Concise Report

Lack of circulating autoantibodies to bone morphogenetic protein receptor-II or activin receptor-like kinase 1 in mixed connective tissue disease patients with pulmonary arterial hypertension

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\textbf{Objectives.} To examine whether autoantibodies against bone morphogenetic protein receptor-II (BMPR-II) or activin receptor-like kinase 1 (ALK-1) are associated with pulmonary arterial hypertension (PAH) in patients with mixed connective tissue disease (MCTD).

\textbf{Methods.} We studied sera from 37 MCTD patients with or without PAH, six patients with idiopathic PAH, and 30 healthy controls. Circulating anti-BMPR-II and anti-ALK-1 antibodies were detected using immunoprecipitation of recombinant antigens generated by \textit{in vitro} transcription/translation and indirect immunofluorescence of cultured cells that were induced to express these antigens by gene transfer. Anti-BMPR-II antibodies were further examined by immunoprecipitation and immunoblotting using a recombinant fragment of the extracellular domain of BMPR-II.

\textbf{Results.} Serum anti-BMPR-II and anti-ALK-1 autoantibodies were not detected in MCTD patients irrespective of the presence or absence of PAH, or in patients with idiopathic PAH.

\textbf{Conclusions.} Our finding does not support the hypothesis that autoantibody-mediated dysregulation of signals through BMPR-II or ALK-1 contributes to the development of PAH in patients with connective tissue diseases.

\textbf{KEY WORDS:} Pulmonary arterial hypertension, Autoantibody, Mixed connective tissue disease, Bone morphogenetic protein receptor-II, Activin receptor-like kinase 1.

Pulmonary arterial hypertension (PAH) is a life-threatening organ involvement in patients with various connective tissue diseases, including scleroderma [1]. PAH also occurs without an underlying disease, and is then termed idiopathic pulmonary arterial hypertension (IPAH; formerly primary pulmonary hypertension) [2]. Recent genetic analyses in IPAH patients have identified germ-line mutations in the gene for bone morphogenetic protein receptor-II (BMPR-II), a type II receptor for transforming growth factor \(\beta\) (TGF-\(\beta\)) superfamilies, in both familial and sporadic cases [3–5]. In addition, families with hereditary haemorrhagic telangiectasia are known to develop PAH frequently, and mutations in the gene for activin receptor-like kinase 1 (ALK-1), a type I receptor for TGF-\(\beta\), have been identified as a genetic factor responsible for this hereditary disease [6]. Both BMPR-II and ALK-1 are receptors for TGF-\(\beta\) family proteins, with intracellular serine/threonine kinase domains that are preferentially expressed on vascular endothelial cells [7, 8]. Upon ligand binding, these receptors participate in the phosphorylation of a series of Smad proteins that regulate the growth and functional properties of vascular endothelial and smooth muscle cells. It is currently believed that dysregulated Smad signals through BMPR-II and ALK-1 are involved in the pathogenesis of IPAH [9, 10].

IPAH and PAH accompanied by connective tissue diseases have similar characteristics [1], including medial hypertrophy and plexogenic arteriopathy in the pulmonary artery histology; progressive clinical course and poor prognosis; and therapeutic responses to continuous infusion of the prostacyclin analogue and endothelin-1 receptor antagonist. Therefore, we hypothesized that dysfunction in BMPR-II or ALK-1 also contributes to the pathogenesis of PAH in patients with connective tissue diseases. However, it seemed unlikely that genetic mutations would be found as the underlying cause because two independent research groups failed to detect mutations in the BMPR-II gene in patients with scleroderma spectrum disorders and PAH [11, 12]. Another potential mechanism includes the presence of autoantibodies that functionally interfere with ligand binding to BMPR-II or ALK-1, which is reasonably likely, because patients with connective tissue diseases, especially those with systemic lupus erythematosus (SLE), are intrinsically prone to producing autoantibodies. To test this hypothesis, we developed assay systems to detect autoantibodies that react with BMPR-II and ALK-1. Since the majority of Japanese patients with connective tissue disease and PAH are positive for antibodies to U1 small nuclear ribonucleoprotein (U1RNP) and have a diagnosis of mixed connective tissue disease

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(MCTD) [13, 14], we examined MCTD patients with or without PAH in this study.

Materials and methods

Patients and samples

We examined 37 patients who were positive for anti-U1RNP antibodies and fulfilled the diagnostic criteria for MCTD proposed by Porter et al. [15]. MCTD patients represented heterogeneous clinical characteristics: 21 patients met the American College of Rheumatology (ACR) classification criteria for SLE [16], 24 met the ACR preliminary classification criteria for scleroderma [17], and six met the diagnostic criteria for definite polymyositis or dermatomyositis as proposed by Bohan and Peter [18]. Fourteen patients satisfied two of these three sets of criteria (SLE and scleroderma in nine, and scleroderma and polymyositis/dermatomyositis in five). Echocardiogram was performed on all patients, and those with a mean pulmonary artery pressure ≤25 mmHg at rest were regarded as non-PAH [9]. The remaining patients, with a mean pulmonary artery pressure >25 mmHg, were further divided into two groups: patients with definite PAH in whom the presence of PAH was confirmed by right ventricle catheterization, and those with questionable PAH in whom right ventricle catheterization was not carried out. Serum samples from six patients with IPAH [2] and 30 healthy individuals were used as controls. Written consent was obtained according to the Declaration of Helsinki, and the study design was approved by Keio University Institutional Review Boards.

IgG fraction

The immunoglobulin G (IgG) fraction was isolated from sera by affinity chromatography using a HiTrap™ Protein G HP Column (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer’s protocol. The protein concentration was measured with the Bradford assay using serial concentrations of bovine serum albumin (BSA) as a standard.

cDNA constructs

Full-length cDNAs encoding human BMPR-II and ALK-1 were kindly provided by Dr Kohei Miyazono (University of Tokyo Graduate School of Medicine, Tokyo, Japan). These cDNAs were subcloned into pcDEF3-FLAG (BMPR-II) or pcDNA3-HA (ALK-1), which add FLAG- and HA-epitope tags, respectively, at the carboxyl terminal end [19, 20].

Immunoprecipitation of recombinant polypeptides generated by in vitro transcription and translation

Recombinant BMPR-II-FLAG and ALK-1-HA were produced by in vitro transcription and translation of full-length cDNAs using the Single Tube Protein™ System 3 (Novagen, Darmstadt, Germany) in the presence of 35S-labelled methionine (ICN Biomedicals, Irvine, CA, USA). 35S-labelled BMPR-II-FLAG and ALK-1-FLAG were applied to immunoprecipitation assays as described previously [21]. Mouse monoclonal antibodies (mAbs) to BMPR-II (clone 18; Becton Dickinson, San Jose, CA, USA) and FLAG (clone M2; Sigma-Aldrich, St Louis, MO, USA) were used as positive controls for BMPR-II-FLAG, and rat anti-HA mAb (clone 3F10; Roche Applied Science, Mannheim, Germany) was used as a positive control for ALK-1-FLAG. Mouse anti-His mAb (Amersham Biosciences) was used as a negative control. The immunoprecipitated materials were separated on sodium dodecyl sulphate (SDS)-6% (for BMPR-II-FLAG) or 8.5% (for ALK-1-HA) polyacrylamide gels, and subjected to autoradiography using a Fuji BAS 5000 Bio-Imaging Analyzer (Fuji Photo Film, Tokyo, Japan).

Immunoprecipitation and immunoblotting using recombinant BMPR-II as an antigen

Anti-BMPR-II antibodies were detected using a recombinant chimeric protein consisting of the extracellular domain of human BMPR-II and the histidine-tagged Fc region of human IgG1 (BMPR-II/Fc chimera; R & D Systems, Minneapolis, MN, USA). Serum samples (10 μl) were incubated with protein A-Sepharose™ CL-4B beads (Amersham Biosciences) for 2 h at room temperature. Goat anti-BMPR-II polyclonal antibodies (R & D systems) and anti-His mAb were used as positive controls, and anti-HA mAb was used as a negative control. The beads were incubated with BMPR-II/Fc chimera (50 ng) for 2 h at 4°C in 10 mM Tris–HCl, 500 mM NaCl, 0.1% Nonidet P-40, pH 8.0, containing 60% fetal bovine serum to block non-specific protein A binding to the Fc region of the BMPR-II/Fc chimera. The immunoprecipitated materials were subsequently separated on SDS-8.5% polyacrylamide gels, and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk, and incubated with anti-BMPR-II polyclonal antibodies and then with alkaline phosphatase-conjugated anti-goat IgG (Sigma-Aldrich). Positive signals were visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Indirect immunofluorescence of cultured cells expressing exogenous genes

COS-7 cells grown on fibronectin-coated eight-well chamber slides (Becton Dickinson Labware, Bedford, MA, USA) were transfected with plasmids harbouring BMPRII-FLAG or ALK-1-HA, using the Effectene transfection reagent (Qiagen, Hilden, Germany). COS-7 cells transfected with the empty vector were used as a control. After a 24-h culture in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, the cells were fixed with cold acetone/methanol for 5 min. Each slide was blocked with 5% BSA, and incubated with purified IgG (40 μg/ml) and then with Alexa Fluor 488-conjugated goat anti-human IgG antibodies (Molecular Probes, Eugene, OR, USA). The cells were further incubated with biotinylated anti-FLAG mAb and streptavidin-conjugated Alexa Fluor 568 (for BMPRII-transfectants) or anti-HA mAb and Alexa Fluor 568-conjugated goat anti-rat IgG antibody (for ALK-1-transfectants). Anti-BMPRII mAb with Alexa Fluor 488-conjugated goat anti-mouse IgG antibodies were used as a positive control for BMPRII-transfectants. Nuclei were counterstained with TO-PRO-3 (Molecular Probes). The cells were observed under a confocal laser fluorescent microscope (LSM5 PASCAL; Carl-Zeiss, Göttingen, Germany).

Results

Of 37 patients with MCTD, 15 and two had definite and questionable PAH, respectively, while 20 did not have PAH. There was no difference in the distribution of clinical diagnosis between patients with PAH and those without. Nine of 15 patients with definite PAH died within 3 yr after the serum sample collection. Autopsy in seven patients revealed a typical medial hypertrophy with or without plexiform lesions in pulmonary arteries. Of the remaining six patients, five are being treated with epo prostenol and one is on bosentan.

Sera from 37 MCTD patients with and without PAH, six patients with IPAH and 30 healthy controls were screened for antibodies to BMPR-II and ALK-1 by immunoprecipitation.
FIG. 1. Screening of anti-BMPR-II and anti-ALK-1 autoantibodies in representative sera from healthy controls and MCTD patients with or without PAH. (A) Detection of anti-BMPR-II antibodies using immunoprecipitation of 35S-labelled BMPR-II-FLAG generated by in vitro transcription/translation. Lane 1, 14C-labelled molecular weight markers; lane 2, recombinant BMPR-II-FLAG without immunoprecipitation; lane 3, anti-BMPR-II mAb; lane 4, anti-FLAG mAb; lanes 5 and 6, healthy control sera; lanes 7–11, sera from MCTD patients with definite PAH; lanes 12–15, sera from MCTD patients without PAH. Arrow denotes intact recombinant BMPR-II-FLAG. (B) Detection of anti-ALK-1 antibodies using immunoprecipitation of 35S-labelled ALK-1-HA generated by in vitro transcription/translation. Lane 1, 14C-labelled molecular weight markers; lane 2, recombinant ALK-1-HA without immunoprecipitation; lane 3, anti-ALK-1 mAb; lane 4, anti-His mAb; lanes 5 and 6, healthy control sera; lanes 7–11, sera from MCTD patients with definite PAH; lanes 12–15, sera from MCTD patients without PAH. Arrow denotes intact recombinant ALK-1-HA. (C) Detection of anti-BMPR-II antibodies by immunoprecipitation and immunoblotting using a recombinant BMPR-II/Fc chimera. Lane 1, molecular weight markers; lane 2, BMPR-II/Fc chimera without immunoprecipitation; lane 3, anti-BMPR-II polyclonal antibodies; lane 4, anti-His mAb; lane 5, anti-ALK-1 mAb; lanes 6 and 7, healthy control sera; lanes 8–12, sera from MCTD patients with definite PAH; lanes 13–15, sera from MCTD patients without PAH. Arrow denotes recombinant BMPR-II/Fc chimera and arrowheads denote non-specific IgG bands.

Discussion

This is the first report examining the autoantibody response to BMPR-II or ALK-1 in patients with connective tissue disease complicated by PAH and in IPAH patients. Contrary to our expectations, we failed to detect IgG autoantibodies that reacted with BMPR-II or ALK-1 in sera from MCTD patients, irrespective of the presence or absence of PAH. Since the recombinant antigens used in our assays are predicted to have a native conformation, our findings indicate a lack of circulating IgG antibodies capable of binding to BMPR-II or ALK-1 expressed on the endothelium. However, there is no human control serum positive for anti-BMPR-II or anti-ALK-1 antibody, although non-human antibodies to human BMPR-II or tags expressed on the recombinant proteins were used as positive controls in the assays. The possibility that our assays were less sensitive in
detecting human autoantibodies cannot be excluded. In addition, because the endothelium consists of approximately $10^{13}$ endothelial cells, occupying almost 7 m$^2$ in adults [22], it is still possible that patients have pathogenic autoantibodies to BMPR-II or ALK-1 that are present on endothelial cell surfaces but not in the circulation.

The mutations in the BMPR-II and ALK-1 genes that were detected in patients with IPAH included frameshifts, deletions, nonsense and missense mutations, and splice-site variations, most of which impaired the proteins’ functional properties or transcriptional activities. This is consistent with a model of haploinsufficiency [23] or a dominant-negative effect [24]. The expression level of the BMPR-II protein is in fact reduced in the lungs of patients with severe IPAH, most of whom have an underlying mutation in the BMPR-II gene [25]. Based on these findings, it is widely accepted that a mutation in the BMPR-II and ALK-1 genes presents a major risk of the loss of TGF-$\beta$ family-mediated growth inhibition of pulmonary arterial endothelial and smooth muscle cells, which leads to the development of the typical IPAH plexiform lesions in the pulmonary arteries [9, 10]. Taken together with the lack of germ-line mutations in the BMPR-II genes of patients with scleroderma spectrum disorders and PAH [11, 12], our findings strongly suggest that the underlying mechanisms for developing PAH are different between patients with connective tissue diseases and those without. This is supported by a recent paper reporting that the endothelial cell proliferation in the plexiform lesions is monoclonal in IPAH and polyclonal in scleroderma [26]. In this regard, the maintenance of the endothelial vascular network is regulated by several factors other than TGF-$\beta$ superfamily proteins and their signalling pathways, including the angiopoietin-Tie system [27]. It has been reported recently that the expression of angiopoietin-1 and the phosphorylation of Tie-2 are higher than normal in the lungs of patients with non-familial PAH, including IPAH and scleroderma [28]. Further studies are necessary to identify the mechanisms contributing to the development of PAH in patients with connective tissue diseases.

**Key messages**

- Circulating autoantibodies to BMPR-II or ALK-1 were undetectable in connective tissue disease patients irrespective of the presence or absence of PAH.
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