Heparin-like molecules inhibit pulmonary vascular pericyte proliferation in vitro

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Heparin-like molecules inhibit pulmonary vascular pericyte proliferation in vitro. Am J Physiol Lung Cell Mol Physiol 279: L252–L261, 2000.—Proliferation of vascular pericytes (PCs), smooth muscle-like cells found in the distal microvasculature, contributes to vascular remodeling in pulmonary hypertension. The factors controlling lung PC quiescence in normal states are poorly understood. We demonstrate that exogenous heparin and heparan sulfate proteoglycans inhibit rat lung PC proliferation in vitro as does pulmonary vascular subendothelial matrix, particularly its heparan sulfate component. Heparin inhibits the intracellular alkalization essential to proliferation, and we show that inhibition of alkalinization by 5-(N,N-dimethyl)amiloride also reduces PC proliferation. As shown by DNA staining and fluorescein-activated cell sorting analysis, heparin does not induce apoptosis in PCs. However, heparin maintains lung PCs in the G0/G1 growth phase. Heparin induces production of p21, a potent inhibitor of cyclin-dependent kinases, thereby potentially identifying a fundamental mechanism by which heparin inhibits proliferation in smooth muscle-like cells. These studies establish additional similarities between lung PCs and smooth muscle cells and provide further understanding of growth control in the lung microvasculature. They also further support the rationale that heparin-like molecules might be therapeutically beneficial in pulmonary hypertension.

pulmonary hypertension; extracellular matrix; cell cycle; smooth muscle cell; proteoglycans

THE PROLIFERATION and phenotypic maturation of pericytes (PCs), smooth muscle-like cells found in the distal pulmonary arterial microvasculature, is thought to be a component of the vascular remodeling and muscularization seen in pulmonary hypertensive states (24, 36). Mural cellular changes and narrowing of the vascular lumen increase the resistance to blood flow in a variety of illnesses, including hypoxic and hyperoxic lung injury and acute respiratory distress syndrome (25, 36, 47, 50), leading to pulmonary hypertension and right heart failure.

PCs are found in normal pulmonary vascular beds, and the available evidence (36, 44) suggests that they are maintained in a relatively nonproliferative state under normal conditions. The factors that maintain PC quiescence are poorly understood. PCs have many characteristics in common with smooth muscle (15, 27–29, 45), but it is unknown to what degree their proliferative control is similar to that of mature smooth muscle. PCs are found just beneath the endothelial layer, and they share the endothelial cell (EC) basement membrane (BM). It has been recognized for some time that endothelium-synthesized extracellular matrices inhibit vascular smooth muscle proliferation (20). Furthermore, in an initial study (12), a similar effect has been described for pulmonary vascular PCs. However, the component(s) of the BM responsible for this inhibition has not been identified.

Proteoglycans containing glycosaminoglycans (GAGs) of the heparin and heparan sulfate families are produced by many cell types, including vascular ECs, and are integral components of the subendothelial matrix. These sulfated GAGs contribute to the regulation of a wide variety of biological functions, including cell proliferation, differentiation, and migration (22). Exogenous heparin markedly inhibits smooth muscle cell proliferation in vivo after arterial injury (9) and the pulmonary vascular remodeling caused by hypoxia (18). Heparin and heparin-like molecules, including heparan sulfate proteoglycan (HSPG), bind to vascular smooth muscle cells and strongly inhibit their growth in vitro (1, 8, 20, 21). Although heparin has been reported to inhibit retinal PC proliferation in vitro (37), it is not known whether heparin-like molecules individually or as part of a subendothelial matrix will inhibit lung PC proliferation.

Intracellular alkalization, mediated by increased Na+/H+ exchange, appears to be an essential event leading to smooth muscle proliferation (2, 3, 42). Heparin inhibits Na+/H+ exchange in vascular smooth muscle cells (11, 51), and 5-(N,N-dimethyl)amiloride (DMA), a potent inhibitor of the Na+/H+ antiporter, also inhibits vascular smooth muscle cell proliferation (42, 43). The effects of DMA on PC proliferation are unknown.

The signaling pathways affected by the inhibitory effect of heparin on smooth muscle cell proliferation...
have been incompletely identified. Moreover, it is not known if heparin induces apoptosis in smooth muscle. Heparin appears to block the G_0/G_1 → S transition in the cell cycle (6). It inhibits protein kinase C-dependent pathways for gene expression (7, 19, 41) and mitogen-activated protein (MAP) kinase (38). Transcription of c-fos and c-myc is reduced as is sgk, an early-response gene (13). The p21 (Waf1/Cip1) gene has recently been described, and its product is considered to be one of the major regulators of the cell cycle in G_1, acting as a potent inhibitor of cyclin-dependent kinases (10). The effects of heparin on p21 expression are unknown.

The present experiments were designed to describe the effects of heparin-like molecules on lung PC proliferation in vitro, to explore some mechanisms of action, and to establish whether the effects were similar to those previously described for heparins and smooth muscle cells (1, 8, 20, 21, 42, 43). We studied heparins from various commercial sources to identify varying potencies and studied structurally modified heparins to assess the importance of structural elements to the growth inhibitory effect. The effects of HSPGs were explored. Other sulfated GAGs may be present in the pericellular milieu, and we examined their effect on proliferation. Then we used specific enzymes to degrade subendothelial BMs and evaluated the importance of heparan sulfate in this matrix as an inhibitor of PC proliferation. Because heparin inhibits Na^+/H^+ exchange in smooth muscle cells and this may represent an important antiproliferative mechanism, studies with DMA were performed to investigate the effect of inhibition of Na^+/H^+ exchange on PC proliferation. We also studied the effects of heparin on apoptosis and the production of the p21 protein.

**MATERIALS AND METHODS**

**Culture of Rat Lung PCs**

With a modification of previously described methods (12, 30, 31), PCs were cultured from the lungs of male Sprague-Dawley rats (Charles River Laboratories, Saint Constant, PQ) weighing 175–300 g. All animal care and handling complied with the guidelines of the Canadian Council on Animal Care. Briefly, the rats were anesthetized with chloroform, the heart and lungs were excised en bloc, and peripheral subpleural pieces of lung were chopped on a McIlwain mechanical tissue chopper (Brinkmann, Rexdale, ON) and rinsed in PBS (Cellgro, Montreal, PQ). The tissue was digested in Ca^{2+}- and Mg^{2+}-free Hanks’ balanced salt solution (HBSS; Life Technologies, Grand Island, NY) containing 0.02% EDTA, and the trypsin was inactivated with 10% platelet-poor plasma and centrifuged (also described in Culture of Rat Lung PCs). The pellet was resuspended with MCDB-131 plus 10% FBS, and the trypsin was inactivated with medium containing serum after passage. Cells in the fourth to sixth passages were used for experiments, and they maintained their phenotypic characteristics throughout the passages. The ECs demonstrated typical cobblestone morphological characteristics and maintained a monolayer pattern even at high densities. The cells produced factor VIII coagulant as assessed by immunoperoxidase staining (DAKO) and showed angiotensin-converting enzyme activity (Hycor Biomedical), but they did not contain smooth muscle actin (Enzo Biochemical). They demonstrated previously described morphological characteristics (12) including ragged margins, a lack of contact inhibition, and formation of macroscopic mounds at high density. PCs did not have angiotensin-converting enzyme activity (assay from Hycor Biomedical, Portland, ME). Immunoperoxidase staining identified smooth muscle actin (antibody MA-935, assay from Enzo Biochemical, New York, NY), but the cells did not produce factor VIII coagulant (assay from DAKO, Santa Barbara, CA). Compared with smooth muscle cells, PCs express nonmuscle actin, and when viewed by electron microscopy, they lack the dense bodies seen in smooth muscle. Bovine pulmonary artery endothelial and smooth muscle cells and human skin fibroblasts were used as controls for the characterizations.

**EC Culture**

Rat microvascular ECs were cloned from lung digests. Lung tissue that had been collagenase digested as described in Culture of Rat Lung PCs was washed in MCDB-131 plus 5% platelet-poor plasma and centrifuged (also described in Culture of Rat Lung PCs). The pellet was resuspended with MCDB-131 plus 0.1% platelet-poor plasma, plated on gelatin-coated T25 plastic tissue culture flasks, and incubated in 5% CO_2 in air at 37°C. Within a few days, clusters of ECs formed, were passaged with cloning rings, and were plated in T25 plastic tissue culture plates with MCDB-131 plus 10% FBS, thus yielding a pure culture of lung microvessel ECs. Culture media were subsequently changed twice weekly, and ECs were allowed to grow to confluence before being passaged for experiments. ECs were passaged using 0.01% trypsin and 0.02% EDTA in HBSS, and the trypsin was inactivated with medium containing serum after passage. Cells in the fourth to sixth passages were used for experiments, and they maintained their phenotypic characteristics throughout the passages. The ECs demonstrated typical cobblestone morphological characteristics and maintained a monolayer pattern even at high densities. The cells produced factor VIII coagulant as assessed by immunoperoxidase staining (DAKO) and showed angiotensin-converting enzyme activity (Hycor Biomedical), but they did not contain smooth muscle actin (Enzo Biochemical). They demonstrated previously described morphological characteristics (12) including ragged margins, a lack of contact inhibition, and formation of macroscopic mounds at high density. PCs did not have angiotensin-converting enzyme activity (assay from Hycor Biomedical, Portland, ME). Immunoperoxidase staining identified smooth muscle actin (antibody MA-935, assay from Enzo Biochemical, New York, NY), but the cells did not produce factor VIII coagulant (assay from DAKO, Santa Barbara, CA). Compared with smooth muscle cells, PCs express nonmuscle actin, and when viewed by electron microscopy, they lack the dense bodies seen in smooth muscle. Bovine pulmonary artery endothelial and smooth muscle cells and human skin fibroblasts were used as controls for the characterizations.

**Sulfated GAGs, Proteoglycans, Heparin Lyases, and DMA**

Heparin sodium salt (bovine lung) was obtained from Sigma. Heparin sodium salts from Laboratoire Choay (porcine mucosal), Elkins-Sinn (Cherry Hill, NJ), and Upjohn (bovine lung; Kalamazoo, MI) were generous gifts from Dr. Arthur Perlin (Department of Chemistry, McGill University, Montreal, PQ). Carboxyl-reduced heparin, de-N-sulfated heparin, chondroitin sulfate C, and dermatan sulfate were also gifts from Dr. Perlin. Heparins and the other sulfated GAGs were kept desiccated at 4°C and were added to MCDB-131 medium at various concentrations just before use. Pulmonary arterial HSPG (a generous gift from Dr. William Benitz, Stanford University, Stanford, CA) (1) and a commercial preparation of HSPG from BMs of Engelbreth-Holm-Swarm mouse sarcoma (Sigma) were stored in 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.4) at −20°C until used. The enzymatic activity of heparin lyases, expressed in units per milligram, relates to 1 U forming 0.1 μmol uronic acid/h at pH 7.5 at 25°C. Heparin lyase I (heparinase I; EC 4.2.2.7),
heparin lyase II, and heparin lyase III (EC 4.2.2.8) from *Flavobacterium heparinum* were purchased from Sigma (478, 193, and 238 U/mg, respectively). Heparin lyase I and heparin lyase III were resuspended in 20 mM Tris–HCl with 50 mM NaCl, 4 mM CaCl₂, and 0.01% BSA, pH 7.5, to a final concentration of 0.1 U/µl. Heparin lyase II was resuspended in 100 mM C₇H₂O₆Na with 0.01% BSA, pH 7.0, to a final concentration of 0.1 U/µl. All heparin lyases were divided into aliquots and stored at −20°C until used. DMA (Sigma) was diluted to 10⁻² M with water and stored at −20°C until used.

**Experimental Design**

**Effects of heparin and other sulfated GAGs on PC proliferation.** PCs were passaged into 24-well plates (n = 8 wells/experimental condition unless otherwise stated) with MCDB-131 plus 10% FBS and allowed to attach for 24 h before onset of the experiments. In all experiments, once the medium containing the agent to be tested was added, it was not changed for the 7-day period. For dose-response studies, the medium was then removed, and fresh MCDB-131 plus 10% FBS containing 0–100 µg/ml of heparin from the various sources or carboxyl-reduced heparin, de-N-sulfated heparin, chondroitin sulfate, or dermatan sulfate was added. The HSPGs were added at 0–1 µg/ml. Subsequent experiments employed heparin and other sulfated GAGs at 100 µg/ml and HPSGs at 1.0 µg/ml. After the 7-day period, the cells were detached with 0.25% trypsin plus 0.02% EDTA in HBSS and counted with a hemacytometer. In the experiment that examined the 7-day growth curve, after addition of the test medium on day 0, cell counts were obtained on subsequent days 1, 2, and 4–7 for eight culture dish wells in each group.

**Effects of EC BM on PC proliferation.** ECs were passaged into 24-well plates in MCDB-131 plus 10% FBS and allowed to grow to confluence. The medium was changed twice weekly. The ECs were then removed by exposure to 2 M urea or 0.2 M NH₄OH in PBS (BioShop Canada, Burlington, ON) for 5 min, leaving an intact BM on the 24-well plate surface. Wells that did not contain ECs and were washed with either PBS, 2 M urea, or 0.2 M NH₄OH were used to control for any effects of urea or NH₄OH. The EC BM was washed three times with PBS. PCs were then passaged onto the EC BM at ~15,000 cells/well, and MCDB-131 plus 10% FBS was added. After 7 days, the cells were detached and counted with a hemacytometer as described in *Effects of heparin and other sulfated GAGs on PC proliferation.*

**Effects of heparin lyase degradation of EC BMs on PC proliferation.** EC BMs were generated as described in *Effects of EC BM on PC proliferation* and then were heparin degraded with heparin lyase I, II, or III at 0.3 U/ml in MCDB-131 plus 10% FBS for 24 h. For controls, empty wells were used. The medium was then removed, the EC BM was rinsed with PBS three times, and PCs were passed into the wells at ~15,000 cells/well. After a 7-day period, the cells were counted as in *Effects of heparin and other sulfated GAGs on PC proliferation.*

**Effects of DMA on PC proliferation.** PCs were passaged into 24-well plates at ~15,000 cells/well and allowed to attach for 24 h in medium containing serum. After the attachment period, the medium was replaced with MCDB-131 plus 10% FBS with varying doses of DMA (0–200 µM). The cells were then counted after a 7-day period.

**Microscopic analysis of apoptosis by assessment of nuclear condensation.** PCs were passaged onto 25-mm glass cover slips within 6-well dishes at ~40,000 cells/well and allowed to attach for 24 h in medium with serum. After the attachment period, the medium was replaced with fresh medium with serum alone or with 100 µg/ml of heparin, 100 µM DMA, 0.2 µM As₂O₃ (as a positive apoptosis control), or 0.3% BSA (as a growth-arrest control) for 72 h. The cells were then rinsed three times in PBS and loaded with Hoechst 33258 for 5 min followed by three more washes in PBS. The cells were then viewed with a fluorescence microscope and photographed.

**Effect of heparin and DMA on the cell cycle.** PCs were plated onto 6-well dishes at ~40,000 cells/well and allowed to grow to subconfluence in medium containing serum. The medium was then replaced with fresh medium containing either serum alone or with 100 µg/ml of heparin or 100 µM DMA and returned for incubation for 3 days. The PCs were then detached in PBS with a rubber policeman and briefly centrifuged. The cell pellet was then resuspended in 500 µl of PBS and 500 µl of propidium iodide solution containing 0.1 mg/ml of propidium iodide (ICN, Costa Mesa, CA), 2 mM EDTA, and 0.2% Triton X-100 in PBS, and monoparametric conventional cell cycle analysis with propidium iodide was performed with an EPICS XL-MCL flow cytometer (Coulter, Miami, FL). Standard forward scatter versus side scatter criteria were used to establish analysis gates for PCs, and fluorescence-activated cell sorting (FACS) analysis was performed for propidium iodide-labeled DNA content.

**Western blot analysis of p21 protein production.** PCs were passaged as described in *Microscopic analysis of apoptosis by assessment of nuclear condensation.* Fresh medium was added either with serum alone, with serum with 100 µg/ml of heparin or 100 µM DMA, or in serum-free condition for 2, 5, 20, 28, 48, or 72 h. The medium was removed, and the plates were washed with PBS. The cells were detached in PBS with

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Fig. 1. *Day 7* cell count of lung pericytes (PCs) exposed to various heparins (Hep) at a concentration of 100 µg/ml and to Engelbreth-Holm-Swarm mouse sarcoma-derived heparan sulfate proteoglycan (HSPG) at 1 µg/ml. The heparin was added on *day 0,* and the medium was not changed for the duration of the experiment nor was the heparin replenished. E-S, Elkin-Sinn. Values are means ± SE; *n* = 8 wells/group. All the compounds tested significantly inhibit pericyte proliferation in vitro. ÷ *P < 0.001 vs. control. There was no significant difference between any of the other treatment groups.
a rubber policeman and then formed into a pellet by centrifugation. Total protein was extracted from the cells with a radioimmunoassay precipitation assay buffer [Tris, pH 7.4, 10 mM NaCl, 100 mM deoxycholic acid, 1% SDS, 0.1% Nonidet P-40, and 1% protease inhibitors (2 μg/ml of aprotinin, 2 μg/ml of leupeptin, 1 μg/ml of pepstatin A, and 100 μg/ml of phenylmethylsulfonyl fluoride)]. Protein (10 μg) was loaded onto a 10% SDS-polyacrylamide gel and electrophoresed at 100 V for 1.5 h. It was then transferred to a nitrocellulose membrane. The membranes were hybridized with monoclonal antibodies against p21 (Santa Cruz Biotechnology, Santa Cruz, CA) or α-tubulin (Amersham). After addition of anti-mouse or anti-rabbit IgG (Amersham), the protein levels were analyzed with a chemiluminescence system (ECL, Amersham). To examine for a dose-response pattern in p21 protein production, PCs were prepared as in Microscopic analysis of apoptosis by assessment of nuclear condensation and then exposed to fresh medium with serum alone or with Elkin-Sinn heparin at concentrations of 1, 10, and 100 μg/ml for 20 h; then the protein was extracted, and Western blot analysis was performed as above. The p21 protein levels were normalized to the protein levels for β-actin for each sample lane.

Statistical Analysis

Data are presented as group means ± SE. To compare groups, one-way ANOVA was used and was followed, where appropriate, by Tukey-Kramer analysis for multiple comparisons. P values < 0.05 were considered significant.

RESULTS

Effects of Heparin and Other Sulfated GAGs on PC Proliferation

Heparin sodium salts from the different suppliers significantly inhibited PC proliferation (Fig. 1) at a concentration of 100 μg/ml compared with that in control cells (P < 0.001). The cells were exposed to the heparins for the full 7 days with no change of the medium or replenishment of the heparin. The heparins from Choay and Upjohn had the greatest antiproliferative effect despite the fact that they were of different origin (porcine mucosa versus bovine lung). When the growth curve was studied over 7 days (Fig. 2), Elkin-Sinn heparin (100 μg/ml) significantly inhibited PC proliferation at all time points after day 1 compared with that in the control group (MCDB-131 plus 10% FBS). There was no catch-up of proliferation in the heparin-treated group. A subsequent dose-response experiment with Elkin-Sinn heparin (Fig. 3) showed that the inhibition of PC proliferation by heparin was dose dependent, with 65% inhibition at 100 μg/ml for that experiment (P < 0.001 for all groups compared with control group). In another experiment (Fig. 4), although heparin sodium salt from Choay inhibited PC proliferation by 46% at a concentration of 100 μg/ml, de-N-sulfation and carboxyl reduction of heparin eliminated most of the inhibitory effects on proliferation, with the former having only a 12% inhibition of control values (P < 0.01). Although chondroitin sulfate and dermatan sulfate had lesser effects than Choay heparin, both significantly (P < 0.01) inhibited PC proliferation, with dermatan sulfate reducing PC growth by 32% versus the control group. The HSPG derived from the BM of Engelbreth-Holm-Swarm mouse sarcoma was as effective an inhibitor of PC proliferation as was heparin (Fig. 1) but at one-hundredth the concentration. Similarly, the HPSG derived from pulmonary artery ECs showed a dose-dependent effect (Fig. 5), with significant inhibition at 0.1 and 1.0 μg/ml (P < 0.05).
Effects of EC BM and Its Degradation by Heparin Lyases

Urea (data not shown) or NH₄OH treatment of plastic wells (Fig. 6) did not affect PC proliferation compared with that in control (untreated) plastic wells. The proliferation of PCs plated onto EC BM that had been produced by removing the endothelial monolayer with NH₄OH was significantly reduced compared with those plated onto plastic (control) \( P < 0.001 \). Heparin lyase I did not alter the inhibitory effects of EC BM on PC proliferation. However, treatment with heparin lyase II and heparin lyase III reduced the inhibition of proliferation by EC BM, with heparin lyase II-treated EC BM inhibiting proliferation by 16% versus control value and heparin lyase III-treated EC BM inhibiting by only 6% (in both cases \( P < 0.05 \) vs. EC BM).

Effects of DMA on PC Proliferation

As seen in Fig. 7, DMA inhibited PC proliferation in a dose-dependent fashion, reducing it by 62% of control levels at 200 \( \mu \)M \( P < 0.001 \). All doses except for 10 \( \mu \)M had significant inhibitory effects \( P < 0.001 \).

Microscopic Analysis of Apoptosis

With the use of the Hoechst 33258 stain, apoptosis was detectable in the As₂O₃-treated PCs as highly fluorescent condensed nuclear material with misshapen nuclei that were smaller than those in the control group. Growth arrest by exposure to serum-free conditions in serum albumin did not cause apoptosis. Neither heparin nor DMA induced apoptosis.

Effect of Heparin and DMA on the Cell Cycle

By FACS analysis (Fig. 8), the control PC cultures contained <1% apoptotic cells, with 43.7% being in the G₀/G₁ phase and 47.2% being in the S phase. In the PC cultures exposed to heparin, there was a significant shift in the proportion toward the G₀/G₁ phase and away from the S phase. A similar pattern was seen in the cultures exposed to DMA. Neither heparin nor DMA exposure caused an increase in the proportion of apoptotic PCs.

Western Blot Analysis of p21 Protein Production

When a specific antibody against p21 was used, both heparin and DMA induced p21 protein production, which was seen 20 h after the onset of exposure (Fig. 9) and peaked at a maximum 28 h after exposure. When a dose-response curve was examined (Fig. 10), p21 production 20 h after the onset of exposure to heparin (expressed as a ratio to β-actin levels) rose progressively with increasing heparin concentration.
**DISCUSSION**

Several elegant and detailed studies (25, 32, 36) have confirmed that cells of mesenchymal origin are involved in the vascular remodeling seen in several models of pulmonary hypertension. Some of these cells migrate into the vessel wall from surrounding tissue areas, but others are already present in the vessel wall as dedifferentiated smooth muscle-like cells, including the PCs in the microvasculature (24, 25, 36). Proliferation and phenotypic maturation of these cells contributes to the muscularization and narrowing of the pulmonary microvasculature, greatly increasing pulmonary vascular resistance (36). PCs normally contain the cytoskeletal elements essential to the generation of contraction (15, 28, 29, 45), and they constrict in response to stimuli such as endothelin-1, thromboxane A2, and endotoxin (14, 31). However, it is their proliferation, hypertrophy, and phenotypic maturation in disease states that probably represents their greatest contribution to the vascular remodeling in disease.

PCs are normally quite quiescent in the pulmonary microvasculature, with a low mitotic rate, but this rate increases greatly during the vascular remodeling process caused by hypoxia (35). The factors that normally minimize proliferation of smooth muscle-like cells in the pulmonary microvasculature are poorly understood. Useful parallels may be found by examining factors that prevent intimal muscularization in systemic atherosclerosis, with heparin-like molecules being prominent inhibitors (9). Heparin is also known to inhibit the development of pulmonary hypertension in vivo (18). ECs incorporate HSPGs into the subendothelial matrix (20, 22), and PCs lie in intimate contact with this matrix. Our studies examined the effects of exogenous heparin-like molecules and those endogenous to endothelium-derived matrices on lung PC proliferation in vitro. We further clarified the mechanism of the antimitotic effect of heparin by identifying the expression of p21, an important regulator of the cell cyclin system (10). Another similarity of PCs to smooth muscle cells, their need for intracellular alkalinization in order to initiate proliferation, was also studied.

The present experiments demonstrated that heparin and heparan sulfate GAGs and proteoglycans inhibit rat lung PC proliferation in vitro in a potent, dose-dependent manner. These molecules demonstrate antiproliferative effects on PCs similar to those reported for heparin and smooth muscle cells (1, 8, 9, 20, 21). Heparins from different commercial sources may have variable antiproliferative potencies (11, 21). We found that the heparins studied had similar degrees of antiproliferative effects, with heparin from Choay and Up-
Having a slightly greater inhibitory activity. However, we compared the heparins at concentrations of 100 μg/ml, and previous studies (5, 46) suggest that at this concentration, as opposed to lower concentrations, the differences between heparins from different sources may be harder to detect. The structural determinants of the antiproliferative activity of heparin have been previously studied (4, 16, 26, 48), and it is as yet unclear which are the essential elements. In our experiments, both de-N-sulfation and carboxyl reductation of heparin reduced its growth inhibitory activity. Other sulfated GAGs, chondroitin sulfate and dermatan sulfate, also inhibited PC growth but they were less effective than heparin. Heparin has a higher degree of sulfation than the other sulfated GAGs studied (22), and this may explain its more potent antiproliferative effect. Furthermore, pentosan polysulfate, which is even more highly sulfated than heparin, has a more potent antiproliferative effect on smooth muscle cells (39).

GAGs organize into proteoglycans by alignment along a protein core. Proteoglycans are found as con-

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Fig. 8. Fluorescence-activated cell sorting (FACS) analysis of cell cycle with accompanying cell cycle phase proportions in PCs exposed to control conditions (top), heparin (middle), and DMA (bottom). Both heparin and DMA induced a shift in cell cycle toward G0/G1 phase and reduced the proportion of cells in S phase.
Heparin might also affect expression of other genes that are potent inhibitors of cell cycle progression toward DNA replication. In the G1 phase, p21 is a potent inhibitor of the cyclin-dependent protein kinases that are the principal regulatory proteins of the cell cycle (10). We therefore explored the effects of heparin on p21 levels in PCs and showed that both heparin and DMA stimulate p21 production 20–28 h after exposure. This finding potentially identifies a potent and fundamental mechanism by which heparin might prevent proliferation of smooth muscle-like cells. We studied

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the effects of heparin on p21 in actively proliferating, non-serum-deprived PCs. Allowing the cells to proliferate implied that at any given time a certain fraction within the culture dish would be proceeding through the cell cycle. Thus this method would provide low and uniform background levels of p21 expression against which a change induced by heparin would be more easily detectable than if the cells had been growth arrested and then returned to serum-containing medium. The latter would provide large synchronous spikes in p21 that would be more difficult to distinguish from a heparin effect. When we examined the effect of heparin concentration on p21 production, there was a clear increase in p21 levels with increasing heparin dose. This again is suggestive, although not conclusive, evidence that some of the antiproliferative effects of heparin may be through increasing levels of p21.

In conclusion, we have demonstrated further similarities between PCs and smooth muscle cells in that heparin-like molecules either given exogenously or as found in subendothelial matrix inhibit lung PC proliferation in vitro. Heparin does not act via apoptosis but synchronizes and maintains the cells in the G0/G1 phase, at least in part, by stimulating p21 production. Inhibition of Na+/H+ exchange by DMA also inhibits PC proliferation. These studies offer novel understanding of growth control in the distal microvasculature and offer further promise that heparin-like compounds may prove to be of great use in the treatment of vascular remodeling in pulmonary hypertension.

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