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Evidence of a Role for Osteoprotegerin in the Pathogenesis of Pulmonary Arterial Hypertension

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Pulmonary artery smooth muscle cell (PA-SMC) migration and proliferation are key processes in the pathogenesis of pulmonary arterial hypertension (PAH). Recent information suggests that abnormalities in the bone morphogenetic protein (BMP) receptor 2 (BMP-R2) signaling pathway are important in PAH pathogenesis. It remains unclear whether and how this pathway interacts with, for example, serotonin (5-HT) and inflammation to trigger and/or sustain the development of PAH. The secreted glycoprotein osteoprotegerin (OPG) is emerging as an important regulatory molecule in vascular biology and is modulated by BMPs, 5-HT, and interleukin-1 in other cell types. However, whether OPG is expressed by PA-SMCs within PAH lesions and plays a role in PAH is unknown. Immunohistochemistry of human PAH lesions demonstrated increased OPG expression, and OPG was significantly increased in idiopathic PAH patient serum. Recombinant OPG stimulated proliferation and migration of PA-SMCs *in vitro*, and BMP-R2 RNA interference increased OPG secretion. Additionally, both 5-HT and interleukin-1 also increased OPG secretion. These data are the first to demonstrate that OPG is increased in PAH and that it can regulate PA-SMC proliferation and migration. OPG may provide a common link between the different pathways associated with the disease, potentially playing an important role in the pathogenesis of PAH. (*Am J Pathol* 2008, 172:256–264; DOI: 10.2353/ajpath.2008.070395)

Abnormal proliferation and migration of pulmonary artery smooth muscle cells (PA-SMCs) play a central role in the pathogenesis of pulmonary arterial hypertension (PAH).¹ The association between cases of idiopathic PAH (IPAH) and heterozygous germline mutations in the gene encoding bone morphogenetic protein receptor type 2 (BMP-R2)² or a polymorphism in the serotonin [5-hydroxytryptamine (5-HT)] transporter (SERT)³ have identified candidate pathways that may be involved in disease pathogenesis. Only a subset of patients with either genotype develop IPAH, and secondary pulmonary arterial hypertension affects only a minority of patients with associated primary illnesses such as systemic sclerosis. This suggests that multiple “hits” involving several cellular signaling pathways are required to produce the dysregulated PA-SMC proliferation and migration seen in PAH. The medial hypertrophy, intimal fibrosis, and in some cases, plexiform lesions of PAH are often accompanied by extensive perivascular inflammation.⁴ Moreover, the levels of several inflammatory mediators, including interleukin (IL)-1 and IL-6, are elevated in patients with pulmonary hypertension.⁵ These observations suggest an inflammatory component to the underlying mechanism of disease and that inflammatory cytokines may interact with dysregulated BMP and/or 5-HT signaling to create the multiple hits required for the development of PAH. However, the identity of key downstream molecules remains unknown.

Osteoprotegerin (OPG, TNFRSF11B), a member of the tumor necrosis factor receptor superfamily, is a secreted basic glycoprotein that exists as a 60-kDa monomer and a 120-kDa disulfide-linked homodimer.⁶ OPG is expressed and secreted by a variety of tissues, including the heart and lung,⁷ and has been primarily studied in its role of regulating bone turnover and apoptosis in health and in disease.^{8,9} In these situations, OPG functions as a secreted decoy receptor, competing either with receptor

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activator of nuclear factor- κ B (RANK) for the binding of RANK ligand (RANKL) to regulate osteoclast differentiation and activation⁷ or with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), preventing binding to membrane-associated death receptors (DR4 and DR5) to trigger apoptosis of multiple cell types.¹⁰

There is increasing evidence that OPG may play an important role in vascular disease. OPG knockout mice exhibit increased vascular calcification,¹¹ and OPG concentrations are elevated in plasma of patients with peripheral and coronary artery disease and systemic hypertension.^{12–14} Additionally, OPG has recently been shown to contribute to plaque progression in an atherosclerotic ApoE^{−/−} mouse model.¹⁵ In the context of a potential link between OPG and PAH, BMPs, 5-HT, and the inflammatory cytokine IL-1 are all known to up-regulate OPG in a variety of cell types.^{16–18} Taken together, these observations suggest that OPG may play an important role in the control of vascular cell behavior, regulated at least in part by signaling pathways implicated in PAH pathogenesis. Because the role of OPG signaling in PAH has not previously been studied, we hypothesized that these signaling pathways could converge to dysregulate expression of OPG and that this in turn would lead to increased PA-SMC proliferation and migration. We present data here to support this hypothesis that demonstrates for the first time that abnormalities in BMP, 5-HT, and inflammatory signaling result in heightened expression of OPG in PA-SMCs. Additionally, we show that OPG increases PA-SMC proliferation and migration, indicating the potential to contribute to the pathogenesis of PAH.

Materials and Methods

Lung and Serum Samples from Patients with IPAH

Lung tissue samples ($n = 12$, 4 male and 8 female; mean age, 40 ± 11 years) from patients with familial IPAH associated with mutant BMPR2 ($n = 6$), IPAH without BMPR2 mutation ($n = 6$), and control subjects undergoing lung resection for cancer or from unused donor-lung ($n = 6$, 4 male and 2 female; mean age, 55 ± 21 years) were obtained from the Papworth Hospital NHS Trust tissue bank as previously described.¹⁹ Serum was also obtained from 38 patients with IPAH (7 with, 13 without, and 18 not genotyped for BMP-R2 mutations; 10 male and 28 female; mean age, 44 ± 14 years) and from a control group comprising healthy adult volunteers and patients from an elective orthopedic preadmission clinic ($n = 33$, 20 male and 13 female; mean age, 58 ± 16 years). All subjects gave informed written consent, and the study was approved by the Local Research Ethics Committee.

Immunohistochemistry

Formalin-fixed, paraffin-embedded lung samples were serially sectioned at $5 \mu\text{m}$ and processed using 0.6 mol/L sodium citrate buffer (pH 6) antigen retrieval where re-

quired, as previously described.¹⁹ Serial sections were stained with goat anti-human osteoprotegerin (AF805; R&D Systems, Abingdon, Oxfordshire, UK), mouse monoclonal anti-human TRAIL (Vector Laboratories, Peterborough, UK), or mouse monoclonal anti-human RANKL (MAB626; R&D Systems). To colocalize components to the vascular endothelium and SMCs, immunohistochemical markers against endothelial cells (anti-CD31; DakoCytomation, Ely, Cambridgeshire, UK) and SMCs (anti- α -smooth muscle actin; DakoCytomation) were also used. Antibodies were incubated for 1 hour and labeled using the relevant streptavidin ABC peroxidase technique [DakoCytomation or Vector Laboratories (for OPG as only goat primary)] and visualized with 3,3'-diaminobenzidine (DakoCytomation).

OPG Enzyme-Linked Immunosorbent Assay

OPG concentration in cell culture medium and patient serum was measured by enzyme-linked immunosorbent assay (ELISA) as previously described.²⁰ For *in vitro* studies on cell culture medium, OPG was normalized to picograms of OPG per cell by performing cell counts on the monolayer using a Coulter counter.

Cell Culture and Phenotypic Assays

Human PA-SMCs were purchased from Cascade Biologics (Mansfield, UK) and maintained as previously described.²¹ Before stimulation, PA-SMCs were synchronized by incubating in Dulbecco's modified Eagle's medium containing PSA Solution (Cascade Biologics) and 0.2% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA) for 48 hours. Cell proliferation was assessed using tritiated thymidine incorporation and Coulter Counting, and migration was assessed using a Boyden chamber assay as previously described.²² Where required, cells were stimulated with recombinant human OPG (R&D Systems), recombinant human IL-1 β (R&D Systems), or serotonin creatinine sulfate complex (Sigma, St. Louis, MO) at the stated concentrations. Pre-incubation with the SERT inhibitor fluoxetine (Sigma) was performed as previously described.²¹

Short Interfering RNA Transfections

RNA interference was induced by transient transfection of 100 nmol/L short interfering RNA (siRNA) oligonucleotides targeting either BMP-R2 or cyclophilin B (Dharmacon, Lafayette, CO) complexed with DharmaFECT2 lipid transfection reagent (Dharmacon) according to the manufacturer's instructions. Knockdown of BMP-R2 was confirmed by TaqMan Gene Expression Assay (Assay ID Hs00176148_m1; Applied Biosystems, Foster City, CA) at 72 hours after transfection.

Statistical Analyses

Statistical analysis was performed using either a Mann-Whitney *U*-test when comparing two groups or a re-

Figure 1. OPG expression localizes to medial areas of IPAH lesions. Lung sections were immunohistochemically stained for OPG (**A–C**), TRAIL (**D–F**), RANKL (**G–I**), CD31 (**J–L**), smooth muscle actin (SMA; **M–O**), CD68 (**P–R**), and mouse IgG isotype control (**S–U**). **A, D, G, J, M, P,** and **S** show serial sections of a control donor pulmonary artery. **B, E, H, K, N, Q,** and **T** show staining of serial sections of a concentric lesion and **C, F, I, L, O, R,** and **U**, of a plexiform IPAH lesions. Magnification, $\times 400$. Scale bar = 50 μ m (**A**).

peated measure analysis of variance followed by the Newman-Keuls post hoc test with a 95% confidence level. $P < 0.05$ was deemed statistically significant (Prism 4.0c for Macintosh; GraphPad, San Diego, CA).

Results

OPG Expression Is Increased in Concentric and Plexiform IPAH Lesions

Immunohistochemical analysis of human lung tissue samples demonstrated a very low level of OPG expression in sections of control (transplant donor) lung, localized mainly in a few luminal endothelial cells (Figure 1A). In the IPAH lung sections, diffuse medial staining and strong cellular immunoreactivity was observed within both concentric (Figure 1B) and plexiform lesions (Figure 1C) of remodeled pulmonary arteries. Further immunohistochemical analysis was performed to determine the expression patterns of OPG-binding partners, TRAIL, RANKL, and Syndecan-1. Weak immunoreactivity for TRAIL was observed within the luminal endothelial cells of control vessels (Figure 1D), whereas increased staining was observed in the outer medial layers and luminal endothelium of the concentric IPAH lesions (Figure 1E) and throughout the plexiform lesions (Figure 1F). RANKL expression was barely detectable in the control lung (Figure 1G). In contrast, diffuse immunoreactivity was observed around the luminal edge and the outermost layers of the concentric lesions (Figure 1H). Plexiform lesions showed diffuse immunoreactivity for RANKL throughout the lesions (Figure 1I). Syndecan-1 was not detected in either control or IPAH sections (data not shown). To characterize the cellular composition of the lesions in relation to the expression of these proteins, serial sections were stained for cell-specific markers. CD31 was used to identify endothelial cells (Figure 1, J–L), smooth muscle actin for smooth muscle cells (Figure 1, M–O), and CD68 to detect monocyte/macrophage infiltration (Figure 1, P–R). Staining of control donor lung sections demonstrates a typical thin-walled artery comprising smooth muscle cells, intact endothelium, and small numbers of CD68⁺ monocytes/macrophages (Figure 1, J, M, and P). In contrast, the concentric lesions show characteristic medial thickening due to increased SMCs (Figure 1N) and the consequent reduced lumen with an intact endothelium (Figure 1K); a slight increase in CD68⁺ staining was observed around the artery (Figure 1Q). The medium was shown to consist of primarily SMCs, indicating that the medial expression of OPG was by SMCs. The expression of RANKL and TRAIL appeared to be associated with both endothelium and SMCs of concentric lesions. The plexiform lesions show an increased CD31 staining (Figure 1L) and disorganized areas of smooth muscle actin⁺ cells (Figure 1O) within the areas bordered by CD31 staining. A few CD68⁺ cells were also observed, mainly in the interstitial regions of the lung (Figure 1R). The localization of OPG, TRAIL, and RANKL appears similar to that within concentric lesions.

Figure 2. OPG expression is increased in IPAH patient serum. Serum was collected from IPAH and control patients and analyzed for OPG concentration by ELISA. Each spot represents an individual patient, and the horizontal line marks the mean, $n = 33$ controls and 38 IPH samples.

OPG Expression Is Increased in IPAH Patient Serum

Given that OPG is a secreted molecule and that increased immunoreactivity was observed within IPAH lesions, we next determined OPG levels in patient sera. OPG levels in sera from IPAH patients was significantly elevated by more than threefold (1530 ± 191.3 pg/ml, $n = 33$ control patients versus 4710 ± 369.2 pg/ml, $n = 38$ IPAH patients; $P < 0.0001$) compared with unaffected control subjects (Figure 2). A summary of the patient demographics, including World Health Organization functional class, BMP-R2 mutation status, and treatment, is shown in Table 1. A breakdown of each individual IPAH patient OPG levels, treatment, and hemodynamic profile is shown in Table 2. No correlation was found between OPG concentration and World Health Organization class or hemodynamic measure-

Table 1. Total Population Demographics, WHO Class, BMP-R2 Status, and Treatments

	Controls [<i>n</i> (%)]	IPAH patients [<i>n</i> (%)]
<i>n</i>	33	38
Age at sample collection (years $\pm$ SD)	57.6 $\pm$ 15.9	45.3 $\pm$ 14.5
Sex		
Male	20 (60.6)	10 (26.3)
Female	13 (39.4)	28 (73.7)
WHO functional class		0 (0)
I		13 (34.2)
II		21 (55.3)
III		4 (10.5)
IV		
BMP-R2 mutation status		
Positive		7 (18.4)
Negative		13 (34.2)
Unknown		18 (47.4)
Targeted treatment		
None		8 (21.1)
Bosentan		9 (23.7)
Epoprostenol		7 (18.4)
Iloprost (intravenous)		2 (5.3)
Iloprost (nebulized)		2 (5.3)
Sildenafil		1 (2.6)
Combined and miscellaneous		8 (21.1)
Osteoprotegerin (pg/ml) (mean $\pm$ SEM)	1530 $\pm$ 191.3	4710 $\pm$ 369.2

WHO, World Health Organization.

Table 2. Individual IPAH Patient Demographic Profile and OPG Expression

ID	BMP-R2 mutation	Sex	Disease duration*	WHO [†]	Rx [‡]	RAP	mPAP	CO	CI	TPR	OPG
29	—	F	6.6	II	None	N/A	N/A	N/A	N/A	N/A	6043.0
35	—	M	3.5	III	Neb I	10	55	4.9	2	11.2	6191.7
39	—	F	6.7	IV	E	N/A	N/A	N/A	N/A	N/A	7058.4
43	—	F	5.5	IV	E	21	43	1.91	1.31	22.5	8240.6
44	—	F	0.2	III	None	6	37	5.44	2.93	6.8	2351.3
45	—	F	0.2	III	E	11	46	3.2	1.9	14.4	8197.1
46	—	F	1.4	III	E	7	45	2.9	1.7	15.5	7399.3
51	—	F	0.0	II	None	4	35	6.3	3.1	5.6	3769.2
62	—	M	0.9	II	B and Neb I	8	69	3.7	1.8	18.6	4577.9
84	—	M	1.4	II	B	18	51	2.7	1.3	18.9	4505.4
104	—	F	0.4	III	None	6	41	2.8	1.6	14.6	6282.3
114	—	F	0.3	III	E	6	30	1.9	1.3	15.8	5125.5
115	—	F	0.1	III	IV I	11	55	2.4	1.3	22.9	2993.2
20	+	F	6.5	III	B and E	12	48	4.09	2.26	11.7	4904.3
60	+	F	1.2	II	E	5	66	3.2	2.2	20.6	6518.0
94	+	M	0.0	III	E	11	70	3.3	1.9	21.2	4157.2
112	+	M	0.3	II	None	4	71	3.9	1.8	18.2	1861.7
140	+	F	0.0	III	B	6	54	3	1.6	18.0	3312.3
153	+	F	0.2	III	B	22	51	4.6	2.2	11.1	4679.4
162	+	M	0.1	IV	IV I	17	54	2	1.1	27.0	6329.5
269	Un	M	1.5	III	B	10	76	4.7	2.3	16.2	2141.0
288	Un	F	0.0	III	None	0	50	3.3	2.3	15.2	2264.3
291	Un	F	0.3	III	None	4	65	3.7	2.4	17.6	2405.7
301	Un	F	0.8	II	Ambisentan	0	54	7	4	7.7	1662.3
302	Un	F	5.8	III	Sildenafil	9	30	5	2.6	6.0	3852.6
304	Un	F	1.0	III	S and E	16	55	1.9	1.2	28.9	8987.6
311	Un	F	1.8	III	B	16	62	3.2	1.7	19.4	2434.7
326	Un	M	0.2	II	B	N/A	N/A	N/A	N/A	N/A	3689.4
327	Un	M	0.8	IV	Neb I	5	60	3.4	1.9	17.6	1600.6
348	Un	M	2.7	II	N/A	9	62	4	2.3	15.5	3703.9
371	Un	M	1.6	III	B and Triamterene	6	58	3.5	1.9	16.6	4033.9
377	Un	F	0.0	III	None	8	53	3.42	2.03	15.5	4777.4
395	Un	F	2.7	II	Remodulin	8	49	2.5	1.6	19.6	8773.7
423	Un	F	0.7	III	S	11	72	3	1.9	24.0	2764.7
428	Un	M	1.2	II	B	N/A	N/A	N/A	N/A	N/A	10470.8
442	Un	F	0.1	III	S and Neb I	18	80	5.07	2	15.8	3852.6
443	Un	F	0.3	II	B	2	70	4.1	2.8	17.1	2724.8
450	Un	F	0.0	II	B	N/A	N/A	N/A	N/A	N/A	4342.2

CI, cardiac index (L/min/m²); CO, cardiac output (L/min); N/A, not available; OPG, circulating serum levels of osteoprotegerin (pg/ml); RAP, right atrial pressure (mmHg); TPR, total pulmonary resistance (mmHg/L/min); Un, unknown.

*Denotes time from first presentation to sample time in years.

[†]World Health Organization (WHO) Functional Class for Pulmonary Arterial Hypertension.

[‡]Treatments for PAH were Epoprostenol (E), intravenous Iloprost (IV I), nebulized Iloprost (Neb I), Bosentan (B), and Sildenafil (S).

ments, although group numbers are small. Further studies on larger patient groups will be required to provide definitive information on these aspects. Further subanalysis also revealed no significant difference in the serum concentration of OPG between those IPAH patients who carried a BMP-R2 mutation compared with those without (4537 ± 619 pg/ml, $n = 7$ BMP-R2 mutation versus 5595 ± 526.6 pg/ml, $n = 13$ BMP-R2 normal; 18 patients were of unknown BMP-R2 status, 4138 ± 615.6 pg/ml).

Recombinant OPG Induces Proliferation and Migration of PA-SMCs

Having shown that OPG is present within IPAH lesions and increased in IPAH serum, we next looked to determine whether the addition of recombinant OPG had any effect on PA-SMC proliferation and migration. Proliferation as measured by fold increase in tritiated thymidine

incorporation increased >2-fold ($P < 0.05$) with 10 ng/ml platelet-derived growth factor (PDGF)-BB as a positive control. Recombinant OPG (1–100 ng/ml) induced proliferation in a dose-dependent manner with significance reached at 50 ng/ml ($P < 0.05$) (Figure 3A). These effects of OPG on tritiated thymidine incorporation were mirrored by a significant 30% increase in cell number after 72 hours. Migration of serum-starved PA-SMCs was similarly increased twofold with 10 ng/ml PDGF-BB. Recombinant OPG (1–100 ng/ml) induced a dose-dependent increase in migration, maximal at 50 ng/ml ($P < 0.01$) (Figure 3B).

Reduced BMP-R2 Expression Is Associated with Increased OPG Secretion

We next investigated whether signaling pathways implicated in PAH pathogenesis regulate OPG expression/secretion in human PA-SMCs *in vitro*, beginning with the BMP-R2 pathway. In an effort to mimic the loss of function

Figure 3. Recombinant OPG induces proliferation and migration of human PA-SMCs. Human PA-SMCs were serum starved for 48 hours before stimulation with recombinant OPG or 10 ng/ml PDGF-BB. **A:** Proliferation was assessed at 36 hours by tritiated thymidine incorporation and expressed as fold increase over unstimulated cells (ctrl). **B:** Migration was measured at 6 hours using a Boyden Chamber assay and normalized relative to unstimulated cells (ctrl). Bars represent mean $\pm$ SEM from four different experiments; ** $P < 0.001$, * $P < 0.05$ compared with 0.1% fetal calf serum-treated control cells (ctrl).

of BMP-R2 caused by the heterozygous mutation,² we used an siRNA oligo targeting BMP-R2. BMP-R2 expression levels were reduced by <80% after transfection with siRNA oligonucleotides targeting BMP-R2 compared with control cells, whereas transfection with a control siRNA oligo, Cyclophilin B, had no significant effect on BMP-R2 expression (Figure 4A). OPG was barely detectable in the culture medium of quiescent, unstimulated, untransfected human PA-SMCs. Transfection with siRNA targeting BMP-R2 increased the concentration of secreted OPG by fourfold ($P < 0.05$) (Figure 4B), whereas transfection with siRNA targeting the control gene Cyclophilin B had no significant effect.

5-HT Increases OPG Secretion in a SERT-Dependent Manner

OPG secretion by quiescent PA-SMCs was increased by 5-HT in a dose-dependent manner with maximal stimulation seen at 1 μ mol/L ($P < 0.05$) (Figure 5A). To determine whether SERT activity was required for the effect of 5-HT on OPG secretion, cells were pre-incubated with 5 μ mol/L fluoxetine or dimethyl sulfoxide vehicle.²³ Treatment with dimethyl sulfoxide alone had no effect on 5-HT-induced OPG secretion, whereas pre-incubation with fluoxetine significantly reduced OPG secretion after treatment with 1 μ mol/L 5-HT (Figure 5B).

Figure 4. Reduced BMP-R2 expression increased OPG secretion. Human PA-SMCs were either mock transfected (Ctrl) or transfected with 100 nmol/L Cyclophilin B siRNA (CycloB si) or BMP-R2 siRNA (BMP-R2 si) oligos. **A:** RNA was collected 72 hours after transfection, assayed for BMP-R2 gene expression by TaqMan PCR, and normalized to expression of β -2-microglobulin (B2M). BMP-R2 siRNA significantly reduced BMP-R2 gene expression 80% compared with mock-transfected cells. **B:** Conditioned media was collected 72 hours after treatment, assayed for OPG via ELISA, and normalized for effects on cell number by Coulter Counting. Bars represent mean $\pm$ SEM from four replicate experiments. * $P < 0.05$ versus nontreated control cells.

Interleukin-1 Stimulates OPG Secretion

Because increased levels of inflammatory cytokines have been reported in PAH patients,⁵ we next looked to see whether treatment of PA-SMCs with IL-1 would induce OPG secretion. Treatment with recombinant human IL-1 α (Figure 6A) or IL-1 β (Figure 6B) significantly increased OPG secretion ($P < 0.05$) in a dose-dependent manner.

Discussion

The reason why only a minority of individuals with specific BMP-R2 genotypes or who have chronic conditions that predispose to PAH actually develop the disease remains unclear. This has resulted in a significant research effort to dissect the underlying mechanisms of disease and identify potential new therapeutic avenues. By analogy with cancer pathogenesis, it is not unreasonable to speculate that multiple hits in several biological pathways might be required to trigger a final common event, namely dysregulated PA-SMC proliferation and migration. In the present study, we demonstrate for the first time that OPG concentrations are increased in IPAH patient serum and lung sections and that OPG protein localizes in pulmonary vascular lesions. Our hypothesis for a potential role for OPG in PAH pathogenesis is further supported by our studies on PA-SMCs grown in culture. We demonstrate that BMP-R2, 5-HT, and inflammatory signaling pathways, all of which have been implicated in PAH pathogenesis, are important regulators of OPG expression and secretion in PA-SMCs. Moreover, recombinant OPG induces a similar phenotype to the SMC mitogen PDGF in terms of both proliferation and migration of PA-SMCs.

Figure 5. 5-HT stimulation increases OPG secretion in a SERT-dependent manner. **A:** Human PA-SMCs were serum starved for 48 hours before stimulation with increasing concentrations of 5-HT (0.3 to 3 μ mol/L). Conditioned medium was collected 72 hours after treatment, assayed for OPG via ELISA, and normalized for effects on cell number by Coulter Counting. Maximal stimulation was observed at 1 μ mol/L 5-HT. **B:** To determine the effect of SERT, the cells were treated with 5 μ mol/L fluoxetine, a SERT inhibitor, or with dimethyl sulfoxide (vehicle) alone before stimulation with 1 μ mol/L 5-HT. Bars represent mean $\pm$ SEM from four replicate experiments. * P < 0.05 versus nontreated control cells.

These data identify excessive OPG as a potential final common mediator in PAH pathogenesis, triggered by inappropriate and/or dysregulated signaling through 5-HT, BMP, inflammatory, and, potentially, as yet unidentified pathways.

Interestingly, contrary to a previous report describing PDGF-induced OPG expression in human aortic SMCs,²⁴ in our PA-SMCs, PDGF did not induce OPG. Whether this difference reflects a phenotypic difference in the arterial source of cells or merely that Zhang et al²⁴ studied intracellular OPG expression is at present unclear. Altered proliferation responses to transforming growth factor β 1 and the presence of mutations in the transforming growth factor- β receptors have also been described in IPAH.²⁵ Interestingly, we also observed increased secretion of OPG in these cells after stimulations with transforming growth factor- β 1 (data not shown), further linking OPG regulation to the important molecules associated with PAH pathogenesis.

The signaling events leading from IL-1, 5-HT, and loss of BMP-R2 to OPG secretion are unclear. Multiple signaling pathways, including both p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase, have been shown to regulate OPG gene expression in vascular SMCs.²⁴ It is well known that both 5-HT and IL-1 induce mitogen-activated protein kinase signaling in vascular SMCs, and previous studies have also highlighted an increase in mitogen-activated protein kinase signaling associated with the loss of SMAD signaling in BMP-R2 mutant PA-SMCs from IPAH patients.¹⁹ This suggests that these independent ligand/receptor interactions may share common intermediate signaling pathways to induce OPG expression.

Figure 6. IL-1 signaling stimulates OPG secretion. Human PA-SMCs were serum starved for 48 hours before stimulation with IL-1 α (0.1 to 3 ng/ml) (**A**) or IL-1 β (3 to 30 ng/ml) (**B**). Conditioned media were collected 72 hours after treatment, assayed for OPG via ELISA, and normalized for effects on cell number by Coulter Counting. Maximal OPG secretion was observed at 1 ng/ml IL-1 α and 10 ng/ml IL-1 β . Bars represent mean $\pm$ SEM from three replicate experiments. * P < 0.05 versus nontreated control cells.

The data presented here justify further investigation into the role of OPG in PAH pathogenesis and systemic vascular biology. OPG is widely expressed in vascular tissues and has been implicated in the protection of endothelial cells from apoptosis,²⁶ but until now, no effects have been reported on PA-SMC phenotype, or how expression is regulated within these cells. To date, the primary function of OPG was thought to be as a soluble decoy receptor for both RANKL and TRAIL, both of which have been previously reported to be expressed by endothelial and SMCs.^{26,27} Moreover, we have demonstrated that all three molecules are expressed within both concentric and plexiform PAH lesions. The mechanism by which OPG exerts its signaling effect on PA-SMC proliferation and migration is therefore still to be elucidated. Whether OPG mediates these effects through the interaction with membrane-bound or soluble forms of TRAIL or RANKL ligand remains unclear, although the fact that all three molecules are expressed within PAH lesions is intriguing. Another proposed binding partner for OPG is syndecan-1, and data suggest that OPG can mediate the migration of monocytes via interaction with syndecan-1.²⁸ Interestingly, the expression of syndecan-1 has been shown to be increased in a rabbit aortic neointimal model;²⁹ however, we failed to detect expression of syndecan-1 within the pulmonary artery (control or lesions) despite a detectable level of syndecan-1 mRNA

in PA-SMCs in culture (A. Lawrie and M. Southwood, unpublished data).

The effect of TRAIL on vascular homeostasis is a debated topic with conflicting reports on endothelial cell apoptosis (reviewed by Zauli and Secchiero³⁰) and data suggesting that TRAIL may be a survival, mitotic, and migratory factor for SMCs.³¹ Both TRAIL and RANKL, as with all tumor necrosis factor superfamily ligands, are initially expressed in a membrane-bound state and subsequently enzymatically cleaved to release a soluble form.³² Which form of TRAIL, secreted or membrane bound, which receptor these effects are mediated through, and whether OPG levels affect or regulate this response are unknown but currently being investigated.

Recent reports have demonstrated an increase in T-cell RANKL expression during atherosclerosis and propose a link with the plaque destabilization.^{33,34} A previous study demonstrated both RANKL and OPG expression in nondiseased and early atherosclerosis. Interestingly, in calcified areas of advanced atherosclerotic lesions, OPG was expressed within the calcified areas with the RANKL in the areas surrounding them.³⁵ To date, the role of RANKL has not been investigated in PAH.

Interestingly, our hypothesis that heightened OPG expression is a contributing factor in the pathogenesis of PAH does appear to fit with a number of recent studies investigating the effect of statins on PAH. A recent study by Ben-Tal Cohen et al³⁶ demonstrated that addition of Atorvastatin reduced both tumor necrosis factor- α - and IL-1 α -induced OPG expression in both endothelial and smooth muscle cells. This further supports the notion of OPG being regulated by inflammatory mediators and, therefore, a potentially important new therapeutic target in PAH.

In addition, the peroxisome proliferator-activated receptor- γ has previously been shown to regulate OPG expression,³⁷ and peroxisome proliferator-activated receptor- γ is decreased in PAH patients.³⁸ Activation of peroxisome proliferator-activated receptor- γ with rosiglitazone in a mouse model of PAH, in which ApoE^{-/-} mice were fed a high-fat diet, reversed disease.³⁹ By extrapolating these data to those of Fu et al,³⁷ it can be suggested that OPG is likely involved in the pathogenesis of PAH. Whether reduced or loss of peroxisome proliferator-activated receptor- γ expression results in an increased expression of OPG has not been addressed and is an avenue of research that deserves further study.

Given the data reported herein describing the increased secretion of OPG after stimulation of multiple pathways associated with PAH, it is clearly important to investigate further whether OPG overexpression is causal or merely a bystander response in PAH. Additionally, studies on the role of TRAIL and RANKL in PAH are currently underway to investigate whether TRAIL and/or RANKL either on their own or via an interaction with OPG influence or mediate the biological activity and response of this molecule.

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