

Original Article

Characterization of human septic sera induced gene expression modulation in human myocytes

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Received February 18, 2009; accepted May 31, 2009; available online June 5, 2009

Abstract: To gain a better understanding of the gene expression changes that occurs during sepsis, we have performed a cDNA microarray study utilizing a tissue culture model that mimics human sepsis. This study utilized an *in vitro* model of cultured human fetal cardiac myocytes treated with 10% sera from septic patients or 10% sera from healthy volunteers. A 1700 cDNA expression microarray was used to compare the transcription profile from human cardiac myocytes treated with septic sera vs normal sera. Septic sera treatment of myocytes resulted in the down-regulation of 178 genes and the up-regulation of 4 genes. Our data indicate that septic sera induced cell cycle, metabolic, transcription factor and apoptotic gene expression changes in human myocytes. Identification and characterization of gene expression changes that occur during sepsis may lead to the development of novel therapeutics and diagnostics.

Key words: Septic sera, gene expression modulation, human myocytes

Introduction

Septic shock (shock due to infection) and sepsis associated multiple organ failure are the number one cause of death in North American intensive care units with an incidence which continues to increase [1, 2]. Approximately 800,000 cases of sepsis are admitted every year to hospitals in North America and despite aggressive antibiotics and supportive care, over 200,000 patients per year succumb to this disorder [1]. The typical human cardiovascular response to septic shock is characterized by hypotension, decreased systemic vascular resistance and elevated cardiac index. In addition, myocardial depression manifested by reversible biventricular dilation and reduction of ejection fraction has been shown to be common in spontaneous human septic shock [3, 4]. Deaths are typically due to early refractory cardiovascular failure (hypotension or shock)

or later multiple organ failure. Shock and organ failure may occur and progress to death despite the fact that complete eradication of the invading organism can usually be achieved with antibiotic support [3, 4]. Severe infection is able to initiate a conserved inflammatory genetic/metabolic cascade in the host that progresses to organ injury, organ system dysfunction and death despite elimination of the initial trigger.

Elevated levels of various cytokines have been observed in sera from patients with sepsis and septic shock [5]. Tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) [6] and migration inhibitory factor (MIF) [7], play a critical role in the development of cellular dysfunction observed during sepsis [8]. Multiple processes have been identified in mediating myocardial dysfunction which include; apoptosis [9-11], mitochondrial dysfunction [12, 13], alterations in calcium current, decrease in myofilament

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Table 1. Subject information, infecting organism and serum cytokine concentrations (TNF- α , IL-1 β , IFN- γ)

Patient	Gender	Age	Infecting organism	Outcome	TNF- α (pg/mL)	IL-1 β (pg/mL)	IFN- γ (pg/mL)
SE	Female	81	<i>Staphylococcus aureus</i>	Died	43.9	6.1	22.9
SF	Male	73	<i>Haemophilus influenzae</i>	Survived	13.4	3.6	6.8
SG	Male	68	<i>Escherichia coli</i>	Survived	19.0	7.7	10.3
SH	Male	54	<i>Streptococcus pneumoniae</i>	Survived	39.0	1.8	6.0
Normal	Healthy individuals				--	--	--

Data are for the 4 septic individuals and the healthy volunteers. Cytokine concentrations were determined using a sandwich ELISA

calcium sensitivity [14, 15], autonomic dysfunction due to neuronal apoptosis [16], metabolic alterations [17-19] and inducible nitric oxide synthase (iNOS) induction [20].

The high-throughput of DNA microarrays allows for the monitoring of thousands of genes and the identification of transcriptional changes that occur in gene expression. In a novel approach to diagnose sepsis, a recent study described a distinct expression profile of patients with early sepsis that differs from critically ill systemic inflammatory response syndrome (SIRS) patients [21]. Tang *et al* [22] provided data which was used to distinguish between sepsis and SIRS patients by utilizing neutrophil gene expression profiling.

In this study, we used a 1700 human cDNA microarray to analyze the gene expression patterns in a tissue culture model which mimics human sepsis. In this model human fetal cardiac myocytes were incubated for 12 hours with 10% human septic sera and 10% sera from healthy volunteers. We showed that human fetal cardiac myocytes responded to human septic serum through the repression of 178 genes and the up-regulation of 4 genes. Septic sera treatment of human myocytes induced the differential expression of several metabolic, transcriptional, cell cycle and developmental genes that contribute to cellular dysfunction observed in septic patients.

Materials and methods

Study patients from whom human septic sera was obtained

Serum was derived from 4 patients who were in acute phase of septic shock as defined by modified ACCP/SCCM Consensus Conference criteria [23]. Patients were required to have all (rather than a minimum of two) of the following four criteria for systemic inflammatory response syndrome 1) a body temperature greater than 38°C or less than 36°C; 2) a heart rate greater than 90 beats per minute; 3) tachypnea, manifested by a respiratory rate greater than 20 breaths per minute or hyperventilation, as indicated by a PaCO₂ of less than 32 mm Hg; 4) an alteration in the white blood cell count (i.e. WBC greater than 12,000/mm³, less than 4,000/mm³, or the presence of more than 10% immature neutrophils). In addition, all patients whose serum samples were utilized exhibited positive blood cultures with a defined focus of infection (e.g. peritonitis or pneumonia) and required substantial pressor therapy (>0.5 μ g/kg/min norepinephrine) to maintain mean arterial pressure > 65 mm Hg. Serum samples were obtained within 24 hours of presentation with septic shock. Human septic serum was obtained after informed consent under an approved Institutional Review Board (Rush University) approved protocol. Subjects contributing human septic sera for this study were not known to have pre-existing structural heart disease. 10 cc of blood was drawn from the patient and centrifuged for 10 min at 1700xg. The supernatant representing the serum was aliquoted and stored at -70°C. Key characteristics and cytokine profiles of sera donors with septic shock is shown in **Table 1**. Normal human sera was harvested from healthy lab volunteers.

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Sandwich ELISA assays

Human septic and normal sera concentrations of TNF- α , IL-1 β and IFN- γ were determined by ELISA. For the detailed protocol, refer to the DuoSet ELISA Development System on the website www.RnDSystems.com or in the following product manuals: Human TNF- α /TNFSF1A, Catalog Number: DY210; Human IL-1 β /IL-1F2, Catalog Number: DY201; and Human IFN- γ , Catalog Number: DY285. The following modifications were made to the ELISA protocol. Four washes were performed instead of three in order to reduce the background. Furthermore, the reagent diluents were optimized for the quantification of each cytokine. The diluents consisted of 10% FCS with PBS, 2% FCS with PBS and 0.5% FCS with PBS for TNF- α , IL-1 β and IFN- γ , respectively.

Treatment of human myocytes with human septic sera

Human fetal myocytes (ScienCell Research) were grown to 2×10^6 cells per 10 cm plate and treated with either 10% septic sera or 10% normal sera.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from all cultured human fetal cardiac myocyte cells using a Qiagen RNA miniprep kit (Qiagen, Hilden, Germany). Isolated RNA was used as a template for cDNA generation using reverse transcriptase. RNA was gel checked for integrity.

Microarray preparation

cDNA preparations from both normal and septic sera treated cardiac myocytes were labeled with either Cy3- or Cy5-conjugated deoxyribonucleoside triphosphates, and were arbitrarily matched and allowed to hybridize to microarray slides as described previously [24]. Changes in gene expression in fetal cardiac myocytes responding to septic serum were detected using 1.7k human cDNA microarray (University Health Network Microarray Center, Toronto, ON, Canada).

Data analysis

Hybridized and washed arrays were scanned on an Axon 4000B dual laser scanner (532

nm/633 nm wavelengths) as described previously [25]. The fluorescence intensities for each feature (spot) on the array were determined using GenePix Pro array software (version 3.0) from Axon Instruments. The GenePix result files (.gpr) were imported into Microsoft Excel worksheets (.xls). The data from duplicate spots on the 8 independent array experiments (4 with reverse fluorescence) were corrected for background and normalized using a previously described method [26]. Filtering of significant genes was performed using a confidence matrix that compared the average ratio between septic and normal sera for each individual from both groups and the total ratio average between the septic group and the healthy individual group. A heat map of this gene list was created using the TM4 microarray software suite version 2.19 [27]. Functional gene classification and ontology analyses were performed by uploading specific gene expression lists to the web-based application D.A.V.I.D. (Database for Annotation, Visualization and Integrated Discovery) [28].

Real-time PCR

Verification of the differential gene expression results detected by the cDNA microarray was conducted using Q-PCR. Duplicate Q-PCR reactions were performed as described previously [25]. A 6 point standard curve was constructed for each gene. GAPDH was used as the reference gene. The gene-specific oligonucleotide primers to confirm the array data using Q-PCR were: API5: forward primer, 5'-CGACAGTAGAGGAGCTTTACCG-3', reverse primer, 5'-GCTGCTAATCGCTTTTCCTTAGT-3'; FECH: forward primer, 5'-GTGGAGCACTATTGACAGGTG-3', reverse primer, 5'-CCACAGACATCGGCAGTGA-3'; STMN1: forward primer, 5'-GCCCTCGGTCAAAGAATCTG-3', reverse primer, 5'-TGCTTCAAGACCTCAGCTTCA-3'; GADD45A: forward primer, 5'-GAGAGCAGAAGACCGAAAGGA-3', reverse primer, 5'-CACAAACACCACGTTATCGGG-3'; RGS4: forward primer, 5'-CAAGCCGGAACA TGCTAGAG-3', reverse primer, 5'-CGGGTTGACCAAATCAAGATAGA-3'; GAPDH: forward primer, 5'-CATGAGAAGTATGACAACAGCCT-3', reverse primer, 5'-AGTCCTTCCACGATACCAAAGT-3'. To verify the changes in gene expression detected in fetal cardiac myocytes treated with serum from septic patients, we conducted Q-PCR analysis on five differentially expressed transcripts. RNA was isolated from fetal cardiac myocytes treated with either 10%

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serum from healthy volunteers or 10% human septic serum, reverse transcribed, cDNA was used in the Q-PCR reaction using the previously described reaction [25]. Using gene-specific primers for API5, STMN1, FECH, RGS4 and GADD45A, the relative expression of these genes to the housekeeping gene GAPDH was calculated. Fold change in gene expression in septic sera treated samples as compared to normal sera treated samples was consistent with the changes as detected by the microarray analysis. Q-PCR showed an increase in the expression of API5, STMN1, and FECH and a decrease in the expression of RGS4 and GADD45A.

Results and discussion

Sera Cytokine Analysis

The levels of TNF- α , IL-1 β and IFN- γ in the sera of the four septic patients and in the serum of healthy individuals were quantified using the Enzyme-Linked Immunosorbant Assay (ELISA) kit from R&D Systems. Patient SE was a female of 81 years old that had *Staphylococcus aureus* as the infecting organism during sepsis. Patient SE succumbed to the disease. The levels of TNF- α , IL-1 β and IFN- γ for this patient were 43.9 pg/ml, 6.1 pg/ml and 22.8 pg/ml respectively (**Table 1**). Patient SF a male of 73 years, with the infecting organism *Haemophilus influenzae* survived from sepsis. This patient had levels of TNF- α , IL-1 β and IFN- γ of 13.4 pg/ml, 3.6 pg/ml and 6.8 pg/ml respectively. Patient SG was infected with *Escherichia coli*. This patient, a male of 68 years old also survived sepsis and the levels of cytokines measured from the serum of this patient were 19.0 pg/ml, 7.7 pg/ml and 10.3 pg/ml for TNF- α , IL-1 β and IFN- γ respectively. The fourth serum SH was from a male of 54 years of age with *Staphylococcus pneumoniae* as the initial infecting organism for sepsis. This patient also survived sepsis and had levels of 39.0 pg/ml, 1.8 pg/ml and 6.0 pg/ml for TNF- α , IL-1 β and IFN- γ in his serum. Normal sera from healthy lab volunteers did not have measurable levels of TNF- α , IL-1 β and IFN- γ .

Modulation of gene expression

We have shown that the human fetal cardiac myocytes responded to human septic sera through the repression of 178 genes and the up-regulation of 4 genes (**Table 2** and see the

corresponding heat map **Figure 1**). The observed general down regulation of gene expression in response to septic sera may be attributed to the elevated levels of the cytokines, TNF- α , IFN- γ and IL-1 β (**Table 1**) in the septic sera which may be inducing cellular dysfunction in the myocytes. Elevated levels of cytokines in septic sera have been previously shown to induce cellular dysfunction in human myocytes [29]. A key element of this tissue culture model of sepsis is that septic myocardial depression induced by septic serum has been shown to correlate with the amount of myocardial depression seen in intact humans using radionuclide measures of ejection fraction [30].

Cell Cycle Genes

Eight cell cycle related genes were differentially expressed. Seven of these genes were down-regulated ranging from 1.6- to 6.1-fold decrease (including MCM2, MCM6, DUSP1, DUSP6, CCNA2, SESN2, and NHP2L1) and one gene was 1.88-fold up-regulated (HDAC5). Dual specificity protein phosphatases (DUSP) are involved in cellular responses to environmental stress as well as negative regulation of cell proliferation. DUSPs play a role in negatively regulating cell cycle and innate immunity. For example, DUSPs are known to dephosphorylate members of the MAP kinase superfamily and two major kinases which are involved in mediating the inflammatory signaling pathways, the p38 MAP kinase [31] and the JNK [32].

Multiple studies have addressed the critical role of DUSP1 (also known as MAPK phosphatase-1 [MKP-1]) in mediating innate immune responses and cytokine production. [32-35] DUSP1 deficiency was associated with significant increase in mice mortality in LPS-induced septic shock [32, 33]. Chi *et al* found that DUSP1 deficiency resulted in the sustained activation of p38 MAPK and JNK and was also associated with IL-10 hyperproduction in mice with endotoxin shock [32]. Atrial natriuretic peptide (ANP)-induced DUSP1 activation inhibits cardiac reprogramming and the re-expression of fetal protein genes by direct inactivation of p38MAPK [36, 37]. Cyclin A2 (CCNA2) plays an important role in cardiomyocyte mitosis [38] and repair [39]. Expression of CCNA2 is normally silenced in 14 days after birth, suggesting its major role in controlling cardio-

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Table 2. Fold change in gene expression of septic to normal sera treated cardiac myocytes using the 1.7K human cDNA array

Genbank Accession ID	Gene Symbol	Description	Fold Change
Apoptosis			
AA136799	GADD45A	growth arrest and DNA-damage-inducible, alpha	0.28
N34233	MAGEH1	melanoma antigen family H, 1	0.33
BG620906	CSE1L	CSE1 chromosome segregation 1-like (yeast)	0.46
T78285	CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	0.51
N91060	VEGF	vascular endothelial growth factor	0.53
AA011445	CASP3	caspase 3, apoptosis-related cysteine peptidase	0.59
W92108	DHCR24	24-dehydrocholesterol reductase	0.60
H79188	ERCC2	excision repair cross-complementing rodent repair deficiency, complementation group 2 (xeroderma pigmentosum D)	0.65
BI824641	BTK	Bruton agammaglobulinemia tyrosine kinase	0.70
BG387747	API5	apoptosis inhibitor 5	2.21
Catalysts			
W31103	BRD2	bromodomain containing 2	0.53
AA039228	FNTB	farnesyltransferase, CAAX box, beta	0.54
H11807	CTPS	CTP synthase	0.57
H62727	CRYM	crystallin, mu	0.58
N77157	PTPN12	protein tyrosine phosphatase, non-receptor type 12	0.61
AA031513	MMP7	matrix metalloproteinase 7 (matrilysin, uterine)	0.64
BE738657	PBEF	pre-B-cell colony enhancing factor 1	0.65
N50000	MAT1A	methionine adenosyltransferase I, alpha	0.65
W92066	RFC4	replication factor C (activator 1) 4, 37kDa	0.66
Cell adhesion			
H29191	APC	adenomatous polyposis coli	0.08
BI496175	ALCAM	activated leukocyte cell adhesion molecule	0.47
H16046	ITGA6	integrin, alpha 6	0.52
H30141	SELP	selectin P (granule membrane protein 140kDa, antigen CD62)	0.60
T80274	CD34	CD34 antigen	0.64
W01300	ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	0.66
Cell cycle			
T65624	DUSP6	dual specificity phosphatase 6	0.16
H71112	MCM2	MCM2 minichromosome maintenance deficient 2, mitotin (S. cerevisiae)	0.46
H14471	MCM6	MCM6 minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)	0.46
BG424365	SES2	sestrin 2 (SES2)	0.52
H04421	DUSP1	dual specificity phosphatase 1	0.55

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AA001329	CCNA2	cyclin A2	0.62
BG403841	NHP2L1	NHP2 non-histone chromosome protein 2-like 1 (S. cerevisiae)	0.62
R14363	HDAC5	histone deacetylase 5	1.88
Cell proliferation			
AA128253	PMP22	peripheral myelin protein 22	0.30
AI819719	PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	0.30
AA114957	MXI1	MAX interactor 1	0.45
W95000	CDC25C	cell division cycle 25C	0.47
W47595	TGFB2	transforming growth factor, beta 2	0.48
AA028183	TGFBR2	transforming growth factor, beta receptor II (70/80kDa)	0.53
H29950	TOB1	transducer of ERBB2, 1	0.58
BG565696	PRDX1	peroxiredoxin 1	0.60
AA055757	CSRP2	cysteine and glycine-rich protein 2	0.60
AA037107	TGFA	transforming growth factor, alpha	0.60
AA044049	GNRH1	gonadotropin-releasing hormone 1	0.61
H12419	USP8	ubiquitin specific peptidase 8	0.63
W17355	DTR	heparin-binding EGF-like growth factor (HBEGF)	0.63
AA007492	RBBP4	retinoblastoma binding protein 4	0.64
W19744	CDK6	Cyclin-dependent kinase 6 (CDK6)	0.67
W78793	EPS8	epidermal growth factor receptor pathway substrate 8	0.69
Cellular component organization and intracellular transport			
AA056151	SEC24C	SEC24 related gene family, member C (S. cerevisiae)	0.27
BG753663	TUBB5	tubulin, beta 5	0.52
H59171	SEC23B	Sec23 homolog B (S. cerevisiae)	0.52
W01720	SRPR	signal recognition particle receptor ('docking protein')	0.57
W92260	RAB11A	RAB11A, member RAS oncogene family	0.58
W33064	TUBA1	tubulin, alpha 1 (testis specific)	0.58
BG169044	ARL6IP	ADP-ribosylation factor-like 6 interacting protein	0.58
W48577	GARS	glycyl-tRNA synthetase	0.60
R20063	TPR	translocated promoter region (to activated MET oncogene)	0.62
W16514	RHO6	Rho family GTPase 1 (RND1)	0.63
AA053988	ARF1	ADP-ribosylation factor 1	0.64
N31521	BPAG1	dystonin (DST)	0.64
BG770889	Rab11-FIP2	RAB11 family interacting protein 2 (class I)	0.64
H28534	AQP1	aquaporin 1 (channel-forming integral protein, 28kDa)	0.64
N34169	NF2	neurofibromin 2 (bilateral acoustic neuroma)	0.65

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Developmental processes

BG740719	DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	0.31
AA037738	HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	0.38
H09372	PPP2CA	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	0.41
T75267	FRAP1	FK506 binding protein 12-rapamycin associated protein 1	0.43
N40420	CCND1	cyclin D1	0.51
W48569	BDNF	brain-derived neurotrophic factor	0.52
H52752	HSPA9B	heat shock 70kDa protein 9B (mortalin-2)	0.55
W49766	WNT5A	wingless-type MMTV integration site family, member 5A	0.55
W01469	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	0.61
N25434	MSX2	msh homeo box homolog 2 (Drosophila)	0.62
W38673	ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	0.63
BI198063	IGFBP2	insulin-like growth factor binding protein 2, 36kDa	0.67

DNA Repair

AA134026	UBE2A	ubiquitin-conjugating enzyme E2A (RAD6 homolog)	0.41
T78280	HAT1	histone acetyltransferase 1	0.43
AA028978	ERCC5	excision repair cross-complementing rodent repair deficiency, complementation group 5 (xeroderma pigmentosum, complementation group G (Cockayne syndrome))	0.56
H30857	MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	0.63

Integral to plasma membrane

H29706	GPC5	glypican 5	0.37
W05301	CD1E	CD1E antigen, e polypeptide	0.55
BF212425	RAI3	G protein-coupled receptor, family C, group 5, member A (GPC5A)	0.59

Metabolism

BG762226	PCSK2	proprotein convertase subtilisin/kexin type 2	0.40
N63511	DPYD	dihydropyrimidine dehydrogenase	0.55
T99689	MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	0.55
H15303	PCCB	propionyl Coenzyme A carboxylase, beta polypeptide	0.57
H19387	ME2	malic enzyme 2, NAD(+)-dependent, mitochondrial	0.57
BI193156	SDF2	stromal cell-derived factor 2	0.58

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AA029397	PSMA6	proteasome (prosome, macropain) subunit, alpha type, 6	0.58
BE254368	FDPS	farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltranstransferase, geranyltranstransferase)	0.59
AA029803	HDLBP	high density lipoprotein binding protein (vigilin)	0.59
BI160119	RPL27A	ribosomal protein L27a	0.59
R98094	BPGM	2,3-bisphosphoglycerate mutase	0.60
R52371	SLC35A1	solute carrier family 35 (CMP-sialic acid transporter), member A1	0.61
H30710	BAT1	HLA-B associated transcript 1	0.63
R01732	AMPD3	adenosine monophosphate deaminase (isoform E)	0.63
W68191	CLU	clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)	0.63
N31054	MPV17	MpV17 transgene, murine homolog, glomerulosclerosis	0.64
W94208	FDFT1	farnesyl-diphosphate farnesyltransferase 1	0.64
W92014	ADH6	alcohol dehydrogenase 6 (class V)	0.64
W52843	ALDH3B1	aldehyde dehydrogenase 3 family, member B1	0.65
W31637	LPL	lipoprotein lipase	0.65
H82585	NT5E	5'-nucleotidase, ecto (CD73)	0.65
W37213	HEM1	NCK-associated protein 1-like (NCKAP1L)	0.66
Metal binding			
H93255	MT1E	metallothionein 1E (functional)	0.62
R18562	ATP7B	ATPase, Cu ⁺⁺ transporting, beta polypeptide	0.62
R99208	MT1B	metallothionein 1B (functional)	0.64
Mitochondrial			
W37672	HK1	hexokinase 1	0.60
AA133258	HADHB	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit	0.62
W21228	UQCRC2	ubiquinol-cytochrome c reductase core protein II	0.65
N72504	FECH	ferrochelatase (protoporphyrin)	2.4
Nervous System(Development)			
R15219	EPHA7	EPH receptor A7 (EPHA7)	0.45
H17696	MBP	myelin basic protein	0.46
BI826246	PLP1	proteolipid protein 1 (Pelizaeus-Merzbacher disease, spastic paraplegia 2, uncomplicated)	0.52
Protein (folding / unfolding / proteolysis)			
H18866	CHRM3	cholinergic receptor, muscarinic 3	0.31
H03673	KRT7	keratin 7	0.48

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AA036988	CCT3	chaperonin containing TCP1, subunit 3 (gamma)	0.53
H62639	HSPA8	heat shock 70kDa protein 8	0.56
W90284	HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	0.57
H53489	C4BPA	complement component 4 binding protein, alpha	0.62
R48054	CCT5	chaperonin containing TCP1, subunit 5 (epsilon)	0.62
AA031513	MMP7	matrix metalloproteinase 7 (matrilysin, uterine)	0.64
T74000	BLMH	bleomycin hydrolase	0.64
Regulation of Translation (Initiators / Inhibitors)			
H79856	UK114	(HRSP12) heat-responsive protein 12	0.29
AA098888	ETF1	eukaryotic translation termination factor 1	0.58
N79725	MTIF2	mitochondrial translational initiation factor 2	0.58
BG178139	WBSCR1	Williams-Beuren syndrome chromosome region 1	0.63
Signaling			
T74308	MAPK6	mitogen-activated protein kinase 6	0.20
AI267373	RGS4	Regulator of G-protein signalling 4 (RGS4)	0.28
H18866	CHRM3	cholinergic receptor, muscarinic 3	0.31
AA055349	ADORA2B	adenosine A2b receptor	0.46
T97105	TXNRD1	thioredoxin reductase 1	0.52
W79493	PDE4B	phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)	0.56
W56577	VLDLR	very low density lipoprotein receptor	0.56
AA043909	PTK2	PTK2 protein tyrosine kinase 2	0.57
T75436	MAPK10	mitogen-activated protein kinase 10	0.59
N31391	AKAP12	A kinase (PRKA) anchor protein (gravin) 12	0.62
AA001104	RGS3	regulator of G-protein signalling 3	0.62
W35223	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	0.62
H15389	NPY1R	neuropeptide Y receptor Y1	0.63
W52156	OXTR	oxytocin receptor	0.63
AA133259	GEM	GTP binding protein overexpressed in skeletal muscle	0.64
AA056159	RHO	rhodopsin (opsin 2, rod pigment) (retinitis pigmentosa 4, autosomal dominant)	0.64
R52300	GABRA1	gamma-aminobutyric acid (GABA) A receptor, alpha 1	0.65
BF971558	STMN1	stathmin 1/oncoprotein 18	1.78
Transcription factors and transcriptional regulators			
W05657	ELF1	E74-like factor 1 (ets domain transcription factor)	0.04
AA044787	CNOT8	CCR4-NOT transcription complex, subunit 8	0.12
AA127003	HSZFP36	ZFP-36 for a zinc finger protein	0.18
R59543	NRIP1	nuclear receptor interacting protein 1	0.23
AA005274	ZNF7	zinc finger protein 7 (KOX 4, clone HF.16)	0.24

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R35310	MEIS2	Meis1, myeloid ecotropic viral integration site 1 homolog 2 (mouse)	0.24
R20489	ZNF16	zinc finger protein 16 (KOX 9)	0.25
R80355	BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1	0.31
R13988	EGR3	early growth response 3	0.32
T77424	FHX	(FOXJ2) forkhead box J2	0.38
T84358	ZNF135	zinc finger protein 135 (clone pHZ-17)	0.41
H14414	ZNF195	DKFZp434A2314 5', mRNA sequence.	0.42
N31546	IFI16	interferon, gamma-inducible protein 16	0.43
AA043380	HOXD10	homeo box D10	0.45
N23578	TCF12	transcription factor 12 (HTF4, helix-loop-helix transcription factors 4)	0.49
W90498	SSB	Sjogren syndrome antigen B (autoantigen La)	0.52
AA035361	STAT1	signal transducer and activator of transcription 1, 91kDa	0.53
N94714	YY1	YY1 transcription factor	0.54
H50875	FOXA3	forkhead box A3	0.56
W32908	FOXO1A	forkhead box O1A (rhabdomyosarcoma)	0.61
W38673	ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	0.63
AA043457	ZNF137	zinc finger protein 137 (clone pHZ-30)	0.64
H27140	ZNF35	zinc finger protein 35 (clone HF.10)	0.65
N34004	STAT6	signal transducer and activator of transcription 6, interleukin-4 induced	0.65
R66676	ETV5	ets variant gene 5 (ets-related molecule)	0.65
W16724	MLL	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila)	0.67
Others			
W03376	NNMT	nicotinamide N-methyltransferase	0.34
T78107	GRM5	glutamate receptor, metabotropic 5	0.59
W39552	BMPRI1A	bone morphogenetic protein receptor, type IA	0.61
BI457624	PPIL2	peptidylprolyl isomerase (cyclophilin)-like 2	0.62
H92049	LRBA	LPS-responsive vesicle trafficking, beach and anchor containing	0.63

Table indicates differentially regulated genes from fetal cardiac myocytes treated 12 hours with sera from either healthy or septic individuals. Fold changes are the average ratios between the 4 septic and the 4 healthy individuals.

myocyte cell cycle and is associated with the decline in cardiac myocyte mitosis [40]. Targeted expression of CCNA2 improved cardiac hemodynamics in myocardial infarction (MI) mouse model [41]. Moreover,

CCNA2 transgenic mice showed a remarkable ability to induce cardiac mitosis and repair after MI [39]. Re-expression of DUSP1 and CCNA2 represents a promising therapeutic target that may contribute to improved heart

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impair mitochondrial function and cellular oxygen utilization [12]. It was reported that TNF- α induces mitochondrial DNA damage and causes mitochondrial dysfunction in cardiac myocytes via an increase in intracellular oxidative stress [13]. Accordingly, the inhibition of inducible nitric oxide synthase and inducible mitochondrial nitric oxide synthase activities through the administration of melatonin prevented mitochondrial function impairment, restores ATP production and improves survival in CLP mice [50].

Four mitochondrial genes were differentially regulated, three genes were 1.54-to 1.66-fold down-regulated (UQCRC2, HADHB, HK1) and one gene was 2.39-fold up-regulated (FECH). The up-regulation of ferrochelatase (FECH) to our knowledge is not known to contribute to sepsis but its partial deficiency and down-regulation is linked to erythropoietic protoporphyria (inherited heme biosynthesis disorder) [51].

UQCRC2 is a part of the mitochondrial respiratory chain. HADHB, encodes the beta subunit of the mitochondrial trifunctional protein (TFP) and is essential for mitochondrial beta-oxidation of long chain fatty acids. Mutations in this gene are associated with TFP deficiency. Among its clinical phenotypes is severe neonatal presentation with cardiomyopathy. Mild myopathy is the most common phenotype due to this deficiency [52]. Hexokinases commit glucose to the glycolytic pathway through glucose phosphorylation. One member of this family is HK1 and its deficiency is associated with hemolytic anemia [53]. Its expression prevents mitochondrial mediated cell death via permeability transition pore (PTP) closure as well as the accompanying cytochrome c release [54].

Our data indicate that impaired mitochondrial function was evident as HADHB and UQCRC2 were down-regulated. The down-regulation of HK1 may contribute to the activation of mitochondrial-mediated apoptotic pathway observed during sepsis.

Apoptosis Genes

Apoptosis plays a key role in sepsis. In a transgenic mouse model, it was discovered that apoptotic rates of 23 cardiac myocytes per 10^5 nuclei are sufficient to induce a lethal, dilated cardiomyopathy [55]. In addition,

apoptosis leads to a profound immune-paralysis, accompanied by high morbidity and mortality in septic patients as reviewed by Hotchkiss [10].

We observed a 2.2-fold up-regulation of the AP15 gene. Its role in suppressing E2F-dependent apoptosis has been identified and its depletion is lethal to tumor cells [56]. Moreover, cardiac myocytes showed down-regulation of nine genes involved in the positive regulation of apoptosis. One of these is VEGF which is an important cytokine that contributes to sepsis mortality. Increased levels of VEGF has been reported in patients with severe sepsis however lower levels (although higher than healthy controls) were associated with organ dysfunction and poor survival outcome [57]. Two members of the cysteine-aspartic acid protease (caspase) family were also among these nine down-regulated genes (CASP2, CASP3).

Genes related to signaling transduction and transcription

Key signaling molecules such as kinases and transcription factors are modulated after the onset of sepsis. For example, activated p38 MAP kinase/NF- κ B signaling molecules play a pivotal role in the sepsis induced conversion of the cardiac myocytes to a proinflammatory phenotype [58]. The pro-inflammatory phenotype is characterized by increased myocyte production of CXC chemokines, specifically keratinocyte-derived chemokine (KC) and LPS-induced chemokine (LIX) and the subsequent transendothelial migration of the polymorphonuclear neutrophils (PMN) [58]. In addition, activation of the JNK pathway in a human *in vitro* model of sepsis-induced cardiac myocyte apoptosis was evident [11]. Septic sera induced the activation of transcription factors STAT1, IRF1, and NF- κ B in human cardiac myocytes [11]. This activation was associated with sepsis induced cellular dysfunction [11].

In our current study, seventeen signaling genes were found to be down-regulated with fold change ranging from 1.55- to 4.96-fold below the normal gene expression in the control samples. One signaling gene, stathmin 1 (STMN1) was 1.78-fold up-regulated, while 26 transcription related genes were down-regulated.

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The transcription factor E1f1, a member of Ets family of transcription factors was the most down-regulated gene in our study with 26.49-fold decrease. It was reported that E1f1 plays an important role in regulating transcription and gene expression in lymphoid tissues [59]. E1f1 is also expressed in normal and malignant mammary tissues [60]. Later studies found that endogenous expression of E1f1 and its phosphorylation vary depending on cell type and condition, with higher levels in the hematopoietic cells [61]. In a breast carcinoma study, E1f1 was significantly down-regulated in mouse tumors. Another cancer study reported its role along with p65 in the induction of NFkB1 gene expression and subsequent NFkB activation in specific chemoresistant human T leukemic cell line [62]. E1f1 role in hematopoiesis and transcription regulation in lymphoid cells was previously reviewed [63] but its role in cardiac myocytes and sepsis has yet to be determined.

Among the other down-regulated transcription factors in our study, two belong to the signal transducers and activators of transcription family, STAT1 and STAT6. It is evident that STAT1 plays a pivotal role in cell sensitivity to stress stimuli and stress-induced apoptosis [64]. STAT6 has been shown to have anti-apoptotic activities [65]. CNOT8, also known as POP2, is a transcription factor. It has been previously shown that CNOT8 negatively regulates NF- κ B signaling in TNF- α stimulated HEK-293 cell line as it alters the nuclear distribution of p65 [66]. In addition, CNOT8 interferes in the inflammasome-mediated procaspase 1 activation and subsequently obstructs IL-1 β secretion [67]. CNOT8 may play a role in protecting the cells from the drastic effects of IL-1 β overproduction. CNOT8 is 8.6-fold down-regulated in our study and this may partially account for the increased production and activity of IL-1 β in septic patients.

Stathmin 1 (STMN1) plays a pivotal role in mitosis and cell cycle regulation through regulating microtubule dynamics [68]. Dysregulation of stathmin expression has been linked to several pathological conditions. Over-expression has been documented in multiple types of cancer [69-71] and other neuronal disorders [72]. Down-regulation of stathmin has been reported in Alzheimer's disease and Down syndrome [73]. Low levels of STMN1 expression is essential for the proper function

of neuronal cells [72, 74], Yamashita *et al.* conducted a study that addressed the role of activated MAP kinases and the subsequently phosphorylated STMN1 in oxidative stress-induced neuronal cell death [75]. Vancompernelle *et al.* suggested a role of STMN1 as a downstream target molecule in TNF-induced cell death signaling pathway [76]. We observed a 1.78-fold up-regulation of the STMN1 gene. Increased expression of the STMN1 gene may provide a mechanism for the autonomic dysfunction observed during sepsis as it triggers apoptosis via activated MAP kinases and TNF signaling pathways.

In addition, RGS3, RGS4 and TXNRD1 are important signaling genes, contributing to cardiac hypertrophy and the progression of heart failure by their reduced expression. Recently, the role of RGS4 and RGS3 in the inhibition of ERK1/2 phosphorylation has been confirmed [77]. RGS4 expression is essential in guanylyl cyclase-A (GC-A) - mediated inhibition of cardiac hypertrophy [78]. Heart cells show high levels of VLDL-R expression and its role in lipid metabolism is evident [79]. VLDL-R down-regulation has a crucial role in alterations of heart lipid metabolism observed during sepsis. This alteration is a result of IL-1 β signaling and its downstream signaling molecule, Hsp90 [80]. Moreover, enhanced levels of TXNRD1 improve cardiomyocyte survival via its antioxidative activity [81, 82].

Metabolism related genes

Impaired metabolic processes have been observed in septic hearts. Increased expression of iNOS and TNF- α in septic hearts has been linked to distinct structural changes, including lipid accumulation and the disruption of the actin/myosin contractile apparatus [83]. Oxidative damage as well as the altered glucose and lipid metabolism account for these structural changes. Our results confirmed these altered metabolic processes through the down-regulation of 22 metabolism related genes and select members of this group are responsible for lipid and carbohydrate metabolism. We observed the down-regulation of the following lipid metabolism genes; VLDL-R, FDPS, HDLBP, CLU, FDFT1, ALDH3B1, LPL, PCCB. The altered free fatty acid metabolism and uptake by the myocardium in septic patients [18] has been linked to IL-1 β mediated down-regulation of genes involved in lipid metabolism [19].

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Increased concentrations of free intracellular Ca^{2+} ions observed during sepsis [46, 47] partially contribute to these metabolic disorders. Sepsis-induced LPL repression contributes to sepsis-associated lipid metabolic disorders in $\text{TNF-}\alpha$ -induced Ca^{2+} -dependent manner [84]. The involvement of other lipid metabolism related genes in this study may be of critical importance and requires further investigations. We observed the down-regulation of genes which are involved in carbohydrate metabolism which include; ME2, SLC35A1, and BPGM however the roles that these genes play during sepsis has not been characterized.

Other Genes

Cardiovascular homeostasis is dramatically affected during sepsis. A previous study reported the role that iNOS plays in inducing apoptosis in cardiovascular autonomic centers [16]. As we have previously discussed, activated MAP kinases phosphorylate STMN1 in oxidative stress conditions and induces neuronal cell death [75]. We observed three nervous system related genes that were down-regulated; PLP1, MBP and EPHA7. These three genes have been implicated in nervous system development and maintenance. Two previous studies determined the differential gene expression profile in blood cells derived from children with septic shock and confirmed the role of altered zinc homeostasis in mortality [85, 86]. Consistent with these studies, our results showed a repression in three metal binding genes (MT1E, MT1B, ATP7B).

Our data indicate that septic sera induced cell cycle, metabolic, transcription factor and apoptotic gene expression changes in human myocytes. Mediators in septic sera influence changes in gene expression that lead to organ dysfunction and potentially death. An important mediator is $\text{TNF-}\alpha$, which showed a consistent elevated level in septic sera. In addition, gene expression changes in LPL, STMN1 and mitochondrial genes are shown to be $\text{TNF-}\alpha$ -induced. Sepsis-induced differential gene expression of LPL, STMN1 and mitochondrial genes contribute to lipid metabolic disorders, autonomic dysfunction and impaired oxygen utilization, respectively. Moreover, these genes are involved in the activation of mitochondria-mediated apoptotic pathway and subsequently cardiac dysfunction. Members of DUSP and HDAC

families, play a key role in protecting cardiac cells from re-programming and the re-expression of fetal gene programs. Targeting DUSP family members, YY1 transcription factor along with HDAC5 may represent a therapeutic approach to improve cardiac function in septic patients. IL-1 β , another mediator in septic sera, showed consistent higher levels in septic sera than normal sera. IL-1 β was associated with altered lipid metabolism in cardiac myocytes. The role of CNOT8 and Elf1 in NF- κ B regulation should be investigated. Furthermore, CNOT8 down-regulation, has shown to contribute to IL-1 β increased production in septic patients.

Taken together, our data provide new insights in relation to the changes in gene expression that contribute to cardiac depression and dysfunction observed in septic patients. Identification and characterization of gene expression changes that occur during sepsis may lead to the development of novel therapeutics and diagnostics.

Acknowledgements

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