MYOFIBROBLAST DEPOSITION AND ALTERED PULMONARY VEIN REACTIVITY CONTRIBUTE TO DISEASE PROGRESSION IN A SURGICAL MODEL OF PULMONARY VEIN STENOSIS

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Introduction

Pulmonary vein stenosis (PVS) is a rare pediatric disease that is associated with high mortality (1, 2). Despite aggressive surgical and catheter-based treatment, survival at 1 year is approximately 50% for patients with severe stenosis (3). PVS can first occur as a localized stenosis; however, it typically progresses retrograde ('upstream') into the lung parenchyma (4). Patients with progressive stenosis develop pulmonary hypertension and right heart failure. For end-stage disease, heart-lung or lung transplantation is the only treatment option, but it is limited by availability of donor organs (5, 6). Currently, there is no known medical therapy which slows the progression of pulmonary vein stenosis, and identification of a novel therapeutic adjunct for upstream PVS could have a dramatic impact on survival.

We have previously reported that pulmonary vein banding in piglets replicates the clinical presentation of PVS with pulmonary hypertension, neointimal hyperplasia and extracellular deposition. In this study, we investigated mechanisms contributing to the pathophysiology and progression of PVS in our model. Specifically, we sought to 1) understand the signaling pathways contributing to PVS progression and myofibroblast deposition, 2) characterize the physiological effects on PVS on vascular function and 3) understand how losartan therapy modulates molecular mechanisms leading to PVS progression.

Methods

Pig Model: 1 week old Yorkshire piglets underwent bilateral sequential PV banding (7) and were studied at 3, 5 and 7 weeks post banding (PVS animals). Losartan-treated animals were given losartan (1mg/kg/day) at the time of banding and were studied at 7 weeks post banding.

Histology/Immunofluorescence and Immunohistochemistry: Tissue was harvested, fixed in formalin and stained with H&E and Movats for morphological studies. IHC and IF with antibodies for ki-67, CD31 and alpha-smooth muscle actin were performed on formalin fixed tissues.

Gene Expression: RNA was isolated from frozen pulmonary vein tissue using Trizol reagent (Invitrogen) and cDNA was synthesized using first stand cDNA kit (Qiagen). Real time PCR template detection was carried out with SYBR green (Qiagen) and specific porcine primers for genes of interest. Gene expression was expressed as relative mRNA expression or fold change in expression compared to sham, where appropriate.

Biochemistry: Pulmonary vein tissue was homogenized in RIPA buffer with protease inhibitors. Quantification was carried out using BCA assay (Biorad) and 20ug of protein was boiled in 4x sample buffer, resolved by SDS PAGE and transferred to PVDF. Membranes were blocked and incubated with the appropriate primary antibody overnight, washed and incubated with appropriate HRP conjugated secondary antibody for 2 hours. Blots were visualized with chemiluminescence reagents.
**Primary pulmonary vein endothelial cell (PVEC) culture:** Pulmonary veins were harvested in sterile fashion and incubated with collagenase II (1mg) for 45 mins. Cultures were maintained in DMEM with endothelial growth factor and 10% FBS. PVECs (Passage 2-3) were used in experiments. Cells (1x10^5) were serum starved overnight (0.5% FBS) and then incubated with TGF-β (5ng/ml) or vehicle for 48 hours. Cells were harvested for RNA, biochemistry and immunofluorescence studies.

**Pulmonary myograph studies:** 3-4th order intrapulmonary veins were dissected, mounted on a wire myograph, bathed in Krebs-Henseleit, bubbled with air/6% CO₂, and maintained at 37°C. Vessels were equilibrated for 1 hour and optimal resting tension and KCl challenges were performed to ensure viability. Pulmonary vascular muscle force generation was evaluated by stimulating with thromboxane A₂-mimetic U46619. Relaxation was induced with the endothelium-dependent and -independent agonists Acetylcholine and S-Nitroso-N-acetylpenicillamine (SNAP) respectively, following precontraction with U46619 at concentrations equivalent to 75% of the maximal agonist-induced contraction (EC₇₅). Contractile responses were normalized to the tissue cross-sectional area as follows: (width × diameter) × 2 and expressed as mN/mm².

**Results**

**Surgical model of progressive PVS:** Our model demonstrates progressive pulmonary vein stenosis with increased pulmonary artery pressures and development of neointimal lesions (7). Over the duration of the 7 week protocol, lesions show histological evidence of increased complexity, with fragmentation of the elastic lamina, deposition of cells and extracellular matrix (ECM) in the sub-endothelium (Fig 1A). Lesion progression can be subcategorized into stages: stage 1 lesions have subtle changes in the endothelium with little ECM deposition. Stage 2 lesions have less than 3 cellular layers comprising the sub-endothelial layer with rounded endothelial cells. Stage 3 layers have greater than 3 layers within the neo-intima and have extensive ECM deposition and elastic lamina fragmentation.

**Subendothelial lesions:** Characterization of the hypercellularity within the sub-endothelium revealed myofibroblast-like cells; these cells were positive for alpha-smooth muscle actin (SMA) and negative for CD31 (endothelial marker) (Fig 1B). Compared to sham animals, pulmonary veins from PVS animals also had increased expression of fibronectin (FN), collagen 1A1 (Col1A1) and matrix metalloprotease 2 (MMP2), indicating myofibroblast synthetic function (Fig 2C and D). Together, this data suggests myofibroblasts are the main cell type within PVS lesions of our surgical model, which is in agreement with human PVS specimens [8].

Supporting myofibroblast deposition, we found evidence of alterations in signaling pathways known to contribute to vascular remodeling and fibrosis. Specifically, we found increased expression of TGFβ1, Angiotension II type 1 receptor and various growth factors (Fig 2E).

**Origin of myofibroblasts:** The role of proliferation in PVS neointimal lesions is unknown. In our model, pulmonary veins from PVS animals had increased mRNA expression of ki-67, a marker of proliferation, compared to sham animals. Immunohistochemistry data localized proliferation to the intimal and adventitial layers. These data supports a role for proliferation in the progressive phenotype in our surgical model (Fig 2A and B).

Activation of TGF-β, Angiotensin II and growth factors can stimulate proliferation and can also induce endothelial to mesenchymal transition (EndMT). To understand if EndMT contributes to cells in the subendothelium, we stimulated PVECs with TGF-β, a known inducer of EndMT. We found that EndMT can be induced in PVECs and results in enhanced expression of fibronectin...
and alpha-SMA, down regulation of CD31 and induction of Snail, a key transcription factors for EndMT (Fig 2C). In agreement with these findings, we found increased expression of Snail and Twist in pulmonary veins from PVS animals when compared to shams. The identification of transitional cells in PVS animals which co-express CD31 and SMA supports EndMT as an important process in vascular remodeling in PVS (Figure 2D and E).

**Pulmonary vein reactivity in PVS:** To understand the physiological effects of PVS banding on 3rd-4th order intraparenchymal pulmonary veins, we performed pulmonary vein myograph studies. Pulmonary veins from PVS animals showed increased force generation in response to U46619, a thromboxane A2 agonist (Fmax 0.144±0.02 (PVS) vs. 0.099±0.017(Sham) mN/mm²; p<0.05). In response to SNAP, an endothelial independent vasodilator, PVS animals had a similar response to sham animals; however, acetylcholine induced endothelial-dependant relaxation was impaired in pulmonary veins from PVS animals compared to shams (Fig 3A).

We hypothesized that eNOS uncoupling may lead to impaired endothelial dependant relaxation in our model of PVS. eNOS uncoupling is common process in vascular pathology and results in nitric oxide combining with end products of upregulated NADPH oxidases to induce reactive oxygen damage and vasoconstriction [9]. In pulmonary veins from PVS animals, we found increased mRNA expression of various NADPH oxidases and their regulatory subunits and increased protein carbonylation, a marker of global ROS modification (Fig 3B and C). In addition, increased mRNA expression of eNOS (2.8 fold increase (B3 and B5) and 2.3 fold increase (B7) compared to shams; p<0.05) and downregulation of Snail and Twist expression, suggesting attenuated EndMT (Fig 3D). In addition, Losartan leads to a reduction in NADPH oxidase signaling. The attenuation of key signaling pathways may contribute to the reduction in lesion progression in losartan-treated animals (Fig 3D).

**Conclusions**

Our study provides insights into the pathophysiology and progression of PVS in our surgical porcine model. Myofibroblast deposition in the subendothelium is likely driven by elevated TGF-B and growth factor signaling. Sources of myofibroblasts include EndMT and proliferation of cells in the neointimal and adventitial layers. Progression of PVS into upstream intraparenchymal pulmonary veins likely involves endothelial dysfunction and impaired relaxation that triggers subsequent signaling leading to myofibroblast accumulation.

**Losartan and PVS:** We previously showed that losartan, an angiotensin II receptor antagonist, ameliorated neointimal lesions in our model of PVS (10); however, the molecular mechanisms of losartan remain unexplored. Losartan-treated animals have attenuated expression of angiotensin II type I receptor, TGF-β, endothelin-1 and FGF2 (Fig 3D and down-regulation of Snail and Twist expression, suggesting attenuated EndMT (Fig 3D). In addition, Losartan leads to a reduction in NADPH oxidase signaling. The attenuation of key signaling pathways may contribute to the reduction in lesion progression in losartan-treated animals (Fig 3D).

Continuing research investigations are focused on pathway interrogation to address cell autonomous and non-cell autonomous signaling in the different cellular populations and discovery of complementary therapeutic agents aimed at regression of neo-intimal lesions in established PVS. Currently, there is no known therapeutic agent to slow the progression of PVS in pediatric patients. In efforts to translate our findings, we have initiated a multi-centre safety and feasibility pilot trial of losartan in pediatric patients with PVS.
Figure 1. Progressive pulmonary vein stenosis in a porcine model. 1A) Movats staining of intraparenchymal pulmonary veins demonstrating increased size of neointimal lesions with time. Lesions have different stages (1-3) with mature stage 3 lesions having multiple layers of lesional cells with ECM deposition and fragmented elastic lamina. B3, 3 weeks post banding; B5, 5 weeks post banding; B7, 7 weeks post banding. 1B-D) Neointimal lesions are comprised of myofibroblasts. 1B) Neointimal cells stain positive for alpha-smooth muscle actin (FITC, green) and are negative for CD31 (TRITC, red) demonstrating myofibroblast deposition. 1C) The pulmonary veins from banded animals have increased expression of fibronectin (FN) 1D) The pulmonary veins from banded animals have increased mRNA expression of collagen 1A1 (Col1A1) and matrix metalloprotease 2 (MMP2) indicating myofibroblast synthetic function (sham expression set to 1). 1E-F) Altered signaling in progressive pulmonary vein stenosis. 1E) Increased expression of receptors for angiotensin II (ATR1), endothelin-1 (ETR1), platelet derived growth factor (PDGFRB), fibroblast growth factor (FGFR2) and decreased expression of bone morphogenic protein receptor (BMPR1A) were found at 3, 5 and 7 weeks post banding in PVS animals compared to sham animals (sham expression set to 1). 1F) Elevated expression of ligands TGF-B, endothelin-1(ET-1), and fibroblast growth factor 2 (FGF2) and decreased expression of BMP4 were found at 3, 5 and 7 weeks post banding in PVS animals compared to sham animals (sham
qPCR data was normalized to RPL13A and expressed as fold increase from sham expression of 1. * p≤0.05 compared to sham

**Figure 2.** Proliferation and endothelial to mesenchymal transition (EndMT) play a role in neointima formation in PVS. **2A-B)** Proliferation of endothelial and adventitial cells contribute to the neointima lesions. **2A)** PVS animals have increased expression of proliferation marker Ki-67 at 5 and 7 weeks post banding compared to sham animals (sham expression set to 1). **2B)** Representative Immunohistochemistry staining of pulmonary veins demonstrating enhanced Ki-67 staining in banded animals compared to sham. Staining is most prominent in the endothelium and adventitia. **2C)** Pulmonary vein endothelial cells (PVECs) undergo EndMT with stimulation of TGF-B for 48 hours. PVECs have increased expression of smooth muscle actin (SMA), fibronectin (FN) and MMP2 and downregulation of CD31. PVECs have increased expression of Snail, a key transcription factor in EndMT. **2D-E)** PVS animals demonstrate evidence of EndMT. **2D)** Co expression of CD31 (TRITC, red) and alpha SMA (FITC, green) in endothelial cells from PVS animals (B5) supports EndMT. **2E)** PVS animals have increased expression of Snail and Twist, 2 key
transcription factors in EndMT. qPCR data was normalized to RPL13A and expressed as fold increase from sham expression of 1. * p≤0.05 compared to sham

Figure 3. 3A-C) Impaired endothelial dependant relaxation in intraparenchymal pulmonary veins. 3A) Endothelial dependant relaxation is impaired in PVS animals compared shams. After preconstriction of pulmonary veins with U46619, acetylcholine was unable to fully relax PVS pulmonary veins compared to shams pulmonary veins. 3B-C) Elevated expression of NADPH oxidases and the regulatory subunits supports eNOS uncoupling. 3B) At various timepoints, there was increased mRNA expression of Nox2, Nox4 and p22 phox, and p47phox in PVS animals compared to shams (sham expression set to 1). 3C) Increased protein carbonylation as measured by oxyblot supports vascular reactive oxygen damage and eNOS uncoupling. 3D) Losartan therapy slows progression of pulmonary vein stenosis. Losartan treated PVS animals have attenuated expression of TGF-B, ATR1, and FGF2 compared to 5 and 7 week PVS animals (sham expression set to 1). Losartan-treated PVS animals had down-regulation of Twist and Snail compared to 5 and 7 week PVS animals (sham expression set to 1), supporting decreased EndMT
with losartan therapy. Losartan-treated PVS animals had down-regulation of Nox2, p22phox and p47phox compared to 5 and 7 week PVS animals (sham expression set to 1). qPCR data was normalized to RPL13A and expressed as fold increase from sham expression of 1. * p≤0.05 compared to sham; # p≤0.05 compared to Banded (B5, B7).

References:


