Original Article
doi: 10.5115/acb.2011.44.2.98
pISSN 2093-3665 eISSN 2093-3673
Anatomy & Cell Biology acb

Gene expression profiling of mouse aborted uterus induced by lipopolysac charide

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Abstract: To identify genes that participate in the abortion process, normal pregnant uteri were compared to lipopolysaccharide (LPS)-induced abortion uteri. At day 6 of pregnancy, mice were treated with LPS at various time points to induce an abortion. Total RNAs were applied to a cDNA microarray to analyze genes with altered expression. At the early stage (2 hours) of LPS-induced abortion, upregulated genes were mainly composed of immune responsive genes, including *Ccl4*, *Ccl2*, *Cxcl13*, *Gbp3*, *Gbp2*, *Mx2*, *H2-Eb1*, *Irf1* and *Ifi203*. Genes related to toll-like receptor signaling were also overexpressed. At late stages of abortion (12-24 hours), many genes were suppressed rather than activated, and these were mainly related to the extracellular matrix, cytoskeleton, and anti-apoptosis. Altered expression of several selected genes was confirmed by real time reverse transcription-polymerase chain reaction. The results demonstrated that many known genes were altered in the LPS-treated pregnant uterus, implying that the molecular mechanisms of the genes involved in LPS-induced abortion are complicated. Further analysis of this expression profile will help our understanding of the pathophysiological basis for abortion.

Key words: Abortion, Lipopolysaccharide, Microarray, Mouse

Received March 28, 2011; Revised April 14, 2011; Accepted April 15, 2011

Introduction

Recurrent spontaneous abortion (RSA) is defined as three or more spontaneous consecutive pregnancy losses and occurs in approximately 1-2% of women of reproductive age [1]. The causes of repeated pregnancy loss are multifactorial, but they can be divided into two major causes: 1) embryologically driven causes are mainly due to an abnormal embryonic karyotype and 2) maternally driven causes are the result of endometrial and/or placental developmental problems. Known causes of maternal defects include coagulation disorders, autoimmune defects, endocrine disorders, and endometrial defects [2].

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The etiology of 50% of RSAs is unknown but it has been postulated that a proportion of these repeated pregnancy losses may be due to an abnormal immune response [3]. There is likely more than one immunological defect eliciting RSA, one of which may be the recognition of paternal antigens on the feto-placental unit by the maternal immune system followed by fetal destruction. However, actual evidence for this hypothesis is limited in humans. One of the problems in understanding the etiology of RSA immune failure is the lack of information on mechanisms by which the fetus is protected from the maternal immune system during normal pregnancy.

cDNA microarrays reveal altered gene expression patterns that occur in response to various biological stimuli. This technology allows for automated imaging analysis by computers and is well suited for large-scale gene expression pattern studies in tissues or cultured cells [4]. Comparing two or more contrasting points, such as during cell differentiation or cell activation, gives researchers an extremely powerful method to analyze alterations in gene expression [5]. It also

offers a great opportunity to rapidly identify critical genes associated with certain disorders, as well as to observe changes in thousands of genes at the same time.

In the present study, cDNA microarray analysis was performed to investigate the differential gene expression profiles in mouse uteri during lipopolysaccharide (LPS)-induced abortion.

Materials and Methods

Experimental animals

ICR strain mice used in this study were purchased from Damul Animal Laboratory Center (Cheongju, Korea). Before the experiments, animals were housed in cages maintained at 22-24°C and kept on a 14-hour light/10-hour dark cycle for at least 1 week. Food and water were available *ad libitum*. After overnight cohabitation of females with males, females with vaginal plugs (day 0.5 of pregnancy) were segregated. On day 6 of pregnancy, control pregnant mice (n=8) were treated with phosphate buffered saline (PBS), whereas experimental mice (n=32) were treated with an intraperitoneal injection of LPS (60 μg/kg in 0.2 ml PBS; Sigma Chemical Co., St. Louis, MO, USA). LPS-treated mice were divided into four groups and sacrificed at 2, 6, 12, and 24 hours after injection, respectively. The numbers of normal embryos and resorptions were determined and the uteri with an abortus were harvested for further study. All samples were obtained according to protocols for animal use approved by the Institutional Animal Care and Use Committee of Chonnam National University Research Institute of Medical Sciences, which were based on NIH guidelines (Guide for Care and Use of Laboratory Animals).

cDNA microarray fabrication

Experiments were performed using mouse 7.4-K cDNA microarrays, as previously described [6]. This microarray consists of 7,636 cDNA spots including incyte clones, housekeeping genes, and *Arabidopsis* DNA as controls. Polymerase chain reaction (PCR) reaction mixtures were subjected to 35 cycles of amplification. The primers used for amplification were: 5'-AAT TAA CCC TCA CTA AAG GG-3' and 5'-GTA ATA CGA CTC ACT ATA GGG C-3'. After the PCR products were hybridized and spotted onto CMT-GAPS II silane glass slides (Corning Inc., Corning, NY, USA) with a Pixsys 5500 arrayer (Cartesian Technologies, Irvine, CA, USA), the printed slides were processed according to the CMT-GAPS II slide protocol.

Preparation of fluorescent DNA probe and hybridization

Total RNA was extracted from mice uteri using TRI reagent (Molecular Research Center, Cincinnati, OH, USA). Fluorescence-labeled cDNA probes were prepared from 50 μg of total RNA by oligo (dT)18-primed polymerization using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The reverse transcription (RT) mixture included 400 U Superscript RNase H-reverse transcriptase; 0.5 mM dATP, dTTP, and dGTP; 0.2 mM dCTP; and 0.1 mM cyanin (Cy)3 or 0.1 mM Cy5 labeled dCTP (NEN Life Sciences, Boston, MA, USA). Then, sample RNA was degraded with stop solution and incubated at 65°C for 10 minutes. After being resuspended in hybridization solution, Cy3- and Cy5-labeled cDNAs were denatured at 95°C for 2 minutes and incubated in a 45°C water bath for 20 minutes. The cDNA mixture was then placed on a spotted slide and covered with a cover slip. The slides were hybridized for 12 hours in a 62°C hybridization chamber and were further washed with standard sodium citrate solution at room temperature.

Scanning and image analysis

The hybridized slides were scanned with the Axon Instruments GenePix 4000B scanner, and the scanned images were analyzed with GenePix Pro ver. 5.1 (Axon, New York, NY, USA) and GeneSpring ver. 6.1 (Sillicon Genetics, San Carlos, CA, USA) software programs. No data points were eliminated from the initial GenePix image by visual inspection to allow the algorithm to eliminate all bad spots. Housekeeping genes (β-actin) and positive control genes (*Arabidopsis thaliana*) were spotted onto each slide for signal normalization. The spot signals were used for normalization. We spotted a spotting solution on each slide to determine background signal intensity. To filter out unreliable data, spots with a signal-to-noise ratio <100 were not included in the analysis. Data were normalized using global, lowess, print-tip, and scaled normalization.

Statistical analysis

Data were clustered into gene groups that behaved similarly across a time course of experiments using GeneSpring ver. 6.1 software. We used an algorithm based on Pearson's correlation to separate the genes with similar

doi: 10.5115/acb.2011.44.2.98

patterns. Pearson's correlation clustering algorithm is not different, in that finding clusters of similar genes relies on finding and grouping those genes that are "close" to each other. In Pearson's correlation algorithm, calculating a distance between the time-course experiments is fundamental to place them in groups. The distance cutoff was considered statistically significant when there was a two-fold change between the time-course experiments, and the correlation cutoff was 0.95. The Student's *t*-test was used to determine the statistical differences between various experimental and control groups ($P<0.05$ after a Bonferroni correction). The microarrays were analyzed at each time point in duplicate. The microarray showed high reproducibility with a mean coefficient of variation of <20%. The accuracy of the microarray analysis was confirmed by real-time PCR analysis.

Real-time RT-PCR

Equal amounts of cDNA were added to 25 μl of QuantiTect SYBR Green Reaction Mix (Qiagen, Valencia, CA, USA) containing 0.5 M gene-specific oligonucleotide primers (see Table 1 for the oligonucleotide sequences). A parallel reaction was performed with β-actin specific primers for each sample. β-actin was chosen as the endogenous control because of its invariant expression in a range of tissues. Standard curves were generated simultaneously for both the target gene and the endogenous control gene using serial dilutions of one of the cDNA samples. The reaction was run in a RotorGene 3000 instrument (Corbett Research, Sydney, Australia) with the following cycling profile: denaturation/activation at 95°C for 15 minutes, followed by 35-40 cycles of denaturation at 95°C for 20 seconds, annealing at 58-60°C for 20 seconds, and elongation at 72°C for 30 seconds. The presence of a specific and unique PCR product for each pair of gene-specific primers was verified by the RotorGene 5-generated melting curve profile and by conventional agarose gel electrophoresis. Relative quantification of the initial amounts of the target and endogenous control was extrapolated from the respective standard curves using the RotorGene ver. 5 software. Target gene expression values were normalized to the respective β-actin values at each time-point.

Table 1. Primer sequences used for real-time reverse transcription polymerase chain reaction

Target	Oligonucleotide sequence	Reference[a)]
Ccl4	5' - AGT CCC AGC TCT GTG CAA ACC TAA C - 3' 5' - GGA AAT CTG AAC GTG AGG AGC AAG G - 3'	NM_013652
Mx2	5' - CAT AGG ACG CCA GAT CAA GAG ACT C - 3' 5' - ATA CCG TAC TTC TGC AGC TCC TCA C - 3'	NM_013606
Gbp2	5' - GCA GAA GGA GTT CGA GCT GAT GAT G - 3' 5' - GAG AAA CGT ATG GCT GGG CAT GAT G - 3'	NM_010260
Gbp3	5' - TTC ACC AAC GGC AAG ACC AAG ACT C - 3' 5' - GTA GCC CAG CTC AAT CTT CTT CCT G - 3'	NM_018734
β-actin	5' - TGT ACC CAG GCA TTG CTG AC - 3' 5' - AAC AGT CCG CCT AGA AGC AC - 3'	NM_007393

[a)]Genebank accession number.

Results

cDNA microarray

The cDNA microarray set allowed for 7,426 mouse cDNA/expressed sequence tag (EST) clones to be screened at one time. Among these clones, about 90% provided a detectable signal, and 388 genes (5.2%) were either upregulated or downregulated during at least one of the time points. Data were subjected to hierarchical clustering analysis for further investigation (Fig. 1). Among the clusters, nine principal clusters were defined by the Pearson's correlation clustering algorithm (data not shown).

In the LPS 2 hour-treated mouse uteri, 6,779 genes

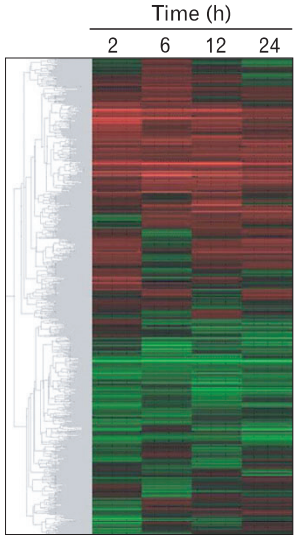

Fig. 1. Hierarchical clustering display of data from a 24-h time course of abortion following treatment with lipopolysaccharide. Hierarchical clustering of the complete set of 7,426 clones. Individual genes or clones are represented in rows, and time points are in columns. Individual cells are colored based on the log of the fluorescent ratio, with black representing a ratio of 1, or no change in expression relative to a control reference. Increasing ratios indicative of increased expression are represented by increasing red intensity, whereas decreasing ratios are represented by increasing green intensity and reveal decreased gene expression.

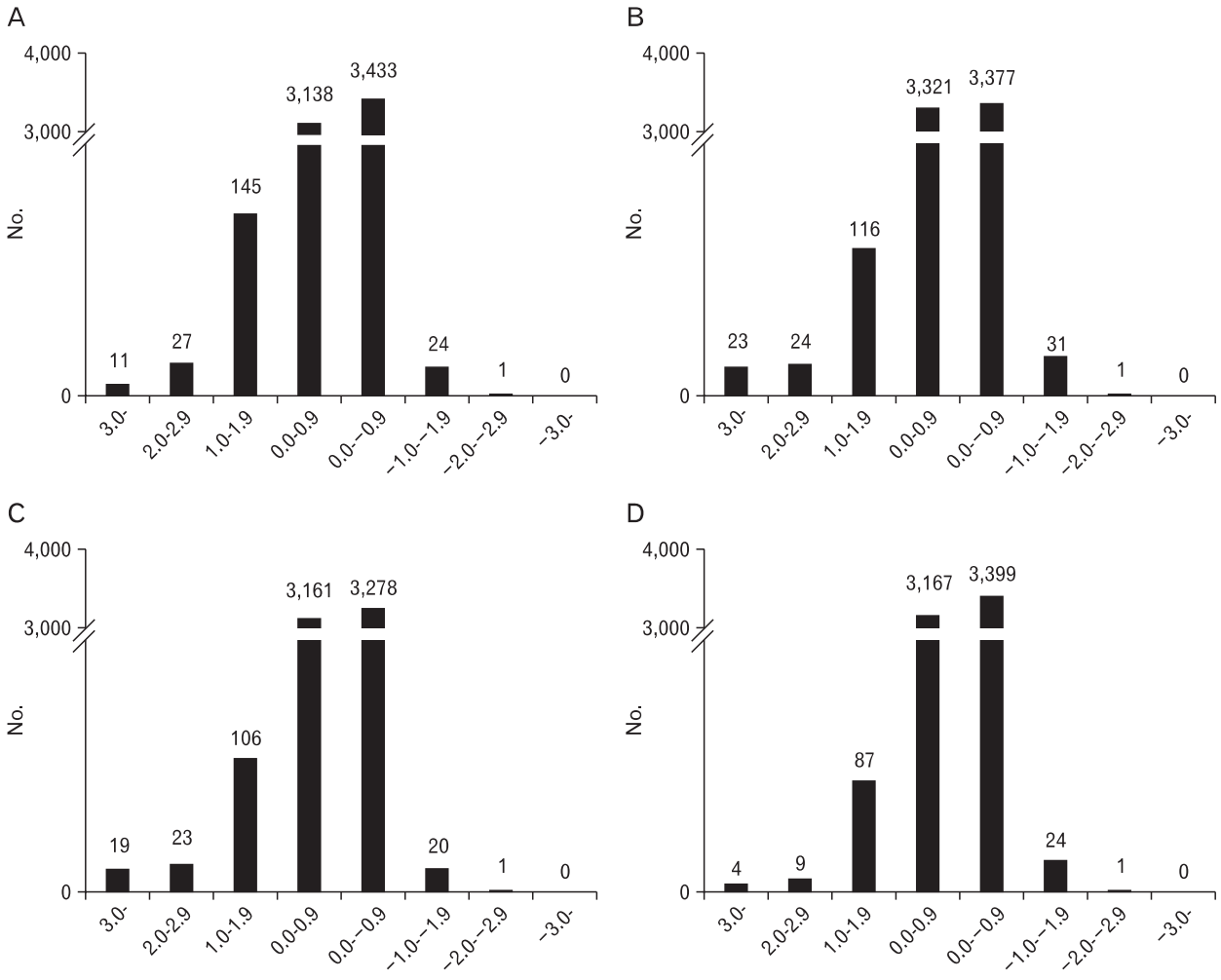

Fig. 2. Intensity ratio histograms showing the numbers (y-axis) of genes up- or down-regulated in lipopolysaccharide 2 h- (A), 6 h- (B), 12 h- (C), and 24 h- (D) treated mice uteri. The x-axis represents the log values for the intensity ratios (e.g., $\log_2 2$ indicates a two-fold change).

(91.3%) were detected and 208 genes (2.8%) were either upregulated or downregulated (Fig. 2A). A total of 183 genes were upregulated. These included nucleus-related genes (*Ifi203, Tgif*, *Pias4*), membrane-related genes (*Cybb*, *H2Eb1*, *Il4ra*), and extracellular and intracellular component genes (*Bcl10*, *H2-Ab1*, *Elk4*, *Maff*). However, 25 downregulated genes were mainly cellular component genes, including *A2m*, *Ly6d*, *Fgb*, *Slc29a1* and *Ptn* (Appendix 1 in the online-only Data Supplement). In the LPS 6 hour-treated uteri, 6,895 genes (92.8%) were detected and 197 genes (2.7%) were either upregulated or downregulated (Fig. 2B). In total, 165 genes were two-fold or greater overexpressed, whereas 32 genes were downregulated, including *Ly6d*, *Itpr5* and *Sox4* (Appendix 2 in the online-only Data Supplement). In the LPS 12 hour-treated uteri, 6,608 genes (89.0%) were detected, and expression was altered in 169 (2.3%); 148 were overexpressed and 21 were downregulated (Fig. 2C, Appendix 3 in the online-only Data Supplement). In the LPS 24 hour-treated uteri, 6,691 genes (90.1%) were detected, and 125 genes (1.7%) were either upregulated or downregulated; 100 genes were overexpressed and 25 were downregulated (Fig. 2D, Appendix 4 in the online-only Data Supplement). Genes that underwent significant changes (>8 times) are listed in Table 2.

Real-time RT-PCR

Real-time PCR analysis was performed with a subset of the differentially expressed cDNAs to confirm the observed differential gene expression. Four genes, representing all nine clustering groups, were chosen. These included chemokine (C-C motif) ligand 4 (*Ccl4*), guanylate nucleotide binding protein 2 and 3 (*Gbp2* and *Gbp3*), and myxovirus (influenza virus) resistance 2 (*Mx2*). The expression patterns of these

doi: 10.5115/acb.2011.44.2.98

Table 2. List of selected genes with significant changes (>8 times) in expression levels between control and LPS-treated uteri

Accession no.	Gene title	Gene symbol	Fold change			
			LPS-2 h	LPS-6 h	LPS-12 h	LPS-24 h
AA178155	Chemokine (C-C motif) ligand 4	*Ccl4*	32.03			
AA240404	Guanylate nucleotide binding protein 3	*Gbp3*	11.36	13.70	28.83	
AA164095	MAD homolog 3 (Drosophila)	*Smad3*	10.39	32.20	39.79	8.88
AI322747	Myxovirus (influenza virus) resistance 2	*Mx2*	9.80			
AA153021	Guanylate nucleotide binding protein 2	*Gbp2*	9.08		38.47	8.01
AA185052	RIKEN cDNA 2510004L01 gene	*2510004L01Rik*	8.89			
AA087193	Lipocalin 2	*Lcn2*	8.54			
AA174620	Histocompatibility 2, class II antigen E beta	*H2-Eb1*	8.52			
AA289657	2'-5' oligoadenylate synthetase-like 1	*Oasl1*	8.18	12.09		
AA152885	Chemokine (C-X-C motif) ligand 13	*Cxcl13*		23.29		20.96
AA210495	Serine (or cysteine) proteinase inhibitor, clade A, member 3G	*Serpina3g*		14.59	11.82	
AI324374	Baculoviral IAP repeat-containing 2	*Birc2*		12.48	20.04	
AA087193	Lipocalin 2	*Lcn2*		11.41	18.91	
AA178100	2'-5' oligoadenylate synthetase 2	*Oas2*		10.97		
AA209640	Histocompatibility 2, complement component factor B	*H2-Bf*		10.60	9.23	
AA023159	Signal transducer and activator of transcription 1	*Stat1*		10.38	14.13	
AA197393	DNA segment, Chr 7, ERATO Doi 458, expressed	*D7Ertd458e*		9.82	11.58	
AA204424	RIKEN cDNA 2700084L06 gene	*2700084L06Rik*		9.49	14.64	
AA276697	Interferon regulatory factor 1	*Irf1*		9.12		
AA145136	Expressed sequence AW111922	*AW111922*		9.10	16.14	
AA260490	Expressed sequence AW111922	*AW111922*		8.86		
AA522165	Chemokine (C-C motif) ligand 22	*Ccl22*		8.42		
AA123837	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	*Tap1*			10.52	
AA172624	Expressed sequence AI595338	*AI595338*			8.46	
AA230451	S100 calcium binding protein A8 (calgranulin A)	*S100a8*				8.87

Fold changes less than 8-fold are not shown in the table. LPS, lipopolysaccharide.

genes by RT-PCR were similar to those of the microarray analysis. The real-time PCR results were also consistent with the microarray data in a time-dependent manner (Fig. 3).

Discussion

LPS is normally present in the environment, whereas it acts as a potent danger signal to the innate immune system. The systemic administration of low-dose LPS, as performed in this experiment, can trigger pregnancy failure during or shortly after administration in mice [7, 8]. An LPS injection can also stress both mice [9] and humans [10]. Thus, data showing a broad diversity of genes modulated during LPS-induced abortion are novel along with the recent reports that have described a microarray-based approach to identify genes in mice uteri during the implantation [11] or post-implantation [12] period.

Current knowledge on the mechanisms of pregnancy maintenance and induced abortion is still somewhat scanty. Previous approaches to investigate pregnancy loss have generally relied on an analysis of individual candidate genes or gene families. With the availability of complete genome sequences for several mammalian species, it has now possible to implement a more comprehensive approach to assess gene expression patterns at the genome-wide level. The present study represents a step in that direction and establishes the basis for further in-depth investigations. While a complete analysis of the known and potential biological significance of the data presented here is beyond the scope of this study, the following discussion selectively highlights certain key points.

Of the 7,426 genes analyzed, 388 genes that underwent significant changes were grouped into nine distinct clusters, with each cluster containing many genes that were not previously known to be involved in uterine function. Genes that were upregulated at the early stages (2 hours) of LPS-induced abortion predominantly belonged to clusters 1, 4, and 7; thus, they represent genes that were highly expressed either at 2 hours (cluster 7), 6 hours (cluster 4), or 12 hours (cluster 1) after LPS injection. As expected, this group

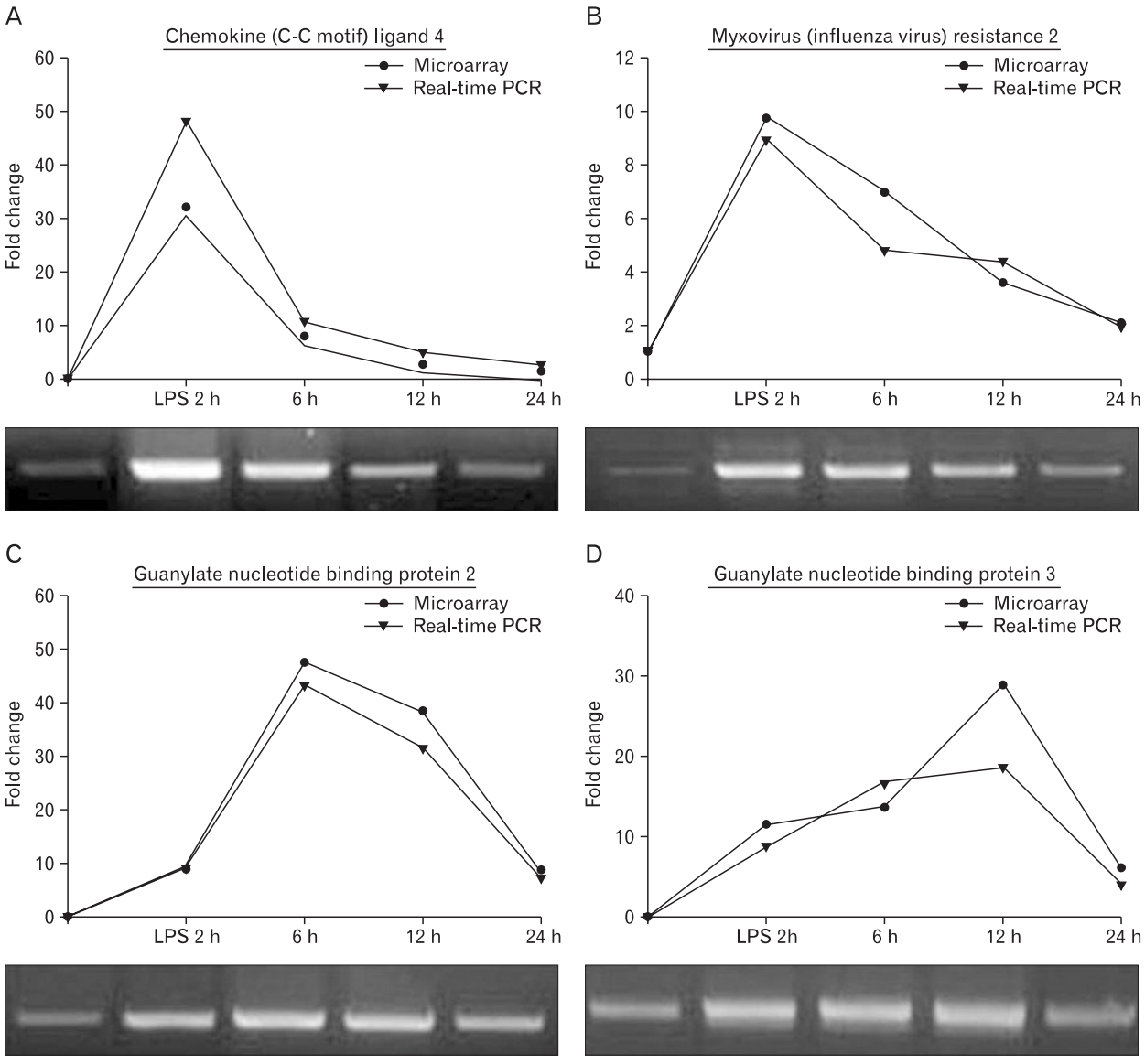

Fig. 3. Validation of the microarray data. Gene expression patterns representing different characteristic patterns were assessed by real-time polymerase chain reaction (PCR), and compared with the expression values obtained by microarray analysis. The genes assessed were: (A) *Ccl4* (Mm244263), (B) *Mx2* (Mm14157), (C) *Gbp2* (Mm24038), and (D) *Gbp3* (Mm1909). The oligonucleotides used for real-time PCR analysis are listed in Table 1. LPS, lipopolysaccharide.

encompassed immune response genes including *Ccl4*, *Ccl2*, chemokine (C-X-C motif) ligand 13 (*Cxcl13*), *Gbp3*, *Gbp2*, *Mx2*, histocompatibility 2, class II antigen E beta (*H2-Eb1*), interferon regulatory factor 1 (*Irf1*), and interferon activated gene 203 (*Ifi203*). Among the novel genes, *Gbp3* and *Gbp2* were identified as genes with a higher induction rate (about 10-fold) (Table 2). *Gbps* are not known to be expressed during pregnancy loss. Instead, they are known to play important roles in various basic cellular processes such as protein synthesis, signal transduction, intracellular protein transportation, cell proliferation and differentiation, and cytoskeletal regulation [13]. However, the physiological roles of these *Gbps* remain elusive. Kim et al. [14] recently reported that interferon (IFN) tau, a type-I IFN produced by the conceptus trophectoderm, stimulates many type-I IFN-stimulated genes, including *Gbp2*, in the ovine uterine endometrial stroma and glandular epithelium using the signal transducer and activator of the transcription 1-dependent pathways. Thus, *Gbps* qualify as potential intracellular mediators of the IFN-induced effect, and the precise role for

doi: 10.5115/acb.2011.44.2.98

the observed expression pattern of uterine *Gbps* and their potential significance for triggering abortion now requires further investigation.

Genes related to the toll-like receptor (TLR) signaling pathway were simultaneously overexpressed in this study. In particular, CD14 antigen (*Cd14*), inhibitor of kappa B kinase epsilon (*Ikbke*), nuclear factor kappa light polypeptide gene enhancer in B-cells 2, p49/p100 (*Nfkb2*), nuclear factor kappa light chain gene enhancer in B-cells inhibitor, alpha (*Nfkbia*), myeloid differentiation primary response gene 88 (*Myd88*), *Ccl4*, and signal transducer and activator of transcription 1 were overexpressed in the LPS 2 hour treated group, and these genes stimulate general upregulation of the TLR signaling pathway, including the MyD88-dependent and JAK-STAT pathways. As a result, the increase in *CD53*, *CD44*, and interleukin 4 receptor alpha gene products described here are novel findings, and this points to their possible function for proinflammatory and chemotactic effects with reference to the complement cascade. This was demonstrated by the result that the genes related to the classical coagulation cascade pathway, including histocompatibility 2, complement component factor B (*H2-Bf*), *C1r*, *C2*, and C3a receptor 1 were generally upregulated from 6 to 24 hours after LPS treatment.

Several genes that were upregulated only at the late stage of LPS-induced abortion mainly belonged to cluster 9, and were upregulated at 24 hours and/or 12 hours. These genes encompassed *H2-K1*, *C2*, *Emr1*, *Lst1*, *Expi*, *Itgb2* and other EST sequences. Many EST sequences also underwent significant upregulation at the early stage and it remains to be determined as to whether these short stretches of nucleotide sequences (200-500 bp) from randomly selected clones in a cDNA library are known corresponding genes and to which genes do the sequences have significant homology (Appendix 5 in the online-only Data Supplement). Analyzing ESTs helps to discover novel genes [15]. However, cloning the full-length cDNA sequences that were represented by the ESTs and clarifying their biological functions requires considerable effort. In contrast, numerous genes were downregulated at least at one of time points, and they encompassed the extracellular matrix, the cytoskeleton, and anti-apoptosis genes, including *Spock2*, *Col3a1*, *Col11a1*, *Epb4.1l2*, *Flna*, *Lamc1*, *Ptn*, *Dsip1*, *Adam 19* and *Nisch*. These results suggest that uterine gene suppression is equally important compared with the more extensively studied mechanisms of gene activation for the induction of pregnancy loss.

Obtaining placental tissue during early on-going human pregnancies is clearly not possible; therefore, studying the abnormal developmental mechanisms that have been postulated to occur during pregnancies destined to miscarry is difficult. Several studies have reported endometrial gene discovery and gene expression results in humans [16, 17], and in mice [11, 12]. The RNA for these studies was mainly derived from isolated and cultured endometrial cells, from biopsied endometrial tissue, or from human surgical specimens. Of critical importance when interpreting data derived from these kinds of specimens is acknowledging that cultured cells may have different responses *in vitro* compared with *in vivo*. Furthermore, endometrial biopsies are a heterogeneous mixture of cell types, and their interpretation must be validated. This is very challenging for experiments conducted with human subjects, whereas animal models provide more experimental uniformity and offer further value for validating the findings obtained using human tissues and cells. However, with mouse studies, the use of the whole uterus to investigate endometrial gene expression requires classical validation for the tissue of interest [18].

In conclusion, we identified genes with recognized roles during LPS-induced abortion, genes having potential roles in this process, and genes whose functions have yet to be defined during LPS-induced abortion. Identifying unique genetic markers for the onset of abortion signifies that a genome-wide analysis coupled with functional assays is a promising approach to understand the molecular pathways involved in successful pregnancy.

Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by the Korea Government (MOEHRD) (KRF-2005-003-E00146).

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