Differential Gene Expression After Hemorrhagic Shock in Rat Lung

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Background: We investigated the differential gene expression in rat lung after hemorrhagic shock (HS).
Methods: A controlled HS model in rats was used. Male Sprague-Dawley rats were randomly segregated into 2 groups, sham and HS. Samples of lung were procured from rats 2 hours after HS and resuscitation. Commercially available gene chips for rat cDNA microarray and software packages were used for the gene expression profile study.
Results: Compared with sham-shock rats, 98 genes were upregulated in HS rat lung. Most upregulated genes were responsible for inflammation (pro-inflammatory or anti-inflammatory cytokines, cognate receptors, and signal transduction for inflammation), protein activation (kinase and phosphatase), oxidation (oxidative and antioxidative enzymes), and apoptosis (apoptosis and anti-apoptosis). Eleven genes were downregulated after HS.
Conclusion: HS may induce upregulation of positive and negative control genes responsible for inflammation, oxidation, protein metabolism and apoptosis, that is, a vulnerable period may develop in the host after HS. Overwhelming inflammatory response or immunosuppression may occur once a second hit, such as infection, ensues. Understanding, on a genome scale, how an organism responds to HS may facilitate the development of enhanced treatment modalities for HS. [*J Chin Med Assoc* 2005;68(10):468–473]

Key Words: gene expression, hemorrhagic shock, microarray

Introduction

Despite recent advances in our understanding of the pathophysiology of trauma-hemorrhage, sepsis and multiple organ failure remain the leading causes of late mortality.[1] Inflammatory mediators, cytokines, oxidative stress, and stress response are responsible for organ dysfunction after trauma-hemorrhage.[2–5] It is evident that trauma-hemorrhage induces a global derangement of cellular function in the host. Recently, a high-throughput technology, microarray, has provided a new tool to investigate gene expression and regulation systematically (the systematic genomic approach),[6] as it is capable of profiling patterns of expression for thousands of genes in a single experiment.[7] Using microarray technology, one can delineate the mechanism of organ dysfunction after hemorrhagic shock (HS). In this communication, we present the findings of a study on differential gene expression in rat lung after HS.

Methods

Animal preparation

Male Sprague-Dawley rats weighing 300–350 g were used. The research protocol adhered to the principles in the Guide for the Care and Use of Laboratory Animals.[8] Pellet food was withheld overnight before the experiment, but animals had free access to tap water. HS was performed as described in our previous report.[9] Animals were anesthetized intraperitoneally with pentobarbital (40 mg/kg). The femoral vessels were isolated using an aseptic technique and

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cannulated with polyethylene tubing (PE50). The left femoral artery was used for continuous monitoring of blood pressure with a pressure transducer and a chart recorder (Model 2107, Gould Inc, Valley View, OH, USA). Blood was withdrawn from the right femoral artery and the right femoral vein was used for drug injection and fluid resuscitation. Blood was withdrawn over 15 minutes into a syringe containing 100 IU of endotoxin-free heparin until the mean arterial blood pressure was reduced to 40 ± 5 mmHg. Blood pressure was maintained at this level for 45 minutes by withdrawal or re-infusion of shed blood. At the end of the shock period, shed blood was re-infused for 15 minutes, followed by a 15-minute infusion of Ringer's lactate solution twice the volume of shed blood.

Experimental groups

Animals were randomly segregated into 2 groups. Sham-shock rats received all of the instrumentations and procedures except that no bleeding was done. HS rats underwent HS and complete resuscitation. Organ samples were obtained 2 hours after sham or HS by excising lung tissue after flushing the carcass with ice-cold Ringer's lactate solution, and these were snap-frozen in liquid nitrogen and stored at –80°C until study. Gene expression in lung tissue, pooled from 5 rats in each group to minimize variations between animals, was compared between groups.

Microarray with fluorescence detection

Total RNA was isolated from lung tissue samples using TRIzol reagent (Life Technologies, Rockville, MD, USA), then loaded onto a Qiagen Rneasy column (Qiagen Inc, Valencia, CA, USA) for purification. Electrophoresis on a 1% agarose-formaldehyde gel was used to determine the integrity of the RNA preparation. Total RNA (2–5 μg) was reverse transcribed with Superscript II Rnase H-reverse transcriptase (Gibco BRL, Grand Island, NY, USA) to generate Cy3- and Cy5-labeled cDNA probes. The labeled probes were hybridized to a commercial cDNA microarray, Rat chip 8K-1 (eGenomix Technology Corp, Taipei, Taiwan) containing 7,500 immobilized cDNA fragments. Fluorescence intensities of Cy3 and Cy5 targets were measured and scanned separately using a GenePix 4000B Array Scanner (Axon Instruments, Molecular Devices Corp, Sunnyvale, CA, USA) and eGenomix V1.0 (eGenomix Technology Corp). The results were normalized to label and detect the efficiencies of 2 fluorescent dyes, then to determine differential gene expression between samples.

Differential gene expression

Primary analysis was performed using the GenePix software package. The fluorescence intensities of Cy5 and Cy3 were analyzed and the intensity ratios of Cy5 to Cy3 were determined for the individual genes along with a quality control parameter (intensity over local background). The intensity of 2 fluorescent signals represented the quantity of the 2 tagged probes. The ratio of Cy5 to Cy3 of certain spots on the slides demonstrated the mRNA abundance of this gene expression in the 2 study groups. The criteria for determination of differentially expressed genes were that the absolute value of the ratio of Cy5 to Cy3 was greater than 2 (upregulated) or less than 0.5 (down-regulated), and the signal value of fluorescence intensity of either Cy3 or Cy5 was greater than 1,000. This differential expression of genes was selected and the accession numbers in the gene bank were provided, from which the gene profile can be found in the NCBI website (www.ncbi.nlm.nih.gov).

Quantitative reverse transcription–polymerase chain reaction

To validate the changes in gene expression, quantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed for the 5 cytokines that were upregulated after HS. In brief, 1 μg of lung RNA was reverse transcribed into first-strand cDNA using a commercial cDNA synthesis kit (SuperScript, Gibco BRL, Life Technologies, Gaithersburg, MD, USA). PCR was performed in 10 μL of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) containing 1 ng of cDNA and 50 μM of forward and reverse primers specific for the genes of interest (Table 1).[10]

Amplification and detection were performed using the GeneAmp 5700 DNA thermal cycle (Applied Biosystems). Relative quantity was calculated in triplicate using the cutoff threshold ($2^{-\Delta\Delta CT}$) method. In brief, mean C_T values were calculated from triplicate reactions. The difference (ΔC_T) between the mean C_T of the samples in the target wells and the mean C_T value of the endogenous control (glyceraldehyde-3-phosphate dehydrogenase) was determined. The difference ($\Delta\Delta C_T$) between the mean ΔC_T values of the samples from HS animals and sham animals was used to determine relative quantity as $2^{-\Delta\Delta CT}$.

Results

The profile of gene expression in HS and sham rats showed a scatter of Cy5 versus Cy3 intensity in the

Table 1. Forward and reverse primers specific for interleukin 1 (IL-1), tumor necrosis factor (TNF), IL-10, macrophage migration inhibitory factor (MIF), intercellular adhesion molecule-1 (ICAM-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[10]

Gene	Size	Forward primer	Reverse primer
IL-1	305 bp	5'-CTTCCTTGTGCAAGTGTCTGAAGC-3'	5'-AAGAAGGTCCTTGGGTCCTCATCC-3'
TNF	209 bp	5'-TGCCTCAGCCTCTTCTCATT-3'	5'-GCTTGGTGGTTTGCTACGAC-3'
IL-10	417 bp	5'-GAACCACCCGGCGTCTAC-3'	5'-AGGGATGAGGGCAAGTGAAA-3'
MIF	470 bp	5'-CGGCCGTCGTTCGCAGTCTC-3'	5'-CCGGAAGGTGGCCATCATTACG-3'
ICAM-1	234 bp	5'-AGACACAAGCAAGAGAAGAA-3'	5'-GAGAAGCCCAAACCCGTATG-3'
GAPDH	384 bp	5'-GCTGGGGCTCACCTGAAGGG-3'	5'-GGATGACCTTGCCCACAGCC-3'

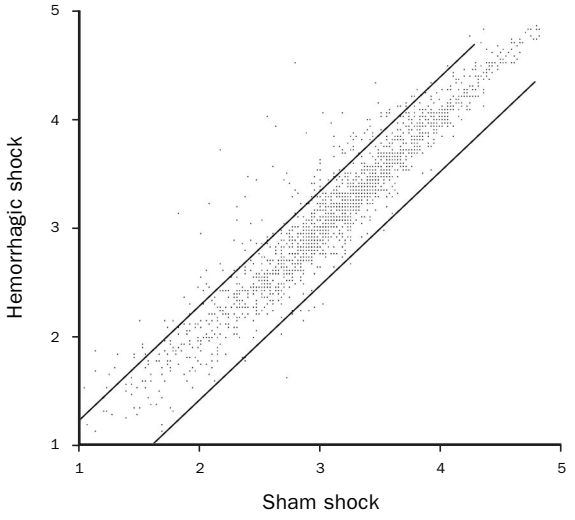

Figure 1. Scatter plot representation of gene expression changes in hemorrhagic-shock versus sham-shock rats. Green (Cy3) intensity is shown in log scale on the horizontal axis and Cy5 intensity is similarly represented on the vertical axis. Most of the differential genes were upregulated in the hemorrhagic-shock versus sham-shock rats.

2 groups (Figure 1). In total, 98 genes were upregulated after HS compared with the sham group. These upregulated genes were grouped according to their function as follows: genes for inflammation (pro-inflammatory or anti-inflammatory cytokines, cognate receptors, and signal transduction for inflammation), genes for protein activation (kinases and phosphatases), genes for oxidation and reduction (oxidative and antioxidative enzymes), genes for apoptosis (apoptosis and anti-apoptosis), genes for metabolism, and other genes that did not fit any of the above categories or had an undetermined function. The upregulated genes with higher intensity (Cy5/Cy3) in each functioning category are shown in Tables 2–4.

Eleven genes were downregulated after HS compared with sham shock, including cadherin 1 (AW916453), best5 protein (AW915965), myelin basic protein (AW141205), L-3-hydroxyacyl-coenzyme A dehydrogenase-short chain (AW142151), methylmalonate semialdehyde dehydrogenase (AW917126), bone morphogenetic protein 2 (AW140758), cysteine-rich protein 2 (AW918405), fibulin 5 (AW142226), chloride intracellular channel 5 (AW917618), proline-rich Gla protein 2 (AW917561), and glutathione reductase (BM986384).

The results of the corroboration between microarray and quantitative RT-PCR of interleukin 1 (IL-1), tumor necrosis factor α (TNF-α), IL-10, macrophage migration inhibitory factor and intercellular adhesion molecule-1 (ICAM-1) are shown in Figure 2. Concordance was found in these genes between 2 tests.

Discussion

We took advantage of recent advances in microarray technologies to perform genome-wide assessment of changes in gene expression.[11] Gene expression profiles were studied in controlled resuscitated HS. The tools used included commercially available microarray expression kits and software packages. We selected the lung as an index organ of dysfunction after HS as lung is frequently injured after HS. Our findings indicated that, relative to sham shock, resuscitated HS significantly altered gene expression in lung tissue. Most of the genes identified have previously been linked to regulation of the inflammatory response. However, some of the genes have not been identified. In addition, gene analysis showed that HS induced coordinate expression of genes that altered cell signaling, apoptosis, and oxidative/antioxidative systems in the lung.

Our study showed that HS induced upregulation of genes responsible for inflammation, oxidation and reduction, protein activity, and apoptosis. The genes with pro-inflammatory effects were often balanced by genes with anti-inflammatory effects, which illustrates the regulatory control embedded in complex

Table 2. Expression of the top 20 inflammation-related genes (cytokines, cognate receptors, and signal transduction) upregulated after hemorrhagic shock compared with sham shock

	Gene bank accession no.
IL-1β	AW916694
ICAM-1	AW312898
TNF-α	BF549278
Macrophage MIF	BM986278
IL-10	NM012854
Fc receptor-IgG-low affinity III	AW917566
Interferon regulatory factor 1	AW917462
TRAF family member-associated NFκB activator	AW140505
Activated leukocyte cell adhesion molecule	AW917593
Interferon induced transmembrane protein 3-like	AW913928
Chemokine (C-C motif) ligand 5	AW917681
NFκB p105 subunit	AW141124
STAT3	AW141118
Mitogen-activated protein kinase 6	AW141988
ATF-4	AW140691
Mitogen activated protein kinase kinase 1	AW916314
Hypoxia inducible factor 1 α subunit	AW915306
IL-1 receptor type 2	NM053953
Janus kinase 2 (a protein tyrosine kinase)	AW143706
Mak11 protein	AW144848

ATF = activating transcription factor; ICAM = intercellular adhesion molecule; IgG = immunoglobulin G; IL = interleukin; MIF = migration inhibitory factor; NFκB = nuclear factor kappa B; STAT3 = signal transducer and activator of transcription 3; TNF = tumor necrosis factor; TRAF = TNF receptor-associated factor.

Table 3. Expression of oxidation-related genes (oxidative and antioxidative enzymes) and genes for protein kinases and phosphatases upregulated after hemorrhagic shock compared with sham shock

	Gene bank accession no.
Oxidation-reduction related genes	
Superoxide dismutase 2	AW141924
Manganese-containing superoxide dismutase	AW141991
Peroxiredoxin 3	AW142671
Cytochrome C	AW140724
Ferredoxin 1	AW140722
Xanthine dehydrogenase	AW144827
Thioredoxin	AW140607
Isocitrate dehydrogenase 1	AW140522
Selenoprotein P-plasma-1	AW140800
Metallothionein-1	AW141679
Genes for proteases, kinases, and anti-proteases	
A kinase (PRKA) anchor protein (gravin) 12	AW144762
Protein tyrosine phosphatase	AW141674
Janus kinase 2 (a protein tyrosine kinase)	AW143706
Protease (prosome-macropain) 28 subunit β	AW917080
Protein kinase-interferon-inducible ds-RNA dependent	AW916915
Protein tyrosine phosphatase-non-receptor type 1	AW917222
Protein-tyrosine kinase FadK	AW914239
Tissue inhibitor of metalloproteinase 1	AW140764
Protein tyrosine kinase	AW141117

Table 4. Expression of apoptosis-related genes, genes for metabolism, and other genes with undetermined function upregulated after hemorrhagic shock compared with sham shock

	Gene bank accession no.
Apoptosis-related genes	
CD24 antigen	AW142164
Fyn proto-oncogene	AW141122
Heme oxygenase 2	AW917100
Nucleolin	BM986279
Cytolysin	AW142167
Prostaglandin F receptor	AW916648
Genes for metabolism	
Acyl-coenzyme A: cholesterol acyltransferase	AW140787
Pyruvate dehydrogenase kinase 1	AW142079
UDP-glucose dehydrogenase	AW142208
UDP-glucose:ceramide glycosyltransferase	AW143799
Uridine-cytidine kinase 2	AW141796
iGb3 synthase	AW918359
Other genes (only 10 of the top expressed genes included)	
Crystallin-alpha B	AW142330
Adenosine A2a receptor	BM982697
UNR protein	AW917589
ATP-binding cassette-subfamily A-member 1	AW918387
Dynein-cytoplasmic-light intermediate chain 1	AW142507
Ly6-B antigen	AW916526
Core promoter element binding protein	AW141955
Ly6-C antigen	AW141822
Immediate early response 5	AW142256
MHC class Ib RT1.S3	AW916757

ATP = adenosine triphosphate; MHC = major histocompatibility complex; UDP = uridine diphosphate.

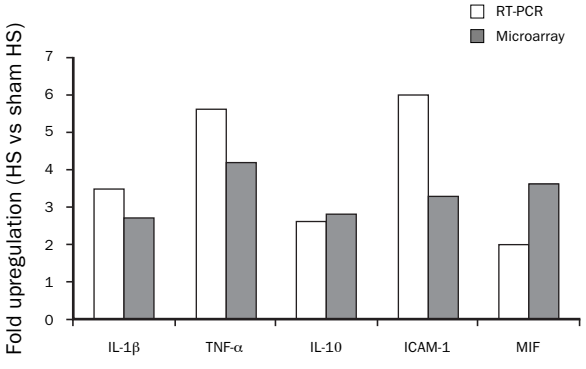

Figure 2. Validation of microarray results by reverse transcription–polymerase chain reaction (RT-PCR) analysis shown as fold upregulation of the respective transcripts in hemorrhagic-shock (HS) versus sham-shock rats as determined by RT-PCR and cDNA microarray analysis. IL-1β = interleukin-1β; TNF-α = tumor necrosis factor α; IL-10 = interleukin 10; ICAM-1 = intercellular adhesion molecule-1; MIF = macrophage migration inhibitory factor.

biochemical pathways. For example, genes for TNF-α and IL-1β were upregulated with IL-10 and IL-1 decoy receptor IL-1 receptor type 2 (Table 2). Genes for reactive oxygen species-generating enzymes were balanced by those for antioxidative enzymes. For example, genes for oxidants, such as xanthine dehydrogenase, were upregulated with genes for antioxidants, such as superoxide dismutase or thioredoxin (Table 3). Genes for protein kinases, such as protein tyrosine kinase, were upregulated with genes for reciprocal regulatory function, such as protein tyrosine phosphatase and tissue inhibitor of metalloproteinase 1 (Table 3). Genes for apoptosis, such as CD24, nucleolin, and cytolysin, were upregulated with genes for anti-apoptosis, such as heme oxygenase (Table 4). Some genes with unknown mechanism were also upregulated after HS, including Ly6-B, Ly6 C antigen, Fyn proto-oncogene, and c-Myc EST2. Their roles in HS need to be further

investigated (Table 4). The induction of counterbalance genes (e.g. anti-inflammation, antioxidation, anti-proteolysis, anti-apoptosis) occurred simultaneously with the induction of inflammatory and stressor genes (e.g. inflammation, oxidation, proteolysis, apoptosis). This indicates that the host defense mechanism was triggered early to attenuate the tissue injury cumulated from systemic inflammatory response after HS. The balance achieved by host response may be fragile. Pro-inflammatory and stress response may be further upregulated once a secondary stress, such as subsequent infection, develops. Increased mortality from infection has been reported during the susceptible period after HS.[12] In our study, 11 genes were downregulated after HS; their significance needs more investigation.

Two major limitations of microarray studies are the relative fidelity of target/probe hybridization and the robustness of signal analysis. The well-documented discrepancies between microarray and real-time PCR data raise concerns about the significance of the small changes in mRNA abundance induced by HS observed in this study. While there were no false positives for selected genes in our study from the corroborative real-time RT-PCR data, we did not perform RT-PCR for all the differentially expressed genes. We did not address the issue of false-negatives (e.g. genes that we would expect to increase in these tissues). Some cytokine genes have been reported to have down-regulated expression after HS. However, their expression did not fulfill the criteria set in this study. For example, IL-2, which has been shown to be downregulated after HS in rats,[13] was not found to be downregulated in this study because the intensity ratio was 0.77. Actually, output variability between microarray analysis strategies is common; it sometimes results from the selection of gene chips, experimental procedures, or inherent genomic variation between animals. However, variation in expression of several genes will not alter the results when the gene profiles are subjected to cluster analysis according to their functions.

In conclusion, this gene profiling study found that controlled resuscitated HS induced expression of positive and negative control genes responsible for inflammation, oxidation, protein metabolism, and apoptosis. High upregulation of positive control genes will induce destruction of tissues and result in organ failure. On the other hand, high upregulation of negative control genes will lead to immunosuppression after HS. Although the roles of some genes were not clarified, the upregulation of these counterbalance genes indicates a vulnerable period in the host after HS, and overwhelming inflammatory response or immunosuppression may occur once a second hit, such as infection, ensues. Understanding, on a genome scale, how an organism responds to HS may facilitate the development of enhanced treatment modalities for HS.

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