Gene array analysis of a rat model of pulmonary arteriovenous malformations after superior cavopulmonary anastomosis

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Objective: Pulmonary arteriovenous malformations commonly develop in children who have undergone a cavopulmonary anastomosis as part of the palliative sequence for single-ventricle physiology.

Methods: We developed a rat model of cavopulmonary anastomosis that results in pulmonary arteriovenous malformations that are angiographically and histologically similar to the human condition. We used this model to analyze the gene expression profile associated with pulmonary arteriovenous malformations developing after cavopulmonary anastomosis.

Results: Six Sprague–Dawley rats underwent right superior cavopulmonary anastomosis, allowing the left lung to serve as a control. Total RNA was isolated from each lung at death 8 months postoperatively and compared by using the Affymetrix Rat Microarray RAE230 2.0 GeneChip (Affymetrix, Santa Clara, Calif). One hundred thirty-seven genes demonstrated altered expression in the lungs after cavopulmonary anastomosis compared with that seen in the control lungs: 55 (40%) genes demonstrated increased expression, and 82 (60%) genes demonstrated decreased expression. Modulation of genes associated with angiogenesis and vascular remodeling was found, including angiopoietin-2, placental growth factor, several matrix metalloproteases, and several collagen subtypes. Genes with vasoactive properties, including endothelin 1 and endothelin receptor type B, demonstrated altered gene expression. Several members of the transforming growth factor β superfamily signaling pathway also demonstrated altered expression.

Conclusions: These changes in gene expression might have causative implications for pulmonary arteriovenous malformations that develop after cavopulmonary anastomosis.

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Children born with single-ventricle physiology undergo a palliative operative sequence with the goal of creating a partitioned circulation. The Fontan procedure is the ultimate step in the sequence whereby all systemic venous blood is directed to the pulmonary arteries while pulmonary venous blood supplies the systemic arterial circulation. In current practice a superior cavopulmonary anastomosis (CPA), which establishes the superior vena cava as the sole source of pulmonary blood flow, is performed as an interval step before the Fontan procedure. At the time of the Fontan procedure, systemic venous blood from the inferior vena cava is routed to the pulmonary arteries, which completes partitioning of the circulation. This staged palliative approach with an interval CPA has been shown to improve outcomes for these children

Abbreviations and Acronyms

Ang	= angiopoietin
CPA	= cavopulmonary anastomosis
ECM	= extracellular matrix
ET	= endothelin
MMP	= matrix metalloproteinase
NPY	= neuropeptide Y
PAVM	= pulmonary arteriovenous malformation
RT-PCR	= real-time polymerase chain reaction
TGF	= transforming growth factor
TPA	= tissue plasminogen activator

and is currently the standard of care for their management. Pulmonary arteriovenous malformations (PAVMs) have been found to develop in children after CPA; these lesions act as an intrapulmonary right-to-left shunt causing progressive cyanosis. There is evidence that PAVMs develop when the superior vena cava serves as the sole source of pulmonary blood flow because of a process characterized by abnormal angiogenesis and vascular remodeling caused by the absence of hepatic-derived factors directly perfusing the lungs; however, the exact cause of these lesions remains unknown.¹⁻³

We have previously developed an animal model of experimental CPA in rats that results in PAVMs that are angiographically and histologically similar to those occurring in human subjects.^{4,5} The present study applies DNA microarray technology to this animal model to determine the profile of genetic expression present in lung tissue after CPA. The results suggest that modulation of a number of genes associated with angiogenesis, vascular remodeling, and control of vascular tone might contribute to the development of PAVMs after CPA.

Materials and Methods**Rat Surgical Intervention**

Six male Sprague–Dawley rats (200–300 g) underwent a right superior CPA, as previously described.⁵ Briefly, animals were intubated and ventilated with a pressure-controlled ventilator (Kent Scientific, Torrington, Conn) after intraperitoneal pentobarbital-induced anesthesia. A right anterolateral thoracotomy was performed, and the right superior vena cava and right pulmonary artery were anastomosed, allowing the left lung to serve as a control. Animals were killed 8 to 9 months postoperatively, at which time lung samples were immediately snap-frozen in liquid nitrogen. All animals used in this study received humane care in compliance with the "Guide for the care and use of laboratory animals." This study was approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

RNA Isolation

Total RNA was isolated from frozen lung tissue by using the RNeasy Lysis Kit (Qiagen, Crawfordsville, Ind), per the manufacturer's instructions. Briefly, samples were pulverized in a prechilled mortar and then mixed with Lysis/Binding Buffer, passed through a 25-gauge needle and centrifuged. Samples were then combined with 64% eth-

anol, mixed, and drawn through a filter cartridge assembly by means of centrifugation. Filters were washed with Wash Solution #1, centrifuged, and washed twice with Wash Solution #2/3. RNA was eluted in a total of 100 μ L of Elution Solution. RNA concentrations were determined by means of ultraviolet absorbance.

Expression Microarrays

Total RNA was prepared from lung tissue of 6 rats for both the right (CPA) lung and the left (control) lung.⁵ RNA samples were processed for use on the Rat Microarray RAE230 2.0 GeneChip (Affymetrix, Santa Clara, Calif), according to the manufacturer's protocol. Briefly, total RNA was used in a reverse transcription reaction (SuperScript II; Life Technologies, Rockville, Md) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an in vitro transcription reaction to generate larger amplified amounts of biotinylated cRNA. After purification and fragmentation, biotin-labeled cRNA was used to produce a hybridization cocktail containing spiked transcript controls, which was then loaded onto microarrays and hybridized. Standard posthybridization washes and double-staining protocols were performed by using an Affymetrix GeneChip Fluidics Station 450. Arrays were then scanned with the high-resolution Affymetrix GeneChip Scanner 3000. GeneChip hybridization reactions and data collection were done at the Gene Expression Array Core Facility, Case Western Reserve University, Cleveland, Ohio.

Data Analysis

To determine those genes with altered expression in the PAVM lung samples, binary quantified comparisons were generated with GeneChip Operating Software 1.3 (Affymetrix, Santa Clara, CA) to yield signal intensities, detection calls, and comparative signal ratios between CPA and control samples for each probe set. To test the overall quality and reproducibility of those chips analyzed, we monitored a number of quality control indices, which included %P values (the percentage of detection calls designated P [for "present"] on a chip, which should typically be consistent across a sample set $\pm 3\%$), background, scaling factor, and 3'/5' signal intensity ratios for reduced glyceraldehyde-phosphate dehydrogenase and β -actin. Signal log ratios from the comparison data were converted into simple fold-change values. Genes with a fold change of between 2 and -2 were removed from further analysis, whereas those genes with fold changes of ≥ 2 or ≤ -2 were collated and grouped based on frequency of occurrence. Further restrictions were added to the analysis by removing those genes that had an occurrence frequency of less than 60%. These genes were then further grouped into functional classifications based on known or implied biologic and molecular functions by using the Database for Annotation, Visualization, and Integrated Discovery (<http://david.abcc.ncifcrf.gov>).⁶ The complete dataset for gene expression in this animal model of PAVMs after CPA was submitted to the Gene Expression Omnibus of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/geo/>) and is accessible through Gene Expression Omnibus Series accession number GSE6212.

Verification of GeneChip Data by Means of Relative/Real-time Polymerase Chain Reaction

Eleven genes were selected for validation by the relative/real-time polymerase chain reaction (RT-PCR) based on either their large fold change, as determined by means of GeneChip analysis, or their

TABLE 1. RT-PCR verification of genes with altered expression levels identified by means of GeneChip analysis

Sample	Forward	Reverse	RT-PCR FC	GC FC
ET-B	agcggctacgggactctc	ctgggcaggtgggaatc	−1.79	−2.1
Efnb1	attactacattacatcaacatcca	tgcccaaccttcataacaa	−1.95	−2.1
Gapj4	tgtgtcagccgggagataaag	ccatggggaggtagaagaaac	−1.48	−2.4
Gli1pr	gggcaattgtgacgggtggaact	ccgagaggaaggctgatgtagggtg	−2.09	−26.0
Plat	Gccctggtgtctatgtct	Gggtgccacggtaagtca	−1.93	−2.3
Ang-2	ccgatcttctccagcccctaca	Ccagcccacctctaccttcac	5.31	4.3
Col18a1	accaccaccaccaccaccagatt	ggaagtcagccccacggataccac	1.80	3.9
Esm1	ttcggatggattgcaaggagact	Ttggctgctgcatctggaagaa	10.33	4.0
MMP-2	gtggccaactacaactcttcc	Gtgacgtcgtccatactttta	1.47	2.3
Myh6	cgggaaggggcaaggctact	Tggctcatgcaggaaggctacg	3.62	422.8
Tnnt2	tgcgaacaggatcaacgacaacc	Attcacagggaaggacacagg	1.83	49.1

Relative expression was calculated by using the $2^{-\Delta\Delta CT}$ method.⁷ Negative fold changes were calculated as follows: $1/(2^{-\Delta\Delta CT})$. *RT-PCR FC*, Fold change determined by means of RT-PCR; *GC FC*, fold change determined by means of GeneChip analysis; *ET-B*, endothelin receptor type B; *Efnb1*, ephrin B1; *Gja4*, gap junction membrane channel protein $\alpha 4$; *Gli1pr*, glucagon-like peptide 1 receptor; *Plat*, tissue plasminogen activator; *Ang-2*, angiopoietin 2; *Col18a1*, collagen, type XVIII, $\alpha 1$; *Esm1*, endothelial cell-specific molecule 1; *MMP-2*, matrix metalloproteinase 2 (Gelatinase A); *Myh6*, myosin heavy chain, polypeptide 6; *Tnnt2*, troponin 2.

suspected involvement in angiogenesis and vascular biology (see below). Total lung RNA, isolated as previously described, was reverse transcribed in a volume of 10 μ L by using TaqMan Reverse Transcription Reagent (Applied Biosystems, Foster City, Calif). cDNA was diluted to a concentration of 2 ng/ μ L. Primers were designed based on mRNA sequences obtained from GenBank by using Lasergene Sequence Analysis Software (DNASar, Inc, Madison, Wis; Table 1). Real-time reactions were carried out by using Platinum SYBER Green quantitative PCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, Calif). Briefly, 1 $\times$ SYBER Green PCR Master Mix was combined with the appropriate primers and 10 ng cDNA to a volume of 25 μ L. Reactions were carried out by using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) with an initial UDG incubation (50°C for 2 minutes) and a Taq activation step (95°C for 10 minutes) followed by an amplification cycle (40 $\times$, 95°C for 15 seconds) at the appropriate optimized temperature for each primer (for 1 minute), as determined by previous experiments (data not shown). Expression fold changes were calculated for each gene by using the $2^{-\Delta\Delta CT}$ method, as previously described, with 18S RNA as a normalizing control.⁷

Results

Gene Expression by Means of Genechip Analysis

A total of 18 samples (2 samples from the CPA lung and 1 sample from the control lung for each animal) were subjected to GeneChip analysis. Two CPA samples that contained outlying %P values were removed from further analytic considerations, which resulted in 16 total samples (10 CPA and 6 control samples) for comparison. PAVM replicates were compared to evaluate GeneChip consistency and then compared with control specimens to generate a list of genes that demonstrated altered levels of expression. As described in the Materials and Methods section, these results were analyzed by a defined set of criteria in which the altered expression of a gene must have an absolute value fold change of at least ± 2.0 and an occurrence rate of 60% or greater. The list of genes with altered expression meeting the criteria defined

above were further evaluated for multiple gene occurrences and expressed sequence tags, which, when identified, were removed from further analysis. These criteria resulted in the identification of 137 genes with significant modulation in the CPA lungs compared with control lungs: 55 (40%) of these genes demonstrated increased expression, and 82 (60%) genes demonstrated decreased expression. Modulated genes were subsequently grouped into one of 4 classifications; according to these classifications, genes that demonstrated significant increases in the CPA lung were involved in growth and development (29%), adhesion/extracellular matrix (ECM)/cell structure (26%), receptor activity/signaling (18%), or other miscellaneous functions (27%). Genes that demonstrated decreased expression were involved in growth and development (17%), adhesion/ECM/cell structure (20%), receptor activity/signaling (36%), or other miscellaneous functions (27%). The entire dataset for gene expression in this animal model of PAVMs after CPA can be found in the Gene Expression Omnibus of the National Center for Biotechnology Information, as described in the Materials and Methods section. For brevity, a selected set of genes of interest with either increased or decreased expression are presented in Tables 2 and 3, respectively.

RT-PCR Verification of Genechip Data

As described above, 11 genes were selected for validation by means of RT-PCR based on either their degree of predicted fold change (eg, glucagon-like peptide 1 receptor, troponin T2, and myosin heavy chain, polypeptide 6) or their suspected involvement in angiogenesis and vascular biology (eg, angiopoietin-2 and endothelin [ET] receptor, type B). The directional fold change of each gene was confirmed in all 11 genes tested, with some variations noted in the fold-change values demonstrated by means of RT-PCR compared with values obtained by means of GeneChip analysis (Table 1).

TABLE 2. Selected genes with increased expression, as determined by means of GeneChip analysis in the lungs of rats after CPA

Gene function	Accession no.	Gene	Description	FC
Development/growth/morphogenesis	NM_012676	Tnnt2	Troponin T2, cardiac	49.1
	NM_022604	Esm1	Endothelial cell-specific molecule 1	4.0
	NM_017239	Myh6	Myosin heavy chain, polypeptide 6	422.8
	NM_053595.1	Pgf	Placental growth factor	4.5
	NM_013130	Smad1	SMAD, mothers against DPP homolog 1 (Drosophila)	3.4
	AF370889	Tpm1	Tropomyosin 1, α	45.3
	BI275292	Agpt2	Angiopoietin 2	4.3
	NM_012715	Adm	Adrenomedullin	2.6
	NM_053963	Mmp12	Matrix metalloproteinase 12	3.0
	NM_134452	Col5a1	Collagen, type V, α 1	2.6
Adhesion/ECM/structural	NM_017066	Ptn	Pleiotrophin	3.6
	U65656	Mmp2	Matrix metalloproteinase 2	2.3
	BI285575	Col1a1	Collagen, type 1, α 1	3.4
	BI288582	Col18a1	Collagen, type XVIII, α 1	3.9
	AI407239	Myom2	Myomesin 2	22.6
	BM386752	Col15a1 predicted	Procollagen, type XV (predicted) (endostatin domain)	3.0
Other				

Genes were grouped into functional classifications based on known or implied biologic and molecular functions by using the Database for Annotation, Visualization, and Integrated Discovery (<http://david.abcc.ncifcrf.gov>)⁶ and are listed only once; however, many genes might have multiple functions that are not listed. FC, Fold change.

Variations in fold-change values between GeneChip and RT-PCR might have been due to different methods of normalization for each technique. GeneChip values were normalized by using a “scaling factor” recommended by the manufacturer to normalize signal intensities to an arbitrary mean value for each individual chip, whereas samples analyzed by using RT-PCR were first normalized against 18S RNA and then subsequently used to generate values indicating the relative change between the test and control samples, thus yielding a complete and more accurate fold-change value.

Discussion

The present study describes the use of DNA microarrays to investigate gene expression profiles in lung tissue from an animal model of PAVMs after experimental CPA. The development of PAVMs after CPA has been a clinically recognized phenomenon for more than 30 years; however, very little is understood regarding the cause or true biologic nature of this condition.^{1,3} Physiologically, these lesions create an intrapulmonary right-to-left shunt that causes progressive cyanosis. Interestingly, PAVMs also develop in patients with advanced liver failure and normal cardiac anatomy that histologically and physiologically appear to be similar to PAVMs that develop after CPA. This condition in patients with liver failure, termed the hepatopulmonary syndrome, is reversible after liver transplantation.⁸ Although the exact cause of PAVM development in these clinical settings is unknown, these lesions appear to develop whenever “functional” hepatic venous effluent no longer directly perfuses the lungs as a result of surgical diversion (after CPA) or as

the result of liver failure. We and others have hypothesized that PAVMs represent a form of abnormal vascular development that can occur due to the absence of a hepatic-derived inhibitor of vascular proliferation and growth.^{1,2,9} Therefore our previous studies have focused specifically on understanding the role of mediators of pulmonary angiogenesis and vascular remodeling in lung tissue from affected children and a rat model of this condition.^{4,5,10-13} In these previous studies we found altered expression of mediators associated with angiogenesis and vascular remodeling by assays for mRNA,¹³ for tissue proteins,^{10,12} and for histologic analyses of vessel number and morphology.^{4,11}

In the animal model used in the present study, a unilateral, right-sided CPA is performed, which results in PAVM development limited to the right lung, allowing the left lung to serve as a control. We have previously documented that this model accurately reproduces the angiographic and histologic manifestations seen in the human condition.⁵ As expected, the transcriptome in the shunted lung after CPA was substantially different from the control lung: 137 genes were substantially modulated, with the majority (60%) demonstrating downregulation. Our previous work focused on mediators of angiogenesis and vascular remodeling in this condition. A number of pertinent genes concerned with these processes demonstrated altered expression; for example, angiopoietin-2 (Ang-2) expression was increased in the CPA lungs compared with that seen in the control lungs. Ang-2 is an endothelial cell-specific growth factor that binds the tyrosine kinase with immunoglobulin and epidermal growth factor homology domain receptor (Tie-2), thus antagonizing

TABLE 3. Selected genes with decreased expression, as determined by means of GeneChip analysis, in the lungs of rats after CPA

Gene function	Accession no.	Gene	Description	FC
Development/growth/morphogenesis	NM_013151	Plat	Plasminogen activator, tissue	−2.3
	AI230625	Efnb1	Ephrin B1	−2.1
	BE105541	Cyp26b1	Cytochrome P450, family 26, subfamily b, polypeptide 1	−6.5
	BF398206	Bmpr2	Bone morphogenic protein receptor, type II	−2.5
	NM_012561	Fst	Follistatin	−12.9
Adhesion/ECM/structural	NM_080776	Mmp16	Matrix metalloproteinase 16	−2.2
	NM_021654	Gja4	Gap junction membrane channel protein α 4	−2.4
	NM_022221	Mmp8	Matrix metalloproteinase 8	−9.4
	NM_031055	Mmp9	Matrix metalloproteinase 9	−5.7
Receptor activity/signaling	NM_022441	Acvr1l	Activin A receptor type II –like 1	−2.5
	NM_012548	Edn1	Endothelin 1	−2.7
	NM_012728	Glp1r	Glucagon-like peptide 1 receptor	−26
	X57764	Ednrb	Endothelin receptor type B	−2.1
	NM_012614	Npy	Neuropeptide Y	−6.6
	NM_012685	Vipr1	Vasoactive intestinal peptide receptor 1	−4
Other	AI058709	Nostrin	Nitric oxide synthase trafficking	−2.9

Genes were grouped into functional classifications based on known or implied biologic and molecular functions by using the Database for Annotation, Visualization, and Integrated Discovery (<http://david.abcc.ncifcrf.gov>)⁶ and are listed only once; however, many genes might have multiple functions that are not listed. FC, Fold change; ECM, extracellular matrix.

angiopoietin-1 (Ang-1)–induced phosphorylation of Tie-2. Ang-2 induces vessel destabilization in preparation for angiogenesis, is increased in areas of vascular remodeling, and appears to assist in the activation of matrix metalloproteinase (MMP) 2.^{14–16} This pattern of vascular destabilization and branching associated with increased Ang-2 and MMP-2 expression might help to explain histologic changes that we have observed in lung tissue of children with PAVMs after CPA, namely the presence of increased numbers of large, thin-walled vessels with complex branching patterns that extend far into the periphery of the lung.⁴

The expression of a number of other genes associated with angiogenesis, vascular remodeling, or both was found to be significantly modulated in the present study, including placental growth factor, insulin-like growth factors 3 and 5, cadherin 13, endothelial cell–specific molecule 1 (Esm1), tissue plasminogen activator (TPA), integrin- β 4, and interleukin 1 β , as well as members of the transforming growth factor (TGF) β superfamily (see below). An important finding was the observed modulation of a relatively large number of factors responsible for the turnover of the ECM, including a number of MMPs demonstrating both upregulation (MMP-2 and MMP-12) and downregulation (MMP-8, MMP-9, and MMP-16) after CPA. In addition, other factors with potential involvement in ECM turnover and tissue remodeling demonstrated altered expression in the CPA lung with either upregulation (mast cell peptidase 2, tissue factor pathway inhibitor 2, and nidogen 2) or downregulation (mast cell protease 8, heparanase, serine protease inhibitor, clade A, member 1, and granzyme A). Several collagen subtypes were upregulated as well, including collagen, type I, α 1; collagen, type

V, α 1; procollagen, type XV; and collagen, type XVIII, α 1. Increased expression of collagen XVIII is of particular interest in that (along with collagen XV) it is a component of the vascular basement membrane and serves as a precursor of the angiogenic inhibitor endostatin.¹⁷ Increased turnover of the ECM is in keeping with mechanisms of angiogenesis and vascular remodeling that have been hypothesized to be of importance in the development of PAVMs after CPA.¹ In addition to the histologic finding of increased numbers of dilated and thin-walled blood vessels in this condition, we found that the basement membrane of these vessels appeared disorganized and contained prominent collagen fibers.⁴

Results of the present study also suggest that the TGF- β superfamily of related polypeptide growth factors might be an important signaling pathway in the development of PAVMs after CPA. Ligand binding of the family of TGF- β receptors activates cytoplasmic signaling proteins, which then translocate to the nucleus to regulate gene transcription and mediate the effects of TGF- β activation.¹⁸ Of particular interest in the present context, primary pulmonary hypertension and hereditary hemorrhagic telangiectasia, which might each manifest PAVMs, are due to mutations in the genes encoding receptors belonging to the TGF- β superfamily.^{19,20} In the present study we found that several members of the TGF- β signaling pathway were modulated, including the receptors activin A receptor type II–like kinase 1 (Acvr1l) and bone morphogenic protein receptor, type II (Bmpr2), as well as the downstream cytoplasmic effector of this pathway, mothers against decapentaplegic homolog 1 (*Drosophila*; Smad1). Furthermore, TGF- β –dependent mechanisms have been found to be responsible for accelerated turnover of components of the

ECM in processes such as angiogenesis and in certain disease states of the lung such as asthma and emphysema.¹⁸ In fact, TGF- β -dependent mechanisms could be responsible for the altered expression we observed in factors associated with increased turnover of the ECM, including MMP-2, MMP-8, MMP-9, MMP-12, and MMP-16. Finally, TGF- β pathway-dependent mechanisms might be responsible for collagen deposition, leading to fibrotic changes observed in emphysema¹⁸; as discussed above, collagen types I, V, and XVIII were all upregulated in the present study.

The expression of a number of other factors associated with mechanisms that have been hypothesized to be of importance in the development of PAVMs after CPA were found to be altered in the present study. For example, factors associated with changes in or maintenance of vascular tone, including neuropeptide Y (NPY), TPA, gap junction membrane channel protein 4 (also known as connexin 37), adrenomedullin, ET-1, and ET-B receptor demonstrated altered expression in the present study. NPY, which appears to play an important role in the control of vascular tone through sympathetic-mediated vasoconstriction,²¹ demonstrated decreased expression. Sympathetic neurons that express NPY have been reported to synthesize, transport, and store TPA, which can then generate plasmin from plasminogen; plasmin can cleave pro-MMPs yielding their active forms, which could promote further ECM turnover.²² Adrenomedullin primarily functions in adrenal development but also appears to play a role in the maintenance of vascular tone, where it functions as a potent vasodilator.²³ Additionally, decreased expression of gap junction membrane channel protein 4 (or connexin 37), which is involved in both the regulation of vascular tone and the remodeling of injured endothelium, might also suggest that vascular remodeling is ongoing in PAVM development.²⁴ Regarding the possible role of ET-1 in this phenomenon, an ET-B receptor-deficient animal model has been shown to develop pulmonary "vascular leak" and pulmonary edema, which is presumably mediated by unopposed action of ET-1 on ET-A receptors through a vascular endothelial growth factor-dependent mechanism.²⁵ Serous pleural effusions are a well-recognized clinical problem after CPA, which occurs because of increases in the systemic venous pressure in the superior vena cava and its branches after this procedure; however, this study suggests that a molecular mechanism mediated by an ET regulatory pathway might also contribute to this phenomenon. Interestingly, we have previously demonstrated histologic evidence of tissue edema in the lungs of children after CPA, as well as ultrastructural changes compatible with increased permeability in the pulmonary capillaries that might corroborate the involvement of ET, its receptors, or both.⁴

Finally, some genes are present that appear to be clustered in terms of function, suggesting their participation in potentially important regulatory pathways that have no clear connection with previously hypothesized mechanisms for PAVM devel-

opment. For example, the expressions of several myocardial muscle proteins were upregulated, including troponin T2, myosin heavy chain polypeptide 6, tropomyosin-1 α , and myomesin-2. The significance of this pattern of gene expression in the lungs after CPA is unclear at present but represents another possible pathway to be explored in the future.

A number of possible limitations exist in the present study. Although altered expression determined by microarray analysis was verified for selected genes by using RT-PCR, expression for any gene of interest should be similarly verified by using an alternate method before definite conclusions are made. In addition, because of posttranslational modifications, protein levels and therefore the biologic effect might differ from measured RNA expression or the protein might be present in inactive or modified forms (eg, MMPs, which might be present in pre-, pro-, and active forms). System-related errors can contribute to erroneous interpretation of these data; for example, significant expression changes might be present for a given gene that does not meet the analytic criteria used, which would then be interpreted as unaltered gene expression compared with control values. Another potential limitation arises because of the diverse cell types present in the analyzed samples; measured alterations in gene expression might come from cell types not involved in PAVM development. Finally, the limitations of the animal model in accurately reproducing the human condition must be kept in mind. In the human condition significant underlying congenital heart disease always exists, whereas these animals have normal cardiopulmonary status before surgical intervention. To validate the present results in an animal model, we are currently proceeding with a similar analysis in human lung specimens in children with PAVMs after CPA and healthy control subjects.

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Discussion

Dr Frank W. Sellke (Boston, Mass). This is a very nice study in a series from the group looking at the potential causal effects leading to the formation of arteriovenous connections in the lungs of patients who have undergone this operation. I have several questions.

This was a unilateral model in which you performed the CPA on one side and you used the opposite, or normally perfused, lung as a control. Is that correct?

Dr Duncan. That is correct.

Dr Sellke. Well, there are other factors involved such that the perfusion to the control lung might not be quite normal. Would you consider performing another control just to verify that there are not additional changes going on in the control lung that ordinarily would not occur?

Dr Duncan. Yes, I think that is a valid point. I think that might be an important control group, especially as we go from general pathways to specific factors. A particular problem with this expression profile technique arises from the great number of multiple comparisons, although it does provide a baseline to start. But certainly going forward, as we look at more specific gene products, I think that is a very good point.

Dr Sellke. What do you think the actual mechanism is? Is it the changes in shear stress or absolute pressure? Do you have any idea what might be causing these?

Dr Duncan. It certainly appears to be a complex process, a true multifactorial process. The direct answer to your question is probably yes, and each factor you named probably contributes. Certainly there does appear to be the presence of a hepatic-derived inhibitor, potentially in the hepatic venous effluent, that might partially control pulmonary vascular development, even in the normal state.

We see the effect of the liver on the pulmonary vasculature in these patients because the CPA isolates the pulmonary arteries from direct hepatic venous perfusion. However, I think it is overly simplistic to say that this hepatic factor is the only causative factor involved. Certainly effects related to alterations in shear stress after CPA might be important; we have done some preliminary work that shows this might play a causative role as well.

Dr Sellke. Listening to your talk and reading the paper, which you gave me quite a while ago, you only examined gene expression at 8 months, yet in disease processes and when you alter perfusion, you can have immediate and midterm and long-term effects. Do you have any idea whether the 8-month expression examination is reflective of the actual mechanism causing the atrioventricular malformations?

Dr Duncan. In our original published description of the model, we reported our attempts at a quantitative assessment of this phenomenon over time. We looked at microvessel density as a quantitative indicator of angiogenesis. We found that the model does give you a fairly linear relationship for microvessel density over time. Applying DNA microarrays over time would be interesting; however, expense involved is the main limitation.

Dr Sellke. Finally, when you are looking at the cDNA microarray tests, even very small changes in gene expression can be statistically significant but really have very little effect on translation to proteins. Have you done studies looking at protein expression to see whether the changes in gene expression really mean anything?

Dr Duncan. Yes, we have; that is really where we started in this. Our initial studies looked at protein expression by using immunohistochemistry. The DNA microarrays have introduced us to a new area. The data that we see here, particularly those suggesting that factors related to the ECM are important, have given us new areas to explore. We are now going back and trying to corroborate these findings in gene expression with changes in protein expression for the MMPs and other ECM components.