Altered Growth Responses of Pulmonary Artery Smooth Muscle Cells From Patients With Primary Pulmonary Hypertension to Transforming Growth Factor-β₁ and Bone Morphogenetic Proteins

Nicholas W. Morrell, MD; Xudong Yang, MD; Paul D. Upton, PhD; Karen B. Jourdan, PhD; Neal Morgan, BSc; Karen K. Sheares, MA; Richard C. Trembath, FRCP

Background—Mutations in the type II receptor for bone morphogenetic protein (BMPR-II), a receptor member of the transforming growth factor-β (TGF-β) superfamily, underlie many cases of familial and sporadic primary pulmonary hypertension (PPH). We postulated that pulmonary artery smooth muscle cells (PASMCs) from patients with PPH might demonstrate abnormal growth responses to TGF-β superfamily members.

Methods and Results—For studies of ³H-thymidine incorporation or cell proliferation, PASMCs (passages 4 to 8) were derived from main pulmonary arteries. In control cells, 24-hour incubation with TGF-β₁ (10 ng/mL) or bone morphogenetic protein (BMP)-2, -4, and -7 (100 ng/mL) inhibited basal and serum-stimulated ³H-thymidine incorporation, and TGF-β₁ and BMPs inhibited the proliferation of serum-stimulated PASMCs. In contrast, TGF-β₁ stimulated ³H-thymidine incorporation (200%; P < 0.001) and cell proliferation in PASMCs from PPH but not from patients with secondary pulmonary hypertension. In addition, BMPs failed to suppress DNA synthesis and proliferation in PASMCs from PPH patients. Reverse transcription–polymerase chain reaction of PASMC mRNA detected transcripts of type I (TGF-βR₁, Alk-1, ActRI, and BMPRIIB) and type II (TGF-βR₂, BMPRII, ActRII, ActRIIB) receptors. Receptor binding and cross-linking studies with ¹²⁵I-TGF-β₁ confirmed that the abnormal responses in PPH cells were due to differences in TGF-β receptor binding. Mutation analysis of PASMC DNA failed to detect mutations in TGF-βR₂ and Act-1 but confirmed the presence of a mutation in BMPRII in 1 of 5 PPH isolates.

Conclusions—We conclude that PASMCs from patients with PPH exhibit abnormal growth responses to TGF-β and BMPs and that altered integration of TGF-β superfamily growth signals may contribute to the pathogenesis of PPH.

Key Words: hypertension, pulmonary ] muscle, smooth ] growth substances ] proteins

Primary pulmonary hypertension (PPH) is a rare disorder that is progressive and often fatal.¹ The disease is characterized by vascular cell proliferation and obliteration of small pulmonary arteries,² leading to severe pulmonary hypertension and right ventricular failure. Typical morphological changes include increased muscularization of small arteries and thickening or fibrosis of the intima, as well as the presence of plexiform lesions, which are tangles of capillary-like channels adjacent to small pulmonary arteries.³ Recently, linkage was established between familial PPH and a region on the long arm of chromosome 2 (2q33).⁴,⁵ Sequencing of positional candidate genes revealed heterozygous mutations involving the gene encoding the type II bone morphogenetic protein receptor (BMPR-II), a member of the transforming growth factor-β (TGF-β) superfamily of receptors.⁶-⁷ The heterogeneous mutations include frameshift and nonsense mutations predicted to cause premature truncation of the 1038 amino acid protein. Missense mutations occur at highly conserved and functionally important sites, predicted to perturb ligand binding or disrupt the kinase domain of the receptor. Interestingly, the same mutations underlie ≈25% of apparently sporadic cases of PPH, some of which are in fact familial, the remainder arising de novo.⁸ Although these recent genetic studies point toward a critical role for the TGF-β superfamily in the regulation of pulmonary vascular cell growth and differentiation, the precise molecular mechanisms leading to disease pathogenesis remain to be elucidated.

The recognized role of the TGF-β superfamily in endothelial⁹ and smooth muscle¹⁰ cell growth, differentiation, and
matrix production reinforces the potential role of BMPR-II in the vascular lesion of PPH. Growth, migration, and excess matrix deposition by endothelial cells, smooth muscle cells, and adventitial fibroblasts all contribute to the process of vascular wall remodeling in pulmonary hypertension. TGF-β, exerts potent effects on vascular smooth muscle cells in vitro, including inhibition of proliferation, extracellular matrix synthesis, and cell differentiation. Bone morphogenetic proteins (BMPs) have been less extensively studied, but BMP-7 has been shown to inhibit proliferation of human aortic smooth muscle cells and increase expression of smooth muscle differentiation markers, and BMP-2 inhibits vascular smooth muscle cell proliferation after balloon injury in rats.

Taken together, these studies provide a platform from which to speculate that disruption of TGF-β superfamily signaling as a consequence of BMPR2 mutation in PPH might contribute to the cellular proliferation and vascular obliteration seen in this condition. The present study demonstrates that cells from patients with PPH but not control subjects or patients with secondary pulmonary hypertension (SPH) exhibit abnormal growth responses to TGF-β and BMPs, which suggests that altered integration of TGF-β superfamily signals may contribute to the vascular lesions characteristic of PPH.

**Methods**

**Isolation of Human Pulmonary Artery Smooth Muscle Cells**

Proximal segments of human pulmonary artery were obtained from patients undergoing lung or heart-lung transplantation for PPH (n = 6) or pulmonary hypertension secondary to congenital heart disease (n = 5). Samples of proximal pulmonary artery were obtained from unused donors for transplantation (n = 9). The study was approved by the Harefield and Papworth Hospital ethical review committees, and subjects gave informed written consent. Explants were processed as described previously. Cells were used for experiments between passages 4 and 8, and the smooth muscle phenotype of isolated cells was confirmed by positive immunofluorescence with antibodies to anti-smooth muscle-α actin antibody (1A4) and anti-smooth muscle-specific myosin (hsm-v), as described.

**Growth Studies**

To determine the serum-stimulated growth rates, cells were seeded at 10^4 per well in 48-well plates in DMEM/10% FBS. Cell numbers were counted with a hemocytometer at days 2, 5, and 7. In additional studies, BMP-2, -4, or -7 (1 to 100 ng/mL) or TGF-β (0.1 to 10 ng/mL) was added to quiescent cells (0.1% FBS) or serum-stimulated cells (10% FBS) to determine the effect on proliferation. Growth of human pulmonary artery smooth muscle cells (PASMCs) in response to individual growth factors was determined by [methyl-^3H]-thymidine incorporation, representing DNA synthesis. Cells were grown to 70% to 80% confluence and made quiescent by incubation with DMEM/0.1% FBS for 48 hours. The media was then exchanged for fresh DMEM/0.1% FBS either alone or containing BMP-2, -4, or -7 (0.1 to 100 ng/mL), TGF-β (0.1 to 100 ng/mL), platelet-derived growth factor-BB (PDGF-BB; 10 ng/mL), or thrombin (3 U/mL) for 24 hours. Then, 0.5 μCi/well [methyl-^3H]-thymidine was added for the final 6 hours. Each isolate was studied at least twice under each condition, and the mean values were taken from all studies conducted with any one isolate.

**Expression Profiling of TGF-β Superfamily Receptors by Reverse Transcription–Polymerase Chain Reaction**

Total RNA was isolated from growth-arrested primary cultures of human PASMCs with TRIzol reagent (Life Technologies) and reverse transcription–polymerase chain reaction (RT-PCR) performed with the Access RT-PCR System from Promega. Primers were taken from previously published sequences, where possible, or designed with the computer program Primer+). The primer sequences and sources are available online in Table I. All primers were synthesized by Sigma-Genosys Ltd. PCR products were visualized by electrophoresis in agarose (2%) gels stained with ethidium bromide. Control reactions were run without the addition of reverse transcriptase. The identity of PCR products was confirmed by direct sequencing.

**Receptor Binding Studies in Cells**

For competition binding studies, cells were seeded in 24-well plates and grown to confluence, then made quiescent by incubation in 0.1% FBS for 3 days. TGF-β competition binding was performed as described previously. Cells were incubated at 4°C for 4 hours with DMEM/0.5% BSA containing 125I-TGF-β (Amersham) in the absence or presence of unlabeled TGF-β (0.01 to 100 ng/mL). The effect of BMP-2, BMP-4, or BMP-7 (10 to 100 ng/mL) on binding was also assessed.

**Receptor Cross-Linking Studies**

For cross-linking studies, cells were seeded in 6-well plates and grown to confluence. Cells were then made quiescent as described above. The initial binding steps were as described above except that the ligand was added at 10,000 to 20,000 cps/well in the absence or presence of 100 ng/mL TGF-β or BMP-4. Cross-linking of 125I-TGF-β to cell surface receptors was performed by a previously described technique, with minor modifications. Samples were separated with 10% PAGE gels with or without 50 mmol/L DTT, stained with Coomassie blue, dried, and exposed to autoradiographic film.

**Screening for Mutations in the ALK1, BMPR-II, and TGF-βRII Genes**

Genomic DNA was extracted from smooth muscle cells by standard techniques (Nucleon Biosciences). Protein coding sequence from the ALK1 gene (exons 2 to 10) was amplified from genomic DNA with primers complementary to the intron sequences, as described previously, and sequenced with a dye-terminator cycle-sequencing system (ABI PRISM 377, Perkin-Elmer Applied Biosystems). BMPR-II sequence (exons 1 to 13) analysis was performed, as described previously and with primer sequences available on the Internet. In addition, we looked for mutations in the A10 microsatellite region of TGF-βRII, as described previously. This region is prone to 1- or 2-bp deletions that lead to premature truncation of the protein and a reduction in gene expression and has been associated with a loss of responsiveness to the growth inhibitory effects of TGF-β, in vascular smooth muscle cells and tumor cells.

**Statistical Analysis**

Data were expressed as mean ± SEM and analyzed with GraphPad Prism version 3.0 (GraphPad Software). Comparisons were made by Student’s t test or 1-way ANOVA with the Tukey post hoc test as appropriate. A value of P < 0.05 indicated statistical significance.

**Results**

**Clinical Characteristics of Pulmonary Hypertensive Patients**

The clinical and demographic information available for pulmonary hypertensive cases is shown in the Table.

**Effect of TGF-β and BMPs on DNA Synthesis**

In control cells, PDGF (10 ng/mL) and thrombin (3 U/mL) led to an increase in [methyl-^3H]-thymidine incorporation of 284±62% and 149±14%, respectively. The response was similar in cells from patients with PPH (PDGF 277±31%; thrombin 189±10%) and SPH (PDGF 367±61%, thrombin 174±12%);
In contrast, clear differences were demonstrated in the response of PASMCs from PPH patients (compared with SPH and normal controls) to TGF-β1 and BMPs. Incubation of serum-deprived donor or SPH PASMCs with TGF-β1 led to inhibition of basal 3H-thymidine incorporation at 24 hours (Figure 1). In PPH cells, TGF-β1 consistently stimulated 3H-thymidine incorporation (Figure 1). In serum-stimulated PASMCs, a similar inhibition of 3H-thymidine incorporation by TGF-β1 was observed in PASMCs from controls and patients with SPH, whereas stimulation was still apparent in PPH cells (data not shown).

Incubation of serum-deprived control and SPH PASMCs with BMP-2, -4, or -7 led to inhibition of basal and serum-stimulated 3H-thymidine incorporation at 24 hours (Figure 2). However, BMPs failed to significantly suppress basal and serum-stimulated 3H-thymidine incorporation in cells from patients with PPH (Figure 2).

**Effect of TGF-β1 and BMPs on Proliferation**

Analysis of growth curves of cells from controls and patients with PPH and SPH revealed no difference in the rate of serum-stimulated proliferation over a 15-day period (Figure 3), which suggests that PASMCs from patients with PPH do not possess an intrinsically enhanced growth response to nonspecific mitogenic stimuli.
also examined. TGF-β1 and BMP-2, -4, and -7 all inhibited the serum-stimulated proliferation of PASMCs from normal subjects and patients with SPH (Figure 4). In contrast, TGF-β1 enhanced the serum-stimulated proliferation of PASMCs from PPH patients. Furthermore, BMPs failed to significantly reduce proliferation rates of PASMCs from patients with PPH (Figure 4).

Expression Profile of TGF-β Superfamily Receptors
mRNA transcripts for TGF-β superfamily type I (ALK-1, TGF-βRI, Act RI, and BMP RIB) and type II (TGF-βRII, BMPR-II, ActR2, and ActR2B) receptors were present in total RNA isolated from PASMCs (Figure 5). We were unable to detect mRNA transcripts for BMP RIA. Specificity of the reactions for RNA was confirmed by the absence of PCR products when samples were run without reverse transcriptase.

Receptor Binding Studies
Specific binding of 125I-TGF-β1 was demonstrated in cells from controls and PPH patients and was ~50% of total binding. Competition binding curves demonstrated concentration-dependent competition by unlabeled ligand (Figure 6). 125I-TGF-β1 binding was not inhibited by an excess of unlabeled BMP-2, -4, or -7 (100 ng/mL; data not shown). The affinity (IC50) of this site was similar in PASMCs from patients with PPH (1.23±0.39 ng/mL; n=3), patients with SPH (2.18±0.26 ng/mL; n=3), and controls (1.07±0.35 ng/mL; n=4). In addition, binding site density was similar in PPH (10 329±1659

Figure 3. Rates of proliferation of PASMCs from control subjects (n=9) and patients with PPH (n=6) and SPH (n=5) in response to 10% FBS.

Figure 5. Detection of TGF-β superfamily receptor mRNA expression by RT-PCR in PASMCs from control subjects and patients with SPH and PPH. Representative ethidium bromide-stained gels demonstrating expression of mRNA transcripts for TGF-βRI, TGF-βRII, Act R2, and BMPR-II.

Figure 6. Characterization of 125I-TGF-β1 binding sites in PASMCs from patients with PPH (n=3) or SPH (n=3) and controls (n=4). Graphs show equilibrium competition binding for 125I-TGF-β1 in presence of increasing concentrations of unlabeled TGF-β1.
mutations were identified.

chromosomal region containing linkage had previously been established with 2q33, the isolate was derived from a patient with familial PPH in whom apparently sporadic PPH. The mutation is predicted to lead to position 1471) mutation in exon 11 in 1 patient with apparent sporadic PPH. This is consistent with the reported frequency of BMPR-II mutations of 26% in patients with "sporadic" PPH. However, it is likely that the actual frequency of BMPR2 mutations in PPH is higher than this, because the direct sequencing methods used in this and other studies may not detect mutations in regulatory regions or large gene deletions or rearrangements. The remaining PPH case in the present study had familial PPH and came from a family in which linkage was established with the BMP2 locus, although again, no mutation was identified. We have recently found that mutations in Alk-1, which encodes an orphan TGF-β type I receptor, may also be associated with PPH in families with the condition hereditary hemorrhagic telangiectasia (R.C.T., unpublished observations, 2001). However, screening of ALK-1 revealed no mutations in the present study.

Our RT-PCR data are consistent with a previous report showing expression of multiple type I receptors for the TGF-β superfamily in rat aortic smooth muscle.22 Although the expression of TGF-βRII by vascular smooth muscle is well documented,10,18 there is little information regarding the expression of BMPR-II by vascular cells. Binding sites for 125I-BMP2 have been identified on a wide variety of cell lines derived from fibroblasts, keratinocytes, astrocytes and kidney epithelial cells, as well as tumor cells from diverse organs,23 although BMP2 can also bind to the type II receptor Act RIIB.

Numerous studies have examined the effects of TGF-β1 on vascular smooth muscle biology. The effects of TGF-β1 on cell growth are context specific in that they depend on the developmental stage at which cells are studied, the tissue of origin, and the presence of other regulatory factors.24 In general, TGF-β1 inhibits the proliferation and migration of smooth muscle and endothelial cells,25 most likely by inhibition of cyclin-dependent kinases and by downregulation of c-myc.24 The effects of BMPs on vascular cell growth have been less extensively investigated, but the emerging consensus is that BMPs inhibit the proliferation of vascular smooth muscle15 and promote differentiation of smooth muscle phenotype,14 although stimulation of vascular smooth muscle cell chemotaxis has also been reported.26 Although the present study showed clear differences in the response to TGF-β1 and BMPs in PPH cells, one potential limitation is that we used cells from large proximal vessels rather than peripheral arteries, which are considered the main site of disease.

Although these studies demonstrate one of the important functional consequences of dysfunctional BMPR-II signaling
in PASMCs, the precise molecular mechanism of the altered responses to BMPs remains to be determined in PPH. Transient transfection of cell lines with mutant BMPR-II led to reduced activation of a luciferase reporter construct containing a Smad binding element, which suggests impaired signaling by Smad proteins.27 The surprising finding in the present study was the stimulation of DNA synthesis and proliferation in response to TGF-β1 in PPH cells. Our data and those of others suggests that TGF-β1 is not a ligand for the BMPR-II receptor,23 which indicates that dysfunctional TGF-β superfamily signaling in PPH cells is more widespread than suggested by mutations in BMPR-II alone. Potential mechanisms for the abnormal response to TGF-β1 include loss of TGF-βRII receptor function, which commonly occurs as a somatic mutation in pancreatic and colonic tumors. However, our mutation analysis, receptor binding, and cross-linking data suggest that an alternative mechanism exists for altered TGF-β responses in PPH cells. An alternative mechanism may involve reduced activation of inhibitory Smads as a consequence of loss of BMPR-II function, which alters the response to other TGF-β superfamily members. In support of this, BMPs are potent inducers of the inhibitory Smads 6 and 7,29 which feed back to inhibit TGF-β receptor signaling. Loss of Smad 6 and Smad 7 negative feedback might have a permissive effect on TGF-β1–induced proliferative responses. In epithelial cells, resistance to the antiproliferative action of TGF-β is a consequence of failure to downregulate c-Myc and to prevent cyclin-dependent kinase inhibitory gene responses.24 The role of these pathways in the abnormal TGF-β1–induced proliferation of PPH cells requires further study. Furthermore, our results do not explain the selectivity of PPH for the pulmonary as opposed to the systemic circulation. Because systemic vascular cells will be similarly affected by germline mutations in BMPR2, additional factors must determine pulmonary vascular selectivity.

In summary, the present study demonstrates that an important functional abnormality of PASMCs isolated from patients with PPH is resistance to the antiproliferative effects of TGF-β1 and BMPs. This abnormality may result directly from mutations/dysfunction in BMPR-II and/or closely related signaling pathways. The TGF-β superfamily plays a critical role in the development and maintenance of the integrity of the normal vasculature. Thus, the altered cellular responses may contribute to the formation of lesions that lead to the vascular obliteration observed in the pulmonary arteries of patients with PPH.

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References
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### Source of primer sequences and conditions used for RT-PCR

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