Airway smooth muscle cell proliferation: characterization of subpopulations by sensitivity to heparin inhibition

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Halayko, Andrew J., Edward Rector, and Newman L. Stephens. Airway smooth muscle cell proliferation: characterization of subpopulations by sensitivity to heparin inhibition. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L17–L25, 1998.—Growth and maturation state of airway smooth muscle cells (SMCs) are determinants of asthma pathophysiology. Heparin reduces airway SMC proliferation and arterial SMC replication and phenotypic modulation. Distinct arterial SMC subtypes, differing in heparin sensitivity, have been characterized. We assessed the cellular mechanisms underlying the growth and phenotype of heparin-treated canine tracheal myocytes in primary culture. Heparin reduced replication by 40%. Immunoblot assay of myosin, actin, and myosin light chain kinase revealed that heparin had no effect on rapid spontaneous phenotypic modulation after the cells were plated. Heparin increased cellular protein and vimentin contents in confluent cultures, suggesting that it may induce hypertrophic growth. Cell cycle analysis revealed that heparin decreased serum-stimulated replicating myocyte number by 40%. Also, G2-M transit was 20% slower for the set of SMCs that proceeded past G2 in the presence of heparin. These data indicate that heparin does not inhibit airway SMC replication by blocking modulation from the contractile state. Moreover, airway smooth muscle is composed of distinct SMC populations differing in mitogen and antiproliferative mediator responsiveness. Identification of functionally divergent subgroups suggests that distinct sets of SMCs may contribute differentially to airway physiology and pathophysiology.

flow cytometry; asthma; airway remodeling; cell cycle; phenotype; smooth muscle cell heterogeneity

HEPARINS ARE HIGHLY ANIONIC glycosaminoglycans composed of alternating residues of either β-D-glucuronic or α-L-iduronic acid and α-D-glucosamine linked by 1,4-glycosidic linkages with O- and N-sulfation and/or acetylation (13). They have been known to possess anti-inflammatory properties for some time (9), and they inhibit arterial smooth muscle cell (SMC) migration and proliferation in vitro and during arterial remodeling after in vivo injury (26).

Mechanisms for heparin-mediated inhibition of proliferation have been studied extensively in vascular smooth muscle. Analysis of cell cycle progression in cultured arterial SMCs revealed that heparin slowed transit to the S phase, but its major effect on proliferating cells was to inhibit the rate of progression from the G2 phase through the M phase (14). Binding and internalization of heparin via receptor-mediated endocytosis appears to be a requirement for antiproliferative activity (24). With internalization, it does not localize in the nucleus but is dispersed throughout the cytosol, with accumulations in perinuclear regions and at sites of cell contact or cellular adhesion (3). Heparins also inhibit the binding of platelet-derived growth factor and the expression of receptors for epidermal growth factor in arterial SMCs (11, 30). A concomitant property of heparins on arterial SMCs is inhibition of spontaneous phenotypic modulation in primary culture and in vivo in response to injury (5, 6).

Arterial smooth muscle is composed of myocytes differing in morphology, electrophysiological properties, and growth characteristics (2, 20, 27). Recent reports indicate that distinct heparin-sensitive and -insensitive arterial SMC subpopulations also exist (3, 4) and that clonal cell cultures of each subtype have been established (32). Heparin-sensitive SMCs appear to differ from their heparin-insensitive counterparts in the ability to upregulate expression of heparin receptors when exposed to exogenous heparins (24, 32). Increased numbers of receptors on heparin-sensitive cells result in increased heparin internalization, with subsequent modulation of gene expression for extracellular matrix proteins, and inhibition of cellular replication and migration (26). Heparin-resistant arterial SMCs display lower levels of immunostaining for α-smooth muscle actin (sm-α-actin), are smaller, and proliferate more rapidly than control SMC cultures in response to serum and phorbol ester stimulation (32).

Asthma is characterized by airway remodeling that may include thickening of the airway smooth muscle layer due to SMC hypertrophy and hyperplasia (10). Factors that regulate airway SMC proliferation have been reported (19). Several inhibitors of airway SMC proliferation, including prostaglandin E2 (12), β2-adrenergic agonists (33), and heparin (21, 23), have been identified. Heparin is stored in considerable quantities in pulmonary mast cells, and the amount released after antigen challenge of sensitized human lung slice preparations appears to be sufficient to inhibit airway SMC proliferation (15). Heparin also prevents acute, antigen-induced airway hyperresponsiveness in Ascaris suum-sensitized sheep (1).

Maximum inhibition of serum-stimulated, cultured airway SMC proliferation and DNA synthesis appears to be ~40% as determined with relatively high doses of heparin (>500 µg/ml) (21, 23). Although the existence of endogenous antimitogenic factors are recognized, the role they play in determining the responsiveness of airway myocytes to mitogenic stimuli in vivo and in vitro is not clearly established. Indeed, other than quantitative information concerning the dose responsiveness of cultured airway SMCs to heparin treatment, no information is known concerning the cellular mechanisms by which heparins mediate reduced SMC proliferation.
Understanding the cellular mechanisms of replication inhibition and regulation of the SMC phenotypic state by heparin may be of benefit in elucidating the role of the endogenous glycosaminoglycan in the pathogenesis of airway remodeling in chronic asthma. We hypothesized that exogenous heparins may inhibit replication of specific, heparin-sensitive subsets of cultured airway SMCs through defined pathways that regulate cell cycle progression. To determine the effects of exogenous heparin on airway myocyte replication and phenotypic modulation, canine airway SMC primary cultures were established. Temporal patterns of phenotypic marker protein expression were determined by quantitative immunoblotting. The effects of heparin on cell cycle progression and DNA synthesis of serum-stimulated airway myocytes were assessed with propidium iodide (PI) DNA staining and 5-bromo-2’-deoxyuridine (BrdU) uptake in conjunction with flow cytometry. The results obtained demonstrated that heparin had no effect on the spontaneous phenotypic modulation of myocytes in primary culture but appeared to induce a hypertrophic state that correlated with increased cellular protein and vimentin content. Moreover, the existence of airway myocyte subpopulations differing in proliferative responsiveness to serum and heparin was identified.

**METHODS**

**Materials**

Sodium heparin, purified from porcine intestinal mucosa (relative molecular weight 6,000–30,000, 184.6 anticoagulant units/mg), was purchased from Life Technologies-GIBCO BRL (Burlington, ON). Fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal anti-BrdU antibody was purchased from Becton-Dickinson (San J ose, CA).

**Canine Airway SMC Primary Culture**

Primary cultures were prepared as previously described by Halayko et al. (18). Tracheae were excised from anesthetized 6- to 12-mo-old mongrel dogs. Myocytes were isolated from cleaned tracheal muscle by enzymatic digestion in Hanks’ balanced salt solution containing 600 units/ml of collagenase, 8 units/ml of type IV elastase, 1 unit/ml of type XXVII Nagarse protease, 100 units/ml of penicillin, 100 mg/ml of streptomycin, and 50 µg/ml of gentamicin. The cells were seeded onto plastic culture plates at 1 × 10⁴ cells/cm² and were grown in culture medium [Dulbecco’s modified Eagle’s medium (DMEM) and 10% fetal bovine serum (FBS)] containing antibiotics as noted above at 37°C in a humidified atmosphere consisting of 95% air-5% CO₂. For all studies, only unpassaged cultures were used. Cell counts were determined with a hemocytometer.

For experiments in which growth-arrested cells were required, the culture medium (DMEM-10% FBS-antibiotics) was aspirated, and the plates were washed with phosphate-buffered saline (PBS). Serum-free arrest medium (Ham’s F-12 containing 5 µg/ml of human recombinant insulin, 5 µg/ml of h-transferrin, 5 ng/ml of selenium, and antibiotics) was then added. All cultures were arrested for 96 h before being used. (Note that flow cytometric analysis revealed that 72 h were required to achieve >80% arrest, and after 96 h, ~95% of all cells were arrested in the G₀/G₁ phase of the cell cycle.)

**Cell Cycle Analysis**

Cell preparation. Primary cultures of tracheal SMCs were grown to 75% confluence, then were growth arrested for 96 h in serum-free medium; sodium heparin was also included in the arrest medium of one-half of the plates. (Note that in preliminary experiments, heparin was not included in the arrest medium and was added only on serum stimulation of arrested cells. The results obtained revealed no qualitative or quantitative differences from those obtained when heparin was included in the arrest medium.) After arrest, the medium of the control cultures was replaced with DMEM-10% FBS to stimulate cell cycle entry; the medium of the treated cultures contained 1–100 µg sodium heparin/ml. Plates for each group were selected thereafter (0–40 h) to assess cell cycle distribution. The cells were lifted from 100-mm culture plates with 0.05% trypsin-0.53 mM EDTA. The cells were pelleted (5 min at 800 g at 4°C), then resuspended in ice-cold PBS and pelleted again. The cells were resuspended in 200 µl of Western Blot Analysis of Marker Protein Content

Phenotypic marker content was assessed by Halayko et al. (18). To assess the effects of heparin on temporal protein expression, 18–24 h after initial cell seeding and thereafter, DMEM was supplemented with 100 µg/ml of sodium heparin. Crude protein homogenates were obtained at various time points after seeding with a buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.6, 0.3% sodium dodecyl sulfate (SDS), 0.6 M β-mercaptoethanol, 20 µg/ml of leupeptin, 50 µM phenylmethylsulfonyl fluoride, 50 mg/ml of soybean trypsin inhibitor, and 10 µg/ml of deoxyribonuclease I. Protein concentration was estimated spectrophotometrically with the BioRad protein assay kit.

**Proteins were size fractionated by discontinuous SDS-polyacrylamide gel electrophoresis as previously described by Halayko et al. (18). The separating gels used were 5% for smooth muscle and nonmuscle myosin heavy chain (sm-MHC and nm-MHC, respectively), 7.5% for myosin light chain kinase (MLCK), and 10% for vimentin and sm-α-actin. Samples were prepared for electrophoresis by diluting 1:3 with 4 × sample buffer (1.0 M Tris-HCl, pH 6.8, 8% SDS, 45% glycerol, 2.86 M β-mercaptoethanol, and 0.02% bromphenol blue) and heating at 95°C for 1 min.**

**Quantitative profiles of phenotypic markers were obtained by electroblotting SDS-polyacrylamide gel electrophoresis fractionated proteins to nitrocellulose (18). Blotting was carried out at 4–8°C in 25 mM Tris-198 mM glycine-20% methanol, pH 8.3. After transfer, the blots were blocked overnight at 4°C in Tris-buffered saline (TBS; 20 mM Tris-500 mM NaCl, pH 7.5) containing 0.1% Tween 20 and 3% nonfat dried milk. The blots were then incubated in primary antibody diluted in TBS-Tween 20–1% nonfat dried milk. A variety of primary antibodies were used: 1) monoclonal mouse anti-sm-α-actin (clone asm1, Boehringer Mannheim Canada, Laval, PQ); 2) monoclonal mouse anti-vimentin (Amershaw Canada, Oakville, ON); 3) polyclonal rabbit anti-nm-MHC (22); 4) monoclonal mouse anti-MLCK (clone K36, Sigma Immunochemicals, St. Louis, MO); and 5) monoclonal mouse anti-sm-MHC (Sigma Immunochemicals).

The blots were stained with biotinylated secondary antibodies and horseradish peroxidase-conjugated streptavidin in the tertiary step, followed by enhanced chemiluminescence substrates. Chemilumigrams were developed on Hyperfilm-ECL (Amershaw Life Sciences, Oakville, ON). An LKB Ultrascan XL laser densitometer was used to scan the films. All values obtained were normalized to total protein load and mean cell number on each plate.

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ice-cold saline and were fixed by adding the suspensions to 5 ml of ice-cold 70% ethanol. The fixed cell suspensions were stored for at least 24 h at −20°C. Five experiments with different primary cultures were completed.

Analysis of cell cycle distribution. The fixed cells were pelleted (10 min at 1,000 g) and then resuspended in 1 ml PBS-20 mM EDTA-0.05% Tween 20-15 µg/ml of PI-50 µg/ml of ribonuclease A to stain for DNA. The cell suspension was triturated through a 24-gauge needle, then filtered through 70-µm nylon mesh. The cells were stained for ~1 h at room temperature (RT) before analysis. An EPICS model 753 fluorescence-activated cell sorter (FACS; Coulter Electronics, Hialeah, FL) with a 488-nm (500-mW) line from an argon laser was used. Forward versus 90° light-scatter histograms were used to gate on intact cells and eliminate debris while peak versus integrated PI-derived fluorescence signals were used for doublet discrimination gating. PI fluorescence was determined through a 610-nm-long pass filter. Fluorescence histograms of 256-channel resolution were collected for at least 5,000 cells that satisfied the light-scatter and doublet discrimination gating criteria. The distribution of cells in phases G0/G1, S, and G2 of the cell cycle was determined with the PARA 1 DNA analysis program (Coulter Electronics).

Cell preparation and BrdU incorporation. The cells were then stimulated to proliferate by adding DMEM-10% FBS to each 100-mm plate. Sodium heparin (100 µg/ml) was included in one-half of the plates. Earlier studies in which only PI staining had been used revealed that cells completed first mitosis ~36–38 h after serum stimulation (see Fig. 3). Because it was our intention to examine cell cycle progression during rapid growth periods, the cells were studied 72 h after serum stimulation. Sixty-eight hours after serum stimulation, the culture medium was supplemented with 10 µM BrdU and 10 µM 5-fluoro-2′-deoxyuridine. The plates were incubated at 37°C for 4 h after the addition of the labeling solution, then the cells were lifted by trypsin-EDTA treatment, fixed in 70% ethanol, and stored overnight at −20°C as described in Cell preparation. Five experiments with different primary cultures were carried out.

Analysis of cell cycle distribution from BrdU incorporation. The fixed cells were pelleted (1,200 g for 12 min at 4°C) and resuspended in 500 µl of ice-cold saline by vortexing. Nuclear DNA was denatured by adding 500 µl of 4 M HCl-1% Triton X-100 at RT; cell clumps were disrupted with gentle trituration through a 24-gauge needle. After 30 min, 1 ml of saline was added, the cells were centrifuged (1,000 g for 5 min), and the pellet was resuspended in 1.5 ml of Na2B4O7 (pH 8.5). After another centrifugation (1,000 g for 5 min), the cells were resuspended in cold PBS-1% bovine serum albumin-0.5% Tween 20. For immunostaining of incorporated BrdU, the cell suspensions were transferred to microcentrifuge tubes and monoclonal FITC-conjugated mouse anti-BrdU was added as per manufacturer’s instructions. The cells were then incubated in the dark for 2 h at RT, then diluted with PBS-1% BSA-0.5% Tween 20 and filtered twice through 70-µm nylons mesh. The cells were centrifuged (1,000 g for 7 min) and stained for DNA by resuspension in the PI-staining solution described in Analysis of cell cycle distribution.

Analysis of immunofluorescence was carried out with an EPICS model 753 FACS (Coulter Electronics) equipped with a 488-nm (500-mW) line from an argon laser. To enable the detection of FITC fluorescence, a 525 ± 10-nm band-pass filter was used. PI fluorescence was determined through a 610-nm-long pass filter as described in Analysis of cell cycle distribution. Bivariate histogram data collection and analysis was carried out with the gating parameters described in Analysis of cell cycle distribution.

Statistical Analyses

All measurements made from the chemilumigrams with laser densitometry were completed in triplicate from samples obtained from four to six different animals. The values reported are means ± SE. Comparisons between individual mean values from different treatments and between mean values of parameters measured by FACS analysis were done with unpaired two-tailed Student’s t-test. Comparisons among multiple values from different treatments on SMCs were done by one-way analysis of variance combined with Duncan’s new multiple range test. Statistical significance for all analyses was accepted at P < 0.05.

RESULTS

Effects of Heparin on Growth Characteristics and Phenotypic Modulation

To measure the proliferative response of canine airway SMCs, cell number and total protein in each culture plate were measured. Cells grown in control conditions (10% FBS) started to increase in number ~5
days after they were plated. At confluence, ~9 days after seeding, cell density was between 7.5 and 10.4 × 10^4 cells/cm² (Fig. 1A). The cultures exhibited typical hill-and-valley morphology and possessed numerous areas of multilayered, focal overgrowth. The total protein extracted from each plate increased temporally in a pattern similar to that seen for the increase in cell number (Fig. 1B).

The inclusion of 100 µg/ml of heparin to the culture medium delayed the onset of SMC proliferation as measured by cell number. Onset occurred 8 days after initial seeding, a delay of 3 days from that seen for the control cultures (Fig. 1A). Confluence was reached 4 days after the cell numbers first began to increase; this period of time was comparable in time to the length of the proliferative phase seen in the control cultures. Interestingly, at confluence (day 12), the mean cell density of the heparin-treated plates was only between 5.4 and 6.8 × 10^4 cells/cm², ~35% lower than that seen in the control plates (P < 0.01; n = 4). At confluence, the heparin-treated cultures did not exhibit a distinct hill-and-valley morphology; instead, the cultures had a cobblestone appearance and lacked the focal, multilayered overgrowths seen in control airway SMC cultures.

Postconfluence, the growth curves for the heparin-treated cultures remained flatter than those in the control cultures, indicating a greater tendency for the cessation of proliferation after cell-to-cell contacts had been reestablished. In contrast to the differences between the heparin-treated and control cells in cell number, the temporal pattern of protein accumulation was nearly the same for the first 10 days in primary culture (Fig. 1B). These data suggest that the cellular protein content was increased in the heparin-treated cultures.

Temporal expression of the airway SMC phenotypic markers sm-MHC, sm-α-actin, MLCK, nm-MHC, and vimentin was assessed by immunoblot assay. In the control cultures, contractile SMCs modulated to an immature phenotype as indicated by a gradual loss of sm-MHC, sm-α-actin, and MLCK and an accumulation of nm-MHC and vimentin (Fig. 2). The cells began reaccumulating sm-MHC, sm-α-actin, and MLCK after confluence was reached, whereas vimentin content began to decrease, indicating that the SMCs reverted partially to their original phenotypic state. Inclusion of heparin had no effect on the loss of sm-MHC, sm-α-actin, and MLCK or on the accumulation of nm-MHC in

Fig. 2. Effects of heparin on temporal expression of phenotypic marker proteins in primary airway SMC cultures. A and B: smooth muscle myosin heavy chain (200–204 kDa) and nonmuscle myosin heavy chain (196–198 kDa), respectively; total protein loaded in each well was 7.5 µg. C and E: α-smooth muscle actin (43 kDa) and vimentin (57 kDa); respectively; total protein loaded in each well was 5 and 10 µg, respectively. D: myosin light chain kinase (138 kDa); total protein loaded in each well was 7.5 µg. Values are means ± SE from 4 different experiments. Data on y-axis are in arbitrary units obtained by normalizing laser densitometric data with total protein loaded per well and with the number of cells on the plates.
the cultured cells (P > 0.6 by analysis of variance; n = 4 cultures). Although the temporal pattern of vimentin content was qualitatively similar in both groups, cellular vimentin content was 50% higher after confluence was reached in the heparin-treated airway SMCs (P < 0.05 by Duncan’s new multiple range test; n = 4 cultures; Fig. 2E). These data strongly suggest that heparin did not inhibit spontaneous modulation from the contractile phenotype on seeding; however, based on differences in vimentin expression postconfluence, it may affect density-dependent phenotypic alterations usually seen in primary cell culture.

Effects of Heparin on Airway SMC Cell Cycle Progression

The effects of heparin on cell cycle progression of airway SMCs were examined by flow cytometry. Subconfluent (75%) cultures were growth arrested for 96 h in serum-free medium, then stimulated with DMEM-10% FBS with or without added heparin. Cell cycle distribution was determined by measuring cellular DNA content by PI staining (Fig. 3). The maximum fraction of tracheal SMCs in the control cultures that traversed the first cell cycle in response to serum stimulation, as determined by the decrease in the number of SMCs in G0/G1, was 70% (Figs. 3A and 4). This finding suggests that 30% of all the cultured tracheal myocyte cells were refractory to the mitogenic factors present in FBS. Hence, hereafter, a distinction will be made between the cells that entered the first cell cycle after serum stimulation, called serum-responsive SMCs (srSMCs), and those cells that did not progress through the first cell cycle after serum stimulation, called serum-insensitive SMCs.

Tracheal srSMCs entered the S phase 18 h after serum stimulation. The cells entered the G2 phase 25 h after stimulation and returned to G0/G1 36–40 h after the serum had been added to the arrested cultures (Fig. 3A). Therefore, the cell cycle time for serum-stimulated tracheal srSMCs appeared to be ~38 h.

Heparin (100 µg/ml) had little effect on the rate of SMC transit through the S phase and into G2; however, the total number of srSMCs that entered the cell cycle was inhibited by 40.3 ± 5.1% (Figs. 3B and 4). This suggests that approximately one-half of the proliferative srSMCs were heparin sensitive and could be blocked from cell cycle entry by exogenous heparin. The remaining srSMCs appeared to be heparin resistant; however, examination of the DNA histograms obtained for the heparin-treated cells indicated that the number of cells in G0/G1 did not begin to increase again until

Fig. 3. Cell cycle analysis of primary cultured tracheal SMC proliferation by flow cytometry. Representative DNA histograms show distribution of propidium iodide (PI)-stained SMCs at various times after serum stimulation. A: progression of control airway SMCs through cell cycle. B: progression of airway SMCs through cell cycle in presence of 100 µg/ml of heparin. Each histogram represents data obtained from 5,000 cells. PI fluorescence is plotted on x-axis with 256-channel resolution.

Fig. 4. Fraction of arrested SMCs that entered cell cycle from G0 to G1 after serum stimulation in presence and absence (control) of heparin. Values are means ± SE of 3 experiments done in triplicate. *Significantly different from control, P < 0.05.
44–48 h after serum stimulation (Figs. 3B and 4). Hence the cell cycle time of srSMCs that at first appeared to be heparin resistant was 6–10 h longer than that seen under control conditions. This finding suggests that proliferation of most srSMCs may be affected by exogenous heparin; however, a heterogeneous response pattern exists in which ~40% of the cells are blocked from cell cycle entry while another subset of cells does enter the cell cycle, but the cells progress more slowly than usual.

The maximum number of heparin-insensitive srSMCs that reached the G2/M phase was attained 28 h after serum stimulation (Fig. 3A). Therefore, to assess the concentration-response relationship between heparin and inhibition of cell cycle entry, the fraction of SMCs in the cell cycle 28 h after serum stimulation in the presence of different concentrations of heparin was compared (Fig. 5A). Concentration-response characteristics of heparin-mediated inhibition of cell cycle entry of srSMCs demonstrated an all-or-none character (Fig. 5B). That is, 1 µg/ml of heparin was as potent as 50 µg/ml of heparin in blocking cell cycle entry. In fact, the lowest dose of heparin used (1 µg/ml) was only 20% less potent than the concentration that exhibited maximum antiproliferative activity (100 µg/ml).

Bivariate Analysis of Cell Cycle Distribution

Bivariate dot blots demonstrated clearly that 68–72 h after serum stimulation, the proportion of SMCs in the latter phases of the cell cycle is increased in the presence of heparin (100 µg/ml; Fig. 6). The proportion of cells between the late S phase and the M phase was doubled in the presence of heparin (Fig. 7). These data suggest that the time for proliferating cells to progress through G2 and into the M phase of the cell cycle is increased by heparin treatment. This corroborates those data observed earlier in which the cell cycle length of srSMCs, which were not blocked from cell cycle entry by heparin, appeared to be increased by 6–10 h.

**DISCUSSION**

The results reported here confirm that exogenous heparins possess antiproliferative activity for airway SMCs. In addition, data suggest that subpopulations, differing in sensitivity to heparin, comprise the total airway SMC population in early-passage primary cultures. With heparin treatment, cultured airway SMCs grew in a cobblestone pattern and confluent cell density was 35% lower than in the control plates. The control cultures exhibited a typical hill-and-valley morphology, characterized by areas of focal overgrowth that were not evident in the heparin-treated cultures. Interestingly, confluent cultures of heparin-resistant arterial SMCs lack a hill-and-valley appearance and have lower cell densities than confluent control cultures (4, 32). This correlation with our observations supports the possibility that heparin-insensitive airway SMCs contribute specifically to the expansion of primary cultures grown in the presence of heparin.

On the basis of electron-microscopic analysis and immunocytochemical data, heparin appears to inhibit arterial SMC phenotypic modulation (5, 14). Although exogenous heparins effectively inhibit arterial SMC proliferation after balloon catheter-induced injury, acute, transient switching from α- to β-actin transcription immediately after injury is not blocked (6). However, heparin treatment induces increased reexpression of sm-α-actin and sm-MHC in vascular SMCs after arterial injury and in culture (6, 8, 14). San Antonio et al. (32) reported that the percentage of heparin-resistant vascular SMCs that expressed sm-α-actin increased dramatically after exposure to heparin. It has been postulated that heparin maintains arterial SMCs in a quiescent state by controlling actin and protein expression (7).
Immunoblot data from our studies indicate that spontaneous, phenotypic modulation of contractile airway SMCs in culture is not inhibited by heparin. Temporal expression patterns of sm-α-actin, sm-MHC, and MLCK suggest that, in addition, heparin does not effect reinduction of these mature-state phenotypic markers. Furthermore, heparin was not effective in inhibiting the accumulation of nm-MHC, a putative marker for immature airway SMCs (17–19). Vimentin, an intermediate filament protein that is expressed in abundance in immature SMCs, was increased in post-confluent, heparin-treated airway SMCs. Vimentin content is also increased in hypertrophic SMCs (34).

Fig. 6. Bivariate fluorescence-activated cell sorter analysis of effects of heparin on cell cycle distribution of serum-stimulated airway SMCs as depicted by dot plots of cell cycle distribution. DNA content is represented by PI fluorescence along x-axis. 5-Bromo-2′-deoxyuridine (BrdU) incorporation is represented by log of fluorescein isothiocyanate (FITC) fluorescence on y-axis. A: negative control; cells were serum stimulated but were not pulsed with BrdU. B: BrdU-pulsed, serum-stimulated cells in absence of heparin. C: BrdU-pulsed, serum-stimulated cells in presence of 100 µg/ml of sodium heparin. Each plot represents data obtained from 10,000 individual cells. Five different experiments were carried out.

Fig. 7. Distribution of airway serum-responsive SMCs that entered cell cycle in presence and absence (control) of heparin. Values represent distribution of cells exclusive of those in G0/G1 phase; n = 5 experiments. *Significantly different between control and heparin at a specific cell cycle phase, P < 0.01.

Fig. 8. Hypothesized distribution of airway SMC subpopulations based on differences in response to serum stimulation and heparin-mediated inhibition of proliferation. Serum-insensitive SMCs (siSMCs) constitute ~30% of cultured canine tracheal SMCs. Remaining serum-responsive SMCs (srSMCs) are divided into heparin-sensitive, heparin-insensitive, and heparin-resistant subpopulations. See text for description.
Interestingly, our data comparing cell number and total protein suggest that cellular protein content is elevated in heparin-treated cultures. This finding may be related to an increase of cell size when cell number and protein content are elevated. These observations suggest that prolonged exposure of cultured airway myocytes to heparin may lead to hypertrophic growth.

The findings reported in this study appear to contrast with some data that indicated that the phenotypic state expressed by vascular SMCs is heparin sensitive. Different smooth muscles differ widely in pharmacological and contractile properties. Indeed, developmental paradigms indicate that arterial SMCs are segregated from airway SMCs on the basis of the site and source of progenitor cells (29). Heterogeneity between different smooth muscles has also been observed at the molecular level in which the content of marker proteins differs and smooth muscle-specific genes are under different transcriptional regulatory control (17, 25). Therefore, differential expression of phenotypic state markers in cultures of airway and arterial SMCs, in response to exogenous heparin, may be a manifestation of such factors.

The data obtained during analysis of the effects of heparin on cell cycle progression support a hypothesis that airway smooth muscle is composed of distinct subpopulations of SMCs that differ in responsiveness to mitogenic and antiproliferative mediators. Heterogeneity in the responsiveness of vascular SMCs to a variety of potent mitogenic stimuli has been reported (27, 31). These observations are in concordance with the heterogeneous response seen for cultured airway SMCs to serum stimulation in our studies. Serum stimulation of growth-arrested airway SMC cultures consistently failed to provoke cell cycle entry in at least 30% of the SMCs. Also, heparin blocked the entry of some srSMCs into the cell cycle, whereas it slowed the transit of others through late cell cycle phases. The biochemical and subcellular mechanisms for heparin-mediated inhibition of SMC proliferation appear to be complex and remain unresolved. Heparin-sensitive and insensitive pathways for arterial SMC proliferation may exist (28). Therefore, a number of mechanisms, impacting at different points in the cell cycle, have been postulated for heparin-induced inhibition of proliferation (14, 28, 30).

Figure 8 is a schematic representation of the hypothetical distribution of the airway SMC subpopulations identified in our studies. The groups include 1) serum-insensitive myocytes (serum-insensitive SMCs; 30% of all cells): these myocytes did not proceed through the cell cycle after serum stimulation; 2) heparin-sensitive srSMCs (40% of srSMCs): cell cycle entry after serum stimulation was inhibited in an all-or-none fashion by heparin; Johnson et al. (21) also showed that heparin inhibited [3H]thymidine uptake by 40% in serum-stimulated human airway myocytes; 3) heparin-insensitive srSMCs (60% of srSMCs): these myocytes traversed the cell cycle in the presence of heparin after serum stimulation; however, heparin caused the myocytes of this subpopulation to accumulate in the G2-M phase, indicating that cell transit was slowed during late cell cycle phases; these data closely mimic those from similar experiments in which the heparin responsiveness of cultured arterial SMCs was studied (14); and 4) heparin-resistant srSMCs: the existence or lack thereof cannot be confirmed by our studies; however, if present, this subpopulation would constitute only a small fraction of the cells.

Heparin released from mast cells in response to antigen challenge is inhibited by >70% in the presence of β2-agonists (15), and it blocks allergen-induced bronchoconstriction in sensitized sheep (1). Hence the effects of heparin on SMC growth (21) have received interest from asthma investigators. We found that airway SMCs are composed of heterogeneous subpopulations in response to exogenous heparin. The possibility that different subtypes of airway SMCs exist raises the possibility that different airway myocytes may contribute in specific ways to altered airway responsiveness, airway remodeling, bronchial muscle thickening, and fibrosis associated with the pathogenesis of bronchial asthma. Indeed, we reported the existence of heterogeneous subpopulations of arterial SMCs differing in phenotypic marker protein content, attachment efficiency to cell culture plates, and proliferative capacity (16, 17). The elucidation of specific characteristics of airway SMC subpopulations and the factors that regulate them will be invaluable in determining future directions in the development of new, more specific therapies for bronchial asthma.