Gene expression profile after cardiopulmonary bypass and cardioplegic arrest

Marc Ruel, MD, MPH^a
 Cesario Bianchi, MD, PhD^a
 Tanveer A. Khan, MD^a
 Shu Xu, PhD^a
 John R. Liddicoat, MD^a
 Pierre Voisine, MD^a
 Eugenio Araujo, PhD^a
 Helen Lyon, MD^b
 Isaac S. Kohane, MD, PhD^c
 Towia A. Libermann, PhD^d
 Frank W. Sellke, MD^a

Objective: This study examines the cardiac and peripheral gene expression responses to cardiopulmonary bypass and cardioplegic arrest.

Methods: Atrial myocardium and skeletal muscle were harvested from 16 patients who underwent coronary artery bypass grafting before and after cardiopulmonary bypass and cardioplegic arrest. Ten sample pairs were selected for patient similarity, and oligonucleotide microarray analyses of 12,625 genes were performed using matched precardiopulmonary bypass tissues as controls. Array results were validated with Northern blotting, real-time polymerase chain reaction, in situ hybridization, and immunoblotting. Statistical analyses were non-parametric.

Results: Median durations of cardiopulmonary bypass and cardioplegic arrest were 74 and 60 minutes, respectively. Compared with precardiopulmonary bypass, post-cardiopulmonary bypass myocardial tissues revealed 480 up-regulated and 626 down-regulated genes with a threshold *P* value of .025 or less (signal-to-noise ratio: 3.46); skeletal muscle tissues showed 560 and 348 such genes, respectively (signal-to-noise ratio: 3.04). Up-regulated genes in cardiac tissues included inflammatory and transcription activators FOS; jun B proto-oncogene; nuclear receptor subfamily 4, group A, member 3; MYC; transcription factor-8; endothelial leukocyte adhesion molecule-1; and cysteine-rich 61; apoptotic genes nuclear receptor subfamily 4, group A, member 1 and cyclin-dependent kinase inhibitor 1A; and stress genes dual-specificity phosphatase-1, dual-specificity phosphatase-5, and B-cell translocation gene 2. Up-regulated skeletal muscle genes included interleukin 6; interleukin 8; tumor necrosis factor receptor superfamily, member 11B; nuclear receptor subfamily 4, group A, member 3; transcription factor-8; interleukin 13; jun B proto-oncogene; interleukin 1B; glycoprotein Ib, platelet, alpha polypeptide; and Ras-associated protein RAB27A. Down-regulated genes included haptoglobin and numerous immunoglobulins in the heart, and factor H-related gene 2, protein phosphatase 1, regulatory subunit 3A, and growth differentiation factor-8 in skeletal muscle.

Conclusions: By establishing a profile of the gene-expression responses to cardiopulmonary bypass and cardioplegia, this study allows a better understanding of their effects and provides a framework for the evaluation of new cardiac surgical modalities directly at the genome level.

From the Division of Cardiothoracic Surgery, Beth Israel Deaconess Medical Center^a and Harvard Medical School, and Divisions of Medical Genetics^b and Informatics Program,^c Children's Hospital and Harvard Medical School, and the Genomics Center,^d Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Mass.

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Address for reprints: Frank W. Sellke, MD, Division of Cardiothoracic Surgery, Beth Israel Deaconess Medical Center, LMOB 2A, 110 Francis St, Boston, MA 02215 (E-mail: fsellke@bidmc.harvard.edu).

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Cardiopulmonary bypass (CPB) and cardioplegic arrest are used in more than 300,000 patients who undergo cardiac surgery in the United States every year. Although off-pump coronary surgery has recently gained in popularity, CPB and cardioplegic arrest are still employed in more than 75% of coronary artery bypass grafting (CABG) procedures and for virtually every other type of adult cardiac operation. Although these modalities are indispensable adjuncts, they are also paradoxically responsible for a large part of the morbidity associated with cardiac surgery. CPB is associated with disturbances of virtually every organ system, mediated by its elicitation of a whole-body inflammatory response.¹⁻⁴ Cardioplegic arrest may result in coronary vasomotor dysfunction, ischemia-reperfusion injury, myocardial edema, perioperative myocardial infarction, and low cardiac output syndrome.⁵⁻⁷

Transcriptional profiling using high-density microarrays provides unique data about disease mechanisms, drug responses, regulatory pathways, and gene function by comparing the level of mRNA transcribed in cells in a given pathologic state versus a control. This allows for the elucidation of complex pathophysiologic mechanisms directly at the gene expression level.⁸ We examined the cardiac and skeletal muscle gene expression profiles of patients undergoing cardiac surgery with CPB and cardioplegic arrest to improve the understanding of these modalities and provide a reference framework for the evaluation of prognostic gene markers and new intraoperative strategies.

Methods

Tissue Samples

After the protocol was approved by the research ethics committee of the Beth Israel Deaconess Medical Center and informed consent was obtained from the patients, samples of right atrium and skeletal muscle were collected immediately before and after CPB in 16 patients undergoing primary elective CABG. Samples were harvested with cold sharp dissection and handled in a nontraumatic fashion.

Right atrial samples consisted of nontraumatized tissue that was not involved in a purse string suture. Pre-CPB tissues were taken from the right atrial appendage before venous cannulation, and post-CPB atrial samples were harvested after the construction of a second purse string suture on the right atrial appendage and decannulation. Skeletal muscle samples were taken from the left intercostal muscle bed, lateral to the internal thoracic artery harvest site. Atrial and skeletal muscle samples ($\sim 10 \times 10 \times 2$ mm) were snap-frozen in liquid nitrogen and immediately stored at -80°C .

To minimize baseline phenotypic variation, 5 patients aged between 55 and 75 years, who were normocholesterolemic, non-smoking, nondiabetic, of the same ethnicity and gender, and who had similar procedural characteristics, were selected from the 16 subjects whose tissues were harvested; 10 atrial (ie, 5 pre- and 5 post-CPB and cardioplegic arrest) and 10 skeletal muscle (ie, 5

TABLE 1. Patient characteristics

	Atrial myocardial (N = 5) and skeletal muscle (N = 5) samples*
Age (yr)†	66 (55, 72)
Men/women, n	5/0
Hypertension, n	5
Current tobacco use, n	0
Cholesterol > 200 mg/dL, n	0
Diabetes, n	0
Redo or combined cardiac procedure, n	0
Number of grafts performed†	3 (3, 4)
Duration of CPB (min)†	74 (67, 99)
Duration of cardioplegic arrest (min)†	60 (44, 83)
Minimum CPB temperature ($^\circ\text{C}$)†	34 (33, 35)
Intraoperative blood transfusion, n	0
Postoperative length of stay (d)†	6 (4, 9)

CPB, Cardiopulmonary bypass.

*One tissue sample was collected from each site before and after CPB in each patient.

†Data expressed as median (minimum, maximum).

pre- and 5 post-CPB) samples obtained from these patients were therefore used for the microarrays analyses and the validation studies (Table 1).

RNA Isolation

Total RNA was isolated with a Trizol-based method, following the manufacturer's protocol (Gibco BRL, Rockville, Md), on samples of approximately 200 mg. Purification was achieved by passing the RNA samples through an RNeasy column (Qiagen, Valencia, Calif). Residual DNA contamination was eliminated by DNase I treatment (Invitrogen, San Diego, Calif). RNA concentrations were determined by spectrophotometry; each sample yielded a minimum of 5 μg of total RNA at a minimum concentration of 0.5 $\mu\text{g}/\mu\text{L}$ with an A_{260}/A_{280} ratio ranging between 1.8 and 2.1.

Microarray Processing

cDNA was prepared according to protocols provided with the Affymetrix U95 GeneChip system (Affymetrix, Santa Clara, Calif).⁹ Five micrograms of total RNA were used in the first-strand cDNA synthesis with T7-d(T)₂₄ primer (GGCCAGTGAATTG-TAATACGACTCACTATAGGGAGGCGG-[dT]₂₄) and Superscript II (Gibco-BRL, Gaithersburg, Md). The second-strand cDNA synthesis was performed at 16°C by adding *Escherichia coli* DNA ligase, DNA polymerase I, and RNase H to the reaction, followed by T4 DNA polymerase to blunt the ends of newly synthesized cDNA. The cDNA was purified through phenol/chloroform and ethanol precipitation. By using a BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY), the purified cDNA was incubated for 5 hours at 37°C in an in vitro transcription reaction to produce cRNA-labeled with biotin.

Microarray Hybridization

cRNA was fragmented by incubating in a buffer containing 200 mmol/L Tris-acetate, 500 mmol/L KOAc, and 150 mmol/L

MgOAc for 35 minutes at 94°C. Fifteen micrograms of fragmented cRNA were mixed with eukaryotic hybridization controls (containing control cRNA and oligonucleotide B2) and hybridized with a pre-equilibrated human U95Av2 Affymetrix chip for 16 hours at 45°C. The chips were washed in a fluidic station with low-stringency buffer (6× standard saline phosphate with EDTA, 0.01% Tween 20, and 0.005% antifoam) for 10 cycles and in high-stringency buffer (100 mmol/L N-morpholino-ethanesulfonic acid, 0.1 mol/L NaCl, and 0.01% Tween 20) for 4 cycles and stained with streptavidin phycoerythrin. This process was followed by incubation with normal goat immunoglobulin G and biotinylated mouse anti-streptavidin antibody, and restaining with streptavidin phycoerythrin.

Statistical Analysis of Microarray Data

The chips were scanned in an HP G2500A ChipScanner (Affymetrix) to detect hybridization signals. Image output files were visually examined for major chip defects and hybridization artifacts, and raw data were processed with GeneChip Microarray Analysis Suite 5.0 software (Affymetrix). The image from each chip was scaled such that the absolute signal intensities were adjusted to target intensity and reported as a non-negative numerical quantity. Data were imported into SAS version 7 (SAS, Cary, NC) for further analysis. Statistical techniques were separately applied to each type of tissue and involved tests that were paired for each patient.

Median post- to pre-CPB and cardioplegic arrest gene expression ratios were computed for each gene by using the relative signal intensities of the probe cells. Two statistical methods were combined to assess differential gene expression between the pre- and post-CPB and cardioplegic arrest tissues.¹⁰ To ensure a minimal number of false positives, only the probe sets commonly yielded by the 2 methods were included in the list of genes differentially expressed in post- versus pre-CPB and cardioplegic arrest samples.

Method 1 identified probe sets with significant intensity differences between pre- and post-CPB and cardioplegic arrest tissues. For each tissue type, a Wilcoxon signed-rank test was applied to the absolute signal intensities of each gene in the pre- versus post-CPB and cardioplegic arrest data set. The use of a nonparametric, paired Wilcoxon test was selected over that of a standard *t* test to avoid a distributional assumption;^{11,12} the threshold was set at a *P* value of .0253 or less, which corresponds to the lowest possible *P* value in the particular experimental setting of the study (observed when 5 of 5 patients show a similar pattern of differential expression). By using this *P* value, the number of genes that could have reached significance by chance alone was determined and compared with the number of observed significant genes, and a signal-to-noise ratio was computed.

Method 2 determined the differentiability of a probe set by its signal intensity fold change. Nonparametric determination of genes with significantly different fold changes between the pre- and post-CPB and cardioplegic arrest states was performed by computing and ordering by increasing size the median relative signal intensities of each probe set. A particular gene was considered to be up-regulated after CPB and cardioplegic arrest in a given tissue type if a median fold change of 4 (4:1) or more was observed in post- versus pre-CPB and cardioplegic arrest samples.

Conversely, a gene was considered down-regulated if the median fold change of post- versus pre-CPB and cardioplegic arrest samples was 0.25 (1:4) or less. Only genes identified by both methods were considered to be differentially expressed and were reported.

Microarray Validation

Northern analysis. cDNA probes of human DUSP1 and CYR61 (2 significantly altered genes), and GADP and β -actin (2 genes that were not altered) were labeled with $\alpha^{32}\text{P}$ -dCTP (New England Nuclear, Boston, Mass) using a random-priming labeling kit (Boehringer, Indianapolis, Ind) and purified from unincorporated nucleotides using G-50 Quick Spin Columns (Boehringer). The blots were hybridized overnight at 48°C using an Ambion Northern Max kit (Ambion, Austin, Tex). After hybridization, the blots were washed twice in 2× standard saline citrate with 0.1% sodium dodecyl sulfate (SDS) for 15 minutes each at room temperature and then washed twice in 0.1× standard saline citrate with 0.1% SDS for 15 minutes each at 60°C. Autoradiography was performed for 20 hours at -80°C. Blots were analyzed after digitization and quantification of x-ray films with the Image-Quant software (Molecular Dynamic, Sunnyvale, Calif), quantified as a ratio of the gene mRNA to loading band density, and presented as a median (minimum, maximum) ratio of post- to pre-CPB and cardioplegic arrest mRNA expression.

Real-time polymerase chain reaction. Reverse transcription was performed on 2 μg of total RNA isolated from atrial and skeletal muscle tissues, using an oligo dT primer (Invitrogen) for 90 minutes at 37°C. DUSP1 primers were forward-TGGAG-GAAGGGTGTGTTGTCC, reverse-CAAGGCAGATGGTG-GCTGA, and TaqMan-FAM-CTGCCAGGCAGGCATTTCCCG-TAMRA (Applied Biosystems, Foster City, Calif). CYR61 primers were forward-ATCAAGGACCCCATGGAGG, reverse-AGGCATCGAATCCCAGCTC, and TaqMan-FAM-CCAG-GACGGCCTCCTTGGCAA-TAMRA (Applied Biosystems). Amplification conditions were as follows: stage 1, 1 cycle for 2 minutes at 50°C; stage 2, 1 cycle for 10 minutes at 95°C; stage 3, 40 cycles for 15 seconds at 95°C and 1 minute at 60°C. Real-time detection was accomplished using SYBR Green I dye and fluorescence quantified with a GeneAmp Sequence Detection System (Applied Biosystems). All experiments were performed in duplicate, and post- to pre-CPB and cardioplegic arrest ratios of polymerase chain reaction (PCR) products are presented as median (minimum, maximum) ratios.

In situ hybridization. Probes for human DUSP1 and CYR61 were generated from PCR fragments containing T3 (forward) and T7 (reverse) promoter sequences incorporated in the amplicon. Frozen sections of 10 μm were cut in a cryostat and captured onto microscope slides. Sections were fixed and acetylated, and hybridized at 68°C to the probe for 3 nights. Hybridized probes were visualized using alkaline phosphatase-conjugated anti-DIG Fab fragments and 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium substrate (Kierkegard and Perry, Gaithersburg, Md). Sections were rinsed several times in 100 mmol/L Tris, 150 mmol/L NaCl, and 20 mmol/L EDTA pH 9.5, and coverslipped with glycerol gelatin. Control sections were incubated in an identical concentration of the sense probe transcript. In situ hybridization autoradiographs were evaluated qualitatively in random high-power fields.

TABLE 2. Genes exhibiting a fourfold or greater increase in expression in post- versus pre-cardiopulmonary bypass and cardioplegic areas atrial samples

Gene name	Symbol	GenBank accession number	Expression ratio (post:pre)*	Function
Proto-oncogene C-FOS	FOS	K00650	20:1	Pro-apoptotic transcription factor, acting with JUN
Nuclear receptor subfamily 4, group A, member 1	NR4A1	L13740	19:1	Steroid receptor; regulates apoptosis
Proto-oncogene JUN-B	JUNB	M29039	17:1	Similar to JUN; pro-apoptotic transcription factor acting with FOS
Nuclear receptor subfamily 4, group A, member 2	NR4A2	X75918	15:1	Coactivator of transcription; stimulated by PGE2, IL1B, and TNF
Dual-specificity phosphatase 1	DUSP1	X68277	12:1	Regulator of the cellular response to stress
Zinc finger transcriptional regulator	ZFP36	M92843	10:1	Component of a negative feedback loop that interferes with TNF production
Transcription factor 8	TCF8	D15050	10:1	Negative regulator of T-lymphocyte IL2 gene expression
Nuclear receptor subfamily 4, group A, member 3	NR4A3	D78579	7:1	Transcription factor
Oncogene FOS-B	FOSB	L49169	7:1	Transcription factor; its truncated form may act to limit the transcriptional effects of FOS and JUN
Solute carrier family 2, member 3	SLC2A3	M20681	6:1	Facilitation of glucose transport in tissues with a high glucose demand
Cellular oncogene C-FOS	FOS	V01512	6:1	Pro-apoptotic transcription factor, acting with JUN
Dual-specificity phosphatase 5	DUSP5	U15932	6:1	Protein-tyrosine phosphatase induced by serum stimulation and heat shock
MADS box transcription enhancer factor 2	MEF2C	S57212	6:1	Transcription factor
Cyclin dependent kinase-like 1	CDKL1	X6358	6:1	Cell cycle regulator
Small inducible cytokine A2	SCYA2	M26683	6:1	Chemotactic factor for monocytes; regulated by NF-kappa-B
Oncogene MYC	MYC	V00568	5:1	Transcription factor
B-cell translocation gene 2	BTG2	U72649	5:1	Cell cycle regulator in response to DNA damage
T-cell cytokine receptor	TCCR	AF053004	5:1	Class I cytokine receptor; involved in the early immune response
Plasminogen activator receptor, urokinase-type	PLAUR	U09937	5:1	Involved in neutrophil recruitment
Activating transcription factor 3	ATF3	L19871	5:1	Transcription factor
Early growth response 2	EGR2	J04076	5:1	Transcription factor
Argininemia gene	ARG1	M14502	5:1	Hydrolyzes arginine to urea and ornithine in the urea cycle
Sortilin 1	SORT1	X98248	5:1	Involved in sorting proteins to endosomal vesicles and golgi
E-selectin	SELE	M24736	4:1	Adhesion of leukocytes to cytokine-stimulated endothelial cells
Kappa casein	CSN10	M73628	4:1	Unknown
Cytokine GRO2	GRO2	M36820	4:1	Chemotactic agent for polymorphonuclear leukocytes; regulated by IL1B, TNF, and LPS
Cysteine-rich angiogenic inducer 61	CYR61	Y11307	4:1	Immediate-early transcription factor; promotes adhesion of endothelial cells, chemotaxis, and angiogenesis
S100 calcium-binding protein A4	S100A4	M80563	4:1	Elevated serum levels have been correlated with cerebral damage after cardiopulmonary bypass and circulatory arrest
Histone H1F3	H1F3	M60747	4:1	Basic nuclear proteins responsible for the nucleosome structure
Fibronectin 1	FN1	AJ001487	4:1	Stimulates endocytosis and clearance of C1q-coated material such as immune complexes, cellular debris, or particulate material from the circulation
mRNA export protein 1	RAE1	U85943	4:1	Nucleocytoplasmic transport of mRNAs
Cyclin-dependent kinase inhibitor 1A	CDKN1A	U03106	4:1	Associated with apoptosis of human endothelial cells

nF, Nuclear factor; *IL*, interleukin; *TNF*, tumor necrosis factor; *LPS*, Lipopolysaccharide.

*Median expression ratio of post-CPB versus pre-CPB samples.

TABLE 3. Genes exhibiting a fourfold or greater decrease in expression in post- versus pre-cardiopulmonary bypass and cardioplegic arrest atrial samples

Gene name	Symbol	GenBank accession number	Expression ratio (post:pre)*	Function
Immunoglobulin gamma 3	IGHG3	Y14737	1:47	Heavy chain immunoglobulin
Annexin A8	ANXA8	X16662	1:23	Vascular anticoagulant beta; function in heart unknown
Immunoglobulin kappa constant region	IGKC	M63438	1:18	Light chain immunoglobulin
Immunoglobulin lambda constant region 1	IGLC1	M18645	1:16	Light chain immunoglobulin
Haptoglobin	HP	X00442	1:16	Binds free hemoglobin and protects against bacterial growth
Immunoglobulin mu	IGHM	X67301	1:16	Heavy chain immunoglobulin
Immunoglobulin lambda-like light chain 3 (incomplete sequence)	—	A1932613	1:9	Light chain immunoglobulin
Keratin 7	KRT7	AJ238246	1:7	Unknown
Protein tyrosine phosphatase, non-receptor, type 2	PTPN2	M25393	1:7	Regulator of cell growth and differentiation
Centrin 1	CETN1	U03270	1:6	Involved in centrosome segregation and microtubule severing
Immunoglobulin alpha constant heavy chain 2	IGHA2	S71043	1:6	Heavy chain immunoglobulin
Properdin factor B	BF	L15702	1:6	Serine protease; C3 proaccelerator
Hexosaminidase B	HEXB	M23294	1:6	Ganglioside breakdown
Cyclin E2	CCNE2	AF091433	1:5	DNA synthesis initiator
Tumor necrosis factor receptor superfamily, member 7	TNFRSF7	M63928	1:5	Cell growth, differentiation, and apoptosis
Immunoglobulin lambda constant region	IGLC	X57809	1:5	Light chain immunoglobulin
Kallikrein 11	KLK11	AB012917	1:5	Serine protease
RAS inhibitor	RIN1	L36463	1:4	Inhibitor of RAS-mediated cell growth and differentiation
Carcinoembryonic antigen-related cell adhesion molecule	CEACAM1	X16354	1:4	Member of the immunoglobulin superfamily
Haptoglobin-related protein	HPR	X89214	1:4	Similar to haptoglobin
Immunoglobulin J chain	IGJ	A1660656	1:4	Synthesized in B lymphocytes; links immunoglobulin monomers
RAL guanine nucleotide dissociation stimulator	RALGDS	AB002349	1:4	RAS-related GTPases; cell signaling
Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	PCK2	X92720	1:4	Gluconeogenesis
Mast cell carboxypeptidase A	MC-CPA	M73720	1:4	Unknown
DMX-like 1 protein	DMXL1	AJ005821	1:4	Protein-protein interactions and regulation
Neuronal nicotinic acetylcholine receptor alpha-7 subunit	CHRNA7	X70297	1:4	Alpha 7 subunit of the neuronal nicotinic acetylcholine receptor

*Median expression ratio of post-CPB versus pre-CPB samples.

Immunoblotting. Total lysate from atrial and skeletal muscle tissues was obtained by 30-second homogenization on ice in lysis buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor, and centrifuged at 12,000g for 10 minutes at 4°C to separate soluble from insoluble proteins. The supernatant protein concentration was measured spectrophotometrically at 595 nm wavelength using a BCA protein assay kit (Pierce, Rockford, Ill). Total protein was fractionated on 10% SDS-polyacrylamide gel electrophoresis and transferred to a poly-

vinylidene difluoride membrane (Millipore, Bedford, Mass) using a semi-dry transfer apparatus. Membranes were stained with Ponceau S and incubated with 5% nonfat dry milk in 50 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, and 0.1% Tween 20 (TBST) buffer for 1 hour at room temperature. Membranes were incubated with antibodies (in 2.5% nonfat dry milk in TBST) (anti-DUSP1 1:1000 [v/v] dilution, rabbit polyclonal 1:1000 [v/v], or anti-CYR61 1/1000 [v/v] dilution), and rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, Calif) for 2 hours. After washing with

TABLE 4. Genes exhibiting a fourfold or greater increase in expression in post- versus pre-cardiopulmonary bypass skeletal muscle samples

Gene name	Symbol	GenBank accession number	Expression ratio (post:pre)*	Function
Interleukin 6	IL6	X04430	20:1	Mediates leukocytosis, thrombosis, and lymphocyte activation
Interleukin 8	IL8	M28130	18:1	Mediates neutrophils chemotaxis and migration
Tumor necrosis factor receptor superfamily, member 11B	TNFRSF11B	AB008822	13:1	Secreted by T cells; mediates inflammation
Nuclear receptor subfamily 4, group A, member 3	NR4A3	D78579	9:1	Transcription factor
Nuclear receptor subfamily 4, group A, member 1	NR4A1	L13740	8:1	Steroid receptor; regulates apoptosis
Transcription factor 8	TCF8	D15050	6:1	Negative regulator of T-lymphocyte IL2 gene expression
Early growth response 3	EGR3	X63741	6:1	Immediate-early transcription activator; induced by stress or injury
Cholesterol 25-hydroxylase	CH25H	AF059214	6:1	Regulation of cholesterol and lipid metabolism
T-cell receptor interacting molecule	TRIM	AJ224878	6:1	Associates with the TCR-CD3 complex in T lymphocytes after T-cell activation
Endothelin 1	EDN1	J05008	6:1	Potent vasoconstrictor produced by endothelial cells
Transforming growth factor-beta receptor, type 1	TGFBR1	AF054598	6:1	Proliferation and differentiation of fibroblasts
Interleukin 13	IL13	U31120	6:1	Expressed in activated human T lymphocytes; synergizes with IL2 in regulating inflammatory and immune responses
Ephrin B2	EFNB2	U81262	6:1	Angiogenesis and vascular remodeling
Activating transcription factor 2	ATF2	L05515	5:1	Transcription factor
T-cell tyrosine kinase EMT	EMT	L10717	5:1	TCR-mediated CD4 stimulation
Oncogene JUN-B	JUNB	X51345	5:1	Pro-apoptotic transcription factor acting with FOS
TAF13 RNA polymerase II	TAF13	X84003	5:1	Mediates the function of gene promoters and transcriptional activators
ATPase, class 1, type 8A, member 2	ATP8A2	AI017981	5:1	Transport of ions across cell membranes
Solute carrier family 2, member 3	SLC2A3	M20681	5:1	Facilitated glucose transporter in tissues that exhibit a high glucose demand
Platelet glycoprotein 1B, alpha polypeptide	GP1BA	J02940	5:1	Platelet adhesion
Signal-induced proliferation-associated gene 1	SIPA1	AB005666	5:1	Involved with IL2 in mitogen-induced cell cycle progression
CD44 surface antigen	CD44	L05424	5:1	Matrix adhesion and migration of lymphocytes from the bloodstream to lymphatic tissues
Inhibitor of kappa light chain gene enhancer in B cells, epsilon	IKBE	U91616	5:1	Inhibits NF-kappa-B transcription
TEC protein tyrosine kinase	TEC	D29767	4:1	Expressed in myeloid, B-, and T-cell lineages
Dual-specificity phosphatase 2	DUSP2	L11329	4:1	A MAP kinase phosphatase
Runt-related transcription factor 1	RUNX1	X90976	4:1	Transcription factor
TAF1 RNA polymerase II	TAF1	X07024	4:1	Mediates the function of gene promoters and transcriptional activators
Protease inhibitor 9	PI9	U71364	4:1	Serine protease inhibitor
Protein-tyrosine phosphatase, receptor-type, J	PTPRJ	U10886	4:1	Modulation of biologic cascades
Activated leukocyte cell adhesion molecule	ALCAM	Y10183	4:1	T-cell receptor; involved in cell adhesion interactions
RAS-associated protein RAB27A	RAB27A	U57094	4:1	Platelet vesicular transport
Interleukin 1-beta	IL1B	M15330	4:1	Cytokine involved in inflammation, cell-cell signaling, signal transduction, and the antimicrobial response

*Median expression ratio of post-CPB versus pre-CPB samples.

TABLE 5. Genes exhibiting a fourfold or greater decrease in expression in post- versus pre-cardiopulmonary bypass skeletal-muscle samples

Gene name	Symbol	GenBank accession number	Expression ratio (post:pre)*	Function
Factor H-related gene 2	FHR2	X64877	1:7	Structurally and immunologically related to complement factor H
T-box 1	TBX1	AF012130	1:5	Transcription factor
Protein phosphatase 1, regulatory subunit 3A	PPP1R3A	AF024578	1:5	Centrally involved in carbohydrate and lipid metabolism; down-regulation or mutation can cause severe insulin resistance
RAS-related protein 2A	RAP2A	X12534	1:5	Unknown
Growth/differentiation factor 8	GDF8	AF104922	1:4	Maintenance of skeletal muscle homeostasis
Chorionic gonadotropin, beta chain	CGB	J00117	1:4	Function in skeletal muscle unclear
Cleavage and polyadenylation specificity factor 6	CPSF6	X67337	1:4	Processing of primary mRNA transcripts

*Median expression ratio of post-CPB versus pre-CPB samples.

TBST, the membranes were incubated for 1 hour in 2.5% nonfat dry milk in TBST diluted with anti-rabbit immunoglobulin G (Jackson Immunolabs, West Grove, Pa), both at 1:3000 (v/v) dilution conjugated to horseradish peroxidase. Peroxidase activity was visualized using enhanced chemiluminescence and exposed to x-ray films (Amersham, Arlington Heights, Ill). Post-CPB to pre-CPB and cardioplegic arrest ratios of protein density are presented as median (minimum, maximum) ratios.

Reporting and Functional Classification

Genes identified to be significant by microarray analysis with both statistical methods were the object of a literature search and reported, along with their GenBank accession number, according to the current nomenclature of Online Mendelian Inheritance in Man.¹³ Functional inferences outlined in the right column of Tables 2 to 5 are based on the authors' interpretation of literature referenced in Online Mendelian Inheritance in Man pertaining to each gene.

Results

Patient Characteristics

Table 1 displays the clinical characteristics of the patients whose tissues were used for the microarray and validation studies. All subjects were Caucasian men aged between 55 and 72 years old, without significant comorbidity, who electively underwent 3 or 4 CABGs with mildly hypothermic CPB and antegrade intermittent (every 20 minutes) hyperkalemic cold blood cardioplegia. Antifibrinolytic drugs were not administered.

Microarray Signal-to-Noise Characteristics

Of 12,625 genes examined, 1,106 and 974 genes were identified by method 1 to have a *P* value of .0253 or less for their differential expression in post- versus pre-CPB and cardioplegic arrest cardiac and skeletal muscle tissues, respectively (Figure 1). The projected number of genes that could have reached significance because of chance alone

was 320 for each tissue type, corresponding to a signal-to-noise ratio of 3.46 in cardiac and 3.04 in skeletal muscle microarrays.

Identification of Significantly Altered Genes

Genes whose expression was found to be significantly altered by using both methods are reported in Tables 2 to 5. Of these, there were 32 up-regulated and 26 down-regulated genes in the heart (Tables 2 and 3), and 32 and 7 such genes in the skeletal muscle (Tables 4 and 5). Most up-regulated atrial genes were transcription factors, and most down-regulated atrial genes were immunoglobulins. In skeletal muscle, transcription factors also represented a large proportion of the up-regulated genes, but no specific functional pattern was observed in the down-regulated genes.

Validation

DUSP1. The microarray results for DUSP1 revealed increased expression (median 12-fold; 3.2, 27.1; *P* = .025) in the atrium, but no significant change in the skeletal muscle after CPB and cardioplegic arrest (median 1.6-fold; 0.3, 6.5; *P* = .68). Validation studies revealed the median mRNA expression of DUSP1 in post- versus pre-CPB atrial samples to be increased 3-fold (1.7, 7.1; *P* = .025) and 6.4-fold (1.5, 16.8; *P* = .05) by Northern blotting and real-time PCR, respectively, with a change in protein level of 4.6-fold (1.3, 5.7; *P* = .05) by immunoblotting (Figure 2). In skeletal muscle, DUSP1 was not significantly up-regulated, with a median differential mRNA expression of 1.6-fold (1.2, 4.6; *P* = .08) and 8.2-fold (2.5, 23.3; *P* = .08) by Northern blotting and PCR, respectively, and a protein level change of 2.1-fold (1.1, 3.0; *P* = .68) by immunoblotting. In situ hybridization revealed a striking increase in DUSP1 expression in atrial tissues, particularly localized to

Figure 1. Genes that were differentially expressed with a P value of .0253 or less after *cardiopulmonary bypass* (CPB) and antegrade cold blood cardioplegia in cardiac (left) and skeletal muscle tissues (right) (of 12,625 genes examined). These genes and the number expected to result from repeat testing alone were identified by method 1 described in the text.

Figure 2. Northern and Western blot analyses of mRNA expression and protein level of DUSP1 and CYR61 in atrial (heart) and skeletal muscle (Sk.M.) tissues, pre- and post-CPB and cardioplegia. mRNA expression and protein levels of DUSP1 and CYR61 were significantly increased in post- versus pre-CPB atrial samples, but not in the skeletal muscle.

the endothelium and myocytes (Figure 3). No increase in expression was detected in skeletal muscle samples.

CYR61. The microarray results for CYR61 revealed increased expression in the atrium (median 4-fold; 1.5, 12.1; $P = .025$), but no significant change in the skeletal muscle after CPB and cardioplegic arrest (median 4-fold; 0.8, 8; $P = .68$). In concordance with these results, the median expression of CYR61 in post- versus pre-CPB atrial samples was 2.5-fold (1.8, 5.3; $P = .025$) and 6.2-fold (1.4, 15.4; $P = .025$) by Northern blotting and real-time PCR, respectively (Figure 2). The amount of protein was also increased 2.7-fold (1.5, 3.2; $P = .05$) in atrial tissues. In the skeletal muscle, CYR61 was not significantly up-regulated, with a median differential expression of 1-fold (1, 2.3; $P = .16$) by Northern blotting, 4.9-fold (1, 15.9; $P = .08$) by PCR, and 2.7-fold by immunoblotting (0.9, 3.2; $P = .68$).

GAPD and β -Actin. Microarray results revealed the differential expression of these genes not to be significantly altered after CPB and cardioplegic arrest in both atrial and skeletal muscle tissues. On Northern blotting, the median differential expression of post-CPB and cardioplegic arrest to pre-CPB samples was 1.2 and 1.1 for GAPD, and 1.0 and 1.1 for β -actin in atrial and skeletal muscle samples, respectively ($P =$ not significant).

Discussion

In this study, the gene expression profile of atrial and skeletal muscle from selected, phenotypically similar patients undergoing cardiac surgery with CPB and cardioplegic arrest was determined. The microarray-based method was feasible and validated with conventional quantification techniques. A large number of genes showing altered transcription with a subthreshold P value were identified, well in excess of the number projected to have reached this P value threshold by multiple testing alone. We determined that CPB and cardioplegic arrest do not cause an indiscriminate change in gene expression, but rather induce a distinct pattern of changes in specific pathways such as up-regulation of inflammation/transcription activators, apoptotic genes, and stress genes, as well as down-regulation of immunoglobulin genes. These results allow a better understanding of the pathophysiology of CPB and cardioplegic arrest on cardiac and peripheral tissues and introduce a new method for evaluating modalities aimed at minimizing the adverse effects of CPB, cardioplegia, and cardiac surgery by directly measuring the overall gene expression profile and response of target groups of genes.

Several of the genes found to be differentially expressed in this study have been implicated in the pathophysiology of CPB and cardioplegia in humans. FOS and JUN were found to be up-regulated in subendocardial myocytes and endothelial cells of right atrial samples after cold crystalloid cardioplegia.^{14,15} Nuclear factor-kappa B activation has

Figure 3. Representative in situ hybridization images of DUSP1, pre- and post-CPB in atrial tissues. A post-CPB and cardioplegic arrest increase in DUSP1 expression was localized to the endothelium (arrowheads) and myocytes (small arrows).

been proposed as an inducer of transcription in right atrial tissue exposed to ischemia-reperfusion,¹⁶ and endothelin 1 receptor blockade has been associated with minimization of left ventricular dysfunction after CPB and cardioplegic arrest in swine.¹⁷ A number of neutrophil adhesion molecules such as CD11 and CD44 have been differentially expressed,^{18,19} and serum levels of IL6, IL8, TNF, IL1B, SCYA2, SELE, and IL13, and CD44 levels have been shown to be increased after CPB.²⁰⁻²⁶ Elevated serum levels of S100 have been correlated with cerebral damage after CPB and circulatory arrest.²⁷

This study also identifies multiple genes that had not been implicated in the pathophysiology of CPB and cardioplegic arrest, such as DUSP1, TCF8, CYR61, SLC2A3, MYC, TCCR, BTG2, TRIM, ALCAM, EFNB2, and many others (Tables 2-5). Although the clinical significance of these new findings is still unknown, further research may characterize the expression of these genes, quantify polymorphisms, and examine their correlation with surgical outcome.

Limitations

Microarray techniques, despite their immense potential to improve disease understanding, have inherent shortcomings related to the selection of tissues, lack of standardized

methods for the statistical analysis of data, presentation of vast amounts of results, and generalizability of the findings.²⁸ Microarray technology also does not allow for the determination of absolute expression patterns and provides a measure of transcribed mRNA rather than a functional gene product. We have attempted to limit these shortcomings by selecting phenotypically and procedurally similar subjects, combining nonparametric statistical methods to minimize false positives, validating the expression patterns, protein levels, and origin of select genes with conventional molecular techniques, and suggesting a potential functional role for genes whose expression was most strikingly altered.

For ethical reasons, we used human atrial biopsies rather than ventricular biopsies to examine the cardiac gene expression changes that result from CPB and cardioplegic arrest. Atrial sampling involves little or no intrinsic risk of morbidity, provides full-thickness samples, and is clinically reproducible. CPB and antegrade cold blood cardioplegia distribute to the right atrium.²⁹ Although atrial myocardium differs from ventricular myocardium with respect to the relative percentage of myocytic, endothelial, connective, and neural elements, these cell types are present in each tissue type and may demonstrate ultrastructural changes in disease.^{30,31}

Conclusion

We believe that this study allows a better understanding of the cardiac and systemic effects of CPB and cardioplegic arrest. We anticipate that these data will serve as a starting point for functional studies and provide a framework for the use of gene expression approaches to better understand and minimize the morbid effects of cardiac surgery on cardiac and peripheral tissues.

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