Interaction of interleukin-6 and the BMP pathway in pulmonary smooth muscle

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1Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Health Sciences Center, Denver, Colorado; and 2Pulmonary and Critical Care Division, Vanderbilt University, Nashville, Tennessee

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Hagen M, Fagan K, Steudel W, Carr M, Lane K, Rodman DM, West J. Interaction of interleukin-6 and the BMP pathway in pulmonary smooth muscle. Am J Physiol Lung Cell Mol Physiol 292: L1473–L1479, 2007. First published February 23, 2007; doi:10.1152/ajplung.00197.2006.—The majority of familial pulmonary arterial hypertension (PAH) cases are caused by mutations in the type 2 bone morphogenetic protein receptor (BMPR2). However, less than one-half of BMPR2 mutation carriers develop PAH, suggesting that the most important function of BMPR2 mutation is to cause susceptibility to a “second hit.” There is substantial evidence from the literature implicating dysregulated inflammation, in particular the cytokine IL-6, in the development of PAH. We thus hypothesized that the BMP pathway regulates IL-6 in pulmonary tissues and conversely that IL-6 regulates the BMP pathway. We tested this in vivo using transgenic mice expressing an inducible dominant negative BMPR2 in smooth muscle, using mice injected with an IL-6-expressing virus, and in vitro using small interfering RNA (siRNA) to BMPR2 in human pulmonary artery smooth muscle cells (PA SMC). Consistent with our hypothesis, we found upregulation of IL-6 in both the transgenic mice and in cultured PA SMC with siRNA to BMPR2; this could be abolished with p38MAPK inhibitors. We also found that IL-6 in vivo caused a twofold increase in expression of the BMP signaling target Id1 and caused increased BMP activity in a luciferase-reporter assay in PA SMC. Thus we have shown both in vitro and in vivo a complete negative feedback loop between IL-6 and BMP, suggesting that an important consequence of BMPR2 mutations may be poor regulation of cytokines and thus vulnerability to an inflammatory second hit.

The pathogenesis of pulmonary arterial hypertension (PAH) remains poorly defined. A significant proportion of idiopathic PAH cases are familial (1), and the majority of hereditary cases are associated with mutations in a transforming growth factor (TGF)-β superfamily receptor, the type 2 bone morphogenetic protein receptor (BMPR2) (17, 29). These patients may have abnormalities in their pulmonary vascular reactivity when clinically asymptomatic (26); however, fewer than one-half of mutation carriers ever develop clinical PAH. This suggests that although there may be baseline abnormalities in vascular tone, the important function of BMPR2 mutation is to cause increased susceptibility to an as yet unknown “second hit.” Patients with PAH are consistently found to have increased circulating inflammatory cytokines, with IL-6, monocyte chemoattractant protein-1 (MCP-1), and TGF-β frequently cited as factors that may be involved in the pathogenesis of PAH. Although a causal role of cytokines in PAH is currently unknown, several animal models of secondary PAH, including chronic hypoxia (15, 32), monocrotaline treatment (2, 12), and chronic air embolism (23), are also associated with increased cytokine production, including increased IL-6, and injection of recombinant IL-6 caused PAH in rats (19). Kaposi’s sarcoma-associated herpesvirus, which may cause PAH in human immunodeficiency virus (HIV)-negative Castleman’s disease, encodes a viral, constitutively active, form of IL-6 (22). In addition, recent studies have suggested the involvement of viral infection or autoimmunity (21) in the development of PAH. Thus there is substantial evidence that the unknown second hit is likely to be inflammatory in character.

Recently, several studies have drawn correlations between regulation of the BMP pathway and regulation of inflammation, in the context of atherosclerosis (9), arthritis (18), kidney disease (6), and even PAH (28). Heterozygous BMPR2 knock-out mice have increased susceptibility to PAH caused by an inflammatory stimulus (28). These findings show that it is plausible that dysregulation of BMP could cause vulnerability to an inflammatory second hit.

In this study, we test the hypothesis that there is a mutual regulatory relationship between IL-6 and the BMP pathway, both in vivo and in vitro. If so, this provides a mechanism by which loss of BMP signaling predisposes to disease resulting from an inflammatory second hit. We chose to concentrate on IL-6 because it is the cytokine most heavily implicated by the literature in PAH.

METHODS

SM22-tet-BMPR2Δ1;4+ mice. SM22-tet-BMPR2Δ1;4+ mice, which when fed doxycycline express a dominant negative BMPR2 only in smooth muscle (34), were fed doxycycline for 8 wk, starting at birth, killed, and lung tissue was collected. Control mice, fed doxycycline but lacking the tetracycline-responsive dominant negative transgene, were killed at 8 wk of age as well. Lung tissue was removed as previously described (34). RNA was prepared from homogenized lung tissue and used for quantitative RT-PCR using standard techniques. All animal experiments were considered and approved by the University of Colorado Health Sciences Center Institutional Animal Care and Use Committee.

Quantitative RT-PCR. Primers were designed using sequences downloaded from GenBank with the Applied Biosystems ABI Primer Express program (Foster City, CA). Each primer was searched against BLAST to ensure that it did not match any known gene aside from the gene for which it was designed (especially other family members). Upon receipt, each primer was tested and titered to ensure it gave a complete amplification product of the expected size.

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Inhibitors
Transducers
Receptors

specifically to BMP activity (14). CAGA-luc plasmid is a luciferase reporter, containing parts of the Id1 promoter, which respond driven by a SMAD-specific promoter (11). pBRE plasmid is a lucif-

amplified lengths are listed in Table 1.

**Table 1. Quantitative RT-PCR primers**

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BMPR, bone morphogenetic protein receptor; PRDC, protein related to DAN and Cerberus; GCSF, granulocyte-colony-stimulating factor; MCP-1, monocyte chemotactic protein-1; TGF, transforming growth factor.

single, clean band of the appropriate size. Primer sequences and amplified lengths are listed in Table 1.

**Cell culture.** Human pulmonary artery smooth muscle cells (PA SMC, Cambrex, East Rutherford, NJ) were plated into 10-cm dishes and grown to ~60% confluence in Smooth Muscle Growth Media 2 (SmGM2) containing 5% FBS. Recombinant human BMP2 (150 ng/ml) or IL-6 (20 ng/ml) (R&D systems, Minneapolis, MN) were added for 1, 2, or 4 h. All time points were done in duplicate. RNA was made using a Qiagen RNeasy mini kit (Qiagen, Valencia, CA). cDNA was made using Invitrogen Superscript II with random hex-

amers (Invitrogen, Carlsbad, CA). Quantitative PCR was carried out in triplicate in an Applied Biosystems ABI 5700, using an unmodified syber-green protocol and the default cycling protocol, with dissocia-

tion curves used to ensure correct product for each reaction. Results were normalized to primers specific to hypoxanthine-xanthine phosphoribosyl transferase (HPRT).

**Plasmids.** pSBE (gift of L. J. Jonk) plasmid contains luciferase driven by a SMAD-specific promoter (11). pBRE plasmid is a luci-

ferase reporter, containing parts of the Id1 promoter, which respond specifically to BMP activity (14). CAGA-luc plasmid is a luciferase reporter containing a SMAD2/3, TGF-β, specific promoter (8).

**Luciferase assay (pSBE).** pSBE was transfected into passage 4 primary human PA SMC using Invitrogen Lipofectamine and Plus reagent in six-well plates. Every well also received plasmid containing a β-galactosidase-expressing plasmid to allow normalization of re-

sults to transfection efficiency. Plasmid quantities were standardized using empty Clontech pIRE2, the parent vector. Starting 48 h after transfection, wells received either no treatment, 150 ng/ml BMP2, or 20 ng/ml IL-6. Each condition was replicated in three wells. After 4 h, the cells were lysed in CCLR lysis reagent (Promega, Madison, WI). Luciferase assays were performed using a Promega luciferase assay kit according to the manufacturer’s instructions using a Monolight 1020 luminometer (Analytical Luminescence Laboratory, San Di-

ego, CA).

Luciferase assay (pBRE and CAGA-luc). PA SMC at passage 4 were transfected using 1 μg of CAGA-luc or pBRE firefly luciferase reporter constructs and 1 μg of renilla luciferase as a transfection control. Transfections were conducted using 2.7 μl of Lipofectamine and 6 μl of Plus reagent (Invitrogen). After 2 h of transfection, media containing 2% FBS was added to recover cells. The media was changed to complete media at 24 h. Forty-four hours posttransfection, 20 ng/ml recombinant human IL-6 (R&D Systems) was added. Cells were harvested at 48 h posttransfection by being scraped into 100 μl of Passive Lysis Buffer (Promega). Luciferase activity was assayed using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol and measured using a single-tube dual lumimeter (Berthold, Bundoora, Australia).

IL-6 adenoviral injections. Sixty microliters at 1 × 10¹¹ particles/ml of ΔE4 adenovirus encoding a cytomegalovirus-driven FLAG-tagged IL-6 was injected into five female 8-wk-old Fv/bn mice via intracardiac injection as described previously (33). As a control, five additional female Fv/bn 8-wk-old mice were injected with a ΔE4 adenovirus encoding LacZ. After 3 days, mice were killed, and whole lung was used to make RNA and cDNA as above.

Western blot and ELISA. Whole mouse lung was homogenized in RIPA buffer (PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS) with proteinase and phosphatase inhibitor cocktails (Sigma), and protein concentration was determined by Bradford assay. Three-

hundred micrograms of total protein was immunoprecipitated with rabbit polyclonal anti-IL-6 (Pepro Tech, Rocky Hill, NJ) or equal total protein quantities were mixed directly with sample buffer containing 2-mercaptoethanol. Samples were boiled, separated on an 8–16% Tris glycine gel (Invitrogen), and transferred to Hybond-P membrane (Amersham, Buckinghamshire, England) in 20% methanol. Membranes were blocked in 1% nonfat milk and 0.1% Tween 20 and probed with goat polyclonal anti-IL-6 (Santa Cruz) or rat anti-mouse TGF-β (RDI, Flanders, NJ). Goat or rat secondary antibodies conjugated to HRP (Santa Cruz) were then used, followed by detection...
using ECL-plus (Amersham). Mouse MCP-1 ELISA kit (Pierce Endogen, Rockford, IL) was used as per the manufacturer’s instructions using the same protein samples as for the Western blots. Rainbow molecular weight markers (Amersham) were used to determine size; blots were stripped and reprobed with β-actin to confirm equal protein loading; β-actin was found just below the 46-kDa marker on all blots.

Small interfering RNA. Human PA SMCs were grown to ~60% confluency on 10-cm cell culture dishes in SmGm2 media containing 5% serum. Transfections in PA SMC were done using 18 nM scrambled small interfering RNA (siRNA) control (Qiagen) or 18 nM siRNA to BMP2 (Ambion). Lipofectamine and Plus reagents (Invitrogen) were mixed with Optimem media (Invitrogen) and siRNA as per the manufacturer’s instructions. SmGm2 media was replaced with Optimem before lipid-RNA complexes were added to the cells and incubated for 2 h at 37°C. A volume of SmGm2 media containing 10% serum equal to that already on the plate was then added to the existing transfection media, and the cells were incubated for an additional 24–48 h.

### RESULTS

IL-6, MCP-1, and TGF-β transcript and protein are elevated in SM22-BMP2delx4+ mouse lung. We previously published a transgenic mouse, SM22-BMP2delx4+, in which overexpression of a dominant negative BMPR2 only in smooth muscle leads to elevated right ventricular pressures and aberrations in smooth muscle function (36) but does not produce the vascular lesions that are hallmarks of PAH (34). We wished to determine whether these mice also exhibited increased pulmonary inflammatory cytokines and chemokines characteristic of human PAH.

Quantitative RT-PCR was performed using primers specific to IL-6, MCP1, and TGF-β using RNA from whole lung from six to eight SM22-BMP2delx4+ mice or SM22-rtTA-only mice. Both groups were fed doxycycline for 8 wk, a time at which these mice exhibit signs of pulmonary hypertension (34). In addition, as a control for the specificity of BMPR2-dependent effect on these genes, we picked several more cytokines from different groups to test, including granulocyte colony-stimulating factor, IFNγ, and TNF-α. All primers were normalized against HPRT expression. We found that the SM22-BMP2delx4+ mice had 15×, 6×, and 2× increased levels of IL-6, MCP-1, and TGF-β, respectively (Fig. 1A), whereas the unrelated cytokines used as controls were unchanged.

We found substantially increased protein levels by Western blot for TGF-β (Fig. 1B) and immunoprecipitation followed by Western blot for IL-6 (Fig. 1C). We performed ELISA to determine MCP-1 protein levels in whole lung and found these were also increased (Fig. 1D).

IL-6, MCP-1, and TGF-β are regulated by BMP signaling in PA SMC culture. Human PA SMC were exposed to 0, 1, 2, or 4 h of 150 ng/ml recombinant human BMP2, a ligand that binds the BMPR2 receptor. Quantitative RT-PCR was performed using primers specific to the inflammatory cytokines, with two experimental replicates per time point. The dose of BMP2 was chosen because it is slightly above the dose found to saturate the dose-response curve in studies of proliferation in rat vascular smooth muscle cells (20). Furthermore, although this is a strong dose, the PA SMC used had high basal BMP pathway activity that needed to be overcome. All data were normalized to HPRT expression to control for loading.

We found that BMP2 decreased RNA levels for all three cytokines of interest (Fig. 2A). IL-6 expression remained the same at 1 h and then dropped fivefold at 2 and 4 h. MCP-1 expression dropped approximately fourfold by 1 h and remained at this decreased level for 2 and 4 h. TGF-β, on the other hand, showed a steady decrease to a twofold drop by 4 h.

**Fig. 1.** IL-6, monocyte chemoattractant protein-1 (MCP-1), and transforming growth factor (TGF)-β are increased in SM22-BMP2delx4+ transgenic mice compared with SM22-rtTA-only controls. A: IL-6 and MCP-1 expression is increased 15× and 6× in whole lung (*P < 0.05 by unpaired t-test, error bars show standard deviation); TGF-β trends toward an increase, whereas other cytokines are not changed. B: TGF-β protein is increased in whole lung by Western blot. C: IL-6 protein is increased in whole lung by IP; equal total protein loading from SM22-BMP2delx4+ transgenic mice compared with SM22-rtTA-only controls. D: MCP-1 protein is increased in SM22-BMP2delx4+ transgenic mice compared with SM22-rtTA-only controls by ELISA. Each point is an individual mouse, *P < 0.01 by unpaired t-test. BMP2R2, type 2 bone morphogenetic protein; GCSF, granulocyte colony-stimulating factor.
BMP2 induced itself, as expected, with a fourfold increase in expression by 4 h (not shown).

Inversely, we tested whether loss of BMPR2 resulted in increased cytokines in cell culture. Primary PA SMC were transfected with either scrambled or BMPR2 siRNA, or mock-transfected, and 48 h later, RNA and protein were prepared from the cell cultures. To verify that the siRNA was effective, we performed Western blot analyses, which showed strong expression of BMPR2 in mock-transfected and scrambled-transfected cells, and reduced expression in the cells receiving siRNA to BMPR2 (Fig. 2B). We also performed quantitative RT-PCR to determine expression levels of relevant cytokines. We found a pattern of increases parallel to those found in the mice, with a threefold increase in IL-6 expression, a lesser increase in MCP-1, and no significant increase in other cytokines checked (Fig. 2C).

BMPR2 signals through at least two alternate pathways, SMAD and p38MAPK (27). Loss of BMPR2 results in increased p38MAPK activity, as demonstrated by phosphorylation of the canonical p38MAPK target, heat shock protein (HSP)-27 (Fig. 2D). Addition of 2.5 μM SB-20358, a p38MAPK inhibitor, results in decreased HSP-27 phosphorylation (Fig. 2D) and returned IL-6 expression to control levels (Fig. 2E). SB-20358 alters p38MAPK activity but not phosphorylation (16, 38).

These experiments show that added BMP ligand results in reduced cytokines, and loss of BMPR2 results in increased cytokines, likely through p38MAPK activation, in PA SMC culture.

IL-6 increases BMP2, suppresses BMP pathway inhibitor transcription, and stimulates BMP activity in cell culture. The previous data show that the BMP pathway regulates expression of IL-6, MCP-1, and TGF-β. Loss of BMPR2 increased these cytokines both in vivo and in vitro, and the addition of exogenous BMP ligand decreased these cytokines. We were interested in testing whether cytokines were also capable of regulating BMP expression; we used IL-6 since it had the strongest response in our earlier tests and has the strongest association with PAH in the literature (2, 4, 7, 10, 19, 32).

IL-6 (20 ng/ml) was added to plates of human PA SMC and lysed, and RNA was extracted at 1, 2, and 4 h, with two replicates for each time point and two untreated controls. We designed primers to 16 BMP pathway genes (Table 1), including ligands, receptors, SMADs, and inhibitors, and performed quantitative RT-PCR. mRNA for all pathway genes examined, with the exception of BMP7, was expressed in cultured human PA SMC (not shown). Whereas most pathway genes were unaffected by treatment, IL-6 increased expression of the

Fig. 2. BMP regulates cytokine expression in vitro in pulmonary artery smooth muscle cells (PA SMC). A: 150 ng/ml BMP decreases IL-6, MCP-1, and TGF-β transcription in PA SMC culture 8-fold, 4-fold, and 2-fold, respectively. Change in all 3 are \( P < 0.05 \) by ANOVA. B: BMPR2 protein levels are decreased by small interfering RNA (siRNA) to BMPR2, but not by mock transfection or scrambled siRNA, by Western blot. The BMPR2 band is found above the 97-kDa molecular weight marker. C: IL-6 and MCP-1, but not other cytokines, are increased by siRNA to BMPR2 in PA SMC (\( P < 0.05 \) by ANOVA). All error bars show SD (D) siRNA to BMPR2 leads to increased heat shock protein (HSP)-27 phosphorylation, indicative of p38MAPK activation, by Western blot. This is reduced using 2.5 μM p38MAPK inhibitor SB-20358. E: IL-6 induction by siRNA to BMPR2 can be normalized through p38MAPK inhibitor SB-20358. HPRT, hypoxanthine-xanthine phosphoribosyl transferase.
ligand BMP2 approximately threefold and inhibited expression of secreted BMP inhibitors Cerberus and PRDC approximately fivefold (Fig. 3 A); other genes tested did not show significant changes.

To determine whether the changes in transcript level brought about a functional change in BMP pathway activity, luciferase reporter assays were performed in PA SMC. pSBE, which measures all SMAD activity, pBRE, which measures BMP-specific SMAD activity, and CAGA-luc, which measures TGF-β-specific SMAD activity, were tested. We found that, consistent with the expression data, 4 h of IL-6 stimulated BMP pathway activity by both pSBE and pBRE. CAGA-luc was unchanged from a low basal activity after addition of IL-6 (Fig. 3B).

**DISCUSSION**

In this study, we have identified the presence of a negative feedback loop between IL-6 and the BMP pathway, in which increased IL-6 induces BMP pathway activity, and increased BMP pathway activity suppresses IL-6. This provides a mechanism by which BMPR2 mutations found in familial PAH cause susceptibility to an inflammatory second hit. A normal role for the BMP pathway may thus be to prevent runaway positive feedback loops in inflammatory cytokines, including IL-6; in the absence of proper BMP signaling, these pathways become uncontrolled and can lead to disease.

Specifically, we show that IL-6 transcript and protein is upregulated with loss of BMPR2 both in vivo (Fig. 1, A and C) and in vitro in PA SMC (Fig. 2, C and E). As expected from this, IL-6 is inhibited in PA SMC by added BMP2 ligand (Fig. 2A). This effect is likely mediated through p38MAPK activity (Fig. 2, D and E). These data show that IL-6 is suppressed by a functional BMP pathway. In addition, we have shown that increased IL-6 leads to transcriptional changes indicative of increased BMP pathway activity in PA SMC (Fig. 3A) and in vivo (Fig. 4). IL-6 is also capable of inducing increased BMP pathway activity via luciferase reporter assay (Fig. 3B).

The specific nature of the changes is interesting as well. IL-6-induced changes in BMP pathway activity in PA SMC involve changes in regulation of paracrine factors, with an increase in BMP2 ligand and a decrease in secreted BMP inhibitors Cerberus and PRDC. These data suggest that the ligand BMP2 approximately threefold and inhibited expression of secreted BMP inhibitors Cerberus and protein related to DAN and Cerberus (PRDC) approximately fivefold (Fig. 3A); other genes tested did not show significant changes.

IL-6 induces Id1 transcript in whole lung in vivo. The experiments above established IL-6 induction of the BMP pathway in vitro; to determine whether this was reflected in vivo, we examined expression of canonical BMP transcriptional target Id1. Wild-type mice were injected with an IL-6-expressing adenovirus, compared with empty vector adenovirus, and expression of canonical BMP target gene Id1 and IL-6 were measured in whole lung after 3 days. This time point was chosen because it represents a maximum activity for adenovirus in vivo (33). We found that IL-6 expression was dramatically increased, at median 27-fold over control adenovirus, as expected (not shown). Id1 activity was increased twofold on average in mice that received the IL-6-expressing adenovirus, indicating that IL-6 causes increased BMP pathway activity in vivo as well (Fig. 4).
dominant effect of IL-6 on smooth muscle is to change BMP signaling in other tissues.

IL-6 is strongly associated with the development of pulmonary hypertension. Multiple studies of serum from patients with PAH have demonstrated increased circulating IL-6 (10). The genome of human herpesvirus type 8 (HHV8), which encodes a particularly potent viral IL-6 analog, is associated with PAH in the setting of HIV/AIDS, Castleman’s disease, and POEMS syndrome, and recently latent HHV8 infection was documented in plexiform lesions of some patients with idiopathic PAH (3, 5, 7). In addition, delivery of recombinant IL-6 protein produced PAH in rats (19), and inhibition of IL-6 with dexamethasone prevented monocrotaline-induced PAH (2). Our data establish the presence of a critical regulatory loop between IL-6 and BMPR2, such that loss of BMPR2 activity results in increased signaling through activin type II receptors when BMPR2 is unknown inflammatory stimulus, likely stimulating the autocrine/paracrine inflammatory loop that leads to clinical PAH.

In addition, the increase in BMP2 and decrease in secreted BMP ligand inhibitor expression found in PA SMC as a result of increased IL-6 results in increased signaling through alternate BMP ligand receptors in the context of defective BMPR2. Some BMP ligands have been shown to have increased signaling through activin type II receptors when BMPR2 is missing (37), currently with unknown consequences.

In summary, we have demonstrated important signaling interactions between BMP, IL-6, and other inflammatory cytokines in the lungs of transgenic mice and in human pulmonary artery cell culture. These are the first studies, to our knowledge, to establish that constitutive PA SMC BMP signaling is anti-inflammatory. With loss of normal BMP signaling, such as SM22-tet-BMPR2Del4+ transgenic mice and humans with BMPR2 mutations, the ability of IL-6 to induce itself may become uncontrolled, contributing to the development of PAH. Similarly, if increased IL-6 signaling is the result of viral homolog expression, as is seen in HHV8 infection, endogenous control by BMP would be ineffective, and IL-6 also unopposed. Since IL-6 is capable, on its own, of causing itself may become uncontrolled, contributing to the development of PAH. These data suggest that specific inhibition of inflammatory cytokines may thus be useful in treating PAH resulting from BMPR2 mutation; conversely, activation of the BMP pathway may be useful as an anti-inflammatory strategy in the pulmonary circulation.

GRANTS

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