Primary Pulmonary Hypertension Is Associated With Reduced Pulmonary Vascular Expression of Type II Bone Morphogenetic Protein Receptor

Carl Atkinson, BSc; Susan Stewart, FRCPath; Paul D. Upton, PhD; Rajiv Machado, PhD; Jennifer R. Thomson, MRCP; Richard C. Trembath, FRCP; Nicholas W. Morrell, MD

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 familial and sporadic cases of primary pulmonary hypertension (PPH).

Methods and Results—Because the sites of expression of BMPR-II in the normal and hypertensive lung are unknown, we studied the cellular localization of BMPR-II and the related type I and II receptors for TGF-β by immunohistochemistry in lung sections from patients undergoing heart-lung transplantation for PPH (n=11, including 3 familial cases) or secondary pulmonary hypertension (n=6) and from unused donor lungs (n=4). In situ hybridization was performed for BMPR-II mRNA. Patients were screened for the presence of mutations in BMPR2. In normal lungs, BMPR-II expression was prominent on vascular endothelium, with minimal expression in airway and arterial smooth muscle. In pulmonary hypertension cases, the intensity of BMPR-II immunostaining varied between lesions but involved endothelial and myofibroblast components. Image analysis confirmed that expression of BMPR-II was markedly reduced in the peripheral lung of PPH patients, especially in those harboring heterozygous BMPR2 mutations. A less marked reduction was also observed in patients with secondary pulmonary hypertension. In contrast, there was no difference in level of staining for TGF-βRII or the endothelial marker CD31.

Conclusions—The cellular localization of BMPR-II is consistent with a role in the formation of pulmonary vascular lesions in PPH, and reduced BMPR-II expression may contribute to the process of vascular obliteration in severe pulmonary hypertension. (Circulation. 2002;105:1672-1678.)

Key Words: hypertension, pulmonary immunohistochemistry receptors

Primary pulmonary hypertension (PPH) is a rare disorder, with an estimated incidence of 1 to 2 per million per year. The disease is characterized by vascular cell proliferation and obliteration of small pulmonary arteries, which leads to severe pulmonary hypertension (PH) and right ventricular failure. Typical morphological appearances include increased muscularization of small arteries and thickening or fibrosis of the intima. The term plexogenic arteriopathy is used because of the existence of plexiform lesions (200 to 400 μm in diameter), which are capillary-like channels adjacent to small pulmonary arteries.

Recently, heterozygous germline mutations that involve the gene encoding the type II bone morphogenetic protein receptor (BMPR2), a member of the transforming growth factor-β (TGF-β) receptor superfamily, have been found to underlie many cases of familial and sporadic PPH. The precise molecular mechanisms of disease pathogenesis remain to be elucidated but are likely to involve altered BMPR-II function. In support of this, we recently found that compared with cells from control subjects or patients with secondary PH, pulmonary artery smooth muscle cells from patients with PPH fail to respond to the growth-suppressive effects of bone morphogenetic proteins (BMPs).

Further insight into the role of mutant BMPR-II in the pathogenesis of PPH would be gained by detailed knowledge of the cellular distribution of this receptor in the normal and PH lung. This information might also be useful to identify the key cell types involved in initiation of the disease. The aim of the present investigation was to determine the pattern of expression and cellular localization of BMPR-II in the human lung. Considerably more is known regarding the expression of TGF-β receptors in the normal and diseased systemic vasculature, where abnormal TGF-β signaling contributes to the pathogenesis of atherosclerosis and restenosis after...
Injury. It has been suggested that an additional acquired somatic mutation may be necessary for the clinical manifestation of PPH in a genetically susceptible individual. This hypothesis remains attractive, because somatic mutations in the TGF-β signaling pathway are relatively common and may contribute to the pathogenesis of certain tumors and atherosclerosis.

In the present study, we demonstrate that BMPR-II mRNA and protein expression is mainly localized to the endothelium in the normal pulmonary circulation, although it is also expressed by myofibroblasts comprising concentric intimal lesions in severe PH. In addition, we report that pulmonary vascular BMPR-II expression is reduced in PPH cases, most markedly in those PPH cases with an underlying mutation in the BMPR2 gene.

Methods

Lung Tissue Preparation

The PH lung tissue used in the present study comprised material from patients undergoing heart-lung transplantation for PPH (n=11) or secondary PH (n=6) at Papworth Hospital, Cambridgeshire, UK. The Huntingdon Local Research Ethics Committee approved this study. Material was fixed in 10% phosphate-buffered formalin by inflation. Adult control lung was obtained from unused donor lungs for heart-lung transplantation.

Immunohistochemistry

Paraffin-embedded lung sections were processed by an antigen retrieval technique. Endogenous peroxidase was quenched by treatment with hydrogen peroxide (Dako). Sections were microwaved (Euroserv 750-W microwave) on full power for 10 minutes in 0.01 mol/L sodium citrate solution (pH 6). Anti-BMPR-II polyclonal antibody designated SMN (a gift from Dr K. Miyazono, Tokyo, Japan; dilution 1:30) was raised against a peptide corresponding to amino acid residues 185 to 202 of the 1038-amino acid BMPR-II sequence. Polyclonal antibodies raised against the human TGF-β type I receptor (TGF-βRI; 10 μg/mL) and TGF-β type II receptor (TGF-βRII; 10 μg/mL) were obtained from Research Diagnostics, Inc. To identify cells of endothelial lineage in serial sections, the monoclonal antiendothelial cell markers (all from Dako) anti-CD31 (1:400), anti-von Willebrand factor (1:20), anti-CD34 (1:80), and protein expression is mainly localized to the endothelium and protein expression is mainly localized to the endothelium in the normal pulmonary circulation, although it is also expressed by myofibroblasts comprising concentric intimal lesions in severe PH. In addition, we report that pulmonary vascular BMPR-II expression is reduced in PPH cases, most markedly in those PPH cases with an underlying mutation in the BMPR2 gene.

Image Analysis

Two methods were used to semiquantify the results of immunohistochemistry. First, PH sections were examined by 2 observers unaware of the underlying diagnosis (C.A. and N.W.M.). The observers identified lesions and classified them as concentric intimal lesions or plexiform lesions. Immunostaining for BMPR-II, TGF-βRII, and CD31 was classified as present or absent and the number of positive lesions expressed as a percentage of the total number of lesions in each case. Second, images were acquired from 5 randomly selected fields and stored as digital image files. Images were imported into an image-analysis software package (Aeqatis). This allowed calculation of the total tissue area and the area of positive immunostaining, which was then expressed as a percentage.

In Situ Hybridization

A 424-bp cDNA fragment (nucleotides 803 to 1226) of human BMPR-II cDNA was ligated into the PstI/HindIII site of the plasmid

### Table: Clinical Data for Patients With PH

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/Age, y</th>
<th>Mean PAP, mm Hg</th>
<th>Cardiac Output, L/min</th>
<th>Underlying Condition</th>
<th>Final Diagnosis</th>
<th>BMPR2 Mutation Analysis</th>
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<tr>
<td>1</td>
<td>M/41</td>
<td>79</td>
<td>3.2</td>
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<td>ND</td>
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<td>Partial deletion</td>
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<td>2q33 Allele sharing</td>
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<td>SPH</td>
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<td>VSD</td>
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<td>Scleroderma</td>
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</table>

PAP indicates pulmonary arterial pressure; NA, not available; FPPH, familial PPH; ND, not detected; VSD, ventricular septal defect; SPH, secondary PH; and CTE, chronic thromboembolism.
vector pBluescriptKS(+) and in vitro transcription from the T3 and T7 promoters yielded antisense and sense probes, respectively. Transcripts were labeled with sulfur-35-uridine triphosphate (35S-UTP; >1200 Ci · mmol⁻¹ · L⁻¹; New England Nuclear). After hybridization with cRNA probe, slides were processed as described previously.15 Slides were exposed for up to 10 weeks at 4°C. After development of the photographic emulsion, slides were stained with hematoxylin and eosin. Silver grains were visualized by light- and dark-field microscopy.

**Mutation Analysis of the BMPR2 Gene**

The entire protein-coding region and intron/exon boundaries of the BMPR2 gene were amplified by polymerase chain reaction, as described previously.5 Amplified fragments were sequenced with a dye-terminator cycle-sequencing system (ABI PRISM 377, Perkin-Elmer Applied Biosystems). In addition, gene dosage analysis of BMPR2 exons 1 and 12 was performed to detect partial gene deletions, as described previously.8 In 1 individual with familial PPH in whom no BMPR2 mutations were identified by these methods, allele-sharing studies of the BMPR2 locus were performed, as described previously.16

### Clinical Data

Clinical data for patients with PPH and secondary PH are shown in the Table.

### Mutation Analysis of the BMPR2 Gene

A total of 3 germline BMPR2 mutations were detected among the 11 PPH patients studied. Direct DNA sequencing was used to identify an exon 3 frameshift mutation (355delA) in 1 patient with familial PPH (patient 8) that created a premature truncating codon, which, if translated, may fail to reach the cell surface.5 BMPR2 gene dosage studies determined heterozygous partial gene deletions in 1 patient with familial PPH (patient 3) and 1 with sporadic PPH (patient 2), the extent of which remains to be determined.8 In the remaining patient with familial PPH (patient 7), no BMPR2 mutations were detected; however, allele-sharing studies in this family were consistent with linkage to the BMPR2 locus at 2q33. No mutations in BMPR2 were identified in patients with secondary PH.

### Cell Types Comprising Vascular Lesions

We performed immunohistochemical studies to determine the main cell types present within vascular lesions in PH. Expression of endothelial cell markers was confirmed to the monolayer of cells lining vascular channels (Figure 1). The remaining cell layers comprising concentric lesions stained heavily for α-smooth muscle actin (Figure 1). Although the vascular channels of plexiform lesions stained for endothelial cell markers, the stroma surrounding these channels also stained for α-smooth muscle actin. Staining with anti-CD68 demonstrated the presence of macrophages in many lesions (Figure 1).

### Expression of BMPR-II mRNA and Protein

In normal lungs, hybridization to BMPR-II mRNA was predominantly observed on vascular endothelial cells, with focal expression in the underlying smooth muscle and interstitial cells, which appeared to be macrophages (Figure 2). The signal was similar in endothelial cells that lined larger pulmonary arteries and alveolar capillary endothelium. Despite repeated attempts, specific hybridization to BMPR-II mRNA was low in PH cases (not shown). Immunohistochemistry confirmed the predominantly endothelial localization of BMPR-II protein in normal lung (Figure 3) but also suggested some focal weaker expression by vascular smooth muscle. BMPR-II immunostaining was also demonstrated in macrophages. In PH cases, BMPR-II protein expression was observed on endothelial cells and myofibroblasts comprising intimal lesions (Figure 3). Endothelial cells lining the vascular channels of plexiform lesions also expressed BMPR-II (Figure 3).

### Immunolocalization of TGF-β Receptors

In normal lungs, TGF-βRI and TGF-βRII colocalized predominantly to vascular endothelium, with focal staining of the underlying smooth muscle (Figure 4). However, airway epithelial staining was also prominent. In PPH and secondary PH, TGF-βRI and TGF-βRII immunostaining remained prominent on endothelial cells, including those of plexiform lesions, as well as on myofibroblast cells comprising intimal lesions.
Semiquantification of Immunostaining
Comparison of the staining intensity of endothelial cells for BMPR-II revealed a generally reduced level of BMPR-II protein expression in PPH cases compared with control or secondary PH cases (Figure 5). Semiquantification of the level of immunostaining by image analysis revealed that staining was significantly decreased in all PH cases, with the most marked reductions observed in PPH cases that harbored mutations predicted to cause loss of protein expression or linkage to the \textit{BMPR2} locus (Figure 6). In contrast, the area of tissue stained by anti-CD31 and anti-TGF-\betaRII was similar between groups (Figure 6).

Because some vascular lesions in PH cases appeared not to stain for BMPR-II, we performed a further analysis to compare the proportion of vascular lesions that were positive for BMPR-II in control or secondary PH cases (Figure 5). The proportion of positive stained lesions was reduced in PPH cases that harbored a mutation or demonstrated linkage to \textit{BMPR2} (Figure 7). In contrast, similar proportions of lesions stained positively for TGF-\betaRII between groups (Figure 7).

Discussion
This study has demonstrated the cellular distribution of BMPR-II in the normal and hypertensive lung. Our observa-
tions suggest that this receptor is predominantly expressed by endothelial cells in the pulmonary circulation, with a lower level of expression in vascular smooth muscle. In addition, BMPR-II is expressed within the characteristic lesions found in PPH, specifically by endothelial cells of plexiform lesions and by endothelial and myofibroblast cells in the intimal lesions. The cellular localization of BMPR-II to key cell types implicated in the vascular remodeling of PPH supports the suggestion from genetic studies that mutations in this receptor play a causal role in disease pathogenesis. Furthermore, the observation that BMPR-II protein expression is reduced in the lungs of patients with severe PH, most markedly in patients harboring heterozygous germline mutations in BMPR2, suggests that reduced BMPR-II signaling may be implicated not only the in the pathogenesis of PPH in which BMPR2 mutations have been identified but also in other mutation-negative cases of PPH, and possibly in secondary forms of PH.

To the best of our knowledge, this is the first study to examine the cellular localization of BMPR-II expression in human lung. Northern blots have revealed that BMPR-II is widely expressed in various human tissues, with a high level of expression in lung tissue. Although BMPR-II mRNA was readily demonstrated in normal lungs, we could not reliably detect hybridization in diseased lungs. In light of our immunohistochemical findings, it may be that reduced expression of BMPR-II protein in severe PH is, at least in part, transcriptionally regulated.

We performed mutation analysis of genomic DNA from all patients with severe PH. Three germline mutations were identified (1 frameshift and 2 partial deletions) that were predicted to cause reduced expression of BMPR-II protein. In another familial PPH case, no mutation was identified, but linkage to the BMPR2 locus at 2q33 was demonstrated. Comparison of the level of immunostaining in known mutants with that in other PH and secondary PH cases revealed an obvious reduction in the level of BMPR-II protein expression in cases with BMPR2 mutations. However, there was also a marked reduction in the level of BMPR-II immunostaining in PPH cases in which no mutation was identified. In these cases, the endothelium remained intact, as judged by a normal level of staining for CD31. Moreover, the observation appeared specific for BMPR-II in that the level of immunostaining for a related receptor, TGF-βRII, was similar between groups. Interestingly, a less marked but significant reduction in the level of BMPR-II immunostaining was also observed in secondary PH. These results suggest that reduced expression of BMPR-II may contribute not only to the pathogenesis of PPH but also to other forms of severe PH.

![Figure 5. Photomicrographs of peripheral lung stained for BMPR-II from control subject (A) and patients with secondary PH (patient 12; B), PPH in which no BMPR2 mutation was identified (patient 4; C), and PPH in which truncating mutation in BMPR2 was identified (patient 8; D). Note reduced alveolar capillary staining for BMPR-II (arrowed) compared with control, particularly in PPH cases. Approximate magnification ×200.](image)

![Figure 6. Semiquantification of lung tissue area positively stained for BMPR-II (A), TGF-βRII (B), and CD31 (C) in controls and patient groups. SPH indicates secondary PH. *P<0.05 vs control, #P<0.05 vs SPH, ##P<0.05 vs PPH, by ANOVA and Tukey multiple comparison test.](image)
noted in hereditary hemorrhagic telangiectasia, with an underlying heterozygous null mutation in the gene encoding endoglin. An intriguing possibility is that additional loss of the wild-type BMPR2 allele or reduction in BMPR2 transcription may be necessary to trigger the formation of vascular lesions in patients harboring a germline mutation. This "second hit" might explain the low penetrance of the condition within families. Additional molecular analysis of individual lesions will be necessary to determine whether such a mechanism occurs. Analysis of tissue from the systemic circulation of PPH patients would be necessary to determine whether the marked reduction in BMPR-II expression is restricted to the lung, which may in part explain the lung-specific nature of this disease.

A recent report identified an increased frequency of somatic mutations that arose as a result of microsatellite instability in the TGF-βRII gene in plexiform lesions of PPH patients. The present study found that ≈20% of vascular lesions in severe PH demonstrated absent expression of TGF-βRII but no difference in the frequency of negative lesions between PPH and secondary PH groups. However, it remains conceivable that germline mutations in BMPR2 and somatic mutation in TGF-βRII may have similar and/or additive effects, removing the growth-inhibitory effect of TGF-β superfamily members and allowing clonal expansion of vascular cells, leading to occlusion of small pulmonary arteries.

Although a limited number of studies have suggested a role for TGF-β in hypertensive pulmonary vascular remodeling, its precise role is poorly defined. Immunostaining for TGF-β isoforms, especially TGF-β2 and TGF-β3, appears to be increased in actively remodeling hypertensive pulmonary arteries in patients with PPH compared with controls. However, TGF-β is not a known ligand for BMPR-II, and the cellular localization of BMP expression in the lung is yet to be determined.

We have recently reported that pulmonary artery smooth muscle cells from patients with PPH exhibit altered growth responses to BMPs in that BMPs suppress proliferation of cells from normal controls and secondary PH patients but not PPH patients. We speculate that the failure of BMP-induced growth suppression may contribute to abnormal proliferation of vascular cells in PPH. Interestingly, in that study, cells from all PPH patients behaved similarly, whether a mutation in BMPR2 was present or not. The findings of the present study provide a possible explanation for this in that markedly reduced BMPR-II expression was a feature of all PPH cases, whether or not BMPR2 mutations were present. Thus, reduced expression of BMPR-II protein, either as a result of underlying mutation or transcriptional repression of BMPR2, may contribute to the abnormal vascular cell responses to BMPs in PPH.

The nature of the cell type responsible for the intimal obliteration of small pulmonary arteries in PPH has been the subject of considerable debate. The evidence, supported by our findings, suggests that the intimal lesion is composed of myofibroblast-like cells, which stain positively for α-smooth muscle actin and vimentin, and that the expression of endothelium-specific markers is confined to cells lining the vascular lumen. The plexiform lesion would appear to consist of a proliferation of endothelial cells supported by a myofibroblast stroma.

In summary, the present study identifies predominantly endothelial cells but also myofibroblast cells comprising intimal lesions as the sites of pulmonary vascular expression of BMPR-II in PPH and secondary PH. The reduced or absent expression of BMPR-II in vascular lesions of PPH patients, especially in those harboring an underlying mutation in the BMPR2 gene, supports the hypothesis that loss of BMPR-II function contributes to the formation of lesions.

Acknowledgments

This study was supported by a grant from the British Heart Foundation (program grant RG/2000012 to Dr Trembath and Dr Morrell). Dr Thomson was a Medical Research Council (UK) Clinical Training Fellow. The authors would like to acknowledge Neal Morgan, University of Leicester, for technical assistance with gene dosage studies.

References


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_Circulation_. 2002;105:1672-1678; originally published online March 25, 2002;
doi: 10.1161/01.CIR.000012754.72951.3D
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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