Upregulation of collagens detected by gene array in a model of flow-induced pulmonary vascular remodeling

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Medhora, Meetha, Michael Bousamra II, Daling Zhu, Lewis Somberg, and Elizabeth R. Jacobs. Upregulation of collagens detected by gene array in a model of flow-induced pulmonary vascular remodeling. Am J Physiol Heart Circ Physiol 282: H414–H422, 2002. First published October 11, 2001; 10.1152/ajpheart.00292.2001.—We recently reported localized increased pulmonary arterial resistance, neointimal lesions, and medial thickening induced by aortopulmonary anastomosis in young pigs. This model was used to investigate changes in expression of genes potentially involved in pulmonary vascular remodeling employing a high throughput Atlas Human Cardiovascular Array carrying ~600 cardiovascular-related cDNA sequences. Data were confirmed by Northern analysis, Western blots, and histological examination. With the use of lower stringency conditions for hybridization, 56% of the 588 human genes on the array showed visible signal after autoradiography. Approximately 10% of the genes with visible hybridization were altered by shunt-induced high flow. Extracellular matrix and cell adhesion molecules were the most highly represented group of upregulated genes. To our knowledge, our data are the first to demonstrate flow-induced changes in gene expression using a combination of cross species cDNA arrays, homologous hybridization, immunospecific protein, and histology. Our observations expand the list of genes as putative candidates in pulmonary vascular remodeling and support the utility of cross-species microarray analysis in such applications.

PULMONARY ARTERIES are generally spared vasculopathic changes secondary to insults like aging, severe hypercholesterolemia, atherosclerosis, or diabetes (4, 43). However, un repaired congenital heart defects or diaphragmatic hernias produce increased shear forces likely transduced by cytoskeletal elements (2), which result in irreversible pulmonary hypertension. Histologically, these conditions as well as hypoxic injury are characterized by intimal and medial hypertrophy in blood vessels of the lung (11, 12, 16, 33). This remodeling must be defined by changes in expression of extracellular matrix protein and growth-related signaling cascades. However, despite the presence of numerous animal models, pathways examining the mechanisms that underlie pulmonary vasculopathy in the lung vessels remain poorly defined at the molecular level (4). A few candidate genes involved in tissue remodeling such as elastase, tenascin (37, 38), tropoelastin, and type I procollagen have been investigated in organ culture and a rat model of pulmonary hypertension (4, 51). However, correlation between histological evidence of flow-induced pulmonary vasculopathy and changes in gene expression in animal models is needed.

Recently, we developed (35) and modified (5) a model of high-flow and/or pressure localized to a single lobe of lung created by a surgical connection between the aorta and pulmonary artery. Anastomosis of the left lower lobe pulmonary artery to the aorta consistently produces pronounced increases in pulmonary arterial resistance within weeks of the surgical connection (5). There are at least two important benefits to this model for systematic analysis of changes in gene expression induced by flow. The first advantage is that pulmonary arteries from an unshunted lobe serve as an ideal control for a shunted high-flow pulmonary artery. The second advantage is that both shunted and unshunted arteries have been exposed to normal or elevated flows in vivo and therefore reflect the responses of vascular endothelial and smooth muscle cells in contact with one another. Histological examination has confirmed neointimal and medial lesions in the shunted lobes with ~10-fold increase in wall-to-lumen area ratio by 8 wk after creation of the anastomosis (5). We used this model to investigate changes in expression of genes involved in remodeling of the pulmonary vasculature. Our hypothesis was that we could detect alterations in expression of a range of genes by using a targeted cardiovascular gene array and that some changes in gene expression induced by high flow might reasonably be related to the vasculopathic alterations observed histologically. However, pig-specific gene arrays were not available, and in fact, very few candidate genes from the
pig have been sequenced to completion. We therefore tested the hybridization of pig RNA-derived cDNA versus human DNA sequences immobilized on nylon filters. The array for ∼600 human genes (Clontech Atlas Cardiovascular Array) relevant to cardiovascular physiology was applied in conjunction with low-stringency hybridization to promote signal detection across the species barrier. Using this approach, we identified and confirmed upregulation of collagen 1 α1 (COL1A1) in shunted pulmonary arteries, which is in accordance with a rat model of pulmonary hypertension (51). In addition, we identified a number of other putative alterations in gene expression and confirmed upregulation at the mRNA as well as protein levels of a second collagen protein, procollagen α1 (III) (COL3A1).

METHODS

Surgical anastomosis. Weanling infant pigs ~6 wk of age underwent creation of an auto-left lower lobe pulmonary artery anastomosis as described in detail in our recent publication (5). The surgical protocol and postoperative care were in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication 85-23, Revised 1985) and approved by the Animal Studies Committee of the Zablocki Veterans Administration Hospital and the Medical College of Wisconsin. Sedation was achieved with acepromazine (1.5 mg/kg) and ketamine (30 mg/kg) intramuscularly and fentanyl (2 μg/kg) intravenously with a halothane mixture to achieve general anesthesia. A left thoracotomy was performed through a midthoracic interspace. The left lower lobe PA was separated posteriorly from the adjacent lower lobe bronchus, and the fissure between the upper and lower lobes was completed using electrocautery. The descending pulmonary artery was ligated just distal to the upper lobe branches and divided. The distal left lower lobe pulmonary artery was sown end to side to the descending thoracic aorta. Patency was confirmed by a palpable thrill within the left lower lobe pulmonary artery. Intramuscular cefazolin (25 mg/kg) and furosemide (1 mg/kg every other day) were given postoperatively over 5 days.

Harvesting lungs, dissecting pulmonary arteries, and preparing histological sections. Three weeks after the operation, the pigs were sedated with intramuscular acepromazine and ketamine as indicated in Surgical anastomosis and then administered pentobarbital (5 mg/kg) to achieve general anesthesia. After intubation, a thoracotomy was performed, and the heart and lungs were removed en bloc. They were transported on ice to the laboratory where the pulmonary arteries (0.5–1.0 mm in diameter and ~2–6 mm in length) were assiduously dissected away from airway and adventitial tissue, microscopically (also on ice), for extraction of RNA or homogenization for immunospecific protein studies. Distal sections of lung from shunted and nonshunted lobes were also immersion fixed in neutral formalin for 2–4 days, after which time samples were embedded in paraffin, sectioned, and reacted with a Movat pentachrome stain (18). Digital images were captured at magnifications of ×200 or ×400 with SPOT Advanced image acquisition software on an inverted Nikon microscope. The protocol was repeated after 1 wk of exposure to high flow to study earlier changes of gene expression.

RNA isolation. RNA was isolated from shunted and nonshunted pulmonary arteries with TRizol (GIBCO BRL; Gaithersburg, MD) as recommended by the manufacturer. The RNA concentration and yield were determined by spectrophotometry (A260/A280), and 5 μg each from nonshunted and shunted vessels were used for the labeling reactions described below. For DNase I treatment, 25 μg of RNA were digested in 100 μl with 25 units of DNase I (Amersham Pharmacia Biotech; Piscataway, N.J.) for 30 min at 37°C. The reaction was terminated in a final concentration of 10 mM EDTA, extracted with phenol:chloroform:i soamyl alcohol (25: 24:1; vol/vol/vol, pH 4.5), and precipitated in the presence of 0.2 M sodium acetate and 10 μg glycogen by adding 2.5 vol of RNase-free ethanol. The RNA was allowed to precipitate overnight at −20°C and was recovered by centrifugation. The pellet was washed with 70% ethanol and then resuspended in 20 μl of RNase-free water. The RNA concentration was measured as before, and 5 μg were taken for the labeling reaction.

Synthesis of labeled cDNA probes. The first-strand cDNA synthesis was carried as specified by Clontech (Clontech Laboratories; Palo Alto, CA) with reagents in their kit. A few modifications in temperature of the reactions were introduced and will be described together with a brief outline of the procedure. The RNA (5 μg) from nonshunted and shunted pulmonary arteries was heated briefly at 70°C to remove secondary structure and allowed to anneal in the presence of the CDS primers supplied in the kit. Reaction buffer, dNTPs, 35 μCi of [α-32P]dATP (specific activity 3,000 Ci/mmol, Amersham Catalog no. PB10204) and MMLV Reverse Transcriptase were added from the Clontech kit after the RNA-primer mix cooled to 50°C. The reactions were allowed to incubate at this temperature for 5 min followed by 10 min at 45°C and 15 min at 40°C to allow cDNA extension from primers that may not be entirely homologous due to species differences. Reactions were terminated with 1/10 vol of 100 mM EDTA and purified to remove the unincorporated bases by Atlas Nucleospin Columna. An aliquot of the eluted cDNAs was counted to determine 32P incorporation after the addition of the liquid scintillant. Control mRNA from human tissue provided in the kit was labeled, purified, and counted to compare incorporation against RNA from the pig.

Hybridization of labeled cDNA to filters. Nylon arrays were prehybridized at 68°C with sheared salmon sperm DNA (Sigma) and ExpressHyb solution. Equal counts of labeled probe from nonshunted and shunted vessels (~65,000 counts/ min) were added independently after denaturation with 0.1 M NaOH at 65°C and neutralization with 0.1 M NaH2PO4 onto two gene array filters. The reaction was allowed to proceed overnight at 50°C in roller bottles. The next day the filters were washed twice with 2× saturated sodium citrate (SSC) and 1% SDS at 50°C followed by one wash with 0.5× SSC and 0.5% SDS at the same temperature. The filters were exposed to X-ray film for 18–36 h in the presence of an intensifying screen at ~70°C. The autoradiographs were scanned in a Molecular Dynamics Personal Densitometer SI and analyzed using ImageQuant Software by constructing a grid with a window for each gene. The data were converted to a spreadsheet format for further processing.

Northern analysis with oligonucleotide probes. Total RNA was extracted from microdissected nonshunted and shunted pulmonary arteries using the TRizol reagent (GIBCO-BRL) as specified by the manufacturer. Equal amounts of denatured RNA from each sample (30 μg) were loaded on a formaldehyde agarose gel (1% agarose containing 0.6% of 37% formaldehyde) and electrophoresed in 3-(N-morpholino)propanesulfonic acid buffer (20 mM MOPS, pH 6.8, 5 mM sodium acetate, and 1 mM EDTA) at 10 V/cm (31), visualized under ultraviolet light, and documented in a Vistra Fluorimag. The RNA in the gel was denatured with 50 mM NaOH in 10 mM NaCl for 20 min, neutralized with 0.1
M Tris (pH 7.5), and blotted onto Nytran Plus Membrane (Schleicher and Schuell; Keene, NH) using a TurboBlotter (Schleicher and Schuell). The blots were prehybridized at 55°C for 3 h with 5 ml of buffer A (6X SSPE, 3X Denhardt’s solution, 10% dextran sulfate, 0.5% SDS, and 100 µg/ml tRNA) and probed overnight in the same conditions with labeled oligonucleotide, which was prepared as follows: the pig oligonucleotides shown in Fig. 2 (10 pmol) were denatured by heating to 90°C for 5 min, cooling to room temperature, and incubating at 37°C for 45 min with 5 µl [γ-32P]ATP (3,000 Ci/mmol, NEN Life Science Products; Boston, MA) and 10 units of T4-polynucleotide kinase (Amer- sham). The labeled products were separated from unincorpo-
rated nucleotides by precipitation in 70% ethanol using tRNA 
sham). The labeled products were recovered by centrifugation, resuspended in sterile water (0.1 ml), and heated to 90°C for 5 min, and at least 10^6 counts/min were put in fresh buffer A and applied to the membrane. After overnight hybridization at 55°C, the filter was washed three times at room temperature with 6X SSC and 0.1% SDS followed by one wash at 55°C. The membranes were exposed to X-ray film (Kodak X-Omat) for autoradiography for 18–36 h using an intensifying screen.

The ethidium bromide-stained gels and autoradiographs were scanned in a VISTRA fluorimeter and densitometer, respectively. Quantitative values for the 28S rRNA in each lane were obtained and used to normalize the corresponding densitometric value of the mRNA bands.

Immunospecific protein identification. Homogenates of microdissected pulmonary arteries containing 20 µg of total protein were separated by electrophoresis on a 10% denaturing sodium dodecyl sulfate-polyacrilamide gel and trans-
ferred to a nitrocellulose membrane. Nonspecific binding was blocked by incubating the membrane in Tris-buffered saline in 10% nonfat milk overnight, followed by three washes with Tris-buffered saline. The nitrocellulose membrane was incu-
bated for 2 h at room temperature with a primary antibody to procollagen (III) (N-18) (catalog no. sc-8779; Santa Cruz Biotechnology). In some cases, the primary antibody was reacted with the blocking peptide supplied by Santa Cruz for 30 min on ice before and during exposure to the membrane to verify the specificity of bands identified by the primary anti-
body. The membrane was rinsed three times before incubating with horseradish peroxidase-labeled goat anti-rabbit sec-
ondary antibody (1:1,000) and then visualized using enhanced chemiluminescence. The X-ray film was developed on the Kodak XOMAT developer, and the X-ray image of the gel was scanned on a densitometer. The bands corresponding to the one eliminated by the blocking peptide were selected on the computer representation of the scan and, after background correction, the pixel density within each band was determined by the computer, providing a means for relative quantitation.

RESULTS

Hybridization of atlas human cardiovascular array with RNA from pig pulmonary vessels. Experiments were performed with microdissected pulmonary arteries from two separate animals, using RNA that was predigested with DNase I in the second experiment, to make sure the substrate used in the reaction was RNA and not chromosomal DNA. The interspecies cDNA synthesis was found to be 10% as efficient as that using human RNA as the substrate, when measured by comparing the incorporated radioactivity in both reactions. With the use of lower stringency conditions detailed in METHODS for cDNA synthesis, hybridization, and washing, at least 56% of the 588 human genes on the array showed visible signal after autoradiography (Fig. 1).

Alterations in gene expression associated with high-
flow. The densitometric readings of the autoradiographs from two independent experiments were analyzed and the results tabulated in Table 1. Normalization of values

of expression between the two filters in the same exper-
iment was accomplished by deriving a factor from the ratio of the sum of expression of all genes on each filter. The data were inspected for consistent changes in expression in both experiments. Only genes that were upregu-
lated more than 1.2-fold by high flow or attenuated <0.8 in both experiments were included. Approximately 10% of the genes with visible signals were altered by shunt-
induced high flow, with the upper and lower limits for the changes being 2.5- and 0.2-fold, respectively. The exper-
iment was repeated with vessels of a pig studied after 1 wk of exposure to high flow to document earlier changes in gene expression induced by high flow. Approximately half of the genes that were upregulated by 3 wk also showed increases above the selected cutoff at 1.2-fold. Only 30% of the genes that were downregulated showed similar results at 1 wk of high flow. These genes that showed early changes reflecting those seen at 3 wk of high flow are marked with an asterisk in Fig. 1.

Confirmation of two genes by Northern blot analysis using homologous oligonucleotides as probes. It was necessary to confirm the fidelity of hybridization of the
Table 1. High flow-induced changes in pulmonary arterial gene expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ratio Shunted Pig 1</th>
<th>Ratio Nonshunted Pig 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein E precursor*</td>
<td>2.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Apolipoprotein A-II precursor*</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>LDL receptor-related protein 1 precursor</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Types 1A &amp; 1B angiotensin II receptor</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Caveolin 2</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Caveolin 3</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Vascular ATP-disphosphohydrolase*</td>
<td>1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Integrin β8 precursor</td>
<td>1.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Integrin β4*</td>
<td>1.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Desmocollin 3A/3B precursor + desmocolin 4</td>
<td>2.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Desmocollin 1A/1B precursor*</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Collagen 1 α1-subunit precursor</td>
<td>2.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Procollagen 2 α1-subunit precursor</td>
<td>1.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Procollagen 3 α1-subunit precursor*</td>
<td>2.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Collagen 4 α2 subunit precursor*</td>
<td>1.4</td>
<td>2.0</td>
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<tr>
<td>Bullous pemphigoid autoantigen 180*</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Cardiac muscle troponin T</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Ezrin; villin 2*</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>α-Catenin-related protein</td>
<td>1.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ratio Shunted Pig 1</th>
<th>Ratio Nonshunted Pig 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF 165 receptor*</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Sterol carrier protein-2*</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Oxyyster-binding protein*</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>E-selectin precursor</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>P-selectin precursor</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Neural-cadherin precursor</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>HMG-coenzyme A reductase</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Platelet activating factor acetyl IB</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>γ-subunit</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Fibrinogen A α-polypeptide</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Fibrinogen B β-polypeptide</td>
<td>0.2</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Individual genes whose expression was altered by high flow for over 2 wk in 2 independent animals are shown. Change is depicted as the ratio of expression of the gene in vessels from the shunted lobe of the lung versus those from the nonshunted lobe of the same animal. Values used for these calculations were normalized for variations in hybridization intensity between array filters (see METHODS and RESULTS). *Genes with the same up- or downregulation by 1 wk of exposure to high flow. LDL, low-density lipoprotein; VEGF, vascular endothelial growth factor.

array by using homologous probes. The results in Table 1 indicate upregulation of four types of collagen, including COL1A1, which had been previously reported to be induced in a rat model of pulmonary hypertension (4, 51). A partial sequence of the corresponding pig homologue was available along with a partial sequence of pig COL3A1. Specific oligomers were designed, labeled (see METHODS), and hybridized (see Fig. 2) to immobilized RNA from control and shunted porcine vessels used for the array (COL1A1) and from two independent experiments (COL3A1). Figure 3, A and B, shows the predominant hybridizing mRNA for both types of collagen. The molecular weights are in accordance with those described from other species (42). The graph below each figure demonstrates increase in signal in mRNA from three shunted compared with nonshunted lung pulmonary arteries after background subtraction and normalization with rRNA loaded in the same lane. There is an increase in the steady-state message level for both COL1A1 and COL3A1 in pulmonary arteries exposed to high pressure/flow, confirming the results obtained from the array.

*Genes with the same up- or downregulation by 1 wk of exposure to high flow. LDL, low-density lipoprotein; VEGF, vascular endothelial growth factor.

**Fig. 2.** Sequence homology between *Sus scrofa* oligonucleotide sequence for COL1A1 (National Center for Biotechnology Information accession no. AF201723, submitted by Y. W. Wang, A. E. Baer, and L. A. Setton, 2000) and COL3A1 (GenBank accession no. AU058685, submitted by N. Hamasima, 1999) versus their *Homo sapiens* homologues. Nucleotide sequences that differ are shown in bold. Pig sequences were used as probes for Northern blot analysis.
of the same animals used for gene array studies showed qualitative increases in extracellular deposition of collagen inside and around the outer periphery of the vessels in the shunted lobe (Fig. 5). Neoproliferative lesions characteristically observed in pulmonary arteries exposed to high-flow shear stress were also evident. Vasculopathic changes were evident in sections from shunted lungs of both animals used for array studies but were more advanced (grade III and IV) in one specimen than the others (grade I and II changes) (20, 22). Left lobes shunted for 1 wk reveal scattered neointimal and muscular changes in the medium-sized arteries, with some loss of small to medium size arteries.

DISCUSSION

The earliest pulmonary vascular responses to increased flow are prodilatory and include augmented release of nitric oxide and prostacyclin (4, 16). Days to weeks later, vascular wall hyperplasia and/or hypertrophy, neointimal lesions and chronic pulmonary hypertension develop (1, 4, 52). Very little is known about factors that underlie the vasculopathic changes of pulmonary arteries exposed in vivo to high-flow, but they are critically important clinically, in that pulmonary hypertension complicates the treatment of a variety of congenital heart diseases (16, 25). The mechanisms that underlie high-flow-induced remodeling in the lung are likely to be important to a wide range of other pulmonary vascular conditions despite differences in pathophysiology and histology. Disorders that exhibit increased shear forces include hypobaric (altitude) hypoxic vasculopathy, perpetuation or amplification of emphysematous vascular “drop out,” leading to significant reductions in pulmonary vascular bed, postpneumonectomy changes, primary pulmonary hypertension, and others (6, 17, 28, 49, 56).

Much of the work on vascular remodeling in the lung has focused on the long-term effects of hypoxia-induced vasoconstriction, globally increased pulmonary vascular flow (e.g., produced by ligation of the ductus arteriosus in fetal lambs), or chemically induced pulmonary hypertension (e.g, monocrotaline) (4, 44, 48, 50, 51, 53). Whereas useful, each of these models has significant limitations, such as difficulty separating the effects of high flow from the constrictive agent,
lack of “control” and “high-flow” pulmonary arteries in the same host, or extrapolation of data obtained from cells exposed in vitro to shear or stretch to in vivo forces.

We have developed a model of high flow and/or pressure in an isolated lobe of young pigs characterized by neointimal lesions and medial thickening within weeks of anastomosis of the left lower lobe artery to the aorta (5). These histological changes are characteristic of clinical pulmonary hypertension but are infrequently a feature of animal models of hypertension (e.g., see Ref. 1). Moreover, unshunted pulmonary arteries serve as ideal controls for shunted, high-flow pulmonary arteries in the same animal, although this advantage is potentially compromised or complicated by factors that are circulating between the two lobes of the animal. Another limitation of our model includes the inability to distinguish between the effects of pressure and flow. Furthermore, despite the attempts to control the size of the anastomosis, shunted lungs demonstrated a range of pulmonary arterial resistances (5). However, even with an optimal model, molecular analysis of gene regulation for vascular remodeling that accompanies experimental pulmonary hypertension remains challenging due to cross species issues. Pigs have often been used for research of the cardiopulmonary system (14) because they resemble the human system more closely than other nonprimate species. The first step in characterizing gene expression in control versus pathological vasculature is to detect the changes induced by the disease. Subtraction libraries (8), differential display, and, more recently, high throughput gene analysis have been used for this purpose (46, 55). The gene array method is increasing in popularity because of the large number of gene sequences now available for this analysis, which has made it technically simpler than the other two approaches. High-density microarrays of genes have proven to be a valuable, semiquantitative tool in evaluating expression of genes in conditions such as metamorphosis involving sequential transcriptional activation of hundreds of genes (55). They have also been applied to examine injury-induced transcriptional or posttranscriptional regulation of gene expression (46). We therefore attempted this technique using interspecies homology by low stringency hybridization, a method that has been employed widely in the past to characterize homologues from diverse species across bacteria, yeast, Caenorhabditis elegans, drosophilia, mouse, and humans (27, 54). To increase our chances of success, we used the most sensitive approach to array technology available, filter hybridization with [32P]cDNA instead of high-density gene chips (glass slide microarrays) analyzed with competing dual fluorescently labeled probes.

The starting material for our study was total RNA (5 μg), which gave a distinctive hybridization profile for pig lung vessels (30) with over 50% of the genes showing visible signals (Fig. 1). We used mRNA from peripheral lung tissue during our preliminary experiments (data not shown), which produced a sharper image with less background after autoradiography. However, generation (or extraction) of mRNA was not efficient from the dissected vessels. Hypertensive pulmonary arteries were more resistant to solubilization

![Non-shunted lung](image1)

![Shunted lung](image2)

Fig. 5. Representative lung sections from nonshunted (top) and shunted lobes (bottom) show an increase in extracellular deposition of collagen inside (solid arrows) and around outer periphery of vessels and marked neoproliferative changes (dotted arrows) in pulmonary arteries from shunted lobes.
by detergents used to make mRNA than the non-shunted controls. The yields of total RNA using chaotropic agents was also low but sufficient for the array protocol. We therefore confirmed that the total RNA we obtained was informative for gene expression analysis by treatment of the material from an independent experiment with DNase I to eliminate chromosomal DNA contamination. The resulting array profile was very similar in experiments with DNase I-treated and untreated RNA isolated with the TRIzol reagent. The major disadvantage of using the interspecies approach was probably the loss of sensitivity due to weaker hybridization by partially homologous DNA sequences. Confirmation of our expression profile (Fig. 1) using two genes from the array supports the potential utility of DNA array techniques even in circumstances where species-specific cDNAs are not available.

Analysis of expression of genes that were altered by high flow in two separate experimental animals showed 24 genes to be upregulated. Extracellular matrix and cell adhesion molecules were the most highly represented group of upregulated genes, which includes at least four types of collagen, cardiac muscle troponin T, two β-subunits of integrins, desmocins, and α-catenin-related protein. These molecules could account for some of the structural consequences of remodeling, like collagen deposition inside the lumen of the vessel as well as in the perivascular regions. Integrins affect the adhesion of cells to the matrix and basement lamina (41). Desmocins are adhesive proteins in desmosome type of cell junctions and belong to the cadherin superfamily (40). The caveolins are the principal protein components of caveolae, which are invaginations of the plasma membrane (3, 32, 36). The caveolins generally inhibit tyrosine kinases and mitogen-activated protein kinase cascades. Ecto-ADPase metabolizes ADP released from stimulated platelets, thereby limiting platelet activation and recruitment (13). Other extracellular signaling and communication molecules, apolipoproteins (Apo) A-II and E had increased mRNA. Whereas ApoA-II is proinflammatory, ApoE plays a protective role in atherosclerosis (7, 10). The low-density lipoprotein (LDL)-receptor-related protein is expressed in atherosclerotic tissue enhancing uptake of LDL (23). The angiotensin II receptor type I is involved in the constrictive action of the peptide hormone angiotensin II (39). One cell structural protein, ezrin villin 2, out of the 19 present on the filter showed consistent upregulation by high flow.

A number of genes were downregulated, including extracellular and cell adhesion molecules fibrinogen Aα and Bβ polypeptides, E- and P-selectin, and neural cadherin. The conversion of fibrinogen to fibrin in the arterial walls stimulates migration of smooth muscle cells from the media to the intima (34). The selectins participate in inflammatory disorders promoting rolling and subsequent adhesion of leukocytes onto vascular endothelium (21, 24, 29). The mRNA for proteins related to transport and metabolism of sterols, sterol carrier protein-2, and oxysterol were downregulated (26, 43, 45). Vascular endothelial growth factor (VEGF) 165 receptor, which may regulate VEGF-induced angiogenesis (19, 47), was also downregulated by high-flow conditions. Gene products from pulmonary vessels that had increased expression just 1 wk after surgery included the ApoA-II and ApoE precursors, vascular ATP disphosphohydrolase, integrin β3, desmocollin IA/IB precursor, procollagen 3A1 (the largest increase of all), collagen 4α3 precursor, bullous pemphigoid autoantigen 180, and ezrin. Interestingly, procollagen 1A1 and caveolin 2 and 3 expression was not increased at the 1-wk time point. Caveolins inhibit growth pathways and so the upregulation later in the remodeling may represent a response to the proliferation of vascular cells in the blood vessels induced by increased blood flow. The mRNA with decreased early expression that was sustained to the 3-wk time point included the VEGF 165 receptor sterol carrier protein and oxysterol-binding proteins. The mRNA for P-selectin, neural cadherin precursor, HMG-coenzyme A reductase, and platelet-activating factor acetyl 1B γ-subunit were increased at 1 wk after surgery.

Inspection of the list of genes altered by high flow suggests that extracellular matrix proteins, particularly collagens, were upregulated and might plausibly be related to remodeling. However, the list also implicates activation of protective pathways in the lung that seem to attenuate formation of proinflammatory molecules or induce protective species to reduce the consequences of the insult. This list may not be exhaustive of the 588 genes tested, because some molecules may not have participated in pig-human hybridization. Also, observed changes in gene expression may have been less dramatic in this model because control as well as shunted vessels were taken from the same animal. Therefore, opposing circulating mediators/factors released from both types of tissue could blunt the responses triggered in each other.

To address the authenticity of this hybridization, we tested two procollagen types, 1A and 3A, with increased steady-state mRNA after exposure to high flow. Our choice was limited by the availability of pig-derived cDNA sequences, and we obtained only a partial sequence of both genes from the pig Sus scrofa. The COL3A sequence overlapped the cDNA fragment that was immobilized on the Atlas array, so we constructed a 70-mer oligonucleotide that would hybridize with the pig mRNA within this region. The 70-mer probe was 100% homologous to the pig sequence and 90% similar to the Homo sapiens probe. The Northern hybridization showed increased mRNA after high flow, which was very similar to that noted on the filter (×1.4) corresponding to a very high fidelity of hybridization. The known S. scrofa sequence for COL1A1 was not within the sequence included on the array, but from a different region 5′ of that in the COL1A1 sequence. The 70-bp oligo designed to probe the pig COL1A1 mRNA hybridized to a single band of mRNA of molecular weight ~6.0 kb. This is in keeping with the size of the human products that showed two bands in the range of 5–7 kb. The difference in expression in the pig mRNA was over threefold, whereas the array
showed more modest upregulation of the gene. This disparity could be due to different extents of homology in the nonoverlapping regions of the gene sequence on the array versus the oligonucleotide probe used for the Northern analysis. In addition, the Northern blot had more controlled conditions for hybridization as well as signal detection, including accurate background correction as well as normalization. However, both COL1A1 and 3A1 showed the same direction of regulation by array as well as Northern blotting, giving us confidence that the interspecies homology was an appropriate tool for this type of experimentation.

In summary, our data are unique in utilization of a combination of cross-species gene arrays, Northern blots, Western blots, and histological analysis to demonstrate flow induced altered expression of extracellular matrix collagens in the lung. Our gene array data expand the list of genes as potential candidates in vascular remodeling in the lung, either as pathophysiologically important in the remodeling process or in response to primary changes. These observations support the potential application of cross-species gene array analysis to examine in vivo injury-induced changes in gene expression within a single experimental animal. Changes in expression as detected by probing the array of human genes were confirmed by Northern analysis with homologous probes, a necessary second step given the experience with these types of questions and methodologies at this point. Finally, we also demonstrate increased protein for one of the upregulated genes in shunted pulmonary arteries COL3A1. The status of COL1A protein after anastomosis in the pig model of pulmonary hypertension is not known, although collagen deposition in the vessels and perivascular areas is increased. These data can be extended to earlier time points to identify candidates for regulatory genes that show altered expression even before histological changes are evident by light microscopy. Our data provide rationale for pursuit of these experiments utilizing this powerful combination of methods.

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