Role of the TGF- β /Alk5 Signaling Pathway in Monocrotaline-induced Pulmonary Hypertension

Ari L. Zaiman¹, Megan Podowski¹, Satya Medicherla², Kimberley Gordy¹, Fang Xu³, Lijie Zhen⁴, Larissa A. Shimoda¹, Enid Neptune¹, Linda Higgins², Alison Murphy², Sarvajit Chakravarty², Andrew Protter², Pravin B. Sehgal³, Hunter C. Champion⁵, and Rubin M. Tuder^{1,5}

¹Division of Pulmonary and Critical Care Medicine, Department of Medicine, Johns Hopkins School of Medicine, Baltimore, Maryland; ²Scios, Inc., Fremont, California; ³Department Cell Biology and Anatomy, New York Medical College, Valhalla, New York; and ⁴Division of Cardiopulmonary Pathology, Department of Pathology, and ⁵Division of Cardiology, Department of Medicine, Johns Hopkins School of Medicine, Baltimore, Maryland

Rationale: Pulmonary arterial hypertension is a progressive disease characterized by an elevation in the mean pulmonary artery pressure leading to right heart failure and a significant risk of death. Alterations in two transforming growth factor (TGF) signaling pathways, bone morphogenetic protein receptor II and the TGF- β receptor I, Alk1, have been implicated in the pathogenesis of pulmonary hypertension (PH). However, the role of TGF- β family signaling in PH and pulmonary vascular remodeling remains unclear. **Objectives:** To determine whether inhibition of TGF- β signaling will attenuate and reverse monocrotaline-induced PH (MCT-PH).

Methods: We have used an orally active small-molecule TGF- β receptor I inhibitor, SD-208, to determine the functional role of this pathway in MCT-PH.

Measurements and Main Results: The development of MCT-PH was associated with increased vascular cell apoptosis, which paralleled TGF- β signaling as documented by psmad2 expression. Inhibition of TGF- β signaling with SD-208 significantly attenuated the development of the PH and reduced pulmonary vascular remodeling. These effects were associated with decreased early vascular cell apoptosis, adventitial cell proliferation, and matrix metalloproteinase expression. Inhibition of TGF- β signaling with SD-208 in established MCT-PH resulted in a small but significant improvement in hemodynamic parameters and medial remodeling.

Conclusions: These findings provide evidence that increased TGF- β signaling participates in the pathogenesis of experimental severe PH.

Keywords: pulmonary hypertension; transforming growth factor- β ; apoptosis; proliferation; matrix metalloproteinase

Genetic studies of familial idiopathic pulmonary hypertension (PH) revealed that a germline mutation in one copy of bone morphogenetic protein (BMP) receptor II occurs in about 80% of patients with familial idiopathic PH (1, 2). The importance of transforming growth factor (TGF) signaling is underscored by the association of loss-of-function mutations of TGF- β receptor I, Alk1, with pulmonary arterial hypertension (PAH), as well as somatic microsatellite instability of the TGF- β receptor II gene in plexiform lesions present in pulmonary arteries of patients

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Transforming growth factor (TGF)- β signaling pathways have been implicated in the pathogenesis of pulmonary hypertension (PH); however, the mechanism by which altered signaling leads to PH is unknown. To understand its role, animal models have been used, but the level of TGF- β varies.

What This Study Adds to the Field

Increased TGF- β signaling participates in the pathogenesis of experimental severe PH.

with idiopathic PAH (IPAH) (3, 4). On the other hand, there is also evidence of increased expression of TGF- β isoforms (5), TGF- β and BMP receptors, and enhanced TGF- β -dependent signaling in both familial PAH and IPAH lungs. These findings suggest that PH might develop due to unbalanced TGF- β signaling in pulmonary vascular cells rather than a simple loss of TGF- β signaling. The concept of imbalanced TGF- β signaling has been supported by the findings of enhanced TGF- β signaling in the setting of TGF- β receptor mutations in systemic vascular abnormalities (6–8).

Activation of TGF- β , which is stored as an inactive dimer bound to the extracellular matrix, leads to its interaction with TGF- β receptor II, a constitutively active serine/threonine kinase that subsequently recruits and phosphorylates TGF- β receptor I. Two distinct forms of the TGF- β type I receptors with distinct cellular distributions and downstream gene targets, Alk1 and Alk5, undergo a conformational change resulting in the phosphorylation of the receptor smad (R-smad) proteins (9). Phosphorylated R-smads 1, 2, 3, 5, and 8 interact with co-smad 4, leading to the translocation of the paired smads to the nucleus where they can complex with other proteins and alter transcription. The inhibitory smads 6 and 7, when activated by the ligand, compete for binding to type I receptors, targeting them for degradation (10–12). In addition to the smad-dependent signaling pathway, both TGF- β and BMPs have been demonstrated to activate mitogen-activated protein kinases (MAPKs) in a smad-independent manner (13, 14).

Several studies using experimental models of PH have provided an unclear picture of the role of TGF- β family signaling in the disease. There are abundant data documenting both increased and decreased TGF- β signaling in the disease (15–25). Significantly, no functional studies have been performed to address TGF signaling in animal models of severe PH.

To elucidate the role of TGF- β signaling in the development of severe PH, we inhibited TGF- β signaling in the monocrota-

(Received in original form July 23, 2007; accepted in final form January 16, 2008)

Supported by the National Institutes of Health, National Heart, Lung and Blood Institute grants K08 HL076297 (A.L.Z.), R01 HL 66554 (R.M.T.), HL 084946 (project 2 to H.C.C. and project 5 to R.M.T., and Pathology Core to R.M.T.), and HL077301 (P.B.S.); H.C.C. is a recipient of the Shin Chun-Wang Young Investigator Award and the Giles F. Filley Memorial Award from the American Physiological Society. This work was also supported in part by the Bernard A. and Rebecca S. Bernard Foundation, a scientist development grant from the American Heart Association, and the W.W. Smith Foundation.

Correspondence and requests for reprints should be addressed to Ari L. Zaiman, M.D., Ph.D., Johns Hopkins School of Medicine, Division of Pulmonary and Critical Care Medicine, 1830 East Monument Street, 5th Floor, Baltimore, MD 21205. E-mail: azaiman1@jhmi.edu

Am J Respir Crit Care Med Vol 177, pp 896–905, 2008

Originally Published in Press as DOI: 10.1164/rccm.200707-1083OC on January 17, 2008
Internet address: www.atsjournals.org

line (MCT) model of severe PH using an orally active Alk5 inhibitor, SD-208. Using a comprehensive set of physiologic and pathologic endpoints, we documented an early protective effect, associated with decreased smooth muscle cell proliferation, and increased rate of medial cell apoptosis.

METHODS

Animals

The Animal Care and Use Committee of the Johns Hopkins University School of Medicine approved the animal protocol. Male Sprague-Dawley rats (200–250 g) were randomly divided into control and MCT-treated groups. The MCT-treated rats (MCT from Sigma-Aldrich, St. Louis, MO) received a single subcutaneous injection of 60 mg/kg, and were randomly assigned to receive 20 mg/kg SD-208 or 60 mg/kg SD-208, or an equal volume of 1% methylcellulose (vehicle) by oral gavage twice a day. The number of rats in each experimental group is highlighted in RESULTS and in the figure legends.

TGF- β Inhibition

SD-208 is a TGF- β receptor I (TGF β RI) kinase inhibitor developed by Scios, Inc. (Fremont, CA). The ability of SD-208 to inhibit the respective kinase was determined by comparing counts incorporated in the presence of compound with those incorporated in the absence of compound. SD-208 has a half-maximal inhibitory concentration (IC₅₀) of 49 nmol/L based on direct enzyme assay of TGF β RI kinase activity with specificity of at least more than 17-fold over members of a panel of related protein kinases (26, 27). SD-208, which has previously been used in an orthotopic xenograft mouse model of pancreatic cancer, revealed a dose-dependent decrease in the incidence of metastasis at 20 and 60 mg/kg (28). Dosing was based on these and other preliminary studies performed by Scios, Inc.

MCT-induced Megalocytosis of Hepatocytes and Endothelial Cells

Human HepG2 and bovine pulmonary artery endothelial cells (PAECs) were cultured in T-75 flasks and in 6-well plates as described previously (29). MCT was purchased from TransWorld Chemicals (Rockville, MD) and either used as such or chemically converted to MCT pyrrole (MCTP) as described previously (29, 30).

For experiments with HepG2 cells, freshly seeded cultures in 6-well plates (2×10^5 cells/well; cultures were used 4–5 h after plating) were exposed to SD-208 at the indicated concentrations and then, 1 hour later, were also exposed to MCT (1.5 mmol/L). These were then imaged using phase-contrast microscopy at daily intervals for 2 days (each well was photographically sampled at each time point in three random locations using camera frames corresponding to $350 \times 250 \mu\text{m}$). For experiments with PAECs, cultures in 6-well plates (3×10^5 cells/well were used 16–20 h after seeding) were exposed to SD-208 at the indicated concentrations and to MCTP (29, 30) in various temporal combinations, and observed for up to 48 hours. The PAEC cultures were imaged as described for HepG2 cells. At the conclusion of each experiment, all cultures were fixed in cold paraformaldehyde (29, 30). All experiments were replicated at least twice.

Image analyses were performed using the NIH Image J software (National Institutes of Health, Bethesda, MD) in two ways: (1) cell number was evaluated in terms of the number of cells per $350 \times 250\text{-}\mu\text{m}$ frame and (2) megalocytosis was quantified based on the increase in the nuclear diameter expressed in arbitrary area units.

Hemodynamic Monitoring

In vivo hemodynamic analysis was performed by placing a four-electrode pressure–volume catheter (Millar Instruments, Houston, TX) through the right ventricular (RV) apex in the open-chest, anesthetized rat to record chamber volume by impedance and pressure micromanometry (31).

Heart and Lung Processing

After hemodynamic measurements were obtained, the cardiopulmonary system was flushed with saline and the right bronchus was ligated. The left lung was then inflated with melted agarose in phosphate-

buffered saline as described (32), and the heart and lungs removed en bloc.

Pulmonary Vascular Morphology and Morphometry

Briefly, the lung was stained for α -smooth muscle actin. Using the Image-Pro Plus software (Media Cybernetics, Bethesda, MD), arteries between 50 and $200 \mu\text{m}$ with a circular or quasi-circular outline were examined. Averages of at least 20 vessels from each animal were taken for medial thickness. Percentage of medial thickness was calculated as (medial thickness $\times$ 2/external diameter) $\times$ 100.

Western Blot Analysis

Membranes were incubated with the primary antibody for 1 hour at room temperature. Detection was by the Pierce West Dura system (Pierce Biotechnology, Rockford, IL). Primary antibodies were as follows: α -smooth muscle actin (1:1,000; Sigma-Aldrich), matrix metalloproteinase (MMP)-2 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and MMP-9 (1:100; Santa Cruz Biotechnology).

Immunohistochemistry

Immunohistochemistry was performed as previously described (33). Antibodies were used at the following concentrations: active caspase 3 (1:50; Cell Signaling, Danvers, MA), smooth muscle actin (1:500; Dako, Carpinteria, CA), proliferating cell nuclear antigen (PCNA) (1:25; Santa Cruz), MMP-2 (1:1,000; Santa Cruz Biotechnology) phospho-Smad2 (1:1,000; Cell Signaling).

Statistical Analysis

Data are expressed as means $\pm$ SEM. Comparisons between groups were performed with one-way analysis of variance (ANOVA) and Tukey multiple-comparisons test, followed by Dunn's multiple-comparison test, as appropriate. At least five animals were used per group. A *P* value less than 0.05 was considered significant. Sigma Stat software (Systat Software Inc., San Jose, CA) was used for all analyses. In the cell culture studies, statistical significance was determined by the two-tailed Student's *t* test.

RESULTS

SD-208 Does Not Inhibit Activation of MCT in Cultured Liver Cells and Does Not Prevent Endothelial Cell Damage *In Vitro*

MCT-induced PH follows pulmonary endothelial cell injury by the MCTP generated in the liver (34). Liver cells metabolize pyrrolizidine alkaloids such as MCT to the bioactive pyrrolic derivative by the cytochrome P450 system, thereby causing megalocytosis (enlarged cells with enlarged nuclei) (29, 35–38). To ensure that a selective 2,3-disubstituted pteridine-derived ATP competitive inhibitor of TGF β RI kinase, SD-208, did not interfere with the generation of MCT-PH, metabolic activation of MCT was evaluated in cultured HepG2 liver cells. This compound has been successfully used *in vivo* and *in vitro* to prevent TGF- β -induced Alk5 phosphorylation and subsequent smad2 phosphorylation, TGF- β -dependent myofibroblastic differentiation, and pulmonary fibrosis (27, 39, 40). Cultures of HepG2 liver cells, when exposed to unmodified MCT, developed marked megalocytosis 48 hours after addition of MCT (Figures 1A–1C). Prior exposure of these cultures to SD-208 at concentrations as high as 1 or $5 \mu\text{M}$ did not affect the development of MCT-induced megalocytosis (Figures 1A–1C). SD-208 alone had little effect on HepG2 cell morphology (Figures 1A and 1B) but led to a partial decrease in cell proliferation (Figure 1C). Taken together, these data suggest that SD-208 does not affect the metabolism of MCT and does not directly interfere with the liver cell toxicity of the MCTP.

Although SD-208 does not affect the MCT metabolism or megalocytic cellular changes in hepatocytes, a potential interference of SD-208 on MCTP toxicity in endothelial cells was evaluated in PAECs exposed to chemically synthesized MCTP. Figures 2A and 2B demonstrate the development of megalocy-

Figure 1. SD-208 does not affect monocrotaline (MCT)-induced megalocytosis in cultured liver HepG2 cells. HepG2 liver cells in 6-well plates were treated with SD-208 for 1 hour at the indicated concentrations and then with MCT (A and B). Phase-contrast microscopy and image analyses were used to quantitate megalocytosis expressed as nuclear size based on arbitrary area units and cell number per 350×250 - μm frame as described in METHODS. (A) and (B) represent independent experiments; data in (C) are derived from the experiment in (B). Scale bar = $50 \mu\text{m}$; not significant (n.s.), $P > 0.05$ in comparison with the SD-208-free MCT-treated group; $*P < 0.05$ in comparison with the SD-208- and MCT-free group.

tosis in cultures of PAECs exposed to MCTP (29). The prior exposure of these cells to SD-208 did not affect subsequent MCTP-induced megalocytosis (Figures 2A and B). Moreover, the addition of SD-208 to PAEC cultures 1 day after MCTP had only a minimal effect on the persistence of these cytopathic changes.

Taken together, these cell culture-derived studies strongly suggest that SD-208 does not interfere with the generation of MCTP and MCTP-induced cell toxicity.

SD-208 Attenuates MCT-induced PH

Sprague-Dawley rats treated with a single subcutaneous injection of MCT (60 mg/kg) developed severe and irreversible PH at 4 weeks, characterized by elevated pulmonary arterial pressures, RV peak pressures, and tau (time constant of isovolumetric relaxation), and diminished RV function with increased RV end-diastolic pressure and decreased cardiac output. Furthermore, there was significant RV and pulmonary artery medial hypertrophy (Figure 3).

Fourteen days after the administration of MCT, there was evidence of increased TGF- β signaling as documented by enhanced psmad2 staining in pulmonary artery vascular cells. To address the role of enhanced TGF- β signaling in the initiation and progression of MCT-PH, we administered SD-208 to rats treated with MCT. On the day of MCT administration, rats began twice-daily treatments with 60 mg/kg SD-208 (maximum inhibition), 20 mg/kg SD-208 (partial inhibition), or 1% methylcellulose (vehicle) for 4 weeks. The development of PH was markedly attenuated in a dose-dependent manner by SD-208 (Figure 3). There was a significant improvement of hemodynamic parameters, including a 27% reduction in RV systolic pressure (53.4 vs. 38.9 mm Hg), a 53% reduction in RV diastolic pressure (12.2 vs. 5.7 mm Hg) with a concurrent 23.5% increase in cardiac output (91.4 vs. 112.9 mm/min), and a 25% reduction in RV hypertrophy calculated by right ventricle/left ventricle + septum (0.44 vs. 0.33), supporting an important role of TGF- β signaling in the development of MCT-induced PH.

Figure 2. SD-208 does not affect monocrotaline pyrrole (MCTP)-induced megalocytosis in cultured pulmonary artery endothelial cells (PAECs). PAECs plated onto 6-well plates were treated with MCTP alone or in combination with SD-208 at the indicated concentrations 1 hour before or 20 hours after MCTP (A). Megalocytosis was quantified in phase-contrast microscopic images based on nuclear size expressed in arbitrary area units and cell number per 350×250 - μ m frame as described in METHODS (B). Scale bar = 50 μ m; not significant (n.s.), $p > 0.05$ in comparison with the SD-208-free MCTP-treated group; * $P < 0.05$ in comparison with the SD-208- and MCTP-free group. Cont. = untreated control.

To determine whether SD208 indeed inhibited TGF- β signaling, we evaluated psmad2 activation, a validated approach to measure TGF- β signaling in histologic preparations (41). Immunohistochemical analysis revealed significantly decreased psmad2 staining within the vascular and alveolar cells of SD-208-treated lungs, consistent with marked inhibition of TGF- β signaling (Figure 4).

Smooth Muscle Proliferation in MCT-PH: Effect of SD208

MCT-treated rats developed marked medial thickening, which was attenuated with the administration of SD208 (Figure 5). Furthermore, SD-208 attenuated vascular smooth muscle cell proliferation based on the expression of PCNA assessed by immunohistochemistry (Figure 6).

Because TGF- β can be released from the endothelium undergoing apoptosis or from extracellular matrix breakdown, potentially resulting in vascular cell proliferation (18, 42–45), we investigated whether there was an association between endothelial cell apoptosis and vascular smooth muscle cell proliferation after MCT treatment. We found an increased level of vascular cell apoptosis within the same time frame as we observed increased smooth muscle cell proliferation (Figure 7).

Extracellular matrix breakdown can cause TGF- β release from its latent binding protein and thus contributes to the available pool of active TGF- β . This process may result from MMP activation, which has been implicated in the progression of MCT-induced PH (44, 46, 47). To evaluate the role of TGF- β inhibition on lung MMP levels, lung homogenates were probed for MMP-2 and MMP-9 expression (Figure 8A). MCT treatment resulted in an increased expression of MMP-2 and MMP-9, which was attenuated by SD208 treatment. This increased expression of MMP-2 was partly localized to the pulmonary arteries (Figure

8B). In addition, MMP-2 activity, when assessed by zymography, was markedly increased over that of MMP-9, despite increased protein expression of both MMP-2 and MMP-9 (Figure 8C).

Inhibition of TGF- β Activity Causes Partial Improvement of Established PH

To determine whether ongoing TGF- β signaling was required to maintain the pulmonary arterial pressures caused by MCT treatment, we inhibited TGF- β signaling in rats with established PH (i.e., 21 d after MCT injection). The experiment was terminated early when both the control and treated animals began to die at Day 30. Despite the lack of survival benefit, there was a 15% decrease in the mean pulmonary artery pressure and a 26% increase in cardiac output with SD-208. In addition, measurements of systolic and diastolic functions measured by tau and (dP/dt)/IP (maximum rate of increase in ventricular pressure over instantaneous pressure), respectively, improved (Figure 9). Although these improvements of indices of heart function may have resulted from decreased pulmonary pressures, inhibition of TGF- β signaling with SD-208 could also have had a direct beneficial effect on cardiac function. Although inhibition of TGF- β did not reverse established disease, it improved several hemodynamic parameters. This suggests an important, but diminished, role of TGF- β signaling through the progression of MCT-induced pulmonary vascular disease.

DISCUSSION

We have hypothesized that the predisposition to PH results from an “unbalanced” TGF signaling, in which enhanced TGF- β /Alk5 signaling might contribute to experimental PH caused by MCT. To directly address the role of TGF- β signaling in MCT-induced

Figure 3. Development of monocrotaline-induced pulmonary hypertension (MCT-PH) and right ventricular (RV) dysfunction are attenuated by SD-208. (A) Right ventricle/left ventricle + septum (RV/LV+Sep); (B) RV systolic pressure (RVSP); (C) RV diastolic pressure (RVDP); (D) Mean RV pressure; (E) cardiac output (co); (F) tau; (G) (dP/dt)/IP (maximum rate of increase in ventricular pressure over the instantaneous pressure). Statistical significance determined by ANOVA and pairwise multiple comparisons performed with the Tukey test; $n = 7$ for all conditions except control where $n = 4$. * $P < 0.05$ compared with control; # $P < 0.05$ compared with MCT.

PH, SD208, a small-molecule inhibitor of TGF β RI Alk5 was used. These studies have relevance to human IPAH, as we observed evidence of TGF- β signaling and expression of TGF- β receptors and signaling smad(s) in endothelial and smooth muscle cells in remodeling pulmonary arteries in lungs with IPAH (33).

SD-208 has been previously used to address the role of TGF- β signaling pathway in models of pulmonary fibrosis. Although this small molecule specifically inhibits Alk5 signaling, others have shown that inhibition of Alk5 prevents both Alk5- and Alk1-dependent TGF- β signaling within endothelial cells (48). Our findings indicated that Alk5 signaling alone does not suffice to promote PH or pulmonary vascular remodeling, but enhanced Alk5 signaling plays a central role in the development of

Figure 4. SD-208 inhibits transforming growth factor (TGF)- β activity assessed by p-smad2 expression. Histologic sections of agarose-inflated lungs from animals treated with monocrotaline (MCT) alone or MCT and 60 mg/kg SD-208 were assessed for psmad2 expression by immunohistochemistry. Positive cells were counted at $\times 40$ original magnification and normalized to total tissue area (μm). Ten to 15 images were captured from each animal ($n = 6$). Statistical significance determined by analysis of variance and pairwise multiple comparisons performed with the Tukey test.

MCT-induced PH. In addition to correlating increased psmad2 staining, a marker of TGF- β signaling, with enhanced pulmonary vascular cell apoptosis and initiation of vascular smooth muscle cell proliferation, we also documented that these alterations appear to be directly related to TGF- β signaling early in the development of PH. Importantly, our *in vitro* studies support a direct role of TGF signaling in the development of MCT-PH and make it unlikely that the observed SD-208 inhibition of MCT-induced PH is due to an interaction of SD-208 with the liver in processing of MCT to its active metabolite or MCTP-induced endothelial cell toxicity. Inhibition of TGF signaling resulted in a marked attenuation of the pulmonary vascular remodeling and right heart failure. In contrast to the recent documentation of reduced expression of TGF- β recep-

A % Medial Thickness

Figure 5. Pulmonary vascular remodeling (medial thickness) is attenuated by SD-208. Histologic sections of agarose-inflated lungs were immunostained for endothelial cells with CD31 and for smooth muscle cells with smooth muscle cell α -actin antibodies. At least 10–15 medium-sized vessels (50–200 μ m) were analyzed from each animal ($n = 6$). Random circular vessel profiles were selected and the internal and external diameter was determined with Image-Pro (Media Cybernetics). Medial thickness was calculated as (external diameter – internal diameter)/external diameter. Statistical significance determined by analysis of variance and with pairwise multiple comparisons performed with the Tukey test. CON = control.

tor(s) and smad(s) in this model (25), our study showed early increase in pulmonary vascular TGF- β signaling due to MCT. This difference may result from the use of whole lung lysates for the quantification of early psmad2 by Zakrzewicz and colleagues (25), which cannot assess TGF- β signaling in pulmonary vascular cells. Importantly, this study did not address the functional significance of altered TGF- β signaling in MCT-PH. Notably, we demonstrated partial reversal/improvement of several hemodynamic parameters when TGF- β signaling was inhibited after the PH was established. The finding that the inhibition of Alk5 signaling was not as effective in reversing established MCT-induced PH suggests that additional Alk5-independent mechanisms might contribute to the progression of the disease in treated rats.

On the basis of the investigation of rat models of severe PH, early endothelial cell apoptosis of muscular pulmonary arteries is required for the initiation of advanced pulmonary vascular

remodeling (49). This observation has been extended to MCT-PH, as administration of a broad-spectrum active caspase inhibitor at the initiation of MCT treatment prevents PH and markedly attenuates pulmonary vascular remodeling (D. Stewart, Ottawa Health Research Institute, Ottawa, ON, Canada, personal communication). Our data support the association of MCT-PH with early vascular cell apoptosis because there was a temporal correlation between endothelial cell apoptosis and increased TGF- β signaling as assessed by psmad2 staining. These findings correlate well with a previous report showing early and sustained increases in the expression of TGF- β genes (50) in MCT-PH presenting in two pulmonary vascular subcompartments, medial smooth muscle and adventitial fibroblastic cells. Endothelial cells involved in efferocytosis (clearance) of adjacent apoptotic cells might represent alternative cellular sources of TGF- β (51, 52). Compartmentalized TGF- β activation may contribute to vascular smooth muscle cell growth (18), as

Figure 6. Time course of smooth muscle cell proliferation during the development of monocrotaline-induced pulmonary hypertension (MCT-PH). Histologic sections of agarose-inflated lungs were costained for proliferating cell nuclear antigen (PCNA), a marker of proliferation, and smooth muscle cell α -actin. Ten to 15 random circular vessels (50–200 μ m) were analyzed from each animal ($n = 6$). The number of PCNA-positive smooth muscle cells was normalized for vessel size. Statistical significance determined by analysis of variance and with pairwise multiple comparisons performed with the Tukey test.

Figure 7. Inhibition of transforming growth factor (TGF) signaling by SD-208 attenuates intimal apoptosis. Histologic sections of agarose-inflated lungs were costained for activated caspase-3 and α -smooth muscle cell actin. At least 15 random circular vessels (50–200 μ m) were analyzed from each animal ($n = 6$). The number of overlapping DAPI- and activated caspase-3-positive cells was determined. Overlapping positive cells internal to the smooth muscle cell layer were normalized to vessel size. Statistical significance determined by analysis of variance and with pairwise multiple comparisons performed with the Tukey test. Con = control.

demonstrated with multiple growth factors, including platelet-derived growth factor and its receptors, epidermal growth factor signaling, and matrix proteins such as tenascin (17, 53–56).

Extracellular matrix proteolysis by matrix proteases plays a significant role in experimental PH. Both MMP-2 and MMP-9 have been localized to remodeled pulmonary arteries in IPAH (46). Extracellular proteases contribute to remodeling by promoting release of vascular elastase and matrix proteins, because a serine protease inhibitor prevents and reverses MCT-PH (47). Not only can MMP activity, most notably MMP-2, activate

TGF- β , but it is apparent that the converse is true in MCT-PH, because SD208-treated rats had significantly reduced MMP expression and activity. These effects may explain the reduction of adventitial fibroblastic cell proliferation seen in SD208-treated hypertensive rats.

In conclusion, our study revealed a specific pathogenetic role of TGF- β activity early in the course of experimental severe PH caused by MCT. The small improvement in hemodynamic and remodeling argues for a continued role late in the disease, but suggests that TGF- β /Alk5-independent mechanisms operate in

Figure 8. Monocrotaline (MCT)-induced pulmonary hypertension is associated with increased expression of matrix metalloproteinase (MMP)-2 and MMP-9, which are decreased with SD-208 treatment at Days 14 and 28. Whole lung lysates were prepared from animals treated with MCT and MCT + SD-208 at Days 14 and 28. (A) Western blot expression of MMP-2 and MMP-9. (B) Immunohistochemistry of lung tissue demonstrating arterial localization of MMP-2 at selected time points. (C) Zymographic assessment of MMP activity. Representative image of $n = 5$ animals.

Figure 9. Evidence that SD-208 partly reverses pulmonary vascular hemodynamics and right ventricular (RV) function. Twenty-one days after injection of monocrotaline (MCT) (60 mg/kg), rats were gavaged twice daily with transforming growth factor (TGF) inhibitor SD-208 (60 mg/kg) for 9 days, $n = 6$. RV mass and hemodynamics: (A) right ventricle/left ventricle + septum (RV/LV+Sep); (B) RV systolic pressure (RVSP); (C) RV diastolic pressure (RVDP); (D) mean RV pressure; (E) cardiac output (CO); (F) tau; (G) dP/dt/IP; (H) medial thickness. Statistical significance determined by analysis of variance and with pairwise multiple comparisons performed with the Tukey test.

advanced disease. Given that the therapeutic options are limited in patients suffering from severe disease, there has been a recent focus on reversal of disease. Although the majority of patients with PH present late in the disease, the triggers of early disease and factors mediating progression are unknown. A better understanding of the molecular events that lead to the development of PH will ultimately benefit patients by providing biomarkers for early detection and therapeutic tools for early intervention.

Conflict of Interest Statement: A.L.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. F.X. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.A.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.H. was employed by Scios, a Johnson & Johnson (J&J) company. A.M. received an annual salary and stock options as part of his compensation package at Scios, Inc., as a full-time employee. S.C. was an employee at Scios, Inc. (May 1992–August 2006); he received several patents while an employee at

Scios, Inc., and owns J&J stocks. A.P. was employed by Scios, a J&J company at the time this work was initiated; he left Scios/J&J in August 2006; he owns shares of J&J, but no options; he receives no money or stock from J&J. P.B.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H.C.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.M.T. received an unrestricted postdoctoral support grant from Quark Biotech for studies involving RTP801 in cigarette smoke-induced emphysema; he received \$2,500 for speaker fees in an international conference sponsored by AstraZeneca; he received \$1,500 from the Rush Medical Center's CME speakers training workshop titled "Simply Speaking PAH: An Expert Educators CME Lecture Series."

Acknowledgment: The authors thank James Watkins for his outstanding technical expertise.

References

1. Lane KB, Machado RD, Pauculo MW, Thomson JR, Phillips JA III, Loyd JE, Nichols WC, Trembath RC. Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. The International PPH Consortium. *Nat Genet* 2000;26:81–84.
2. Machado RD, Pauculo MW, Thomson JR, Lane KB, Morgan NV, Wheeler L, Phillips JA III, Newman J, Williams D, Galie N, *et al.* BMPR2 haploinsufficiency as the inherited molecular mechanism for primary pulmonary hypertension. *Am J Hum Genet* 2001;68:92–102.

3. Harrison RE, Flanagan JA, Sankelo M, Abdalla SA, Rowell J, Machado RD, Elliott CG, Robbins IM, Olschewski H, McLaughlin V, *et al*. Molecular and functional analysis identifies ALK-1 as the predominant cause of pulmonary hypertension related to hereditary haemorrhagic telangiectasia. *J Med Genet* 2003;40:865–871.
4. Shamberger RC, Sherins RJ, Ziegler JL, Glatstein E, Rosenberg SA. Effects of postoperative adjuvant chemotherapy and radiotherapy on ovarian function in women undergoing treatment for soft tissue sarcoma. *J Natl Cancer Inst* 1981;67:1213–1218.
5. Botney MD, Bahadori L, Gold LI. Vascular remodeling in primary pulmonary hypertension: potential role for transforming growth factor-beta. *Am J Pathol* 1994;144:286–295.
6. Loeys BL, Schwarze U, Holm T, Callewaert BL, Thomas GH, Pannu H, De Backer JF, Oswald GL, Symoens S, Manouvrier S, *et al*. Aneurysm syndromes caused by mutations in the TGF-beta receptor. *N Engl J Med* 2006;355:788–798.
7. Loeys BL, Chen J, Neptune ER, Judge DP, Podowski M, Holm T, Meyers J, Leitch CC, Katsanis N, Sharifi N, *et al*. A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. *Nat Genet* 2005;37:275–281.
8. Ng CM, Cheng A, Myers LA, Martinez-Murillo F, Jie C, Bedja D, Gabrielson KL, Hausladen JM, Mecham RP, Judge DP, *et al*. TGF-beta-dependent pathogenesis of mitral valve prolapse in a mouse model of Marfan syndrome. *J Clin Invest* 2004;114:1586–1592.
9. Derynck R, Zhang Y, Feng XH. Smads: transcriptional activators of TGF-beta responses. *Cell* 1998;95:737–740.
10. Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;113:685–700.
11. Izzi L, Attisano L. Regulation of the TGFbeta signalling pathway by ubiquitin-mediated degradation. *Oncogene* 2004;23:2071–2078.
12. Moustakas A, Pardali K, Gaal A, Heldin CH. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. *Immunol Lett* 2002;82:85–91.
13. Moustakas A, Heldin CH. Non-Smad TGF-beta signals. *J Cell Sci* 2005;118:3573–3584.
14. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425:577–584.
15. Mata-Greenwood E, Meyrick B, Steinhorn RH, Fineman JR, Black SM. Alterations in TGF-beta1 expression in lambs with increased pulmonary blood flow and pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L209–L221.
16. Morrell NW, Yang X, Upton PD, Jourdan KB, Morgan N, Sheares KK, Trembath RC. Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-beta(1) and bone morphogenetic proteins. *Circulation* 2001;104:790–795.
17. Merklinger SL, Jones PL, Martinez EC, Rabinovitch M. Epidermal growth factor receptor blockade mediates smooth muscle cell apoptosis and improves survival in rats with pulmonary hypertension. *Circulation* 2005;112:423–431.
18. Sakao S, Taraseviciene-Stewart L, Wood K, Cool CD, Voelkel NF. Apoptosis of pulmonary microvascular endothelial cells stimulates vascular smooth muscle cell growth. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L362–L368.
19. Sheares KK, Jeffery TK, Long L, Yang X, Morrell NW. Differential effects of TGF-beta1 and BMP-4 on the hypoxic induction of cyclooxygenase-2 in human pulmonary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L919–L927.
20. Perket EA, Lyons RM, Moses HL, Brigham KL, Meyrick B. Transforming growth factor-beta activity in sheep lung lymph during the development of pulmonary hypertension. *J Clin Invest* 1990;86:1459–1464.
21. Perket EA, Pelton RW, Meyrick B, Gold LI, Miller DA. Expression of transforming growth factor-beta mRNAs and proteins in pulmonary vascular remodeling in the sheep air embolization model of pulmonary hypertension. *Am J Respir Cell Mol Biol* 1994;11:16–24.
22. Botney MD, Parks WC, Crouch EC, Stenmark K, Mecham RP. Transforming growth factor-beta 1 is decreased in remodeling hypertensive bovine pulmonary arteries. *J Clin Invest* 1992;89:1629–1635.
23. Song Y, Jones JE, Beppu H, Keaney JF Jr, Loscalzo J, Zhang YY. Increased susceptibility to pulmonary hypertension in heterozygous BMPR2-mutant mice. *Circulation* 2005;112:553–562.
24. Beppu H, Ichinose F, Kawai N, Jones RC, Yu PB, Zapol WM, Miyazono K, Li E, Bloch KD. BMPR-II heterozygous mice have mild pulmonary hypertension and an impaired pulmonary vascular remodeling response to prolonged hypoxia. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L1241–L1247.
25. Zakrzewicz A, Kouri FM, Nejman B, Kwapiszewska G, Hecker M, Sandu R, Dony E, Seeger W, Schermuly RT, Eickelberg O, *et al*. The transforming growth factor-beta/Smad2,3 signalling axis is impaired in experimental pulmonary hypertension. *Eur Respir J* 2007;29:1094–1104.
26. Leung SY, Niimi A, Noble A, Oates T, Williams A, Medicherla S, Protter AA, Chung KF. Effect of TGF-beta receptor I kinase inhibitor, SD-208, in chronic allergic airway inflammation and remodeling. *J Pharmacol Exp Ther* 2006;19:586–594.
27. Leung SY, Niimi A, Noble A, Oates T, Williams AS, Medicherla S, Protter AA, Chung KF. Effect of transforming growth factor-beta receptor I kinase inhibitor 2,4-disubstituted pteridine (SD-208) in chronic allergic airway inflammation and remodeling. *J Pharmacol Exp Ther* 2006;319:586–594.
28. Gaspar NJ, Li L, Kapoun AM, Medicherla S, Reddy M, Li G, O'young G, Quon D, Henson M, Damm DL, *et al*. Inhibition of transforming growth factor beta signaling reduces pancreatic adenocarcinoma growth and invasiveness. *Mol Pharmacol* 2007;72:152–161.
29. Shah M, Patel K, Sehgal PB. Monocrotaline pyrrole-induced endothelial cell megalocytosis involves a Golgi blockade mechanism. *Am J Physiol Cell Physiol* 2005;288:C850–C862.
30. Sehgal PB, Mukhopadhyay S. Dysfunctional intracellular trafficking in the pathobiology of pulmonary arterial hypertension. *Am J Respir Cell Mol Biol* 2007;37:31–37.
31. Takimoto E, Champion HC, Li M, Belardi D, Ren S, Rodriguez ER, Bedja D, Gabrielson KL, Wang Y, Kass DA. Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat Med* 2005;11:214–222.
32. Halbower AC, Mason RJ, Abman SH, Tudor RM. Agarose infiltration improves morphology of cryostat sections of lung. *Lab Invest* 1994;71:149–153.
33. Richter A, Yeager ME, Zaiman A, Cool CD, Voelkel NF, Tudor RM. Impaired transforming growth factor-beta signaling in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2004;170:1340–1348.
34. Wilson DW, Segall HJ, Pan LC, Lame MW, Estep JE, Morin D. Mechanisms and pathology of monocrotaline pulmonary toxicity. *Crit Rev Toxicol* 1992;22:307–325.
35. Harris PN, Anderson RC, Chen KK. The action of monocrotaline and retronecine. *J Pharmacol Exp Ther* 1942;75:78–82.
36. Bull LB. The histological evidence of liver damage from pyrrolizidine alkaloids: megalocytosis of the liver cells and inclusion globules. *Aust Vet J* 1955;31:33–40.
37. Sullman SF, Zuckerman AJ. The effect of heliotrine, a pyrrolizidine alkaloid, on human liver cells in culture. *Br J Exp Pathol* 1969;50:361–370.
38. Armstrong SJ, Zuckerman AJ, Bird RG. Induction of morphological changes in human embryo liver cells by the pyrrolizidine alkaloid lasiocarpine. *Br J Exp Pathol* 1972;53:145–149.
39. Bonniaud P, Margetts PJ, Kolb M, Schroeder JA, Kapoun AM, Damm D, Murphy A, Chakravarty S, Dugar S, Higgins L, *et al*. Progressive transforming growth factor beta1-induced lung fibrosis is blocked by an orally active ALK5 kinase inhibitor. *Am J Respir Crit Care Med* 2005;171:889–898.
40. Kapoun AM, Gaspar NJ, Wang Y, Damm D, Liu YW, O'young G, Quon D, Lam A, Munson K, Tran TT, *et al*. Transforming growth factor-beta receptor type 1 (TGFbetaRI) kinase activity but not p38 activation is required for TGFbetaRI-induced myofibroblast differentiation and profibrotic gene expression. *Mol Pharmacol* 2006;70:518–531.
41. Rosendahl A, Checchin D, Fehniger TE, Ten Dijke P, Heldin CH, Sideras P. Activation of the TGF-beta/activin-Smad2 pathway during allergic airway inflammation. *Am J Respir Cell Mol Biol* 2001;25:60–68.
42. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 2000;14:163–176.
43. O'Callaghan CJ, Williams B. Mechanical strain-induced extracellular matrix production by human vascular smooth muscle cells: role of TGF-beta(1). *Hypertension* 2000;36:319–324.
44. Lepetit H, Eddahibi S, Fadel E, Frisdal E, Munaut C, Noel A, Humbert M, Adnot S, d'Ortho MP, Lafuma C. Smooth muscle cell matrix metalloproteinases in idiopathic pulmonary arterial hypertension. *Eur Respir J* 2005;25:834–842.
45. Frisdal E, Gest V, Vieillard-Baron A, Levame M, Lepetit H, Eddahibi S, Lafuma C, Harf A, Adnot S, Dortho MP. Gelatinase expression in pulmonary arteries during experimental pulmonary hypertension. *Eur Respir J* 2001;18:838–845.

46. Matsui K, Takano Y, Yu ZX, Hi JE, Stetler-Stevenson WG, Travis WD, Ferrans VJ. Immunohistochemical study of endothelin-1 and matrix metalloproteinases in plexogenic pulmonary arteriopathy. *Pathol Res Pract* 2002;198:403–412.
47. Cowan KN, Heilbut A, Humpl T, Lam C, Ito S, Rabinovitch M. Complete reversal of fatal pulmonary hypertension in rats by a serine elastase inhibitor. *Nat Med* 2000;6:698–702.
48. Goumans MJ, Valdimarsdottir G, Itoh S, Lebrin F, Larsson J, Mummery C, Karlsson S, Ten Dijke P. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGF β /ALK5 signaling. *Mol Cell* 2003;12:817–828.
49. Tudor RM, Yeager ME, Geraci M, Golpon HA, Voelkel NF. Severe pulmonary hypertension after the discovery of the familial primary pulmonary hypertension gene. *Eur Respir J* 2001;17:1065–1069.
50. Lipke DW, Arcot SS, Gillespie MN, Olson JW. Temporal alterations in specific basement membrane components in lungs from monocrotaline-treated rats. *Am J Respir Cell Mol Biol* 1993;9:418–428.
51. Fadok VA, Bratton DL, Guthrie L, Henson PM. Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases. *J Immunol* 2001;166:6847–6854.
52. McDonald PP, Fadok VA, Bratton D, Henson PM. Transcriptional and translational regulation of inflammatory mediator production by endogenous TGF-beta in macrophages that have ingested apoptotic cells. *J Immunol* 1999;163:6164–6172.
53. Cowan KN, Jones PL, Rabinovitch M. Elastase and matrix metalloproteinase inhibitors induce regression, and tenascin-C antisense prevents progression, of vascular disease. *J Clin Invest* 2000;105:21–34.
54. Ihida-Stansbury K, McKean DM, Lane KB, Loyd JE, Wheeler LA, Morrell NW, Jones PL. Tenascin-C is induced by mutated BMP type II receptors in familial forms of pulmonary arterial hypertension. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L694–L702.
55. Liu Y, Li M, Warburton RR, Hill NS, Fanburg BL. The 5-HT transporter transactivates the PDGF β receptor in pulmonary artery smooth muscle cells. *FASEB J* 2007;21:2725–2734.
56. Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, Sydykov A, Lai YJ, Weissmann N, Seeger W, *et al.* Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest* 2005;115:2811–2821.