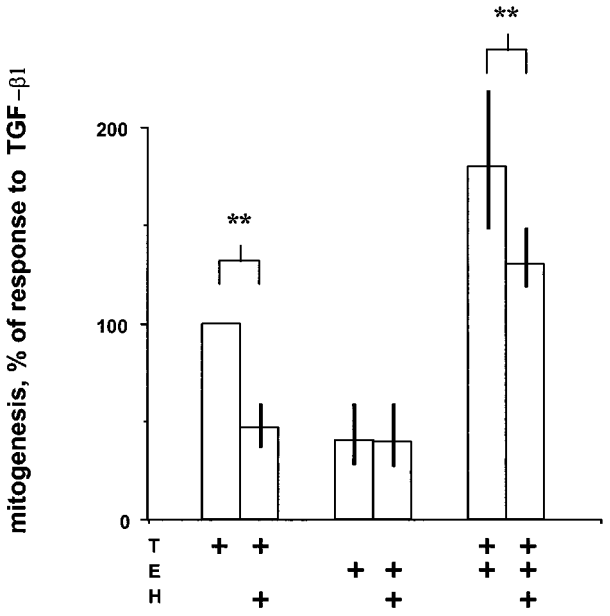

Figure 6 Effect of heparin on ASMC DNA synthesis in response to exposure to TGF-β1, EGF or combined exposure to TGF-β1 and EGF. Cells were plated at 20,000 cells cm^{-2}. Following 24 h in serum-free conditions, cells were exposed to 0.25% BSA (basal), TGF-β1 (T, 100 pM), EGF (E, 70 nM), or combined TGF-β1 and EGF in the presence or absence of heparin (H, 100 μg ml^{-1}) for 24 h prior to measurement of [^{3}H]-thymidine uptake over 24 h. DNA synthesis is expressed as fold-stimulation of control basal (0.25% BSA in the absence of heparin). Bars are geometric means with 95% confidence intervals of data derived from four experiments using ten replicates for each condition. **, $P<0.01$ compared to the absence of heparin.

influences the interaction between TGF-β1 and cell surface heparans. A facilitatory role of heparin has been observed in the interaction between another heparin binding growth factor, fibroblast growth factor (FGF) and corresponding receptor-heparan sulphate complex (Lindahl *et al*., 1994). In the present investigation we have observed an inhibitory effect of heparin against TGF-β1-induced ASMC over a concentration range previously observed for heparin against serum-induced ASMC and vascular smooth muscle cell division (Kilfeather *et al*., 1995a; Wright *et al*., 1989). This suggests that inhibition of interaction between TGF-β1 and a glycosaminoglycan-associated receptor or inhibition of signal transduction underlying mitogenic responses to TGF-β1 predominates in the action of heparin over any potential facilitatory effect that heparin could impart at the cell surface. This reflects the effects of heparin on vascular smooth muscle mitogenic responses to FGF in which heparin facilitates FGF-heparan binding site interaction, but inhibits mitogenic responses to FGF (Nugent & Edelman, 1992; Karnovsky & Edelman, 1994).

The sensitivity of smooth muscle cell mitogenesis to heparin appears to depend on the involvement of protein kinase C-mediated pathways in mediation of the mitogenic signalling (Wright *et al*., 1989). The mitogenic actions of TGF-β1 appear to be dependent upon the expression of platelet derived growth factor (PDGF) and this could contribute to the relative delay in mitogenic signalling by TGF-β1 compared to PDGF in vascular smooth muscle cells (Majack *et al*., 1990). PDGF mediates at least part of its mitogenic activity through protein kinase C-(PKC)-dependent pathways (Karnovsky & Edelman, 1994) and this could contribute to the sensitivity of responses to PDGF and TGF-β1 to heparin.

Our findings of a mitogenic action of TGF-β1 on ASMC are consistent with previous findings in bovine ASMC (Black *et al*., 1996). Cohen *et al*., 1997, however, found that TGF-β1 inhibited human ASMC DNA-synthesis at a similar confluent cell density to that used in the present investigation and following a similar duration of exposure to TGF-β1 (40 vs 48 h). It is difficult to interpret the marked differences in findings apart from a possible species difference in ASMC responses to TGF-β1. The disparities found within studies concerning differential effects of duration of TGF-β1 exposure (Black *et al*., 1996), cell density (Assoian & Sporn, 1986 (VSMC) and present investigation (ASMC) and potential species-related differences emphasise the condition-dependent nature of proliferative responses to TGF-β1. The ubiquitous relationship between TGF-β1 induction of PDGF expression suggests a predominance of a positive mitogenic action of TGF-β1 in general, particularly in VSMC and ASMC where PDGF plays such a significant role in maintaining proliferation (Hirst *et al*., 1992).

We have shown that cells at differing levels of confluence within a plating range of 10,000–45,000 cells cm^{-2} exhibit differences in basal and serum-induced DNA synthesis. This variation in DNA synthesis could reflect increasing levels of contact inhibition with increasing cell density, involving increased differentiation (Thyberg *et al*., 1990) and production of smooth muscle cell-derived anti-mitogenic factors such as heparin-related glycosaminoglycans (Campbell *et al*., 1992). The potential for humoral release from airway smooth muscle tissue to inhibit ASMC DNA synthesis has been demonstrated (Kilfeather *et al*., 1995b) and could involve ASMC-derived glycosaminoglycans. Others have demonstrated ASMC as a source of PGF_2 (Delamere *et al*., 1994), which has been found to inhibit human ASMC mitogenesis (Tomlinson *et al*., 1995), although we have not found anti-mitogenic activity of PGE_2 against bovine ASMC (Kilfeather *et al*., 1996).

The inhibitory effect of heparin on serum and TGF-β-stimulated DNA synthesis was observed at each level of confluence in terms of absolute increases in DNA synthesis above basal (Table 1), and in terms of fold-stimulation of basal DNA synthesis at a cell plating density of 45,000 cells cm^{-2} (Figures 4 and 6). The stability of FBS-induced fold-stimulation of basal DNA synthesis in the presence of heparin at 10,000 and 20,000 cells cm^{-2} indicates similar inhibitory effects of heparin against the underlying basal and FBS-stimulated DNA synthesis at these cell densities. In cells plated at 45,000 cells cm^{-2} the decreases in FBS-induced fold-stimulation of corresponding basal DNA synthesis by heparin demonstrate an effect of heparin on serum-induced responses that is greater than the effect on corresponding basal DNA synthesis.

The expression of growth factors by smooth muscle suggests autocrine stimulation of mitogenesis and release of mitogenic factors from bovine ASMC has been demonstrated (Kilfeather *et al*., 1996). Therefore heparin inhibition of basal DNA synthesis could involve interruption of autocrine growth factor activity, while interruption of both autocrine-derived and exogenously applied mitogen-activated pathways could be involved in the inhibition of FBS-induced DNA synthesis by heparin.

The insensitivity of EGF-induced responses to heparin has previously been observed in vascular smooth muscle cells and has been attributed to the independence of EGF-induced mitogenesis from PKC-dependent pathways (Karnovsky & Edelman, 1994). In the present investigation a submaximal concentration of EGF was used and therefore excludes the possibility that insensitivity of responses to EGF are due to the use of supramaximal concentrations of EGF. The responses to EGF above basal were significantly smaller than those to TGF-β1 or FBS excluding the possibility that responses to EGF were insensitive due to their magnitude. TGF-β1 and EGF have demonstrated synergistic activation of mitogenic signals in other cell types (Ranganathan & Getz, 1990). The net DNA synthesis obtained in the presence of both mitogens in the present investigation, however, is suggestive of an additive rather than synergistic interaction. The possibility that EGF can override effects of heparin on underlying basal DNA synthesis should be considered. Responses to TGF-β1 and EGF combined exhibit a significant reduction in the presence of heparin, despite the heparin-insensitivity of responses to EGF in isolation. Therefore, heparin retains inhibitory activity against exogenously applied TGF-β1 and therefore possibly underlying autocrine mitogenic activity in the presence of EGF. EGF-induced increases in thymidine uptake are relatively small under these conditions compared to those in response to TGF-β1 or serum. The relevance of an approximately 50% increase in thymidine uptake is difficult to estimate, but this relatively small signal from EGF could indicate that other factors inducing higher responses, such as TGF-β1, are more influential over ASMC mitogenesis under conditions in which both growth factors are expressed (Elovic *et al*., 1994; Taylor *et al*., 1994; Ohno *et al*., 1992). On the other hand, exposure *in vivo* to a range of growth factors simultaneously is probable and the summation of growth factor effects and duration of exposure in clinical conditions involving tissue remodelling over decades may be as important as the magnitude of acute responses to a single growth factor.

Concerning use of different cell densities in studies of mitogenesis and proliferation, the present study demonstrates significant qualitative and quantitative differences between events relating to mitogenesis in confluent cell populations of differing densities. In view of the density of cells in tissues, it could be argued that examination of events at the highest possible smooth muscle density in culture, in which cell populations exhibit areas of multi- as opposed to monolayering, would be more likely to reflect events in tissues *in vivo*.

In the context of airway remodelling, the present findings demonstrate that TGF-β1 and EGF could be involved in maintenance of ASMC proliferation. A contribution of TGF-β1 above normal is not supported by the absence of a generalized increase in levels of TGF-β1 expression in airways of asthmatics (Aubert *et al*., 1994). However, these growth factors are expressed in cells involved in airway inflammation in asthma including eosinophils and macrophages (Elovic *et al*., 1994; Taylor *et al*., 1994; Ohno *et al*., 1992) and eosinophils in nasal polyps and chronically inflamed upper airways exhibit elevated levels of TGF-β1 (Elovic *et al*., 1994; Ohno *et al*., 1992). Heparin and heparin-related glycosaminoglycans could provide inhibition of TGF-β1-induced proliferation or responses to other growth factors utilizing similar heparin-sensitive signal transduction, but not EGF-like mitogenic activity. The inhibitory effect of heparin on FBS-induced division could be a demonstration of the possible wide spectrum of activity of heparin-related glycosaminoglycans against ASMC mitogenesis, since serum contains many of the known growth factors for smooth muscle derived from platelets including PDGF, TGF-β1 and FGF (Assoian *et al*., 1983; Gospodarowicz *et al*., 1975) all of which demonstrate sensitivity to heparin (Karnovsky & Edelman, 1994). In addition, serum has a large thrombin content, which accounts for a large proportion of the serum-stimulated mitogenesis of lung-derived fibroblasts (Paris *et al*., 1988). Thrombin is also a mitogen for ASMC (Tomlinson *et al*., 1994) and thrombin receptors utilize both PKC-dependent and independent transduction pathways. Therefore, the actions of heparin against serum could involve inhibition of a range of serum-containing growth factors including thrombin.

In conclusion, the consistent inhibitory effect of heparin against basal levels of ASMC DNA synthesis indicates that heparin-related glycosaminoglycans are active in regulating even autocrine-derived ASMC DNA synthesis. The DNA synthesis induced by TGF-β1, but not of EGF in ASMC is heparin sensitive. TGF-β1 and EGF are potential mitogens for ASMC and expression of TGF-β1 and EGF in airways and infiltrating inflammatory cells suggests that these mitogens could influence tissue remodelling involving airway smooth muscle.

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