Superior cavopulmonary anastomosis suppresses the activity and expression of pulmonary angiotensin-converting enzyme

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Background: Superior cavopulmonary anastomosis is widely used for palliation of various forms of univentricular heart defects. However, clinically significant pulmonary arteriovenous malformations develop in 15% to 25% of patients after surgery.

Objective: To assess altered regulation of pulmonary vascular tone caused by superior cavopulmonary anastomosis in an ovine model.

Methods: Lambs, aged 35 to 45 days, underwent an end-to-end anastomosis of the superior vena cava to the right pulmonary artery. In age-matched controls, a sham operation was performed. Arteriovenous malformations were detectable by contrast echocardiography by 8 weeks after surgery. Animals (n = 24) were studied at various time points after the operations. Expression of angiotensin-converting enzyme messenger RNA, protein levels, and enzyme activity were measured in lung homogenates. Levels of angiotensin II were measured by enzyme-linked immunosorbent assay.

Results: Expression of angiotensin-converting enzyme messenger RNA and protein was significantly reduced at 1 to 5 weeks after superior cavopulmonary anastomosis. Angiotensin-converting enzyme activity in the right lung of animals subjected to superior cavopulmonary anastomosis was reduced 86% ± 1% (standard deviation) compared with control values at 1 week (P = .003) and 77% ± 8.5% at 2 weeks (P < .001) after surgery. This correlated with a 59% ± 3.5% (P = .007) reduction in angiotensin II levels up to 5 weeks after cavopulmonary anastomosis. By 15 weeks after the operations, angiotensin II levels were equivalent to control levels (P = .19).

Conclusions: Superior cavopulmonary anastomosis causes an early reversible reduction in activity and expression of angiotensin-converting enzyme, resulting in decreased circulating levels of the vasoconstrictor angiotensin II. These results suggest that the ability of the pulmonary endothelium to regulate vascular tone is inhibited after superior cavopulmonary anastomosis. Dilation of the affected vasculature induced by cavopulmonary anastomosis may contribute to the disordered vascular remodeling observed in this setting.
A second group of lambs of the same age served as sham-operated controls. The preparation and dissection were the same as described for the SCPA cohort. The right pulmonary artery was occluded with a vascular clamp for 30 minutes. After this time, the clamp was removed and the chest was closed as described above.

All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society of Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health. The protocol was approved by the Committee on Animal Research at the University of California, San Francisco.

**Echocardiography**

Pulmonary AV shunting was demonstrated by bubble contrast echocardiography. Epicardial echocardiography was performed on each animal after median sternotomy. Next, 5 mL of a saline solution/blood mixture was agitated to produce bubbles and then injected sequentially into the superior and inferior venae cavae. The presence of bubbles in the left atrium within 5 cardiac cycles after injection of the superior vena cava indicates pulmonary AV shunting. The absence of bubbles in the right atrium after injection of the superior vena cava excludes systemic venovenous shunting. The absence of bubbles returning to the left atrium after inferior vena caval injection excludes pulmonary AV shunting in the left lung.

**Tissue Harvest**

Lambs were put to death 1, 2, 5, and 15 weeks after surgery to obtain tissue for analysis. At each time point, 3 SCPA animals and 3 sham controls were put to death for a total of 24 sheep studied. Animals were intubated and their lungs were mechanically ventilated. After median sternotomy, the lambs were anticoagulated with sodium heparin (300 U/kg). The superior and inferior venae cavae and the right pulmonary artery and vein were identified and occluded with a vascular clamp for 30 minutes. After this time, the clamp was removed and the chest was closed as described for the SCPA cohort. The right pulmonary artery was divided near the pulmonary bifurcation and the proximal end was oversewn. The superior vena cava was similarly divided at the cavoatrial junction and the atrial end was oversewn. The superior vena cava was then anastomosed to the right pulmonary artery in an end-to-end fashion with running polypropylene suture. The venous canulas were removed and the sternum closed. After skin closure, the lambs were extubated and allowed to recover.

**Methods**

**Surgical Preparation**

Mixed Western lambs (35-45 days old) were anesthetized and their lungs were mechanically ventilated. After median sternotomy and pericardiotomy, the superior vena cava and right pulmonary artery were identified and dissected free from their attachments (Figure 1). Intravenous heparin was administered (300 U/kg) and 16F to 20F venous canulas were placed to bypass the superior vena cava to the right atrium. The right pulmonary artery was divided near the pulmonary bifurcation and the proximal end was oversewn. The superior vena cava was similarly divided at the cavoatrial junction and the atrial end was oversewn. The superior vena cava was then anastomosed to the right pulmonary artery in an end-to-end fashion with running polypropylene suture. The venous canulas were removed and the sternum closed. After skin closure, the lambs were extubated and allowed to recover.

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**Reverse Transcription–Polymerase Chain Reaction**

Total RNA was prepared from homogenates of right lower lobe lung tissue from control and SCPA lambs studied at selected time points after the operations. RNA was extracted with the use of the TRI reagent RNA isolation protocol (Molecular Research Center, Inc, Cincinnati, Ohio). Samples were incubated with oligo (dT) and reverse transcriptase (Superscript Kit; Gibco BRL, Life Technologies, Inc, Rockville, Md) at 42°C for 50 minutes. The resulting complementary DNA underwent polymerase chain reaction (PCR) with the use of the following primers: 5’ AGGCTGATGATTTTCTTCACCTCC 3’ and 5’ GCCGACACGAGGGCTAG 3’. PCR was initiated with 3 minutes of denaturation at 95°C followed by 25 cycles of 30 seconds of denaturation at 95°C, 30 seconds of annealing at 55°C, and 2 minutes of extension at 72°C, followed by 10 additional minutes of the extension step. All reactions were carried out on a Peltier Thermal Cycler (MJ Research, Waltham, Mass). PCR products separated on...
a 2% agarose gel. The PCR products were identified by being sub-cloned with the TOPO TA Cloning kit (Invitrogen Corporation, Carlsbad, Calif) and sequenced. Densitometric analysis was used to quantify the PCR signal (1D Image Analysis Software; Eastman Kodak Company, Rochester, NY).

**Western Blot Analysis**

Homogenates from right lower lobe lung tissue of SCPA and control specimens were obtained as described above. Equal aliquots of solubilized protein were then separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (4%-12% gradient precast gel; Bio-Rad Laboratories, Hercules, Calif). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Corp, Arlington Heights, Ill). The membrane was soaked in Tris-buffered saline solution (10 mmol Tris-HCl, 250 mmol NaCl) containing 5% nonfat powdered milk and 0.1% Tween 20 to block nonspecific binding. The membrane was then incubated overnight at 4°C with a 1:1000 dilution of an ACE monoclonal antibody (clone 9B9; Kamiya Biomedical Company, Seattle, Wash). Blots were washed and incubated for 30 minutes at 4°C with a peroxidase-conjugated anti-mouse secondary antibody (1:10000 dilution; Amersham). Immunoreactivity was visualized with the SuperSignal Chemiluminescent Substrate kit (Pierce Chemical Company, Rockford, Ill). Quantitative assessment of band densities was performed by scanning densitometry as above.

**ACE Activity**

Segments of right lower lobe lung tissue of equal weights (0.25 g) were placed in 1 mL of HEPES buffer, 0.05 mol/L, and homogenized with a Tissuemizer T-25 homogenizer (Tekmar-Dohrmann, Mason, Ohio). Homogenates were centrifuged at 1000 g for 10 minutes and the supernatants at 30,000 g for 30 minutes. The resulting pellet was resuspended in 500 µL 0.05 mol/L HEPES buffer, pH 8.0, in 0.1 mol/L NaCl, and 0.6 mol/L Na₂SO₄. The homogenate was then diluted 1:20 for assay. ACE activity was determined as previously described by Ryan and associates. In brief, ACE activity was measured by the generation of 3H-hippuric acid from the cleavage of hippurlyglycylglyline, an ACE substrate. Homogenates were incubated with 16 mmol/L (0.125 mCi/mmol) 3H-labeled hippurlyglycylglyline for 1 hour at 37°C. The reaction was terminated with 1 mL of 0.1 mmol/L HCl. Then, 1 mL of ethyl acetate was added to extract the radiolabeled fragment cleaved by ACE. Samples were centrifuged at 2000 g for 20 minutes to separate the organic and aqueous phases. Next, 0.5 mL of the upper organic layer was placed in a scintillation vial and counts per minute were measured on a scintillation counter. All reactions were performed in duplicate. ACE activity was normalized for protein content determined in each homogenate with a Bio-Rad protein assay kit (Bio-Rad Laboratories).

**AT-II Determinations**

Blood samples were drawn from the right pulmonary vein in Glenn and control animals when they were put to death (1-15 weeks). Samples were centrifuged at 4000 g at 4°C for 20 minutes. Plasma was removed and stored at –80°C for future analysis. Concentration and purification of AT-II was accomplished by elution over Sep-Pak C₁₈ columns (Waters Corporation, Milford, Mass). AT-II levels were determined by enzyme-linked immunosorbent assay with a commercially available kit (Peninsula Labs, Belmont, Calif).

**Statistical Analysis**

Data were analyzed with StatView software, a commercially available software package (SAS Institute, Inc, Cary, NC). The 4 time points (1, 2, 5, and 15 weeks) were evaluated with 3 animals each in...
the experimental and sham controls for a total of 12 in each cohort. Analysis of statistical differences between groups at each time point was performed by unpaired, 2-tailed Student t tests. Significant differences between SCPA and control groups were confirmed by analysis of variance. Significance was established at the 95% confidence level. All values are expressed as mean ± standard deviation.

**Results**

**Detection of Pulmonary AV Shunting**
Contrast echocardiography detected AV shunting in all animals evaluated 56 days or later (n = 23) after SCPA. No shunting was detected in any animals studied up to 190 days after sham surgery (n = 17). Atrial or ventricular septal defects were not present in any of the animals.

**ACE Messenger RNA Expression**
ACE mRNA expression was significantly reduced after construction of the Glenn shunt. Reverse transcription PCR demonstrated a marked decrease in ACE mRNA in lung homogenates up to 5 weeks after surgery. Densitometry revealed a 72% ± 7.2% reduction in mRNA signal at 1 week (P = .01), 62% ± 4.7% at 2 weeks (P = .01), and a 58% ± 3.2% decline at 5 weeks after surgery (Figure 2). At 15 weeks, the mRNA signal was reduced only 14% ± 5.2% with a confidence interval < 95% (P = .06).

**ACE Protein Expression**
Cavopulmonary anastomosis caused a similar reduction in ACE protein expression. Densitometric analysis of the Western blots revealed that ACE protein expression was reduced 93% ± 1.0% at 1 week (P < .001), 95.3% ± 1.3% at 2 weeks (P < .001), and 58.8% ± 3% at 5 weeks (P < .001) after SCPA (Figure 3). There was no statistically significant difference in protein expression between the experimental and sham groups at 15 weeks after surgery (P = .76).

**ACE Activity**
A substantial early reduction in pulmonary ACE activity was also observed after cavopulmonary anastomosis. This reduction, however, progressively diminished as a factor of time after surgery (Figure 4). One week after SCPA, ACE activity was reduced 7-fold (P = .003). At 2 weeks, the degree of reduction was 4.2-fold (P < .001) when compared with the sham-operated animals. As time progressed after surgery, this reduction was attenuated. At 5 weeks, pulmonary ACE activity in SCPA animals was diminished 2.2-fold (P = .006). At 15 weeks, there was no statistically significant difference in ACE activity between the 2 groups (P = .20) (Table 1).

**AT-II Levels**
The levels of AT-II present in the right pulmonary vein of the SCPA lambs correlated with the initial, drastic decrease in ACE activity and expression. Although levels of AT-II in control lambs were constant over time, AT-II was reduced 73% ± 1% at 1 week (P < .001), 78% ± 4% at 2 weeks (P = .005), and 59% ± 3.5% at 5 weeks after SCPA (P = .007) (Figure 5). After 15 weeks, no significant differences existed between the 2 groups (P = .19).

**Discussion**
The development of pulmonary AV malformations after cavopulmonary anastomosis is a well-documented but poorly understood phenomenon. We have demonstrated a significant impairment of the pulmonary angiotensin system after SCPA in the lamb. In our model, AV shunting is detectable...
by bubble contrast echocardiography by 6 to 8 weeks after SCPA. It is therefore important to recognize that the most significant reduction in ACE expression, activity, and circulating AT-II occurs before AV malformations are detectable, a period of active pulmonary vascular remodeling.

Chronic pulmonary vasodilation is a hallmark of these abnormal AV structures. A recent histologic analysis of pulmonary AV malformations in children after cavopulmonary anastomosis revealed lakes of dilated vessels, as well as thin-walled vessels branching from precapillary arterioles. Other authors have described the appearance of abnormal dilated vascular structures within the pulmonary interstitium after SCPA. These vascular changes undoubtedly contribute to the intrapulmonary shunting responsible for cyanosis. Interruption in the normal function of ACE may contribute significantly to the chronic dilatation of the affected vasculature.

Furthermore, loss of pulmonary ACE expression and function reflects altered endothelial phenotype. ACE is concentrated on the pulmonary microvascular endothelium and serves as an indicator of endothelial integrity. It is known that ACE mRNA expression in endothelial cells is regulated in an inverse relationship to their proliferative state. Down-regulation of ACE may indicate dedifferentiation of the pulmonary endothelium as a result of accelerated proliferation in this setting of surgically induced vascular remodeling. Various forms of endothelial injury such as shear stress and oxidative damage have also been shown to suppress endothelial ACE activity and expression. The altered physiology after SCPA may be responsible for a similar insult to the endothelium, impairing its ability to express ACE.

Development of AV malformations represents pathologic pulmonary vascular remodeling in response to surgically altered pulmonary blood flow. SCPA creates several relevant modifications to the right pulmonary circulation. First, there is a reduction in the blood volume in the right pulmonary circulation. In sheep, blood return from the superior vena cava is approximately one-third less than that from the inferior vena cava. Moreover, blood flow to the right lung is no longer pulsatile, because it bypasses the right side of the heart. It is unlikely that nonpulsatile flow contributes to the development of AV malformations, evidenced by the low incidence of AV malformations after the Fontan operation. Another series demonstrated pulmonary AV shunting after modified Fontan repairs only when hepatic venous drainage was excluded.

Exclusion of inferior vena caval blood from the right lung is likely the most significant factor in the development of AV malformations after the classic Glenn shunt. It has been postulated that exclusion of hepatic venous effluent interrupts the delivery of a mediator of normal vascular development from the pulmonary circulation. Theoretically, this “hepatic factor” would prevent pathologic vascular remodeling. This assertion is supported by reports of AV malformations resolving after inclusion of hepatic venous blood. As yet, no such entity has been identified. Further studies will be necessary to demonstrate whether the absence of inferior vena caval blood flow is responsible for the inhibition of ACE activity and expression after cavopulmonary anastomosis.

**References**


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Discussion

Dr Guo-Wei He (Hong Kong, China). I have 2 questions. First, why did you choose ACE? So many things are related to pulmonary circulation and endothelial function, such as vasoconstrictors like endothelin-1 and vasodilators like nitric oxide and epoprostenol (prostacyclin).

Second, have you tried to measure nitric oxide release, which probably is a more important indicator for endothelial function after cavopulmonary shunt?

Dr Malhotra. Thank you for those 2 important questions. First, with regard to studying the other constrictors and regulators of vascular tone in the pulmonary system, we actually began by examining levels of various vasoconstrictors and dilators in the pulmonary venous system after the Glenn anastomosis. The only one that we found that was significantly altered was AT-II. That led us to ask whether we should be investigating ACE expression and activity.

Second, we have looked at nitric oxide release. It is interesting because we have demonstrated increased endothelial nitric oxide synthase expression, without a corresponding increase in nitric oxide production, suggesting that endothelial dysfunction may occur in this setting.

Dr He. What about epoprostenol (prostacyclin)?

Dr Malhotra. We have not yet evaluated that.

Dr Edward D. Verrier (Seattle, Wash). I have a little difficulty with the concept that vasodilatation alone has a potential to induce a proliferative process. These AV malformations appear to be an angiogenic type, proliferative process. Do you have any evidence that simply lowering pulmonary vascular resistance actually induces proliferation?

Dr Malhotra. I am sorry if my conclusions were unclear. I was trying to illustrate that ACE downregulation and loss of ACE activity may represent dedifferentiation of the endothelium as a result of proliferation. I did not want to suggest that the 2 processes were related.

Dr Verrier. I am not an expert on this topic, but I was under the impression there was evidence that hepatic angiogenic inhibiting factors were released. Because of the uniqueness of the Glenn anastomosis, these inhibitory factors bypassed the lung with a loss of angiogenic inhibition, thereby leading to angiogenic proliferation and AV malformations. Have you looked at the relationship between that and the lung and the vasodilatory pathways? Is there a relationship that can be correlated?

Dr Malhotra. I believe the evidence is substantial that if hepatic venous effluent is excluded from the pulmonary circulation, there is a propensity for these AV malformations to occur. We are currently examining the effect of different exposure to inferior and superior vena caval blood on endothelial cell phenotype. However, whether AV malformations are induced by a hypoxic insult or by the lack of a protective factor derived from the liver is debatable. As yet, no such “hepatic factor” has been identified.

Dr Ludwig K. von Segesser (Lausanne, Switzerland). The Glenn shunt is in general used for problems related to severe cyanosis. Did you try to mimic some of these scenarios or can you speculate on this?

Dr Malhotra. No. We used healthy lambs and did not use that variable. However, in our model all animals with the SCPA had pulmonary AV malformations by 8 weeks after surgery, so I doubt that cyanosis is relevant to the development of AV malformations.

Dr Hikaru Matsuda. My question concerns the relationship between hepatic blood and the development of AV malformations. If pulmonary blood flow is not obtaining hepatic blood directly, AV malformations are likely to develop. However, in a regular Fontan or a regular Glenn operation, this malformation does not occur as often as in the total cavopulmonary shunt operation, in which the total venous flow, except hepatic venous flow, goes directly to the pulmonary flow.

You mentioned that it is very interesting to look for a possible relationship of the hepatic blood flow in this model. You probably have seen some patients in whom AV malformations developed after the total cavopulmonary shunt operation. Are the histologic changes of such patients similar to those which you have seen in this experimental model?

My second question concerns etiology. Do you think this is just the hemodynamic consequences or is some tumoral effect involved?

Dr Malhotra. We have not performed total cavopulmonary anastomosis on these animals, so I cannot comment on your first question.

Regarding the second question, inhibition of ACE expression at the mRNA level suggests that pulmonary vasodilation after cavopulmonary anastomosis is related to altered cellular physiology due to the exclusion of hepatic venous effluent.