Effect of covalent antithrombin-heparin complex on developmental mechanisms in the lung

Nagina Parmar,1 Leslie R. Berry,2 Martin Post,1 and Anthony K. C. Chan1,2

1Department of Lung Biology, Hospital for Sick Children, Toronto; and 2Henderson Research Centre, McMaster University, Hamilton, Ontario, Canada

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Respiratory distress syndrome (RDS) is a debilitating illness associated with underdevelopment of lung structures in premature newborns (47). Among preterm infants, RDS is characterized by the presence of intra-alveolar fibrin deposition, which inhibits surfactant function (71). Preterm infants exposed to O2 with mechanical ventilation often develop bronchopulmonary dysplasia (BPD), a form of chronic lung disease (CLD) (8, 46, 80). The pathogenesis of BPD/CLD involves dysmorphic microvasculature and disrupted alveolarization, potentially due to impaired vascular endothelial growth factor (VEGF) and VEGF receptor expression (80).

During recovery in RDS patients, elevated cell proliferation leads to remodeling of the alveolar surface (18, 37, 50) that may assist in the evolution of BPD characterized by areas of increased fibroblast growth (fibrosis) (2, 3, 35) and generalized reticulogranular lung opacities (12). Indeed, for severely premature neonates, BPD/CLD shows progression towards impairment of alveolar septation from enhanced proteolysis of extracellular matrix components (30, 77), coinciding with increased appearance of a number of enzymes such as neutrophil elastase and matrix metalloproteinase (MMP)-8 (20, 77, 79). Recent data suggest that disruption of angiogenesis (36) prevents alveolarization and development of other distal lung air space architecture (81, 82), which is a major hallmark of BPD. Furthermore, current work has revealed that misregulation of VEGF and/or VEGF receptor expression (5, 80) critically disturbs endothelial cell growth and survival (52), resulting in the inhibition of vascular development and the concomitant arrested pulmonary maturation of BPD. Overall, a key feature of BPD may be seen as impairment of normal lung development (1, 49), in which factors inhibiting pulmonary angiogenesis may play a vital role.

Intrapulmonary coagulation has significant involvement in the transformations of lung structures from RDS to BPD. Fibrin has been found in the pulmonary microcirculation and small airways due to the reaction of fibrinogen with thrombin generated by coagulation system activation (15–17) in preterm infants with severe RDS (42, 78). Besides inhibiting surfactant function, fibrin monomer products formed in the air space assist in alveolar surface remodeling (18, 25, 37, 50) and fibrosis (14, 34) due to recruitment of fibroblasts (18, 26). Other studies have shown that binding of integrins to the COOH-terminal globular domain of fibrinogen degradation products induces endothelial apoptosis and blockage of tube formation that suppresses angiogenic vessel formation in vivo (4). Ongoing antiangiogenic effects may be sustained by either persistent intra-alveolar procagulant activity or abnormal removal of fibrin products by extravascular fibrinolytic enzymes (6). Consequently, any fibrin formation by interstitial and alveolar thrombin is a significant factor that may contribute to the developmental sequelae of BPD/CLD through amplification of inflammation and fibrosis (76). Thus, creation of an anticoagulant that would prevent pulmonary fibrin formation through thrombin regulation offers an attractive form of adjuvant therapy for RDS treatment and BPD prevention.

Control of thrombin activity in vivo occurs primarily through inhibition by plasma antithrombin (AT), a reaction that is vastly enhanced by the clinical anticoagulant heparin (64). Therefore, intrapulmonary administration of AT plus heparin may be considered to quench the effects of thrombin-generated fibrin in premature lung disease. Unfortunately, heparin is
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readily lost from the lung air space into the circulation (83). Additionally, some reports have indicated that heparin inhibits angiogenesis (41) and lung branching morphogenesis (70) by disrupting endothelial (38) and smooth muscle cell proliferation (45). Moreover, naturally occurring subtypes of AT have properties that can antagonize organ development (69). A latent form of AT (L-AT), in which the reactive center loop is inserted into the main β-sheet, strongly inhibits growth factor-stimulated proliferation, migration, and capillary tube formation in cultured endothelial cells and induces tumor regression in a mouse model (51, 53, 61). AT, particularly in the latent form, inhibits angiogenesis during development (21, 51, 61). L-AT exerts its antiangiogenic effects by inducing cell apoptosis (53, 67), downregulating endothelial proangiogenic heparan sulfate perlecan proteoglycan expression (87) and global genetic reprogramming of systems involving endothelial ligand-receptor signaling mechanisms (86, 89). Thus, although L-AT would have no anticoagulant effect because of its low heparin affinity and inability to inhibit thrombin (48), even small fractions of AT in the latent form would counteract critical vascular angiogenesis (58) during long-term retention in the developing premature lungs.

Our laboratory has developed a novel covalent AT-heparin complex (ATH) in an attempt to improve on AT plus heparin anticoagulant treatment. ATH reacts directly with thrombin 4–10 times faster (11, 21) than non-covalent AT plus heparin mixtures. Additionally, the heparin chains of ATH can potently catalyze thrombin neutralization by free plasma AT (21). Thus, ATH that contains an AT moiety in the latent or inactivated state still possesses catalytic anticoagulant activity through its heparin chain. Inhibition by ATH of thrombin on fibrin surfaces and thrombin generation on fetal distal lung epithelial cells was significantly more potent than equivalent doses of AT plus heparin (21, 22).

Furthermore, intratracheal instillation of ATH resulted in high anticoagulant activity in lung lavage fluid for over 48 h, with no measurable activity systemically (12). Further preliminary data also indicate that ATH may exert a selective antiproliferative effect on lung fibroblasts. Therefore, ATH has many of the characteristics necessary to alleviate coagulation activity contributing to RDS and BPD in premature lungs. Although significant advances have been made in understanding the mechanisms by which ATH inhibits coagulant activity on pulmonary cells, the impact of this conjugate on important cellular processes and development of the fetal lung is unclear. In particular, the impact of ATH or latent ATH (L-ATH) on vascularization in the developing lung is unknown. Given the adverse effects of L-AT and heparin on lung growth, we assessed the effect of this novel ATH anticoagulant on angiogenesis and morphogenesis in a murine developing lung model.

MATERIALS AND METHODS

Preparation of ATH. ATH was prepared as described previously (11, 21). Briefly, 1 mg/ml of human AT (Affinity Biologicals, Ancaster, ON, Canada) and 56 mg/ml of aldehyde-containing unfractionated heparin (UFH, Sigma, Mississauga, ON, Canada) were heated in PBS at 37°C for 14 days, followed by purification including hydrophobic chromatography on butyl-sepharose (Amersham Biosciences, Uppsala, Sweden) before anion exchange on DEAE-sepharose (Amersham Biosciences). ATH product was assessed with or without exhaustive heparinase treatment (2 μl of ATH incubated with 2 μl of heparinase and 10 μl of 0.15 M NaCl at 37°C for 2 h) by SDS-PAGE separation on gradient NuPage Gel (4–12% Bis Tris gel) under reducing conditions, with staining for protein (Coomassie blue) and heparin (alcian blue-silver) (21). To confirm anticoagulant function, anti-factor Xa assays were performed [by standard methods described previously (21)] that determine the specific activity of ATH heparin to catalyze factor Xa inhibition by AT. The anti-factor Xa activity of ATH preparation used for this study was 459 U/mg heparin. This value was well within the typical range (463 ± 33 U/mg heparin) for ATH preparations derived from these commercial sources of plasma AT and porcine UFH, as reported previously (24).

Preparation of L-AT and L-ATH. Human plasma AT (Affinity Biologicals, 5.1 mg/ml) and ATH (9.0 mg/ml) were used for the preparation of L-AT and L-ATH, respectively, by a modification of the method described previously (11, 21). Briefly, both AT and ATH were dialyzed against 0.25 M sodium citrate, 10 mM Tris·HCl, pH 7.4, for at least 24 h at 23°C. The recovered amount of AT and ATH was heated at 80°C for 8 h. Absorbance at 280 nm was determined for quantification of L-AT and L-ATH genera. L-AT and L-ATH were then further dialyzed against 0.02 M Tris·HCl, 0.15 M NaCl, pH 7.4, at room temperature for 24 h, and absorbance at 280 nm was measured again to quantify the final amount of L-AT and L-ATH products. To confirm that anticoagulant activity was retained in ATH heparin after generating the latent form of the complex, anti-factor Xa assays were carried out. Activity of L-ATH preparations used was 448 ± 12 U/mg heparin, similar to the starting ATH product.

Transgenic mice. Tie2-LacZ mice were obtained from Jackson Laboratory (Bar Harbor, ME) (75). In Tie2-LacZ transgenic mice, the 2.1-kb 5′-flanking region of the murine Tie2 promoter drives the expression of the bacterial LacZ reporter gene exclusively in endothelial cells (33, 75). Cells transcribing the LacZ genes can be viewed by staining for β-galactosidase activity. All mouse protocols were in accordance with Canadian Council of Animal Care guidelines and were approved by the Animal Care and Use Committee of the Hospital for Sick Children, Toronto, ON, Canada.

Whole lung organ culture and treatment. The developmental lung model was based on a well-established, validated design we reported previously (39). Although previous work has suggested that an actively beating heart maintains lung microvasculature and lung architecture (85), our experiments were expressly with isolated lung explants to ensure that any observed angiogenic or morphological effects were from mechanisms within the lung. Further details for the rationale on this issue have been described before (56). Lung buds were dissected from embryonic day (E) 11.5 Tie2-LacZ mouse embryos (day of vaginal plug is E0.5) and placed on a floating (8-μm Whatman Nucleopore polycarbonate) membrane (Fisher Scientific, Mississauga, ON, Canada). Explants were grown in DMEM supplemented with 10% PBS (GIBCO, Grand Island, NY) and maintained at 37°C in an atmosphere of moist room air with 5% CO2. The DMEM was supplemented with either PBS, AT (100 μg/ml and 10 μg/ml), L-AT (100 μg/ml), ATH (100 μg/ml), L-ATH (100 μg/ml), or heparin (25 μg/ml). Other sets of experiments were done to determine the effect of FGF2 on the development of lung cells. Impact of FGF2 on angiogenic mechanism was determined after incubating lungs with 20 ng of FGF2/ml ± PBS, ± L-AT, and ± L-ATH (n = 5).

X-galactosidase staining. Cultured LacZ lung explants at 96 h of growth were fixed (1% formaldehyde, 0.1% glutaraldehyde, 2 mM MgCl2, and 5 mM EGTA in 0.1 M sodium phosphate buffer, pH 7.8–8.0, for 45 min at 4°C), washed (2 mM MgCl2, 0.01% deoxycholate, and 0.02% Nonidet P-40 in 0.1 M sodium phosphate buffer, pH 7.8–8.0) four times for 30 min at 4°C, and stained overnight at 37°C in X-galactosidase (X-gal) staining solution [5 mM K4Fe(CN)6·3H2O and 5 mM K3Fe(CN)6 in wash buffer, mixed 40:1 with X-gal stock solution (40 mg/ml in dimethyl formamide)]. Explants were then copiously washed in 70% ethanol, fixed in 4% paraformaldehyde in PBS overnight at 4°C, and...
stored in 70% ethanol. For imaging, explants were dehydrated by two treatments of 30 min in methanol and cleared in methyl salicylate. Pictures (all at the same exposure time) were taken with a Leica digital imaging system at ×200 magnification using a ×20 objective lens and the same light source intensity for all samples. Vascular area analysis was done using Open Lab Software with quantification restricted to the blue X-gal stain color and image intensity normalized relative to the background. This software allows determination of stained material as blue pixel intensity over the entire lung explant.

RNA isolation and real time RT-PCR. Lung explants from Tie2-LacZ at 96 h, cultured in 3% O₂ with the various treatments mentioned above, were rinsed in ice-cold PBS after 4 days in culture, immediately added into the RNA LATER solution (Ambion, Austin, TX), and stored at either −20°C or −80°C. Explants from each time group were divided into three subgroups, and RNA was extracted using the Micro RNA easy kit (Qiagen, Mississauga, ON). Small amounts of RNA were reverse-transcribed by using SuperScript III platinum two-step qRT-PCR kit with ROX (Invitrogen, Carlsbad, CA). The resulting templates (50 ng of cDNA for target genes and 5 ng for 18S rRNA) were quantified by real-time PCR (ABI Prism 7700; Applied Biosystems, Foster City, CA). Primers and TaqMan probes for total VEGF, platelet endothelial cell adhesion molecule (PECAM)-1, angiopoietin (Ang)-1, Ang-2, and FGF2 were purchased from Applied Biosystems as Assays-on-Demand for murine genes. For each probe, a dilution series determined the efficiency of amplification of each primer-probe set, and the relative quantification method was employed (54). For the relative quantification, PCR signals were compared among groups after normalization using 18S as an internal reference. Relative expression was determined as $2^{-ΔΔCT}$, and the fold-change was calculated as described previously (54).

Statistical analysis. All data are given as means ± SE. Differences between the treatments were assessed by one-way ANOVA followed by a Tukey post hoc test for multiple comparisons, with $P < 0.05$ considered to be significant.

RESULTS

Inhibition of epithelial branching morphogenesis. Lungs from Tie2-LacZ mice were dissected at E11.5 and cultured with either PBS, AT, L-AT (100 μg/ml), heparin, ATH, or L-ATH. Whole mounts revealed a complete image of the developing airways at 4 days of growth under in vitro conditions (Fig. 1). Observations for the effect of agents on global lung development were recorded as number of terminal buds and branching morphogenesis. A decrease in the bud counts were observed in lungs treated with AT and L-AT compared with the buffer control at 4 days of growth. This effect was more pronounced in lungs incubated with L-AT and heparin compared with ATH, L-ATH, and PBS. Branching morphogenesis was quantified by counting the number of terminal buds ($n = 5$, from 5 separate experiments) (Fig. 2). Terminal bud formation was inhibited (relative to PBS control) by treatment with heparin followed in decreasing degree by L-AT, AT, and ATH. No significant inhibition of bud formation was observed with lung grown in the presence of L-ATH. Thus, ATH complex did not have the developmental inhibitory effects of the L-AT and heparin moieties.

Inhibition of lung vascularization. Vasculature analysis of 4-day-old lungs by the X-gal staining procedure is shown in Fig. 3. Whole mount LacZ staining revealed a complete image of the developing airways. At E11.5, the lung rudiments consisted of the developing airways. During culture, evolution of additional branching gave exponential growth and complexity in the distal airways. LacZ staining of explants cultured with PBS, ATH, and L-ATH (Fig. 3) revealed a more elaborate distal branching compared with AT, heparin, and L-AT (Fig. 3). Explants cultured with PBS, ATH, and L-ATH had a more complex vascular network in the mesenchyme surrounding the...
developing lung buds than explants cultured with AT, heparin, and L-AT at 96 h of growth. Arrangement of vessels in the explants also varied depending on the treatment given. Treatment with AT, heparin, and L-AT (Fig. 3) revealed that vascularization was decreased in the lung explants. However, explants incubated with PBS, ATH, and L-ATH (Fig. 3) showed X-gal-positive-stained vessels along the trachea, main bronchi, and smaller airways, up to the distal branch tips (edges) of the developing lungs. These results suggest that the presence of AT, heparin, or L-AT have an antiangiogenic effect on lung development. Effects of the various agents on vascular development were assessed by quantification of X-gal-stained areas in the explants using Open Lab Software. Results are shown as means ± SE (n = 5) in Fig. 4. Analysis of relative vascular area showed that heparin (0.46 ± 0.06) and L-AT (0.34 ± 0.035) inhibited angiogenesis compared with PBS (0.61 ± 0.08), ATH (0.60 ± 0.13), and L-ATH (0.72 ± 0.03); P < 0.05. Moreover, significant antiangiogenic effects were observed by treatment of explants with AT and L-AT, whereas inhibition of vascular development by heparin and ATH occurred to a lesser extent. Treatment with L-ATH revealed X-gal-stained vessel area comparable to that of PBS controls (P > 0.05). Again, as with lung bud morphology, maintenance of AT and heparin in a covalent complex ameliorated disruptive effects of both these moieties on vascularization.

Regulation of growth factor gene expression by latent forms of AT. Since angiogenesis is a complex physiological process thought to be controlled by a wide variety of genes (19), we expected that conformationally altered forms of AT would induce antiproliferative effects on endothelial cells by altering the gene expression profile. Therefore, we determined the
impact of L-AT and L-ATH administration on differential gene expression of Ang-1, Ang-2, FGF (as FGF2), VEGF, and PECAM in Tie2-LacZ mice lung explants after 4 days in culture using RT-PCR (Fig. 5). It was observed that expression of all genes studied was downregulated by exposure to the L-AT product (Fig. 5). A decrease in expression of Ang-1 and Ang-2 due to L-AT (Fig. 5) was much more pronounced relative to that for transcription of the other genes studied. In contrast, expression of Ang-1, FGF2, PECAM, and VEGF all increased relative to PBS control when L-ATH was applied (Fig. 5). Thus, L-AT-heparin conjugation reversed the decreased growth factor gene expression profiles in the developing lung tissue seen with free L-AT. A few short-term experiments (n = 3) with heparin gave intermediate results [log_{10}(2^{-\Delta\Delta Ct})] for expression of Ang-1, Ang-2, FGF2, PECAM, and VEGF in lungs treated with heparin was 0.47 ± 0.17, −0.84 ± 0.09, 0.42 ± 0.01, 0.10 ± 0.07, and 0.01 ± 0.03, respectively.

Inhibition of morphogenesis by L-AT and L-ATH supplemented with FGF2. Treatment with L-AT induces apoptosis of cultured endothelial cells and inhibits cell migration towards FGF2 (53). Thus, we further examined the effects of L-AT and L-ATH on the branching morphogenesis and vasculature of explants in media supplemented with FGF2. Terminal bud count analysis was done on whole mounts of explants by counting the number of buds (n = 5) under the light microscope (Fig. 6). Results show that when L-AT- and L-ATH-containing media were supplemented with FGF2, there was a significant decrease in the bud count number relative to buffer alone. The effect of FGF2 inclusion with L-AT and L-ATH treatments on development of the pulmonary vascular system was also investigated (Fig. 7). Similar to the lung morphology, vasculature analysis showed significant inhibition of vessel development in L-AT and L-ATH experiments containing FGF2 compared with those without FGF2 supplementation. Alternatively, enhanced vascularization was seen in explants bathed in media with either L-ATH or PBS. Interestingly, addition of FGF2 itself inhibited lung growth, particularly in bud number (Fig. 6). Compared with FGF2-supplemented media, combination of FGF2 with latent forms showed trends

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**Fig. 4.** Quantification of pulmonary vascular area. Vessels in murine lung explants were visualized by X-gal staining, and total area was determined using computerized digital analysis of micrographs. Murine lung explants were cultured for 4 days in either PBS buffer, AT, heparin, ATH, L-AT, or L-ATH. Data are expressed as means ± SE (n = 5). *P < 0.05, relative to PBS control.

**Fig. 5.** Effect of latent antithrombins on growth factor gene expression. RNA was extracted from murine lung explants grown for 4 days in culture in either PBS buffer, L-AT, or L-ATH. RT-PCR technology was used to determine mRNA levels for angiopoietin-1 (A), angiopoietin-2 (B), fibroblast growth factor 2 (FGF; C), platelet endothelial cell adhesion molecule-1 (PECAM; D), and vascular endothelial growth factor (VEGF; E). Data are reported as log_{10}(2^{-\Delta\Delta Ct}), which represents the logarithmic change in test sample relative to control of the duplicate threshold cycle value for the target gene normalized relative to the 18S reference gene. Data are expressed as means ± SE (n = 5).

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for decreased lung development that approached significance ($P < 0.1$), except for bud number in the L-AT plus FGF2 group that had an inordinately large SE. Therefore, overall, it is apparent from our work that even if FGF2 protein was added to cell environments, a somewhat negative effect on epithelial branching morphogenesis and vascular growth occurs in the presence of L-AT, with or without bound heparin.

**DISCUSSION**

The potential to inhibit angiogenesis is important when considering therapeutic strategies for drug treatment of neovascular diseases (7). Activities of these agents can affect alterations in the angiogenic process, including vascular atrophy, endothelial migration and proliferation, and three-dimensional restructuring of blood vessels. Recent work has clearly shown that both cleaved and latent forms of the serpin AT display antiangiogenic properties (60). In this study, we demonstrated that antiangiogenic effects of L-AT on lung morphology can be prevented when the protein is permanently bound to heparin. Consequently, covalent ATH may have enhanced safety as an anticoagulant treatment during vascular development. Furthermore, anticoagulant activity of L-ATH doses tested here were comparable to that of ATH studied previously in animals. For example, levels of $\sim 5$–$50$ U/ml anti-factor Xa activity have been measured in lung lavage fluid 48 h after intratracheal instillation of ATH treatment into rabbits (21) or rats (13). Incubation of the lung explants with 100 $\mu$g/ml (equal to 31 $\mu$g/ml in terms of heparin content) of L-ATH at 448 U/mg heparin content corresponds to 13.7 U/ml anti-factor Xa activity, a value well within the range used for intrapulmonary administration.

Previous work has shown that the AT and heparin moieties of ATH affect development of certain structures and functions present in the lung. Different forms of AT (free and latent) are known to hinder angiogenesis (51, 53, 60). Additionally, inhibition of thrombin by heparin may reduce the expression of procoagulant cell receptors (9), and anticoagulant molecules can be mobilized due to displacement from the cell surface by heparin (73). Furthermore, there are a few reports indicating that heparin may also affect surfactant expression by the epithelium (44, 72). If ATH was viable as an anticoagulant to prevent injury from pulmonary fibrin deposition in the developing lung, it was crucial to confirm that components in its structure did not cause interference with normal lung growth. L-AT had an inhibitory effect on lung epithelial branching and pulmonary vascular development compared with L-ATH (Figs. 1–4). In contrast, the number of terminal buds (representing epithelial branching morphogenesis) in explants treated with PBS and L-ATH were found to be comparable ($P > 0.05$). Previous work has revealed that the affinity of heparin is vastly reduced for L-AT compared with native AT (62). Thus, interactions with cell surface heparinoids would not be critical in the antiangiogenic effects of latent forms of AT. More detailed microscopic examination confirmed that the lung immaturity induced by L-AT or heparin was not present with the covalent complex. It has been shown recently that L-AT is effective in halting tumor growth at the low dose of 1 mg·kg$^{-1}$·day$^{-1}$ (53) by exerting its anti-tumor effects through inhibition of angiogenesis. This finding complements data in our study where both L-AT and heparin also displayed antiangiogenic effects with decreased growth in vascular area and less complex branching compared with L-ATH. Heparin has been shown to significantly effect cell proliferation (27, 66) and chemotaxis (74), which may have been a factor in the uneven branching and dilation observed in heparin-treated lungs (Fig. 1). Similar to the morphological studies, since L-AT has low affinity for heparin [$K_d$ of $\sim 10^{-4}$ M (62)], mechanisms involving inhibition of angiogenesis occur without interaction of the serpin with heparinoids (either free heparin or cell surface proteoglycans). However, in the case of ATH, permanent covalent linkage will constrain close association of the heparin chain with L-AT in the complex. The ever-present heparin may thus prevent adverse association(s) of L-AT in L-ATH with cell surface molecules involved in the antiangiogenic mecha-

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**Fig. 6.** Lung morphogenesis in samples supplemented with FGF2. The number of terminal buds are shown for murine lung explants cultured for 4 days in either PBS buffer, PBS buffer supplemented with FGF2 (PBS_FGF), L-AT, L-AT supplemented with FGF2 (L-AT_FGF), L-ATH, or L-ATH supplemented with FGF2 (L-ATH_FGF). Data are expressed as means ± SE ($n = 5$). $*P < 0.05$, relative to PBS control.

**Fig. 7.** Lung vascular development in samples supplemented with FGF2. Total X-gal-stained vessel area is shown for murine lung explants cultured for 4 days in either PBS buffer, PBS_FGF, L-AT, L-AT_FGF, L-ATH, or L-ATH_FGF. Data are expressed as means ± SE ($n = 5$). $*P < 0.05$, relative to PBS control.
nism(s) followed by free L-AT. To a lesser degree, covalent coupling of the AT (or L-AT) protein may hinder heparin’s cell receptor interactions responsible for stunting vessel growth. Considering simple mixtures of AT plus heparin, it is well known that noncovalent AT-heparin complexes variably dissociate to free AT and heparin molecules that bind to other plasma or cell surface proteins (23, 55). Furthermore, significant amounts of heparin molecules in AT plus heparin mixtures are always in the free state since up to 2/3 of pharmaceutical heparin chains have no binding affinity for AT (43). These “no-affinity” heparin molecules can, nonetheless, participate in adverse effects on lung growth. Thus, only ATH with permanently linked AT (L-AT) and heparin was deemed useful to study possible effects of such complexes on the development of lung morphology and vessels.

The early stages of lung vascular development involve both angiogenesis and vasculogenesis (28, 63). Normal vascular growth results from a complex interplay of growth factors, including VEGF and the angiopoietins (29). VEGF has been determined as a selective mitogen for endothelial cells, thus influencing angiogenesis and vasculogenesis during normal development (32), but signaling molecules that control this process are not known. Alternatively, endogenous proteolytic cleavage protein fragments such as angiostatin [plasminogen product (59)] and endostatin [COOH-terminal portion of collagen XVIII (31, 57, 61)] inhibit angiogenesis by specific interference of endothelial cell function (53). Interestingly, angiostatin and endostatin have been shown to arrest tumor expansion in a synergistic manner (10). Similarly, the antiangiogenic activity of conformationally altered forms of AT is also well established, but molecular mechanisms underlying the serpin’s antiangiogenic activity remain to be determined. While the function of proangiogenic growth factors has been traditionally associated with promotion of cell proliferation, these factors are increasingly recognized as important mediators of tissue interactions in the developing and adult lung. Thus, although stimulus of the growth factor mRNA expression is not directly related to angiogenic activity or ability to affect morphology, induction of blood vessel development by these factors may indeed be an ancillary feature affecting the evolution of lung structures. To further understand the integrated relationships between the pro- and anti-stimuli of vascular evolution, we studied expression of Ang-1, Ang-2, FGF2, PECAM, and VEGF in the explants treated with PBS, L-AT, and L-ATH. L-AT downregulated the mRNA expression of all genes studied in the present investigation compared with the L-ATH. As expected, the level of transcripts was not affected by treatment of explants with the PBS buffer vehicle. The study by Zhang et al. (87) has shown that the antiproliferative effects of the cleaved and latent conformations of AT on human umbilical vein endothelial cells, a component of the more complex antiangiogenic biological activity of these AT forms (53, 60), is correlated with the downregulation of a key proangiogenic extracellular matrix heparin sulfate proteoglycan, perlecan. Further reports show that, in addition to perlecan, cleaved forms of AT significantly alter expression of numerous other endothelial genes, strongly implicating an endothelial cell ligand-receptor signaling mechanism in mediating these global changes in gene expression (86). In our experiments, the native form of AT typically induced more moderate changes relative to untreated explants, consistent with the reports of increased antiangiogenic potency for the conformationally altered forms of serpin (89). In this sense, AT is distinguished from other antiangiogenic serpins where antiangiogenic activity is not conformationally dependent (89). Both cleaved and latent AT forms have previously been shown to display similar antiangiogenic activities to disturb cell proliferation, cell migration, capillary-tube formation, FGF2 signaling, and perlecan gene expression (53, 87, 88). Again, our findings indicate that once a conformationally altered AT is permanently attached to a heparin chain, ability of the AT component to induce shutdown of growth factor gene expression is negated. As alluded to earlier, associations of L-AT with cell surface signaling molecules related to gene function may be hindered by conjugated heparin. As a consequence, L-ATH molecules may remain in the unbound free state allowing their heparin to carry out further pulmonary anticoagulation.

Several members of the FGF2 family and their receptors have been implicated in mouse lung development (65). Previous work has indicated that FGF2-induced angiogenesis in the chick embryo was inhibited by treatment with L-AT (53). To further investigate the relationship of L-AT-containing molecules on exogenous FGF2 protein mechanisms, murine embryo lungs were placed in FGF2-containing media with either L-AT or L-ATH. Relative to normal buffer, FGF2-treated explants showed inhibition of both terminal branching morphogenesis (Fig. 6) and pulmonary vascular development (Fig. 7) when supplemented with L-AT or L-ATH. Interestingly, FGF2 treatment itself had an inhibitory effect on branching (Fig. 6). Similar to our experimental system, other studies have reported conditions where FGF has variable effects on lung morphogenesis (40). Nevertheless, neither L-AT nor L-ATH overcame any potential FGF2-mediated effects to return morphology and vascularization to that with normal buffer (Figs. 6 and 7). Previous results have demonstrated that cleaved and latent forms of AT exert their antiangiogenic effects by inducing cell apoptosis (53, 67, 89), inhibiting endothelial cell cycle progression (87), and suppressing HUVEC expression of many proangiogenic products such as perlecan (87, 89). Alternatively, it has been suggested that cleaved and latent AT can compete with proangiogenic growth factors FGF2 and VEGF for binding to “low affinity” heparin sulfate coreceptors, thereby attenuating growth factors binding to “high affinity” endothelial cell protein receptors where they exert their mitogenic effects (68, 84). However, although L-ATH also caused inhibition of angiogenesis with FGF2 supplementation, it is unlikely that the conjugate may compete for perlecan binding since the L-ATH already possesses a heparan sulfate-like heparin chain. A potential alternative is that the heparin moeity in L-ATH may bind the FGF2, which may either prevent FGF2 acting on the cells or cause other unknown effects from the L-ATH-FGF2 complex. Since exogenously added FGF2 alone (no L-AT or L-ATH) did not give significant increases in vessel development relative to the PBS buffer control, it would seem likely that extracellular growth factor is less effective for angiogenic induction. This outcome emphasizes the importance of dampening intracellular growth factor gene expression by L-AT compared with the increase in growth factor transcripts observed with L-ATH. Other work is consistent with
the major deterrent of tissue development relying on upregulation of antiangiogenic genes and/or downregulation of proangiogenic genes in endothelial cells, regardless of extracellular growth factor levels (89). Indeed, changes in intracellular mitogen protein resulting from altered gene activity profiles may be a major contributor to the differential angiogenic effects of the latent AT and ATH serpins. As a result, (L-)ATH may allow/encourage favorable cell propagation as a mechanistic component of the enhanced lung growth. Initial forays into study of effects on lung cell proliferation have shown that native ATH gives significantly increased epithelial growth and a marginal increase in total lung cell number, relative to AT. Complete detailed investigation of the impact on proliferation of all lung cell types by native and latent AT/ATH forms is underway to determine the relationship between effects on cell proliferation and the changes in vascular/branching morphology reported here.

The present study was conducted to evaluate the effect of stable AT-heparin complexation on the inhibition of lung morphological development and angiogenesis by AT and heparin. A primary impetus for this work was to test the potential impact of using ATH and its latent forms as an anticoagulant to treat thrombosis during RDS in the developing lung. Thus, AT, L-AT, heparin, ATH, and L-ATH were compared on an equimolar basis to assess the relative intrinsic effects of these molecules on lung airway and vascular development. In this setting, (L-)ATH had reduced inhibition of lung bud formation and angiogenesis compared with the free serpin or heparin. However, since L-ATH (or ATH) has a significantly higher specific anti-Xa activity than heparin, it could be used at much lower concentrations to achieve the same anticoagulant effect. Consequently, any antiangiogenic effects of a subpopulation of L-ATH molecules would be minimized due to the reduced dosages required to achieve the same effective anticoagulation as with standard heparin. This alternative possibility for lower inhibitory effects on lung development by (L-)ATH over heparin further enhances this drug’s potential application for thrombotic treatment.

In conclusion, these findings indicate that covalent linkage of AT to heparin prevents effects caused by the free moieties on lung morphology, vascularization, and gene expression profiles for common growth factors involved in vascular development. Thus, AT may be safe for use as an intrapulmonary treatment in premature infants. Future work is necessary to determine cell surface molecules involved in antiangiogenic mechanisms of L-AT that are obviated by bound heparin chains. Comparative receptor binding and other studies with AT, L-AT, and various ATH derivatives can then be done to clarify mechanisms underlying the differential developmental effects.

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