Effects of bone morphogenic proteins and transforming growth factor-beta on in-vitro production of endothelin-1 by human pulmonary microvascular endothelial cells

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A B S T R A C T

Background: Altered endothelial cell (EC)-derived mediator levels, including increased endothelin-1 (ET-1), are hallmarks of human pulmonary arterial hypertension (PAH). Gene mutations for receptors for bone morphogenic proteins (BMPs), or transforming growth factor beta (TGF-β), cause heritable PAH. The effects of BMPs and TGF-β on ET-1 production by human pulmonary microvascular EC (HMVEC-LBl) are unknown.

Methods: HMVEC-LBl were exposed in-vitro to BMPs 2, 4, and 7 or TGF-β1 in basal or complete medium. ET production was measured, as well as total cellular protein. Levels of Smad 5 and phosphorylated Smads 1/5 were also measured.

Results: BMP-4 did not increase ET-1 while BMP-2 increased it minimally in basal medium. BMP-7 increased ET-1, but only at 100 ng/ml. By contrast, TGF-β1 increased ET-1 throughout most of the studied dose range. All BMPs and TGF-β1 increased levels of phosphorylated Smads 1/5 without depleting levels of Smad 5.

Conclusions: With the exception of BMP-7 at high-concentrations, the BMPs that interact with BMP receptor 2, the receptor implicated in heritable PAH, do not or minimally modulate in-vitro constitutive ET-1 production by HMVEC-LBl. TGF-β1 increases ET-1 synthesis, and this may have clinical relevance in PAH.

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1. Introduction

Pulmonary arterial hypertension (PAH) is a group of disorders that cause vascular remodeling of the pre-capillary pulmonary microvasculature, leading to increased pulmonary vascular resistance, right heart failure, and ultimately death (Humbert et al., 2004; Simonneau et al., 2004). Endothelial cell (EC) dysfunction is a hallmark of PAH, manifesting most significantly as abnormal EC growth that occludes the microvascular lumen, but also via reduced levels of endothelium-derived mediators that normally help maintain vascular homeostasis, including prostacyclin and nitric oxide (Archer et al., 1998; Christian et al., 1992; Giaid and Saleh, 1995; Humbert et al., 2004; Pietra et al., 2004; Tuder et al., 1999). Moreover, increased plasma and tissue levels of the endothelium-derived vasoconstrictor and mitogen, ET-1, have been described (Giaid et al., 1993; Stewart et al., 1991). Identification of increased ET-1 levels has led to the development of endothelin receptor antagonists that have improved the course of the disease for many PAH patients (Barst et al., 2006; Langleben, 2007; Rubin et al., 2002).

Whether the increased levels of ET-1 in PAH were related to excess local synthesis, or to a reduction in the normal pulmonary clearance of circulating ET-1 from the bloodstream, or both, had been unclear. Histologic study of lungs from patients with PAH established that excess local ET-1 expression and synthesis was a major factor (Giaid et al., 1993). Physiologic measurements of pulmonary ET-1 extraction in patients with PAH subsequently established that the majority of patients have normal or near-normal levels of ET-1 extraction (Langleben et al., 2006). Thus, the increased ET-1 levels in PAH result mainly from excess synthesis. There is at present only limited evidence that ET-1 synthesis can be reduced by therapy (Langleben et al., 1999; Prins et al., 1994). The stimuli for this excess synthesis are unknown. Numerous factors that can alter ET-1 production have been described, including transforming growth factor beta (TGF-β1) (Michael and Markewitz, 1996; Perez del Villar et al., 2005). In vitro, TGF-β1 stimulates ET-1 synthesis in endothelial cells from several vascular origins (Castanares et al., 2007; Kurihara et al., 1989; Lee et al., 2004; Rodriguez-Pascual et al., 2004). Its effects have not been studied in pulmonary microvascular endothelial cells.

Identification of families with PAH has led to the description of genetic mutations in components of receptors for the TGF-β superfamily of molecules (Nohe et al., 2002; Yamashita et al., 1996). These components include a receptor for bone morphogenic proteins, BMPR-II (Deng et al., 2000; Lane et al., 2000; Morrell, 2006) and the TGF-β receptor components activin-like kinase-1 (ALK-1) and endoglin (Trembath et al., 2001). The end-effect of the mutations seems to be a reduction in signaling via the receptor type that is expressed by the mutated gene (haplotypic insufficiency) (Machado et al., 2001). Activation of these receptors affects growth, apoptosis and differentiation in many cell types (Miyazono et al., 2005) and BMP signalling...
promotes pulmonary endothelial cell survival [Teichert-Kuliszewska et al., 2006]. The loss of this survival factor may contribute to the emergence of apoptosis resistant endothelial clones that narrow the microcirculation in familial PAH (Teichert-Kuliszewska et al., 2006). Moreover, ALK-1 is expressed in the circulation solely on endothelial cells, and a mutation in ALK-1 causes hereditary hemorrhagic telangiectasia (HHT), and PAH that is clinically indistinguishable from familial PAH (Trembath et al., 2001). Thus, there is compelling evidence for involvement of the TGF-β superfamily of molecules in the pathogenesis of several types of PAH (Newman et al., 2008). Given normally low ET-1 levels in humans, but high ET-1 levels in PAH, we hypothesized that the TGF-β superfamily of molecules would alter ET-1 production by pulmonary microvascular endothelial cells, the cell critical to the pathogenesis of PAH.

2. Materials and methods

2.1. Cells and reagents

Lung-derived normal human microvascular blood vessel endothelial cells HMVEC-LBI (Lonza, Walkersville MD) were cultured in EGM-2MV medium (supplemented with 5% fetal bovine serum, 0.04% hydrocortisone, 0.4% human fibroblast growth factor, 0.1% vascular endothelial growth factor, 0.1% insulin like growth factor, and 1% GA-1000). These cell lines are purified by double staining and flow cytometry and are 90% pure for cells of vascular origin; they express CD31, but do not express podoplanin, a marker for cells of lymphatic origin (HMVEC — LBI Lung Blood MV Endothelial Cell — Technical Sheet, Lonza, February 2008). BMPs 2, 4, 7 and TGF-β 1 were purchased from Peprotech (Rocky Hill, NJ).

2.2. Experimental techniques

HMVEC-LBI cells (passage 5 only) were grown on 24 well plates until they reached confluence. Twelve wells were used for each experimental condition, including each concentration at each time point. The medium was collected post exposure to the peptides and it was frozen at −70 °C for subsequent measurement of ET-1 levels. At each time point, after aspiration of the medium, the cells were lysed with Reporter Lysis Buffer (Promega) and the protein concentration in the lysate was measured by the BCA method (Pierce, Rockford IL). ET-1 levels in the supernatant were measured with a commercial ELISA kit (Assay Designs, Ann Arbor, Michigan) with antibodies specific for human ET-1. The intra-assay coefficient of variation was 5.8%, with an inter-assay variation of 4.7%.

2.3. Smad signalling

HMVEC-LBI cells were grown to confluence on 6-well plates in EGM-2MV medium. The cells were then serum starved in basal medium (EGM medium +0.1% BSA) overnight. Next, the medium was replaced with either basal medium, complete EGM-2MV medium, or basal medium containing BMP 2, 4, or 7 (100 ng/ml) or TGF-β (2.5 ng/ml) for 5 min. The cells were then lysed directly in sample buffer (2.5 mM Tris–HCl, pH 6.8, 25 °C, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue), homogenized by syringe aspiration through a 26-gauge needle, and separated on a 10% polyacrylamide gel. The proteins were transferred to a PVDF membrane (Pierce, Rockford IL) and blocked in TBS (0.02 M Tris, 0.14 M NaCl, 0.1% w/v Tween) with 5% powdered skim milk. The membranes were incubated overnight at 4 °C with anti-Smad5 (Cell Signalling, Danvers, MA), anti-phosphorylated Smad1/5 (Cell Signalling, Danvers, MA), and anti-GAPDH (Fitzgerald, Concord, MA), all in TBS with 5% BSA. The membranes were then washed in TBS and incubated at room temperature for 1 h with anti-rabbit-HRP (Cell Signalling, Danvers, MA) and anti-mouse-HRP (Pierce, Rockford, IL). After another wash, the membranes were incubated with Western lightening enhanced luminol (Perkin-Elmer, Waltham, MA) and then exposed to photographic film. Density of the bands on the developed film was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

In a separate experiment, the time course of Smad 1/5 phosphorylation was studied. The cells were serum starved overnight in endothelial basal media (EBM, Lonza) containing 0.1% BSA. The medium was then replaced with fresh EBM alone or EBM containing 100 ng/ml of BMP 2, 4, or 7, or 2.5 ng/ml of TGF-β. The cells were exposed to the experimental conditions for 15 min, 45 min and 7 h, after which the cells were lysed in sample buffer. The samples were then processed and analyzed as above.

2.4. Statistical methods

Data were expressed as mean±SD. For each experimental group, at 24 or 48 h, to detect differences in group means, one-way analysis-of-variance was used, followed where appropriate by the Tukey–Kramer multiple comparison test. Two-tailed p values <0.05 are considered significant.

3. Results

3.1. Effect of BMPs on ET-1 levels in complete medium (Fig. 1A)

At the concentrations used, after 24 h of exposure in complete medium, neither BMP-2, BMP-4 nor BMP-7 altered absolute ET-1 levels, normalized ET-1 levels or cell protein levels as compared to controls. Similarly, BMP-2 and BMP-4 did not raise absolute ET-1 levels after 48 h of exposure. However, after 48 h, BMP-2 (10 ng/ml) slightly increased cell protein levels, by 19% over controls (p <0.05). By contrast, BMP-4 (1 ng/ml) slightly reduced cell protein levels (12% less than controls) and this resulted in an 18% increase in normalized ET-1 levels versus controls (p <0.05). At 48 h, BMP-7, only at a dose of 100 ng/ml, increased absolute ET-1 levels by 32% as compared to control (p <0.05), and it also increased ET-1 levels normalized for cell protein by 27% versus controls (Fig. 1A).

3.2. Effect of TGF-β 1 on ET-1 levels in complete medium (Fig. 1B)

TGF-β 1 stimulated ET-1 production by the pulmonary microvascular endothelial cells. At 24h, as compared to controls it significantly increased ET-1 levels at all TGF-β 1 concentrations except 10 ng/ml, with a 47% increase at 2.5 ng/ml, and when normalized to cell protein, ET-1 levels were increased at all concentrations except 5 and 10 ng/ml (40% increase at 2.5 ng/ml). Exposure to TGF-β 1 did not cause any
3.5. Effect on ET-1 levels of BMPs 2, 4, and 7, and TGF-β in basal medium (Fig. 4)

In basal medium, an 8 h exposure to BMPs 2, or 7 or TGF-β significantly increased normalized ET-1 levels as compared to CONTROLS, with TGF-β inducing a 51% increase and BMP-7 a 27% increase. BMP-4 had no effect on ET-1 levels. Absolute ET-1 levels were significantly increased by BMP-7 (24% increase) and TGF-β (20%) as compared to CONTROLS, but not by BMP-2 (data not shown). None of the BMPs affected cellular protein levels, but TGF-β decreased cellular protein by 20%.

4. Discussion

The results of the present studies demonstrate that ET-1 can be modulated by some BMPs and by TGF-β in human pulmonary microvascular endothelial cells in vitro. With TGF-β, this stimulated increase in ET-1 occurs within 4 h, remains at 24 and 48 h and is seen in basal or complete medium. Despite inducing sustained SMADs 1/5 phosphorylation, BMP 2, only in basal medium, induced a slight increase in normalized ET-1 levels, and BMP-4 had no effect in basal medium, while BMP-7 at high doses had stimulatory effects similar to TGF-β at 48 h in complete medium and at 8 hours in basal medium.

An imbalance, with loss of the normal suppressive effects of the BMPs and enhancement of the stimulatory effects of the TGF-β molecules, has been proposed to lead to the vascular abnormalities in PAH (Newman et al., 2008). For example, unlike their normal growth-inhibitory effects seen in controls, BMPs did not inhibit proliferation of pulmonary artery smooth muscle cells derived from patients with idiopathic PAH (Morrell et al., 2001). By contrast, TGF-β1 stimulated proliferation in those same cells. These two effects were not apparent in smooth muscle cells from patients with other causes of pulmonary hypertension. Furthermore, BMPs appear to be essential survival factors for some cell types, preventing apoptosis in pulmonary vascular endothelial cells (Teichert-Kuliszewska et al., 2006). Thus it is essential to characterize the effects of these peptides in the clinically relevant cell population. The pulmonary microvascular endothelial cell appears to be the primary source of the excess ET-1 in PAH (Giaid et al., 1993), although there may be some lesser contribution from other cells in the vessel wall. In this study, we examined cells from individuals without pulmonary hypertension, to examine normal signaling pathways. Previously available commercial microvascular endothelial cell lines were significantly contaminated with endothelial cells of lymphatic origin. The line we used, HMVEC-LBl is vascular...
in origin. Thus, our results provide novel information about the effects of the TGF-β superfamily of molecules on ET-1 synthesis in these cells most relevant to PAH.

The HMVEC-LBl are extremely dependent on adequate culture conditions, including the presence of complete medium. Although experiments in basal medium represent the ideal situation free of endogenous cytokines, the HMVEC-LBl do not survive well in basal medium for extended periods of time. Therefore, our initial experiments were all performed with complete medium as the control. In that medium, only BMP-7 and TGF-β increased ET-1 levels. The time course experiment (Fig. 2) suggests that ET-1 levels can be stimulated to rise within 4 h of exposure to TGF-β, and that the levels continue to rise as compared to control levels. We chose 24 and 48 h time points for our dose-response experiments, since differences at those times would be more clearly detectable. A previous study has also used the 24 h time point (Castanares et al., 2007). Due to the need for serum starvation prior to onset of the experimental period, the studies using basal medium could not be extended beyond 8 h since cell mortality became an issue. However, in the experiments we performed in basal medium, the same pattern was seen, with BMP-7 and TGF-β increasing ET-1, with a lesser effect for BMP-2. Complete medium clearly stimulates ET-1 levels by itself, and this may reduce the ability to detect earlier ET-1 increases induced by BMP-7, such as were seen in basal medium.

Activation of the BMP receptor, or the type 1 TGF-β receptor ALK-1, results in phosphorylation of Smads 1/5 (Yamashita et al., 1996). The effects of BMPs and TGF-β on Smads 1/5 phosphorylation were studied in basal medium. Complete medium induces significant phosphorylation by itself (Fig. 3), likely due to the endogenous cytokine and TGF-β-family molecules present in the growth supplement and fetal bovine serum. Our measurement of Smad levels confirms that the different biologic effect between the BMPs studied
and TGF-β was not related to abnormal ligand-receptor interactions, or to defective BMP molecules. All the BMPs studied and TGF-β1 caused rapid Smads 1/5 phosphorylation, which would be the predicted signalling reaction for effective receptor-ligand interactions. Also, levels of Smad 5 did not change, indicating that the peptides did not affect the levels available for phosphorylation, but this latter finding would have been important only if we had not demonstrated the phosphorylation event. The time course experiments show that phosphorylation is rapid, within 5 min, and sustained at 45 min. By 7 h it has tapered off for TGF-β1 or returned to basal levels for the BMPs. The appearance of increased ET-1 levels in the medium takes several hours. The timing of the phosphorylation and secretion events suggests that they are linked, but separated by the delay necessary for de novo ET-1 peptide synthesis. In future studies, siRNA inhibition of Smad components or the TGF-β family receptors, as has been reported elsewhere, might help clarify this issue in the cells we studied (Castanares et al., 2007).

We studied the effects of BMPs 2, 4, and 7, which interact with BMPR-II, and TGF-β1 which interacts with TGF-β receptors, but not BMPR-II (Morrell et al., 2001). The experiments in Fig. 1 were not all performed simultaneously, and results for a given molecule should only be compared its respective control, not to other molecules or time points. BMP-2 and 4 did not alter ET-1 levels. BMP-7 stimulated ET-1 production, but only at the highest concentration studied (100 ng/ml). Studies of BMPs as endothelial survival factors have employed concentrations of 200 ng/ml (Teichert-Kuliszewska et al., 2006). It is noteworthy that in a previous study relevant to PAH, all these BMPs demonstrated effects on pulmonary vascular smooth muscle cells at concentration from 1 to 100 ng/ml (Morrell et al., 2001). However, it is also noteworthy that some cells, such as osteoblasts, only increase ET-1 production when stimulated with BMP-7 concentration of 100 ng/ml or higher (Kitten and Andrews, 2001). Thus, the microvascular ECs seem to demonstrate a true response to BMP-7, and it may be that endothelial cells are much less sensitive to BMP effects than other vascular cells such as smooth muscle. Indeed, a previous study of endothelial cells of various origins showed origin-dependent variability in responses to a given BMP, in that case BMP-4 (Kiyono and Shibuya, 2006). It is unknown whether local pulmonary levels of BMP-7 reach these high concentrations in vivo in patients with PAH. By contrast, TGF-β did stimulate ET-1 production at most concentrations studied, and these concentrations overlap those that have previously shown biologic effects on pulmonary smooth muscle cells in vivo (Morrell et al., 2001). That previous study, using cells from patients with idiopathic PAH, showed a paradoxical response to TGF-β1 as compared to controls, leading the authors to suggest that abnormalities in TGF-β1 superfamily signalling in PAH might be more widespread than just via BMP receptors, and might also involve TGF-β3 receptors (Morrell et al., 2001). If this concept is true, then one stimulus for increased ET-1 production in PAH might be via TGF-β3.

Our study has several limitations. First, the cells studied were not from patients with PAH. We chose to define “normal” behaviour in these experiments. Second, the cells were studied in isolation, without other vascular cell types being present, and they represent an imperfect simulation of events in a vessel wall. Third, the relevance of in vitro findings to the clinical state is still unclear. Nonetheless, our study does show differing biologic effects of the various TGF-β3 superfamily of molecules, and suggest a potential role for TGF-β1 in controlling pulmonary microvascular ET-1 production in vivo. Clinical studies will be needed to confirm the applicability of these findings to PAH.

5. Conclusions

— BMP 2 and 4 have minimal effects on ET-1 production by human pulmonary microvascular cells in vitro. BMP-7 at high concentra-


