Gene expression profiles in children undergoing cardiac surgery for right heart obstructive lesions

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Background: The global myocardial stress response during cardiac surgery has not been systematically studied, nor is it known whether the response of the neonatal myocardium is intrinsically different from that of older children. To determine the age-related molecular basis of this response, we conducted microarray-based differential gene expression profiling on right ventricular tissue samples acquired in patients of varying ages with right ventricular outflow tract obstruction.

Methods: We studied gene expression profiles in 24 patients during operations for lesions involving right ventricular outflow tract obstruction age stratified into group I (7 patients, aged 5 to 66 days; mean, 30 days) and group II (17 patients, aged 4 months to 12.5 years; mean, 2.8 years). Myocardial samples were taken from the right ventricular outflow tract after aortic occlusion and archived in liquid nitrogen. RNA isolation, fluorescence labeling of complementary DNA, hybridization to spotted arrays containing 19,008 characterized or unknown human complementary DNAs, and quantitative fluorescence scanning of gene-expression intensity were performed at the University of Toronto Health Network Microarray Centre. Data were analyzed with the Significance Analysis for Microarrays program. Minimum Information About Microarray Experiments-compliant, log₂-normalized data sets were compared to ascertain potential statistical differences in gene expression between patient groups.

Results: There were no hospital deaths or major postoperative morbid events. We identified 50 transcripts differentially expressed in the neonatal group (the predicted false discovery rate was <0.8 transcripts). The neonatal pattern of gene expression (group I) was dominated by genes with literature-validated cardioprotective, antihypertrophic, and antiproliferative properties, including increases in atrial natriuretic peptide, protein phosphatase 2A, small GTPase rap1, and protein inhibitor of activated STAT protein, PIASy. Several transcripts have not been previously reported in heart.

Conclusions: Neonatal myocardium has a unique pattern of gene expression, which may result from developmental (age-related) differences or reflect a more severe disease phenotype independent of age effects per se. The neonatal transcript profile seems to reflect a stress-induced protective program composed of genes with functions diametrically opposed to those expected to be related to the pathogenesis of critical right ventricular outflow tract obstruction, thus revealing a novel and compensatory antidisease transcriptional response in the neonatal heart.

Considerable evidence supports the idea that the newborn mammalian heart has intrinsically greater resiliency in response to metabolic and hemodynamic stress than the mature heart, presumably because of a greater repertoire of inducible and cytoprotective gene programs. For example, neonatal rabbit hearts adapted to hypoxia exhibit improved functional recovery in response to a subsequently imposed ischemic stress.¹ This cardioprotective effect has been attributed to in-

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creased activity of endothelial nitric oxide synthase production of nitric oxide in association with increased cyclic guanosine monophosphate (cGMP) levels.² There is also precedence for developmental regulation of hypoxia-inducible factor, a transcription factor that binds under hypoxic conditions to an enhancer element in the 3' region of the erythropoietin gene and for which there are decreasing message levels from fetus to neonate to adult.³ Additional evidence for adaptive hypoxia-induced responses in the neonatal heart include the observed increase in apoptotic threshold in intact rat heart through downmodulation of myocardial ceramide levels,⁴ the energetically favorable reduction in global RNA and protein synthesis in isolated cardiomyocytes,⁵ and the antiapoptotic effects associated with ectopic expression of cdk inhibitors p21CIP and p27KIP1.⁶

The evidence for the developmentally regulated capacity of the immature heart to generate an adaptive response to reduced oxygen availability, although compelling, is derived largely from acute experimental interventions in animal models. Further, the reductionist approach of these studies, which typically address a single target, fails to capture the global complexity of the response. Nevertheless, the general observation that the human neonatal heart copes well with dramatic degrees of hypoxia and hypertrophy is in accordance with these experimental findings. To determine the molecular basis of this putatively adaptive response, we conducted microarray-based differential gene expression profiling on tissue samples acquired in patients of varying ages undergoing repair of right ventricular (RV) obstructive heart lesions, focusing on potential age-related differences.

Methods

Patients

Consent to conduct tissue sample analysis was obtained from the Research Ethics Board of the Hospital for Sick Children, Toronto. Myocardial samples were taken in 24 patients, ranging in age from 6 days to 12.5 years, who were operated on for obstructive heart lesions. The samples were taken from muscular trabeculae of the endocardial surface of the RV infundibulum, were acquired immediately after aortic occlusion and standard blood cardioplegia administration, and were stored in liquid nitrogen. The patients were divided into 2 groups. Group I consisted of 7 patients (2 females and 5 males). Age ranged from 5 to 66 days (mean, 30 days); weight ranged from 2.5 to 4.9 kg (mean, 3.6 kg). The diagnoses included tetralogy of Fallot (TF; $n = 4$), complex transposition ($n = 2$), and truncus arteriosus ($n = 1$). Group II consisted of 17 patients (6 females and 11 males). Age ranged from 4 months to 12.5 years (mean, 2.8 years); weight ranged from 5.18 to 33.5 kg (mean, 11.3 kg). The diagnoses in group II included TF with ($n = 1$) or without ($n = 16$) pulmonary atresia. One patient underwent RV to pulmonary conduit change subsequent to repair of ventricular septal defect and subaortic stenosis by a Damus-Kaye-Stansel procedure.

Gene-Expression Analysis

RNA isolation. Total RNA was isolated from tissue samples by using Trizol reagent according to the protocol outlined by the manufacturer (Gibco/BRL). Briefly, frozen tissues were powdered with a mortar and pestle, cooled in liquid nitrogen, and then further manually homogenized in a microtube by using disposable homogenizers in the presence of the Trizol reagent. RNA concentrations were determined spectrophotometrically at 260 nm, and quality was confirmed by running a 50- to 250-ng aliquot on the Agilent 2100 Bioanalyzer (Palo Alto, Calif). All samples were stored at -70°C until analyzed. Universal Human Reference RNA (Stratagene, La Jolla, Calif) was used as the reference sample for all hybridizations.

Arraying procedure and processing. Microarrays were manufactured at the University of Toronto Microarray Centre (Toronto, Canada) by using complementary DNAs (cDNAs) generated from 19,000 individual cDNAs from Genome Systems (St Louis, Mo). The cDNA inserts were polymerase chain reaction (PCR)-amplified from the pT7T3D-Pac vectors in 96-well format. The name and identification of each of the expressed sequence tags (ESTs) used in the production of the slide can be found at <http://www.microarrays.ca/>.

Hybridization. Resuspended samples of fluorescence-labeled cDNA were added to a hybridization mixture containing 80 μL of DIG Easyhyb (Roche, Mississauga, Canada), 4 μL of yeast transfer RNA (10 $\mu\text{g}/\mu\text{L}$), and 4 μL of salmon sperm DNA (10 $\mu\text{g}/\mu\text{L}$; Sigma, Mississauga, Canada). Samples were heated to 65°C for 2 minutes, cooled briefly, and centrifuged to bring down any condensate that may have accumulated during heating. The entire volume was applied to the microarray, placed in a sealed, humidified hybridization chamber, and incubated overnight at 37°C . Slides were then washed consecutively in $1\times$ standard saline citrate and 0.1% sodium dodecyl sulfate for 30 minutes at 50°C . A final rinse was performed at room temperature in $0.1\times$ standard saline citrate for 5 minutes, and the slides were then centrifuged for 5 minutes at 500 rpm to dry.

Scanning and quantification. Slides were scanned on a scanning laser fluorescence confocal microscope (ScanArray 4000XL; PerkinElmer, Boston, Mass). Individual 16-bit Tagged Image File Format (TIFF) images were obtained by scanning for each of the 2 flours. An overlay image of the 2 images was created and quantified with the QuantArray (version 2.1) program (PerkinElmer). Intensity values for each spot were normalized, and ratios were calculated, resulting in value of patient sample normalized to Universal Human Reference RNA. Individual spots had to pass a number of quality criteria to be included in the data analysis, including a minimum spot/local background intensity greater than or equal to 1, a minimum spot/mean background intensity greater than or equal to 1, and a minimum spot intensity of 100.

Data analysis. Data were stored in and analyzed with the GeneTraffic Microarray Database and Analysis System (Iobion Informatics, La Jolla, Calif), as well as the Significance Analysis for Microarrays program.⁷ Scanned 16-bit TIFF images representing each hybridized microarray slide and the associated quantification data files were entered into the database with a complete annotation of the experiments based on the current Minimum Information About Microarray Experiments standards for microarray experiments (<http://www.mged.org>).

Each hybridization data set was normalized with Lowess subarray normalization (<http://oz.berkeley.edu/tech-reports/>). Lowess normalization uses a local weighted smoother to generate an intensity-dependent normalization function. In subarray normalization, each subarray or grid is normalized individually to correct for variation in local mean signal intensities across the surface of the array.⁸ The resultant normalized log₂ patient/sample intensity ratios were used for statistical analysis. A repeated permutation procedure was performed to ascertain potential statistical differences in gene expression between the 2 age groups.⁷ The median false discovery rate, based on analysis of permuted data sets, was less than 1.0%, and only genes with a minimum 2-fold change in expression were selected. Results from the Significance Analysis for Microarrays analysis were visualized as hierarchical clusters in TreeView (Eisen Lab, Berkeley, Calif).

Validation with real-time PCR. Independent confirmation of increased transcription levels was performed by using real-time quantitative PCR (qPCR) on 4 randomly selected genes that showed increased neonatal expression. Primers were constructed against the 3' ends of fibroblastic growth factor (FGF)-1 (acidic), hepatoma-derived growth factor (HDGF), syntenin, and early growth response (egr-1), and amplicon abundance was determined in real time by SYBR Green Dye (Applied Biosystems, Foster City, Calif) fluorescence measurement during the logarithmic phase and normalized to that of a control gene, cyclophilin. Fold changes for the cyclophilin-normalized value of each transcript were determined as a ratio of sample patient RNA to that of the Universal Human Reference RNA. Multiple regression analysis was performed to compare intergroup differences in transcript fold changes determined by microarray analysis versus qPCR for each of the selected genes.

Two-step reverse transcriptase-PCR was performed as outlined in the SYBER Green PCR Master Mix protocol (Applied Biosystems). To synthesize cDNA, 2 μ g of deoxyribonuclease-treated RNA was added to a reaction mixture containing the following: 8 μ L of 5 $\times$ first-strand buffer (Life Technologies), 1.5 μ L of AncT primer (T₂₀VN), 5 μ mol/L of a 500 μ mol/L deoxynucleoside triphosphate mix (deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate), and 10 mmol/L dithiothreitol. Final reaction volumes were brought up to 40 μ L, and primer annealing was initiated by heating the reaction mix to 65°C for 5 minutes and then 42°C for 5 minutes. Reactions were initiated by the addition of 2 μ L of Superscript II reverse transcriptase (Life Technologies; 200 U/ μ L) and allowed to proceed for 2 hours at 42°C. Primers were designed by using PrimerExpress 2.0 (Applied Biosystems) directed toward the 3' sequence of each clone. Primer sequences were as follows: atrial natriuretic factor (5'-GCTCCTAGGTCAGACCAGAGCTAA; 3'-TTCTTCCAAATGGTCCAGCAA), egr-1 (5'-CCTTCGCTAACCCTCTGTCTA; 3'-TGGGACTGGTAGCTGGTATTGAG), syndecan (5'-AATACATGGCTTGCTGCCTGTT; 3'-GGATGGTACGCTTGCTCTGA), and hepatoma growth factor (5'-CAGTGTCAATTCTCATCCACATACC; 3'-TCTCTCTGTCTCTCAGTGGGTTAC).

Samples were diluted 100-fold and added to the reaction mix containing SYBER Green PCR Master Mix and the appropriate primers to a final volume of 25 μ L. All samples were run in triplicate on the ABI Prism 7900 detection system (Applied Biosystems). All samples were analyzed relative to human cyclophilin.

lin. Calculation of relative quantities of the various products was determined with the relative standard curve method using the ABI Prism 7900 sequenced detection system spectral calibration kit (Applied Biosystems).

Results

All patients survived the surgical procedure and were discharged from the hospital. One neonate with a Taussig-Bing anomaly plus an atrioventricular septal defect required postoperative extracorporeal membrane oxygenation. There were no significant differences in preoperative arterial saturation between the 2 age groups (group I, 79.85% $\pm$ 12.5%; group II, 87.24% $\pm$ 12.9%; $P = .21$). There were no differences in preoperative central venous pressure (group I, 7.2 $\pm$ 2.3 mm Hg; group II, 7.4 $\pm$ 2.6 mm Hg; $P = .85$) or postoperative inotropic support (dopamine: group I, mean, 4.5 $\pm$ 2.74 μ g kg/min; group II, 4.5 $\pm$ 3.01 μ g kg/min; milrinone: group I, 0.53 $\pm$ 0.29 μ g kg/min; group II, 0.26 $\pm$ 0.31 μ g kg/min; $P = .11$).

We found that 38 genes (the term *gene* is used to denote a unique UniGene cluster ID in this discussion) exhibited significantly different expression levels between groups I and II (Figure 1 and Table 1). Thirty-four genes showed increased expression levels in group I, whereas 4 gene elements exhibited repressed transcription in group I in comparison to group II.

Significant genes were searched by using The Stanford Online Universal Resource for Clones and ESTs (SOURCE), which compiles information from several publicly accessible databases, including UniGene, dbEST, Swiss-Prot, GeneMap99, RHDdb, GeneCards, and LocusLink (<http://genome-www5.stanford.edu/cgi-bin/SMD/source/sourceSearch>). Several genes are literature validated as having a protective effect against experimentally induced myocardial ischemia/reperfusion injury, including atrial natriuretic polypeptide (ANP), HDGF, syndecan-binding protein (syntenin), toll-interleukin-1 receptor, and insulin-like growth factor-2 (somatomedin A; indicated with an asterisk in Table 1). Several additional genes have documented antigrowth properties and may be speculated to resist cardiac hypertrophy and promote vasodilation, including the small guanosine triphosphatase rap1, the transcriptional repressor zinc finger protein-7, protein phosphatase-2, ubiquitin-specific protease-15, and egr-1. The finding of decreased transcript expression of FGF-1 (acidic; indicated with a dagger in Table 1) would be predicted to confer additional net antiproliferative effects. Several genes have been previously designated as "fetal" genes, including ANP, HDGF, and keratin, hair, basic, 5. The remaining genes are unprecedented in terms of predicted cardiovascular effects (black typeface in Table 1). Intergroup differences in 4 randomly selected transcripts levels determined by microarray analysis were highly correlated with those determined by qPCR (multiple $R^2 = 0.998$; $P = .001$).

Figure 1. Hierarchical clustering of gene-expression data of the 24 patients operated on for RV outflow tract obstruction. Each row represents a separate cDNA clone on the microarray, and each column represents a messenger RNA (mRNA) sample from a separate patient. Patient mRNA samples are separated in 2 groups as indicated on the top. The results represent the ratio of hybridization of fluorescent cDNA probes prepared from each patient mRNA sample to a reference mRNA sample and are a measure of gene-specific expression levels. Red indicates higher expression and green indicates lower expression relative to the reference sample.

TABLE 1

Confirmed clone ID	Confirmed UniGene cluster ID	Gene name
Up-regulated*		
301819	Hs.75640	Natriuretic peptide precursor A*
245371	Hs.356717	Myosin, light polypeptide 4*
5749628	Hs.356717	Myosin, light polypeptide 4, alkali; atrial, embryonic
4754192	Hs.111779	Secreted protein, acidic, cysteine-rich (osteonectin)
5405119	Hs.119571	Collagen, type III, alpha 1
363968	Hs.111779	Secreted protein, acidic, cysteine-rich (osteonectin)
110787	Hs.226103	Homo sapiens mRNA; cDNA DKFZp564G222
5704539	Hs.75617	Collagen, type IV, alpha 2
301788	Hs.75636	Myosin light chain 2a*
144223	Hs.152931	Lamin B receptor
134272	Hs.89525	Hepatoma-derived growth factor*
6050536	Hs.334842	Tubulin, alpha, ubiquitous
120176	Hs.7940	RAP1, GTP-GDP dissociation stimulator 1†
N/A	Hs.433622	Follistatin-like 1
341679	Hs.182507	Keratin, hair, basic, 5
258999	Hs.119571	Collagen, type III, alpha 1
429072	Hs.2076	Zinc finger protein 7 (KOX 4, clone HF.16)
5740157	Hs.74405	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide
120375	Hs.17681	Toll-interleukin 1 receptor (TIR)*
110331	Hs.108885	Collagen, type VI, alpha 1
502114	Hs.23168	Ubiquitin specific protease 15†
N/A	Hs.75248	Topoisomerase (DNA) II beta 180kDa
182411	Hs.326035	Early growth response 1†
115434	Hs.10739	ESTs
327591	Hs.356350	Hypothetical protein LOC283970
182871	Hs.15725	Immediate early response 5†
111571	Hs.142442	HP1-BP74
184151	Hs.159154	Tubulin, beta, 4
148703	Hs.105779	Protein inhibitor of activated STAT protein PIASy†
4730276	Hs.349109	Insulin-like growth factor 2 (somatomedin A)*
299022	Hs.300711	Annexin A5
154604	Hs.107125	Plasmalemma vesicle associated protein
2463766	Hs.116992	ESTs, moderately similar to A chain A, human homogentisate dioxygenase
271985	Hs.2053	Tyrosinase (oculocutaneous albinism IA)
469697	Hs.80350	Protein phosphatase 2 (formerly 2A)†
N/A	Hs.75975	Signal recognition particle 9kDa
Down-regulated*		
5750213	Hs.177776	Hypothetical protein MGC4276 similar to CG8198
270544	Hs.270956	Chromosome 4 open reading frame 1
282695	Hs.75297	Fibroblast growth factor 1 (acidic)*
138757	Hs.28427	ESTs

*Literature-validated cardioprotective properties.

†Unprecedented cardiovascular properties.

Discussion

Among the estimated 32,000 to 38,000 genes encoded by the human genome, approximately 20,000 to 25,000 are thought to be expressed in the cardiovascular system.⁹ The combinatorial pattern of gene expression in the heart serves to increase the repertoire of responses to pathologic stress, and it is intuitively logical that the capacity for such adaptive responses is inversely related to the fetal-neonatal-adult development gradient. The results of this microarray-based gene expression profiling study seem to confirm the existence of a protective reprogramming response that is most evident in the neonatal myocardium and is subject to the hemodynamic and metabolic stress imposed by structural congenital heart disease.

Disease-specific expression profiles have also implicated unexpected or novel molecular targets in the pathogenesis of human cardiovascular disease, including cardiac hypertrophy,¹⁰ dilated cardiomyopathy,¹¹ and the clinical response to β -blockade in patients with dilated cardiomyopathy¹² (see review¹³). The molecular signatures identified with this approach are typically construed as either mechanistically relevant to the disease pathogenesis or, alternatively, as markers of disease progression. In contrast, we believe that this approach can be used to identify endogenous patterns of gene expression that are activated in response to the primary disease-causing pathway and have the effect of generating a counteracting and highly adaptive pattern of gene activation, which serves to suppress aberrant disease-related molecular pathways. The transcriptional profile in the neonatal group was characterized by increased expression levels of genes with literature-validated antihypertrophic and cytoprotective properties.

Atrial Natriuretic Polypeptide

The effects of ANP are mediated through binding to the A-type natriuretic peptide receptor that activates guanylyl cyclase, leading to the formation of cGMP.^{14,15} Upregulation of ANP expression in occurs in all 4 cardiac chambers in response to acute and chronic hypoxic stress,¹⁶⁻¹⁸ implying that the ANP may represent a hypoxia-inducible gene per se, the regulation of which can occur independently of changes in pulmonary artery pressure and ventricular hypertrophy. The fact that there were no significant differences in saturation levels between the 2 age groups argues that the increased ANP response observed neonatally reflects an age-dependent enhancement to hypoxic signaling rather than a response commensurate with a greater degree of hypoxia. Similarly, the lack of intergroup differences in central venous pressure rules out stretch-induced ANP activation as an explanation for differential expression.

The direct effect of ANP on myocardial ischemia/reperfusion injury is unknown; however, the fact that ANP increases cGMP levels, inhibits proapoptotic p38 mitogen-

activated protein kinase activation, and antagonizes tumor necrosis factor- α -induced changes in the endothelial cell cytoskeleton and prevents macromolecule permeability changes¹⁹ is highly suggestive of a novel cytoprotective effect in this context. Recombinant ANP peptide has been shown to potentiate myocardial ischemic preconditioning through a nitric oxide-dependent mechanism.²⁰ Additional cytoprotective effects may accrue from upregulation of the toll-interleukin-1 receptor²¹ and insulin-like growth factor-2,²² attributable to activation of ischemic preconditioning and antiapoptotic signaling pathways, respectively.

It is unknown whether ANP gene induction in the heart confers a direct cytoprotective effect against excessive, or pathologic, hypertrophic or hypoxic stimuli independently of its vasoreactive and natriuretic properties. Consistent with this prediction, however, are the observations that exogenous or endogenous ANP peptide suppressively regulates the cardiac hypertrophic response in an autocrine/paracrine manner by increasing myocyte cGMP levels in neonatal rat cardiomyocytes *in vitro*²³ and that transgenic mice overexpressing ANP have lower heart weights under normoxic conditions and an attenuated RV hypertrophic response to hypoxia-induced pulmonary hypertension.²⁴

A decline in ventricular ANP gene transcription normally occurs postnatally concurrently with a switch from a right to left ventricular dominant circulation²⁵⁻²⁷; this provides an explanation for neonatal expression of this transcript in the presence of RV outflow tract obstruction. However, the fact that expression levels of 2 other hypertrophy-associated genes, β -myosin heavy chain and endothelin-1, were not found to be differentially expressed in our study, argues that neonatal upregulation of ANP is functionally important and not simply a marker of hypertrophic stress. We speculate that ANP gene activation in the context of neonatal obstructive heart disease may serve to mitigate excessive hypertrophic signaling and protect against the transition from physiological to pathologic hypertrophy.

Protein Phosphatase-2A

Transgenic mice overexpressing protein phosphatase-2A exhibit reduced cardiac contractility and progressive ventricular dilation, an effect that may serve to mitigate the concentric hypertrophic response inherent in neonatal TF²⁸ and that may be attributable to protein phosphatase-2A-mediated antagonism to calcium calmodulin-dependent protein kinase activity.²⁹ In vascular smooth muscle cells, protein phosphatase-2A inhibits platelet-derived growth factor-BB-mediated phosphorylation of BAD and forkhead transcription factor FKHR-L1, and this effect correlates with increased apoptosis.³⁰ Further, protein phosphatase-2A is predicted from sequence similarity to cause deactivating dephosphorylation of the carboxy terminal domain of RNA polymerase II and would be predicted to be limiting to

cardiac hypertrophic growth.³¹ Thus, antihypertrophic signaling described for this phosphatase may thus be predicted to complement favorable cardiac vascular remodeling attributable to increased ANP.

Early Growth Response-1

egr-1 is a zinc finger transcription factor that exerts opposing effects depending on the latency of the measured response and the contextual pattern of co-regulated gene expression. For example, growth factors and cytokines including platelet-derived growth factor, angiotensin II, tumor necrosis factor- α and - β , and interleukin-1 β increase the egr-1 message within 15 minutes,³² which, in turn, activates transcription of several genes implicated in the pathogenesis of vascular diseases, including tumor necrosis factor- δ ,³³ platelet-derived growth factor (PDGF),³⁴ interleukin-2,³² and FGF,³⁵ producing a positive amplification loop that favors smooth muscle cell proliferation.

Conversely, egr-1 exerts a counterregulatory effect through a sustainable transactivation of peroxisome proliferator-activated receptor- γ 1, itself a ligand-activated nuclear transcription factor that potently suppresses growth factor- and cytokine-mediated signaling in vascular smooth muscle,^{32,36} possibly accounting for the reduced FGF message observed in the neonatal group. Thus, in addition to mitigation of hypertrophic cardiomyocyte signaling, coordinated expression of ANP, egr-1, and protein phosphatase-2A would be predicted to favor vascular smooth muscle regression promoting coronary vasodilation and having the effect of augmenting oxygen delivery to hypertrophic, hypoxically perfused myocardium. Although they are not literature-validated as cardiac targets, increased neonatal expression of the small guanosine triphosphatase rap1, which inhibits the extracellular signal-related kinase signaling cascade,³⁷ and the transcriptional repressor zinc finger protein-7³⁸ could plausibly further limit hypertrophic responses in the hemodynamically stressed heart.

Several genes differentially expressed in the neonatal group conceivably augment the capacity for matrix remodeling and cellular regeneration, including the re-expression or, more likely, persistence of "fetal" genes, implying parallels between physiological fetal and stress-induced tissue remodeling. The gene specifying secreted protein, acidic, rich in cysteine (osteonectin) is a developmentally regulated matricellular protein involved in wound healing and tissue repair³⁹ and may promote favorable extracellular matrix remodeling.⁴⁰ Hepatoma-derived growth factor is a nuclear-targeted growth factor conspicuously expressed in embryonic ventricular myocytes, endocardium, and cells of the ventricular outflow tract, implying a role in cardiovascular growth and differentiation.⁴¹ Although not specified as a fetal gene, egr-1 may promote adaptive matrix remodeling by virtue of its capacity to stimulate angiogenesis,⁴² endo-

thelial production of membrane type 1 matrix metalloproteinase,⁴³ and accrual of fibrin stroma through upregulation of plasminogen activator inhibitor-1.^{44,45} egr-1-mediated upregulation of plasminogen activator inhibitor-1 (also known as serpine-1)⁴⁴ may serve an important adaptive function by increasing the fibrin stroma on which neoangiogenesis and tissue repair may take place.⁴⁵

An unexpected finding of global gene analysis of heart tissue in this study was the evidence for antigrowth properties of several transcripts, including the tumor-suppressor genes egr-1, ubiquitin-specific protease-15, and the transcriptional repressor zinc finger protein 7, in concert with reduced levels of FGF-1 messenger RNA. This antigrowth program is thematically consistent with a greater compensatory reaction to hyperproliferative signals in the immature heart, which may serve to protect against the development of pathologic interstitial fibrosis.

Limitations of the Study

Need for independent validation of microarray data. Technical and biological sources of variation are inherent in microarray experiments. Our study design incorporated biological replication (based on analysis of multiple patients) rather than performing multiple technical (array) replicates. This design has been recommended to maximize degrees of freedom and statistical precision in studies of this nature.⁴⁶ Further, the correlation between duplicate spots used in our arrays exceeded 96% among the significant genes, although this does not necessarily guarantee interhybridization reproducibility across different arrays.⁴⁶ The statistically high correlation of transcript levels determined with microarray and qPCR methods provides strong support for the validity of our findings.

We used the false discovery rate, which determines the percentage of positive calls that are false positives. This parameter is computed by permutation analysis, which compares the observed differences in gene-specific intensity values between the 2 age groups with that expected from random assignment of values between the 2 age groups. The false discovery rate is a tunable parameter and was set to less than 1%. This approach is considered preferable to *P* value calculations, which, in microarray data analysis, necessitate the use of multiple comparison adjustment algorithms.⁷

Lack of homogeneous patient diagnosis. Although all patients in the neonatal group had RV outflow tract obstruction, 2 had complex transposition and 1 had truncus arteriosus. The neonatal group thus differed in both age and diagnosis from the older cohort group, and the neonatal patients may represent a more severe disease phenotype. Regardless of whether the transcriptional response in the neonate results from immaturity or, alternatively, disease-specific factors, the biological properties of the neonatal

expression pattern are plausibly suppressive to cardiac hypertrophy. The fact that the differentially expressed gene elements thematically clustered into genes predicted to have cardioprotective properties, often consistent with a less differentiated (fetal) state, itself lends further validity to the biological significance of our findings. It is also noteworthy that unsupervised clustering analysis (ie, without a priori age stratification) correctly classified 6 of the 7 youngest patients (data not shown).

The constituent cell types in the heart that are responsible for the observed gene expression patterns are not discernible from this study, nor is it known whether the tissue composition of cardiomyocytes, fibroblasts, endothelial cells, and trafficking cells is the same in both patient groups. Finally, the interpretation of these microarray data is speculative and requires orthogonal validation through experimentation or in silico comparative analysis by using age- and diagnosis-specific, standards-compliant data sets.

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Discussion

Dr Peter J. Gruber (Philadelphia, Pa). I compliment Dr Konstantinov, Dr Coles, and their Toronto colleagues on their near-comprehensive analysis of age-related gene expression patterns in children with right heart obstructive lesions. The incorporation of contemporary molecular techniques, those that are now standardized in other scientific disciplines, to cardiac surgery provides powerful tools to advance understanding of congenital heart disease and especially its interaction with surgery at a basic level. Genomic, naive approaches such as those just described in comparison to candidate-based techniques allow one to identify unforeseen molecules and thus provide the opportunity for new testable hypotheses.

It is in this hypothesis generation, rather than discrete findings, that expression profiling is generally most valuable. I have 3 brief questions. One limitation of the study is the use of single arrays rather than replicates. Nonrandomization of spotting on arrays in addition to interchip variability can have profound effects on expression levels. Although qPCR confirmation of selected targets was performed, I'm interested to know whether this was considered in other ways.

Second, I reluctantly bring up the issue of correlation, or rather the lack of correlation, between messenger RNA (mRNA) levels, protein levels, and function. Considerable work has been done in yeast and other organisms demonstrating that mRNA levels are in fact insufficient to predict protein expression levels in any way. Additionally, protein levels, although 1 step closer to physiology,

only partially correlate with function because conservatively, there are at least 20 modifications such as phosphorylation, lipidation, glycosylation, or sumoylation per protein. So I would be interested to know how you interpret your work in light of the lack of predictive value of mRNA with respect to function in any individual gene.

Last, given the hypotheses generated with respect to the current study, I would like to know what you think are your most promising leads, and of those, which ones would you follow up in which direction?

Dr Coles. Thank you, Dr Gruber, for your insightful questions. Regarding the use of single arrays, we relied on biological replicate variability by including as many patients as we could, and using that approach effectively eclipses the need for multiple technical arrays.

We also spotted each cDNA array in duplicate or quadruplicate, and we found a very high concordance among the expression levels in each of the duplicate spots, and that, coupled with the PCR results, gave us confidence that we were getting bona fide results.

Your question is very germane regarding protein analysis. As you point out, posttranslational modifications may affect the ultimate phenotype in a way that's not necessarily predicted by the mRNA. We think, however, that the transcriptional response by itself can be informative and clearly was different between the newborn heart and the older cohort patients. Regardless of which particular approach is used, I certainly agree with the need for independent validation and clinical corroboration of any of the putative targets that we found.

In response to your final question as to follow-up, it is a very challenging problem to do target triage to pick the leads that you think are going to be best, recognizing that in many cases, it is really the combinatorial pattern of genes that affects the ultimate phenotype. So it is hard to pick one out in isolation.

But I did mention that ANP has already been identified, and actually brain natriuretic peptide, its analog, turns out to be a blockbuster drug for left ventricular heart failure. So this does suggest, at least by circumstantial evidence, that some of these targets will have a potential clinical impact.

We are intrigued personally with the phosphatase theme. Following up that in terms of drug discovery and downstream analysis, it is a very difficult, specialized project. Delivery of phosphatases, since they are large proteins, becomes an issue. The best

approach is to try and find drugs that are receptors that can be given systemically.

We don't yet have any results with that approach, but that's really phase II target validation, which we look forward to doing.

Dr Richard A. Jonas (*Boston, Mass*). John, did you have enough spread of oxygen levels to get a sense as to whether the gene expression changes that you were seeing were in response to pressure load hypertrophy versus cyanosis?

Dr Coles. No. The saturation levels were a little bit lower in the neonatal group, suggesting that there may be a bias there, but the differences weren't statistically significant.

The other thing is, is it really a complex stress, as you imply it is? There is hypertrophy, there is hypoxia, and there are any number of metabolic factors acting on these patients, and so is it really an oversimplification to say that the differences are due to age per se?

Dr Bradley Allen (*Houston, Tex*). That was sort of my question. Can you really say it is age versus stress since the stresses are so much different in the neonate compared to the older cohort? I would think the only way to prove this would be if the neonates that underwent a greater degree of stress, such as hemodynamic instability, lower O₂ saturation, etc, had more gene expression. Otherwise, you can't say how much of the change might be due to differences in stresses versus age.

Dr Coles. I would certainly agree with that. In fact, I would likely hazard to guess that the newborn hearts were in fact at the more severe spectrum of disease, and that's why they presented in the neonatal period, and therefore they had a higher stress.

Nevertheless, our interpretation was that greater disease severity would elicit a commensurately greater antistress response, and this was reflected, if you will, in the higher dose of protective genes that we found.

The higher stress or the young age, whatever the explanation, is, in a sense, irrelevant. What we are looking for is a way to exploit the differences that would identify cytoprotective properties.

Dr Jonas. John, these arrays are quite expensive. Roughly what sort of cost are you looking at for this type of array?

Dr Coles. Well, this was sort of a homegrown academic thing, and it was free for us through our academic collaborators.

We used spotted glass arrays slides. Most people seem to be converging on the Affymetrix platform, and they are about US\$600 per array.