Losartan ameliorates "upstream" pulmonary vein vasculopathy in a piglet model of pulmonary vein stenosis

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Objectives: Pulmonary vein stenosis (PVS) is a relentless disease with a poor prognosis. Although surgical repair can effectively treat ''downstream'' (near left atrial junction) PVS, residual ''upstream'' (deep in lung parenchyma) PVS commonly dictates long-term survival. Our initial studies revealed an association between PVS and transforming growth factor-β signaling, which led us to investigate the effect of losartan on upstream pulmonary vein vasculopathy in a piglet model of PVS.

Methods: Neonatal Yorkshire piglets underwent sham surgical banding (sham, n = 6), staged bilateral pulmonary vein banding of all pulmonary veins except the right middle pulmonary vein (banded, n = 6), and staged pulmonary vein banding with losartan treatment (losartan, 1 mg/kg/d, n = 7). After 7 weeks, the hemodynamic data were obtained and the piglets killed.

Results: Pulmonary vein banding (compared with sham) was associated with continuous turbulent flow in banded pulmonary veins, pulmonary hypertension (pulmonary artery/systemic blood pressure ratio 0.51 $\pm$ 0.06 vs 0.23 $\pm$ 0.02, $P < .001$), and diffuse pulmonary vein intimal hyperplasia in the upstream pulmonary veins ($P < .001$). Losartan administration decreased the pulmonary artery/systemic blood pressure ratios compared with those in the banded piglets (0.36 $\pm$ 0.08 vs 0.51 $\pm$ 0.06, $P = .007$) but it remained greater than those in the sham group ($P = .001$). Losartan was also associated with diminished pulmonary vein intimal hyperplasia compared with that in the banded piglets ($P < .001$) but still remained more than that in the sham group ($P = .035$). Pulmonary vein banding reduced vascular endothelial-cadherin expression, indicative of diminished endothelial integrity, which was restored with losartan.

Conclusions: Losartan treatment improved PVS-associated pulmonary hypertension and intimal hyperplasia and might be a beneficial prophylactic therapy for patients at high risk of developing PVS after pulmonary vein surgery. (J Thorac Cardiovasc Surg 2014;148:2550-8)

See related commentary on page 2559.

Pulmonary vein stenosis (PVS) occurs in $\leq$18% of patients after repair of total anomalous pulmonary vein drainage, despite improved surgical techniques and perioperative management.[1-4] PVS can also occur congenitally, with or without associated cardiac defects, and occasionally after nonpulmonary vein surgery.[2,5-8] Surgical repair of PVS is often feasible when the stenosis is limited to the pulmonary vein–left atrial junction, but it has been associated with an increased risk of adverse composite outcomes (death, reoperation, or recurrent PVS), which can be as great as 30% to 45%.[1,2,4] The current surgical techniques to repair PVS are limited, because they cannot directly address the upstream (deep in lung parenchyma) spread of pulmonary venous disease, an important mediator of long-term survival. With diffuse disease (eg, severe involvement of all 4 pulmonary veins), the 1-year mortality rate is 80%.[2,3] As a last resort, palliative stenting of the pulmonary veins can be attempted, with a 1-year survival rate of only 62% and an incidence of in-stent stenosis of 63%.[9] Chemotherapy protocols have been suggested to treat diffuse PVS but have had limited success.[10] Thus, currently, no therapy for upstream PVS is available.

We previously reported a piglet model of PVS associated with progressive diffuse obstructive intimal hyperplasia in the upstream pulmonary veins that recapitulates the clinical PVS pathogenesis.[11] Specimens upstream from a banded

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This study was supported by Canadian Institutes of Health Research (CIHR # 312549) and the Saving Tiny Hearts Society.

Disclosures: Authors have nothing to disclose with regard to commercial support.

Read at the 94th Annual Meeting of The American Association for Thoracic Surgery, Toronto, Ontario Canada, April 26-30, 2014.

Received for publication April 9, 2014; revisions received June 26, 2014; accepted for publication July 16, 2014; available ahead of print Aug 27, 2014.

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0022-5223/$36.00

http://dx.doi.org/10.1016/j.jtcvs.2014.07.050

Abbreviations and Acronyms

AT1 receptor = angiotensin II receptor, type 1
PAP = pulmonary artery pressure
PVS = pulmonary vein stenosis
RV = right ventricular
SMA = smooth muscle actin
TGF-β = transforming growth factor-β
VE-cadherin = vascular endothelial cadherin
vWF = von Willebrand factor

pulmonary vein in the piglet model and human specimens from patients with PVS were associated with robust expression of transforming growth factor-β (TGF-β).[11]

Losartan selectively blocks angiotensin II receptor, type 1 (AT1 receptor) and inhibits multiple avenues of TGF-β action both directly and indirectly.[12] Losartan inhibits TGF-β–mediated activation of extracellular signal-regulated kinase[12] and Smad2 phosphorylation, both critical to the canonical signaling pathway of TGF-β. Losartan also decreases tissue expression of TGF-β responsive genes and protein levels of connective tissue growth factor and collagen IV.[12,13] Losartan restores the aortic architecture and root dimensions in a fibrillin-1–deficient mouse model of Marfan syndrome[12,14] and is clinically available for the prevention of dilated aortopathy associated with Marfan syndrome.[13,15] The relationship between losartan and pulmonary vein pathology, however, has not been reported. We hypothesized that losartan might slow the progression of upstream disease in our piglet model of PVS.

METHODS

The Animal Care Committee at the Hospital for Sick Children approved the present study. Neonatal piglets (3.7 $\pm$ 0.6 kg) were divided into 3 groups: sham surgical banding (sham, n = 6), pulmonary vein banding (banded, n = 6), and banded with losartan treatment (losartan, n = 7).

PVS Model and Hemodynamic Measurement

The pulmonary vein banding procedure was modified from that of our previous study.[11] Each piglet underwent a 2-stage banding procedure involving bilateral thoracotomies. In the stage I procedure, the left upper pulmonary vein and the lower common pulmonary vein were banded through the left fifth intercostal space. After a 1-week recovery period, the stage II procedure included banding of the right upper pulmonary vein through the fourth intercostal space, and the right middle pulmonary vein was left unbanded. The unbanded right middle pulmonary vein (which drains a relatively small portion of the right lung) provided an intra-animal control for the banded pulmonary veins and improved survivability in the model. Cotton umbilical tapes (0.125-in. width, and length equivalent to 1.3 times the pulmonary vein circumference) were used to band the pulmonary veins. The sham group underwent identical procedures, including encirclement of the pulmonary veins, with the exception that the bands were not left in place. In the losartan group, 1 mg/kg/d losartan (Cozaar; Merck, Whitehouse Station, NJ) was administered orally from the second postoperative day after the stage I procedure to the day before tissue harvest.

At 7 weeks after the stage II procedure, the pigs underwent a hemodynamic assessment and tissue harvest. The body surface area was calculated using the following formula[16]: body surface area (m^2) = $0.0734 \times (\text{body weight})^{0.656}$. The heart rate, invasive arterial blood pressure, and saturation were monitored. Echocardiography was performed. A 5F Swan-Ganz catheter (Teleflex, Limerick, Pa) was inserted from the right internal jugular vein to measure the central venous pressure, right ventricular (RV) pressure, pulmonary artery pressure (PAP), and pulmonary capillary wedge pressure. Cardiac output was measured by thermodilution (model 9520A, Edwards Lifesciences, Irvine, Calif). After sternotomy, the RV pressure, left ventricular pressure, and left atrial pressure were obtained by direct needle measurement. The heart and lungs were removed en bloc quickly after exsanguination.

The pulmonary veins were dissected as distally as possible from the pulmonary vein–left atrial junction and divided into 3 equivalent segments if long enough. Otherwise, the pulmonary veins were divided into 2 segments. The proximal segment was closest to the site of pulmonary vein banding (1-3 cm from the site of banding) and was termed "downstream." The adjacent "upstream" segments originated >3 cm from the site of banding and extended $\leq$8 cm into the lung. The heart was then harvested, and the ventricle was split into 2 parts: the RV free wall and the left ventricle.

Histologic Examination

Paraffin-embedded pulmonary vein tissues were sectioned and stained with hematoxylin and eosin and trichrome staining. Movat pentachrome staining was performed to quantify intimal hyperplasia in every segment. ImageJ (National Institutes of Health, Bethesda, Md) was used for the quantification. The intimal area was measured between the endothelial layer and lamina interna, and the media area was quantified from the lamina interna to the outside border of the smooth muscle cells. The circumference of the internal lamina was used to calculate the radius of this vessel.

The extent of intimal hyperplasia is reported using the ratio of the intima to media area. However, this ratio can lead to inaccurate interpretation in specimens with irregular hypertrophy contours. A complimentary method was used as a second index to quantify intimal hyperplasia that normalized the measured intimal area to the square of the calculated radius, representing the ratio of the intimal area to an idealized vessel internal luminal area. A third technique measured the media area and normalized this measurement to the square of the calculated radius (media area/R^2) to evaluate the extent of media hypertrophy. All measurements were performed by an observer who was unaware of the specimen grouping.

Immunofluorescence

Paraffin-embedded pulmonary vein slides were deparaffinized in xylene and rehydrated in ethanol. Antigens were re-exposed with sodium citrate buffer (Dako, Glostrup, Denmark), immersed in blocking buffer for 40 minutes, incubated with primary antibodies for 2 hours, washed in phosphate-buffered saline solution, and labeled with secondary fluorophore-labeled antibodies. The nucleus was stained with 46′-diamidino-2-phenylindole-2 HCl (1:1000).

The endothelial markers evaluated included CD31 (1:100; Abcam, Cambridge, Mass), vascular endothelial cadherin (VE-cadherin, 1:50; Thermo Fisher Scientific, Waltham, Mass), and von Willebrand factor (vWF, 1:100; Dako Canada Inc, Burlington, Ontario, Canada). The mesenchymal markers evaluated included fibronectin (1:100; BD Transduction Laboratories, Franklin Lakes, NJ) and α-smooth muscle actin (α-SMA, 1:50; Santa Cruz Biotechnology, Santa Cruz, Calif).

Protein Extraction and Western Blotting

Left upper pulmonary vein samples were homogenized in lysis buffer and centrifuged (20 minutes, 13,400*g*, 4°C), and supernatants were collected. The protein concentrations were quantified using a Bradford protein assay (Bio-Rad, Hercules, Calif). The samples were separated on midi-Protein TGX Precast Gels (Bio-Rad), transferred to nitrocellulose membranes, and blocked with 5% skim milk or 5% bovine serum albumin for 1 hour. The membranes were probed with antibodies of interest, and the blots were then incubated with goat anti-mouse IgG horseradish peroxidase