Arch Pharm Res Vol 29, No 10, 890-897, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

Expression Profiling of Lipopolysaccharide Target Genes in RAW264.7 Cells by Oligonucleotide Microarray Analyses

Hao Huang, Cheol Kyu Park, Ji Yoon Ryu, Eun-Ju Chang, Youngkyun Lee, Sam Sik Kang[1], and Hong-Hee Kim

Department of Cell and Developmental Biology, BK21 Program, Dental Research Institute, Seoul National University School of Dentistry, 28 Yeongon-Dong, Chongno-Gu, Seoul, 110-749, Korea and [1]Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul 110-460, Korea

(Received July 21, 2006)

In inflammatory responses, induction of cytokines and other immune regulator genes in macrophages by pathogen-associated signal such as lipopolysaccharide (LPS) plays a crucial role. In this study, the gene expression profile changes by LPS treatment in the macrophage/monocyte lineage cell line RAW264.7 was investigated. A 60-mer oligonucleotide microarray of which probes target 32381 mouse genes was used. A reverse transcription-in vitro translation labeling protocol and a chemileuminescence detection system were employed. The mRNA expression levels in RAW264.7 cells treated for 6 h with LPS and the control vehicle were compared. 747 genes were up-regulated and 523 genes were down-regulated by more than 2 folds. 320 genes showing more than 4-fold change by LPS treatment were further classified for the biological process, molecular function, and signaling pathway. The biological process categories that showed high number of increased genes include the immunity and defense, the nucleic acid metabolism, the protein metabolism and modification, and the signal transduction process. The chemokine-cytokine signaling, interleukin signaling, Toll receptor signaling, and apoptosis signaling pathways involved high number of genes differentially expressed in response to LPS. These expression profile data provide more comprehensive information on LPS-target genes in RAW264.7 cells, which will be useful in comparing gene expression changes induced by extracts and compounds from anti-inflammatory medicinal herbs.

Key words: Lipopolysaccharide, Microaray, Inflammation, Cytokine, RAW264.7

INTRODUCTION

Inflammation plays a role as the first line of defense in fighting against infection of pathogenic microbes. Inflammatory response is initiated by macrophages that recognize pathogens and become activated to produce cytokines and chemokines. These inflammatory mediators help recruitment of effector molecules and cells to the site of infection and induce endothelial cell activation to increase vascular permeability. The recognition of infected pathogens by the innate immune system including macrophages is mediated by pattern-recognition receptors that bind molecules with repeating patterns in microorganisms (Kaisho and Akira, 2000).

The Toll-like receptor (TLR) family receptors recognize pathogen-associated molecular patterns. To date 10 human and 13 mouse TLR molecules have been identified (Takeda and Akira, 2005). TLR-4 on macrophages recognizes lipopolysaccharide (LPS) of Gram-negative bacteria in association with CD14 (Qureshi *et al.*, 1999; Poltorak *et al.*, 1998). The LPS binding to TLR-4 triggers, through adaptor molecules TIRAP, MyD88 and TRAM, the activation of intracellular signaling pathways that ultimately leads to the activation of transcription factors NF-kB, IRF, and AP-1 (Barton and Medzhitov, 2003). These transcription factors then stimulate the gene transcription of inflammatory cytokines, chemokines, and type I interferons. Activation of TLR-4 by LPS has also been shown to trigger the surface expression of co-stimulatory molecules on macrophages, which is essential for induction of adaptive immune responses (Hoebe *et al.*, 2003).

DNA microarray is an efficient system to examine gene expression changes at a global level. In addition to cDNA

Correspondence to: Hong-Hee Kim, Seoul National University School of Dentistry, 28 Yeongon-Dong, Chongno-Gu, Seoul, 110-749, Korea
Tel: 82-2-740-8686, Fax: 82-2-765-8656
E-mail: hhbkim@snu.ac.kr

form of arrays, arrays with oligonucleotide form of probe sets have begun to be applied to several studies (Luo *et al.*, 2005; Sorlie *et al.*, 2006). Oligonucleotide arrays are divided into a short oligonucleotide type of which probe length ranges 25-30-mer and the oligonucleotide type that has 50-75-mer probes (Greenberg, 2001; Walker *et al.*, 2005). Since the development of these DNA microarray platforms, their application has been expanded to various fields of biological and medical research. However, it has only recently begun to be used in studies on medicinal herbs and natural products (Chen *et al.*, 2006). As gene expression profiling data are being accumulated, DNA microarray will become more useful in predicting effects of drug candidates, purified compounds from medical plants.

In this study, we investigated gene expression changes by LPS in the RAW264.7 macrophage-lineage cell line using an oligonucleotide DNA microarray. The microarray data give a more thorough view on the regulation of gene expression by LPS. In addition, the expression profile information will be useful in future studies examining gene regulatory function of medical herbs and oriental regimens used for anti-inflammatory effects.

MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, MO, U.S.A.). α-MEM, ampicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, U.S.A.). Trizol reagent and Superscript reverse transcription kit were from Invitrogen (Carlsbad, CA, U.S.A.). Mouse Genome Survey Microarray (oligonucleotide microarray chip), chemiluminescent RT-IVT labeling kit v.2.0, and chemiluminescence detection kit were purchased from Applied Biosystems (Foster, CA, U.S.A.).

Cell culture

RAW264.7 cells were grown in a-MEM medium supplemented with 100 U/mL penicillin G, 100 μg/mL streptomycin, and 10% heat-inactivated FBS in an humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37°C. The cells were seeded at a density of 5×10^5 cells/well in a six-well plate and incubated for 24 h before being treated with LPS or the control vehicle (dimethyl sulfoxide) for the time indicated in figures.

Preparation of RNA

Cells were washed with ice-cold PBS trice. One mL of Trizol reagent was added to each well. The extracts were transferred to a microfuge tube and centrifuged at 10,000 *g* for 15 min. The supernatants were collected and 500 μL isopropanol was added. The mixture was incubated for 10 min at room temperature and centrifuged for 8 min. The supernatants were collected and 75% ethanol was added. After centrifugation for 5 min, the precipitated RNA was dissolved in 25 μL nuclease free water. The concentration of RNA in the final solution was determined by spectrophotometry. The quality of RNA was examined by gel electrophoresis and only RNA of good quality was further processed.

Reverse transcription-polymerase chain reaction (RT-PCR)

One μg of total RNA obtained as above was heated at 65°C for 10 min followed by chilling on ice. The denatured RNA was annealed with 0.5 μg of poly dT in a 20 μL volume containing 1 mM dNTP, 25 mM Tris-HCl, 50 mM KCl, 2 mM DTT, and 5 mM $MgCl_2$. One unit of RNase inhibitor and 20 units of reverse transcriptase were added. The mixture was incubated for 50 min at 42°C and 15 min at 70°C for RT reaction. 1 μL of the cDNA was used. The thermal cycle was consisted of denaturing at 94°C, annealing at 58-60°C, and extension at 72°C for 30 sec each. The number of amplification cycles was determined to be in a linear range of amplification. 22 cycles were run for GAPDH and 25 ~ 30 cycles for the others. The PCR primer sequences used are as follows: TNF-α, 5'-ACACCGTCAGCCGATTTGC-3' (forward) and 5'-CCC-TGAGCCATAATCCCCTT-3' (reverse); IL-1α, 5-ATAAC-CTGCTGGTGTGTGAC-3' (forward) and 5'-TGCAGACT-CAAACTCCACTT-3' (reverse); IL-6, 5-TTGTGCAATGG-CAATTCT-3' (forward) and 5'-AGAGCATTGGAAATTG-GG-3' (reverse); GAPDH, 5-ACCACAGTCCATGCCATC-AC-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse).

RT-*In vitro* transcription (IVT) labeling

Digoxigenin (DIG)-labeled cRNA was generated from the total RNA prepared as above by using the chemiluminescent RT-IVT labeling kit as per the manufacturer's instruction. 2 μg of total RNA was reverse transcribed for 2 h at 42°C. The second strand DNA was synthesized for 2 h at 16°C. The double stranded cDNA was purified and mixed with DIG-UTP, IVT enzyme mix, and IVT buffer mix included in the labeling kit. IVT reaction was performed for 9 h at 37°C. DIG-labeled cRNA was purified and quantitated by spectrophotometry.

Hybridization and detection

The microarray chip was prehybridized for 1 h at 55°C. DIG-labeled cRNA was fragmented for 30 min at 60°C following the manufacturer's protocol. The fragmented cRNA was added to prehybridized chip and the chip was incubated for 16 h at 55°C with constant rocking at 100 rpm. The microarray chip was washed and incubated with

anti-digoxigenin-alkaline phosphatase for 20 min at 22°C. After washing, chemiluminescence substrate was added and the chip was immediately scanned with Applied Biosystems 1700 Analyzer.

Data processing and analysis

The data were filtered for values with more than 3 of signal to noise ratio and 0 Flags using the software in the scanner system. Differences in microarray intensities were normalized by regression normalization method and grouped using the Avadis Prophetic 3.3 (Strand Genomics Pvt. Ltd., http://avadis.strandgenomics.com/). Genes showing more than 2-fold increase or decrease were collected. The experiment was repeated three times (biological replicates) and genes showing changes in all three experiments were only considered. The fold change presented is the mean of the three replicates. The genes with more than 4-fold change were processed for categorizing by Panther classification (https://panther.appliedbiosystems.com/).

RESULTS

Effect of LPS on cytokine mRNA induction in RAW264.7 cells

Preparation of biological samples is an important initial step in microarray studies. Determination of appropriate treatment time and dose in cell culture-based experimental designs helps reducing number of microarray chips needed and thus decreases the cost. In order to determine

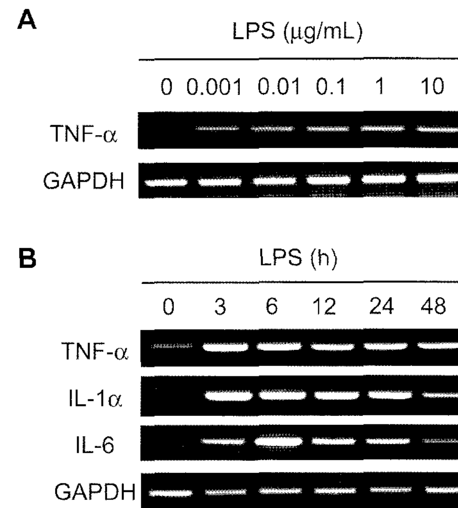

Fig. 1. Effects of LPS on cytokine expression in RAW264.7 cells. (A) RAW264.7 cells were treated with the indicated concentration of LPS for 24 h. The expression levels of TNF-α were determined by RT-PCR. (B) RAW264.7 cells were treated with 1 μg/mL LPS for the indicated time. The mRNA levels of TNF-α, IL-1α, and Il-6 were assessed by RT-PCR.

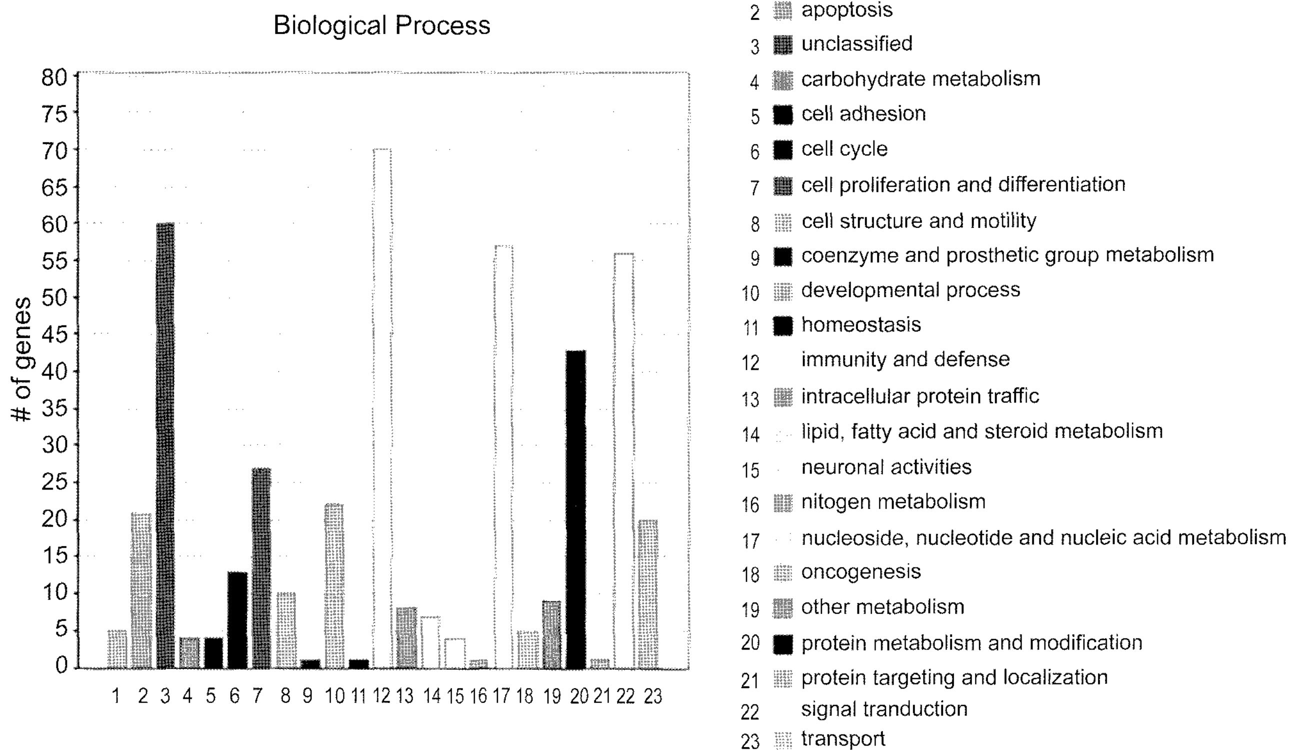

Fig. 2. Analysis of biological process of genes affected by LPS. The genes showing differential expression by 4 or more folds in RAW264.7 cells by LPS treatment at 1 μg/mL for 6 h were classified by Panther biological process.

Table I. Expression fold change of genes in the immunity and defense category

Gene_ID	Gene Symbol	fold	Gene_ID	Gene Symbol	fold
mCG129818.1	Adora2b	4.9	mCG130792.1	Il18	5.6
mCG8184.2	Ccl2	22.5	mCG20997.2	Il1a	21.7
mCG11624	Ccl3	8.3	mCG4837.2	Il1rn	17.6
mCG11627.1	Ccl4	20.8	mCG9125.2	Lgals8	4.5
mCG11684.1	Ccl5	349.1	mCG1597.2	Lgals9	9.6
mCG19020.1	Ccl9	4.9	mCG22374.2	Nfkb1	4.1
mCG15797.1	Ccrl2	141.6	mCG16031.2	Oas1b	10.9
mCG12531.2	Cxcl10	55.5	mCG16029.2	Oas1h	6.0
mCG12536.2	Cxcl11	176.1	mCG142507.1	Oas2	4.4
mCG1710	Cxcl2	200.2	mCG142508	Oas3	4.6
mCG16734.2	Fcgr1	8.9	mCG141547	Oasl1	26.9
mCG8631.2	Fcrl3	6.0	mCG141548	Oasl2	66.6
mCG16028.2	Fln29	7.0	mCG5001.1	Ptgs2	34.5
mCG11767.1	Fosl1	6.0	mCG8770.2	Rel	4.2
mCG12105.2	Gadd45b	11.0	mCG14500.2	Rgl1	4.5
mCG21119.2	Gbp3	105.3	mCG147785	Saa3	88.9
mCG15920.2	H2-Bf	53.4	mCG5428.2	Sdc4	10.0
mCG4997.2	H2-Q5	4.1	mCG20151.2	Slc11a2	7.1
mCG12223.1	H2-T23	4.2	mCG118877.1	Slfn8-pending	9.4
mCG132391.2	H2-T9	8.6	mCG21979.2	Src	9.4
mCG119794	Hck	4.7	mCG140850	Stat2	12.4
mCG120039	Ifi16	108.3	mCG23012.2	Tap1	10.1
NM_008327.1	Ifi202b	26.2	mCG23013.2	Tap2	4.9
mCG120042.2	Ifi203	13.7	mCG23001.3	Tapbp	5.1
mCG21470.1	Ifit1	245.6	mCG15911.2	Tnf	24.5
mCG21468.1	Ifit2	164.8	mCG14931.2	Tnfaip2	6.5
mCG2608.2	Ikbke	10.4	mCG17305.2	Tnfaip3	10.6
mCG2645.1	Il10	9.3	mCG8233.2	Tnfrsf1b	19.8
mCG11250.1	Il13ra2	8.4	mCG17530.2	Tnfrsf5	55.8
mCG119097.1	Il15	25.1	mCG141460	Usp18	34.3

the proper dose and time for LPS treatment prior to starting RNA preparation for microarray experiments, we evaluated dose and time course responses of RAW264.7 cells to LPS. The cells were treated with 0.001-10 μg/mL LPS for 24 h and the mRNA level of TNF-a was determined by RT-PCR (Fig. 1A). The TNF-a mRNA level increased from the lowest dose tested and appeared to reach the maximum level at 1 μg/mL (Fig. 1A). Next, the concentration of LPS was fixed at 1 μg/mL and the treatment time was varied from 3 to 48 h (Fig. 1B). The mRNA levels of TNF-α, IL-1α, and IL-6 were examined. The time point at which the cytokine mRNA levels were highest or nearly highest was 6 h. Therefore, we decided to treat RAW264.7 cells with LPS at 1 μg/mL for 6 h in the microarray sample preparation.

Gene expression changes in LPS-treated RAW264.7 cells

Based on the result shown in Fig. 1, we decided to treat RAW264.7 cells with LPS or the control vehicle (DMSO) at 1 μg/mL for 6 h in the RNA sample preparation for microarray experiments. The mRNA was converted to digoxigenin (DIG) labeled cRNA by RT-IVT reaction. Labeled cRNA was hybridized with a 60-mer oligonucleotide microarray chip that covers complete and annotated 32,381 mouse genes. The hybridized signal was detected by using anti-DIG-alkaline phosphatase and chemiluminescent substrate. This set of experiment was repeated three times.

The reproducibility of triplicates was 98% for the LPS-treated group and 96% for the control group. Among 9,983 genes with meaningful values, 747 genes were up-

regulated and 523 genes were down-regulated by more than 2 folds in response to LPS. 320 genes showing more than 4-fold change by LPS treatment were categorized by the Panther classification of biological process, molecular function, and pathway.

The LPS-responded genes were grouped into 23 biological process categories (Fig. 2). Besides the unclassified biological process category, the immunity and defense, the nucleic acid metabolism, the protein metabolism and modification, and the signal transduction categories contained high number of hits (Fig. 2). The genes belong to the immunity and defense category are listed in Table I. Classification based on Panther molecular function revealed that the nucleic acid binding, the signaling molecule, the transcription factor, and the select regulatory molecule categories have high number of genes (Fig. 3). Among the 32 Panther pathways, the apoptosis signaling, the inflammation mediated by chemokine and cytokine signaling, the interleukin signaling, and the Toll receptor signaling categories displayed high numbers (Fig. 4). The expression fold change of genes in the inflammation mediated by chemokine and cytokine signaling pathway is shown in Table II.

Discussion

Inflammation is a pivotal defense response of our body to infection by pathogenic microbes. Recognition of pathogen-associated certain molecules by macrophages triggers expression of inflammatory cytokines, chemokines, and other mediators (Ozato *et al.*, 2002). Other genes involved in metabolism, cytoskeletal regulation, cell cycle, and cell death have also been documented to be regulated (Albina *et al.*, 1993). In this study, we examined the expression profile elicited by LPS treatment in the macrophage cell line RAW264.7 by using a DNA microarray system.

Among 32381 gene probes, 9983 gene probes were significantly above background when the flag threshold was set at 0. This 30.8% signal detection rate is far lower than a previous report that showed 56% with the flag < 5000 (Walker *et al.*, 2005), which could be attributed to the more stringent flag applied to our study. Among the 9983 significant signals, expression of 1270 genes changed by 2 or more fold. Thus LPS caused a differential expression in 12.7% of genes in RAW264.7

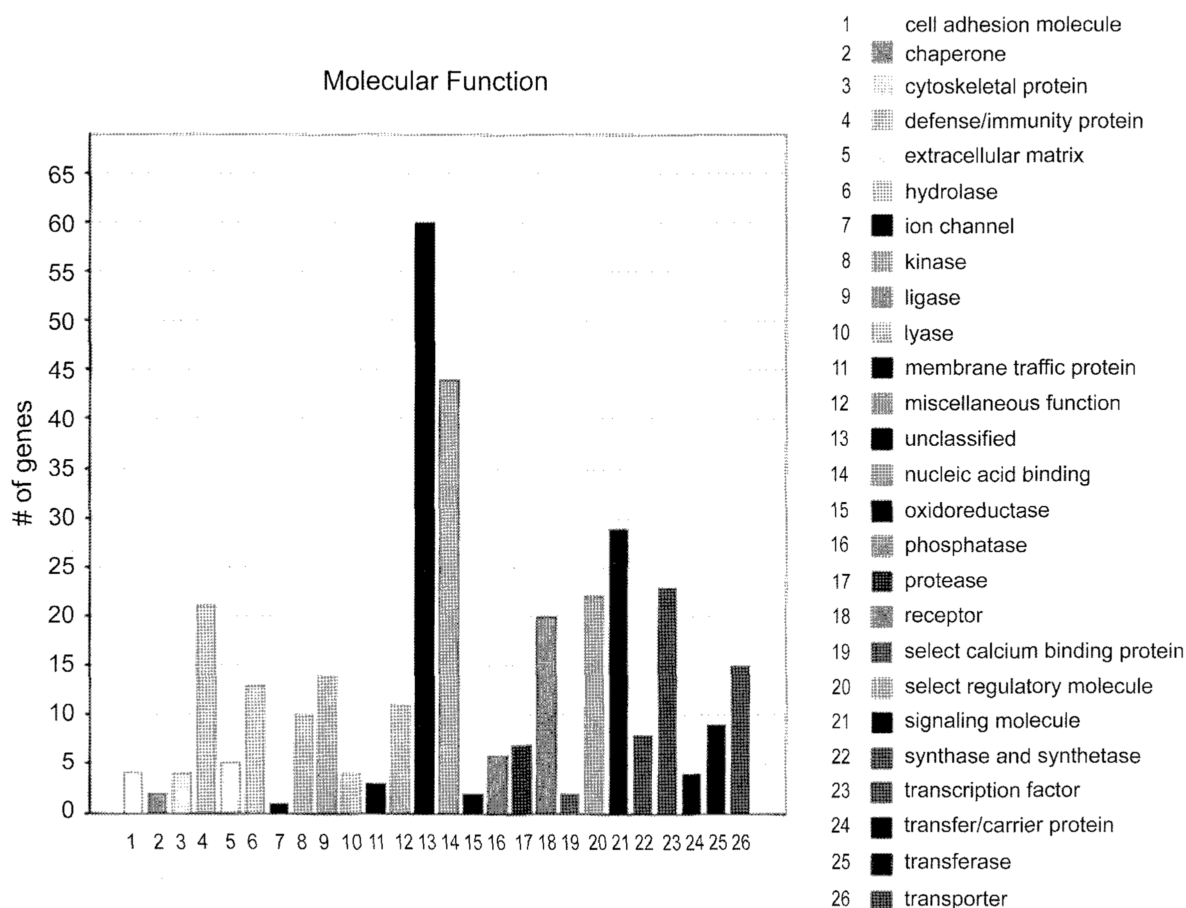

Fig. 3. Molecular function of LPS-target genes. The genes of which expression was changed by 4 or more folds by LPS in RAW264.7 cells were analyzed by Panther molecular function.

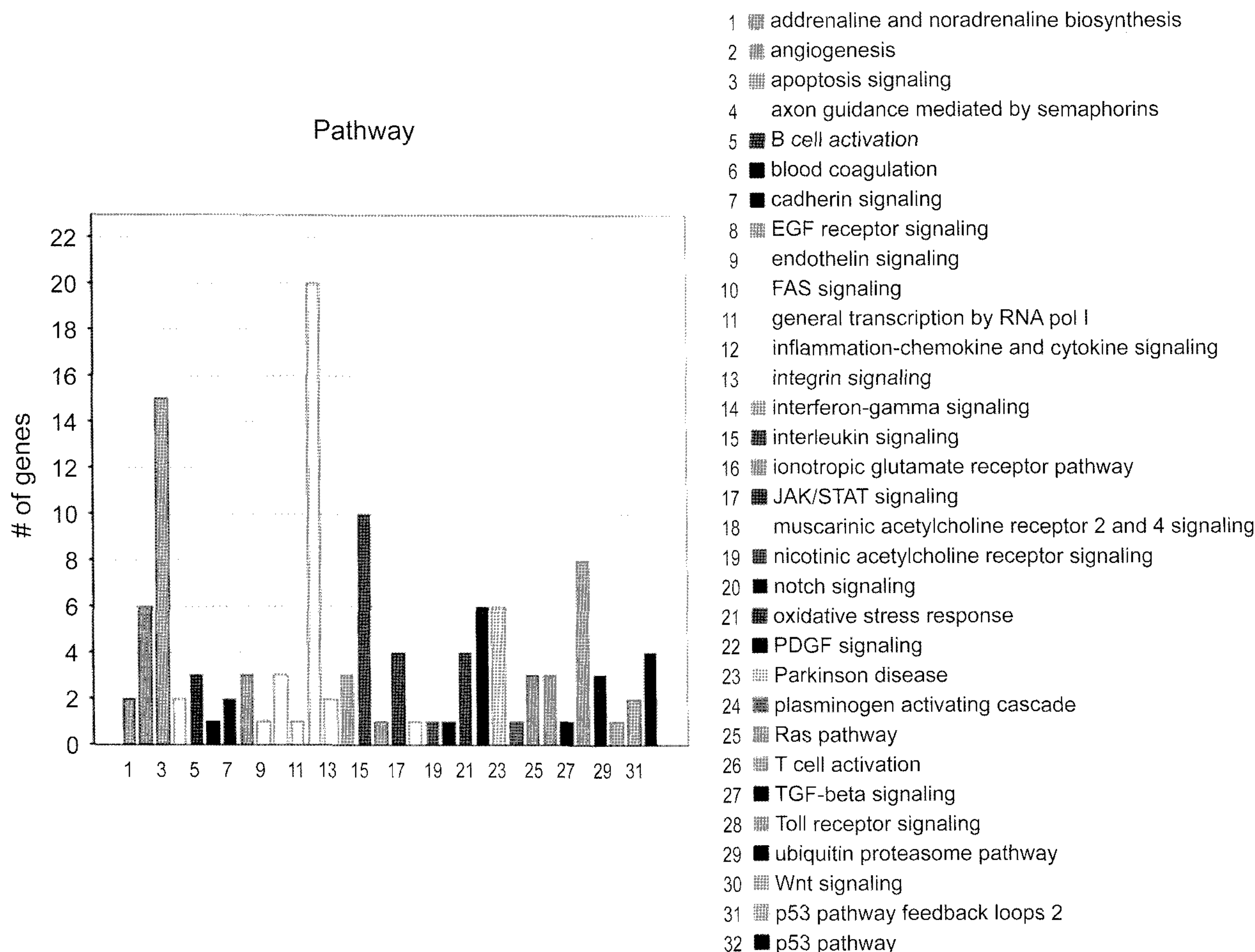

Fig. 4. Signaling pathway of LPS-responsive genes. The genes affected by 4 or more folds in RAW264.7 cells in response to LPS treatment were grouped by Panther pathway.

cells. Expression of 320 genes was affected by 4 or more folds by LPS in RAW264.7 cells. Most of those genes were up-regulated and only 10% (32 genes) were down-regulated, indicating LPS influenced gene induction more than gene suppression.

Analysis of affected genes revealed that genes associated with inflammation and defense were most highly affected. Both the number of genes and the extent of fold change were greatly modulated. The highly affected genes include several Ccl and Cxcl chemokines. Ccl5 has been reported to cause chemotaxis of monocytes, basophils, and mast cells (VanPtteren *et al.*, 1995; Conti *et al.*, 1997; Conti *et al.*, 1998; Volin *et al.*, 1998). The level of several interferon-inducible genes (IFG), Ifi and Ifit family genes, was also increased by LPS. The Ifi family proteins have been shown to interact with Rb and p53 and have been implicated in the regulation of cell growth and differentiation (Asefa *et al.*, 2004). The specific function and molecular characterization of Ifit members await further investigation. Several of the 2', 5'-oligoadenylate synthetase (Oas) family genes were also differentially regulated by LPS in RAW264.7 cells. Oas proteins have been suggested to have anti-viral function by producing 5'-phosphorylated, 2'-5'-linked oligoadenylates that stimulates RNase L, which in turn increases transcription of viral replication suppressors (Kajaste-Rudnitski *et al.*, 2006; Malathi *et al.*, 2005). The genes involved in signal transduction process that were differentially affected by LPS in RAW264.7 cells included dual specificity phosphatase 1 (Dusp1), growth arrest and DNA-damage-inducible 45 beta (Gadd45b), hemopoietic cell kinase (HCK), MARCKS-like protein (Mlp), phosphodiesterase 4b (Pde4b), Rab40b, ral guanine nucleotide dissociation stimulator like 1 (Rgl1), and suppressor of cytokine signaling 3 (Socs3) as well as NFkB and IkB genes.

Signaling pathway analyses with the genes differentially affected by LPS in RAW264.7 cells reveal several pathways had multiple gene expression changes. Besides the genes in the inflammation signaling pathway shown in Table II, apoptosis pathway contained highest number of genes affected. The genes belong to this pathway are Jun

Table II. Expression fold change of genes in the inflammation mediated by chemokine and cytokine pathway category

Gene ID	Gene symbol	fold	Gene ID	Gene symbol	fold
mCG123683.2	Stat1	9.1	mCG5001.1	Ptgs2	34.5
mCG22374.2	Nfkb1	4.1	mCG8770.2	Rel	4.2
mCG21979.2	Src	9.4	mCG19293.2	Socs3	57.9
mCG130792.1	Il18	5.6	mCG119097.1	Il15	25.1
mCG20077.2	Tnc	98.0	mCG11627.1	Ccl4	20.8
mCG15797.1	Ccrl2	141.6	mCG12531.2	Cxcl10	55.5
mCG2608.2	Ikbke	10.4	mCG11624	Ccl3	8.3
mCG20997.2	Il1a	21.7	mCG11684.1	Ccl5	349.1
mCG124090.2	Nfkbie	5.8	mCG8184.2	Ccl2	22.5
mCG119794	Hck	4.7			

dimerization protein 2 (Jundm2), B-cell leukemia/ lymphoma 2 related protein (Bcl2) a1b, Bcl2a1a,;Bcl2a1c, Bcl2-like 1, Fas death domain-associated protein (Daxx), Fas, protein kinase interferon-inducible double stranded RNA dependent (Prkr), Nfkb1, Rel, Tnfrsf1b, and Tnf. LPS was shown to prevent apoptosis of human peripheral blood monocytes (Mangan *et al.*, 1991). Regulation of apoptosis by Jun dimerization protein 2 and Bcl2 have been documented (Lerdrup *et al.*, 2005; Reed, 1995). The NFkB transcription factors play crucial roles in induction of anti-apoptotic genes.

Changes in gene expression patterns can provide an insight into the biological and disease processes. In addition, responses to toxicants, drugs, and nutrients can be monitored by surveying gene expression patterns. The DNA microarray provides an efficient and unique means to explore expression pattern of entire genome at a time. Naturally, since the discovery of DNA microarray (Schena *et al.*, 1995), its use has been widely extended to various studies. However, in the area of natural compound research the DNA microarry has only recently been started (Sharma *et al.*, 2006). Our study provided information on expression profile of entire genome in response to LPS. The inflammatory gene expression data will be useful in evaluating anti-inflammatory natural products. In fact, we found that the extract and certain compound from *Paeoniae radix* had suppressive effects on LPS-induced inflammatory gene up-regulation in the microarray analysis using the system described here. Accumulation of DNA microarray data with natural products will provide information useful in development of new drugs and standardization of the quality of medicinal herbs and products.

ACKNOWLEDGEMENTS

This work was supported by the Korea Food and Drug Administration through the National Center for Standardization of Herbal Medicine.

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