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Genome Wide Expression Profile of *Asiasarum sieboldi* in LPS-stimulated BV-2 Microglial Cells

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Abstract

Recent studies suggest that activated microglial cells play an essential role in the inflammatory responses and neurodegenerative disorders such as Alzheimer's and Parkinson's disease. This study was conducted to evaluate the protective mechanisms of *Asiasarum sieboldi* (AS) on LPS-induced activation of BV-2 microglial cells. The effects of AS on gene expression profiles in activated BV-2 microglial cells were evaluated using microarray analysis. BV-2 microglial cells were cultured in a 100 mm dish (1×10^7/mL) for 24 h and then pretreated with 1 μg/mL AS or left untreated for 30 min. Next, 1 μg/mL LPS was added to the samples and the cells were reincubated at 37°C for 30 min and 1 hr. The gene expression profiles of the BV-2 microglial cells varied depending on the AS. The microarray analysis revealed that MAPK signaling pathway-related genes were down-regulated in AS-treated BV-2 microglial cells. AS can affect the neuroinflammatory-related pathway such as MAPK signaling pathway in activated BV-2 microglial cells.

Keywords: LPS, *Asiasarum sieboldi*, Gene expression profile, Microarray

Microglia activated after brain injury, are macrophages resident in the central nervous system. Activated microglia release neurotoxic factors such as nitric oxide (NO), as well as cytokines and chemokines, such as IL-1β, IL6, and TNF-α[1-4]. NO is known to be an important mediator of acute and chronic inflammation. In addition, NO plays an important role in diverse physiological processes, including smooth muscle relaxation, platelet inhibition, neurotransmission, immune responses and inflammation[5]. Inhibition of microglial activation, therefore, would be an effective therapeutic approach to alleviate the progression of neuroinflammation disorder including Alzheimer's and Parkinson's disease[6-9].

In this study, during a search for new agents from medicinal plants for use in the treatment of neuroinflammation disease, the spray-dried extracts of 270 herbal medicines in a phytolibrary™ kit were tested for their ability to inhibit LPS-induced NO production in BV-2 microglial cells. Of these medicinal plants, *Asiasarum sieboldi* (AS) was selected for this study based on its higher inhibitory activity. AS is a well-known traditional Chinese medicine that is used an anti-allergic agent for the treatment of infections[10]. However, little is known of the inhibitory effects of AS in microglial activation.

Therefore, this study was conducted to determine the protective effects of AS on LPS induced activation in BV-2 microglial cells. Specifically, AS was evaluated to determine if it could prevent LPS induced activation of microglial cells. The anti-neuroinflammation strategies and their possible mechanisms are also discussed herein.

Gene Expression Profiles in BV-2 Microglial Cells

Gene expression profiles were significantly up- or down-regulated in the experimental groups (LPS or LPS plus AS-treated BV-2 microglial cells) when compared with the control (non-treated BV-2 microglial cells). In total, 1,742 differentially expressed genes (26%) were detected in the experimental group using approximately 45,100 oligonucloetide probes. These up- and down-regulated probes revealed that several genes involved in various functional categories were coordinately induced in the experimental groups when compared with those in the control (Figure 1). These genes were involved in processes including

metabolism, signal transduction, transcription, cell cycle, transport, apoptosis, biological process, development, cell adhesion & migration, translation, inflammatory & immune response and proliferation (Figure 1). Genes showing highly altered expression levels were aligned according to the magnitude of the altered expression. The most differentially expressed genes (6 up-regulated, 41 down-regulated) are listed in Table 1 and 2, which shows a comparison of the expression levels of a variety of genes between the experimental group and the control. All genes were grouped into functional categories and metabolic pathways based on the KEGG database.

Discussion

In this study, during a search for new candidate agents for the treatment of neuroinflammation disease using medicinal herbs, 270 herbal medicines in the phytolibrary™ kit were tested for their ability to inhibit LPS-induced NO production in BV-2 microglial cells. This search revealed that AS showed considerable inhibition at a concentration of 1 μg/mL; therefore, it was selected for further analysis to determine the inhibition mechanisms of AS on LPS-induced NO production in BV-2 microglial cells. In addition, we found that the AS non-cytotoxicity affected the viability of BV-2 microglial cells (data not shown). The early signaling events involved in LPS-induced microglial activation are not completely understood. Therefore the effects of AS on the gene expression profiles of BV-2 microglial cells that were treated for different lengths of time (30 min and 1 h) were evaluated. Specific and significant alterations of the expression profile of AS-treated BV-2 microglial cells were observed (Table 1 and 2). The genes found to be differentially expressed were responsible for inflammatory and immune response processes.

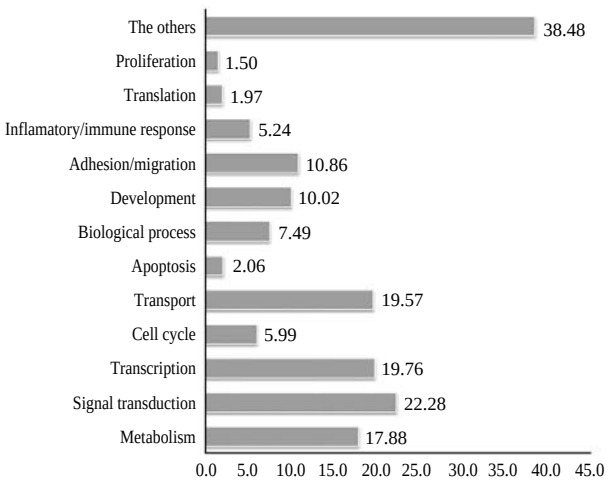

Figure 1. Gene Ontology classifications of genes based on comparison of gene expression between experimental (*Asiasarum sieboldi* (AS)-treated) and control (non-treated or LPS-treated) BV-2 cells.

Signaling from the TGFβ stimulates the MAPK pathways[11]. The MAPK pathways are deeply involved in signaling for various immune responses including apoptosis. MAPKs are serine/threonine kinases, which include the extracellular signal-related kinases (ERKs), p38 kinases, and c-Jun N-terminal kinases (JNKs). Activation of the MAPK pathway often occurs in response to growth factor stimulation of receptor tyrosine kinases, which are coupled to the activation of Ras G-proteins through Src homology 2 domain-containing proteins, such as Shc and Grb2, and quinine nucleotide exchange factors such as SOS[12,13]. In this study, we detected the down-regulation of *Tgfbr1*, *Tgfbr3*, *Mapk4*, *Fgf23*, *Fgf9*, *Fgfr3*, *Tgfbr1*, and *Tgfbr3* in AS-treated BV-2 microglial cells (Table 2). Each of the MAPKs have also been implicated in neuroinflammatory events, including mediation of many of the physiological responses to NO. NO is a signal-

Table 1. Up-regulation of genes based on comparison of gene expression between experimental (LPS plus *Asiasarum sieboldi* (AS)-treated) and control (non-treated or LPS-treated) BV-2 cells.

Gene description	Affimetrix probe set ID	Gene symbol	Regulation profile and ratio[a]			
			LPS		LPS+AS	
			30 m	1 hr	30 m	1 hr
<Cell adhesion-related genes>						
Cadherin 1	1448261_at	Cdh1	−2.7	−3.2	3.6	3.4
Cadherin 13	1454015_a_at	Cdh13	−3.1	−1.4	5.0	2.3
Cadherin 8	1456221_at	Cdh8	−1.4	−3.0	2.6	4.3
Cadherin-like 26	1456937_at	Cdh26	−2.8	−2.7	2.5	1.2
CD4 antigen	1427779_a_at	Cd4	−1.0	−1.2	3.9	1.7
protocadherin beta 22	1438106_at	Pcdhb22	−2.4	−3.7	3.2	1.7

[a]Ratio: log 2 (fold change)

Table 2. Down-regulation of genes based on comparison of gene expression between experimental (LPS plus *Asiasarum sieboldi* (AS)-treated) and control (non-treated or LPS-treated) BV-2 cells.

Gene description	Affimetrix probe set ID	Gene symbol	Regulation profile and ratio[a]			
			LPS		LPS+AS	
			30 m	1 hr	30 m	1 hr
<MAPK signaling pathway-related genes>						
fibroblast growth factor 23	1422176_at	Fgf23	1.3	1.8	−0.7	−0.5
fibroblast growth factor 4	1449729_at	Fgf4	0.5	3.2	−0.9	−3.4
fibroblast growth factor 9	1438718_at	Fgf9	0.9	2.1	−0.6	−0.5
fibroblast growth factor receptor 3	1425796_a_at	Fgfr3	3.3	3.8	−1.0	−0.6
mitogen-activated protein kinase 4	1435367_at	Mapk4	3.4	3.6	−2.0	−1.7
Protein kinase C, alpha	1445028_at	Prkca	4.3	4.7	−4.1	−0.8
Transforming growth factor, beta receptor I	1446946_at	Tgfbr1	0.7	0.9	−1.3	−1.4
Transforming growth factor, beta receptor III	1440041_at	Tgfbr3	1.8	3.1	−1.8	−2.0
<Cytokine-cytokine receptor-related genes>						
chemokine (C-C motif) ligand 20	1422029_at	Ccl20	3.7	4.5	−2.7	−1.8
chemokine (C-C motif) receptor 9	1421920_a_at	Ccr9	2.6	1.0	−2.6	−2.5
interleukin 11	1449982_at	Il11	2.3	2.4	−2.0	−0.7
Interleukin 28 receptor alpha	1446908_at	Il28ra	2.2	1.8	−1.2	−0.8
interleukin 9	1450565_at	Il9	2.8	1.3	−3.6	−0.9
tumor necrosis factor receptor superfamily, member 10b	1422344_s_at	Tnfrsf10b	1.9	1.5	−3.0	−0.8
nerve growth factor receptor (TNFR superfamily, member 16)	1421241_at	Ngfr	1.3	1.6	−0.9	−1.4
<G protein-coupled receptor protein signaling pathway>						
G protein-coupled receptor 110	1421443_at	Gpr110	2.7	0.9	−1.5	−0.7
G protein-coupled receptor 123	1429696_at	Gpr123	1.4	1.1	−1.7	−3.3
G protein-coupled receptor 56	1433485_x_at	Gpr56	0.8	3.0	−2.9	−1.0
G protein-coupled receptor 63	1440725_at	Gpr63	3.1	3.6	−2.0	−3.3
G protein-coupled receptor kinase 1	1421361_at	Grk1	0.7	2.5	−0.7	−2.4
prostaglandin E receptor 1 (subtype EP1)	1445445_s_at	Ptger1	1.4	1.2	−3.6	−0.9
regulator of G-protein signaling 18	1420398_at	Rgs18	3.9	3.5	−4.3	−1.2
Regulator of G-protein signaling 3	1427648_at	Rgs3	2.4	2.3	−2.1	−2.1
<Cell adhesion-related genes>						
fibronectin 1	1437218_at	Fn1	1.7	2.0	−2.7	−2.8
fibronectin type III domain containing 8	1430915_at	Fndc8	1.8	1.8	−2.3	−0.8
Immunoglobulin kappa chain variable 14-111	1427443_at	Igkv14-111	0.9	3.0	−0.6	−1.5
immunoglobulin kappa chain, constant region	1452417_x_at	Igk-C	2.6	0.6	−0.7	−1.0
Immunoglobulin superfamily, member 4	1444664_at	Igsf4d	4.3	4.6	−0.7	−0.9
immunoglobulin superfamily, member 9	1441420_at	Igsf9	1.6	4.4	−0.9	−3.2
Integrin alpha 4	1457376_at	Itga4	1.1	0.9	−0.6	−0.8
integrin binding sialoprotein	1417484_at	Ibsp	4.5	3.1	−1.8	−1.7
vascular cell adhesion molecule 1	1436003_at	Vcam1	3.9	2.3	−0.7	−0.9
<The others>						
C1q and tumor necrosis factor related protein 7	1460163_at	C1qtnf7	2.6	0.5	−1.3	−1.5
complement component 4 binding protein	1418037_at	C4bp	2.1	1.9	−3.4	−1.8
complement component 8, beta subunit	1427472_a_at	C8b	1.8	1.9	−1.4	−1.9
Dimethylarginine dimethylaminohydrolase 1	1438879_at	Ddah1	3.9	3.3	−3.0	−0.5
Ras association (RalGDS/AF-6) domain family 1	1456994_at	Rassf1	4.3	4.5	−3.0	−4.0
Ras association (RalGDS/AF-6) domain family 6	1429262_at	Rassf6	4.8	3.1	−2.5	−1.3
Ras interacting protein 1	1428016_a_at	Rasip1	1.6	0.7	−1.8	−1.2
TNFAIP3 interacting protein 2	1459844_at	Tnip2	1.2	2.0	−0.7	−3.3
Epidermal growth factor receptor pathway substrate 15-like 1	1441844_at	Eps15l1	1.1	1.1	−0.6	-1.3

[a]Ratio: log 2 (fold change)

ing molecule, neurotransmitter, and immune effector[12,14,15]. Also NO is produced by the activity of the family of enzymes nitrix-oxide synthases (NOSs), and the *DDAH* encoded enzyme plays a role in nitric oxide

generation by regulating cellular concentrations of methylarginines, which in turn inhibit nitric oxide synthase activity[16]. Leiper *et al*.[17] suggested that DDAH inhibition could be harnessed therapeutically to reduce the vascular collapse associated with sepsis. The results of this study revealed that *Ddah1* was down-regulated in AS-treated BV-2 microglial cells (Table 2).

TGFβ signaling is often coupled with alteration in cell adhesion and motility[11]. In many of these inflammatory situations, the expression of adhesion molecules is induced by cytokines. Adhesion molecules are shown to play important roles in the induction of inflammation. The interaction of the adhesion molecules (e.g., ICAM1, VCAM1 and E selectin) with their counter-receptors on circulating leukocytes (e.g., β1 and β2 integrins) results in the capture, rolling, and firm adhesion of the leukocytes to the vascular endothelium. The arrested leukocytes then transmigrate the vascular wall and move toward the lesion along the chemotactic gradient[18-21]. In our experiments, we detected the down-regulation of immunoglobulin kappa chain variable 14-111 (*Igkv14-111*), immunoglobulin kappa chain, constant region (*Igk-c*), Immunoglobulin superfamily, member 9 (*Igsf9*), Integrin alpha 4 (*Itga4*), integrin binding sialoprotein (*Ibsp*), and vascular cell adhesion molecule 1 (*VCAM1*) in AS-treated BV-2 microglial cells (Table 2). However, *cardherins* were significantly increased in this study. E-cadherin is predominantly located in adhesions junctions and linked to the actin cytoskeleton via β-catenin. Damage to the epithelium may result in loss of E-cadherin membrane expression and intercellular contacts. Also E-cadherin membrane expression is reduced in bronchial biopsies from asthma patients at the sites of epitherial lesions[22]. In our experiments, we detected the up-regulation of Cadherin 1 (*Cdh1*), *Cdh8, Cdh 26*, and protocadherin beta 22 (*Pcdhb22*).

Taken together, these results indicate that AS may have potential efficacy for the treatment of inflammation disease and other neurodegenerative diseases through anti-neuroinflammation by inhibiting TGFβ signaling and MAPK pathway.

Materials and Methods

Preparation of *Asiasarum sieboldi* (AS)

AS that was purchased from Sun Ten Pharmaceutical (Taipei, Taiwan), powdered to 0.1 g and then extracted by stirring in 10 mL of DW (distilled water) overnight at room temperature. The sample was then centrifuged for 10 min at 3,000 rpm, after which the supernatant was removed and sterilized by passing it through a 0.22 m syringe filter and then used for the experiments.

Cell Culture

The immortalized murine BV-2 microglial cell line, which exhibits both the phenotypic and functional properties of reactive microglia cells, was grown and maintained in 100% humidity and 5% CO_2 at 37˚C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), streptomycin, and penicillin (Invitrogen Life Technologies, Rockville, USA). BV-2 microglial cells were then plated onto 100 mm dish (1×10^7/mL) for 24 h, and then pretreated with 1 μg/mL AS or left untreated and incubated for 30 min. Next, 1 μg/mL LPS was added to the samples, and the cells were then reincubated at 37˚C for 30 min and 1 hr.

RNA Preparation

BV-2 microglial cells were initially cultured in a 100 mm dish (1×10^7/mL) for 24 h, and then pretreated with 1 μg/mL AS or left un-treated. The cells were then incubated for 30 min, after which 1 μg/mL LPS was added. Next, the cells were re-incubated at 37˚C for 30 min and 1 hr. The RNA was then isolated from the BV-2 microglial cells using an Rneasy® mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, after which the RNA was quantified using NanoDrop (NanoDrop Technologies, Inc ND-1000; Wilmington, DE, U.S.A).

Oligonucleotide Chip Microarray

Oligonucleotide chip microarray was performed using single round RNA amplification protocols, following the Affimetrix specifications (Affimetrix GeneChip Expression Analysis Technical Manual). Briefly, 3 micrograms of total RNA were used to synthesize first-strand complementary DNA (cDNA) using oligonucleotide probes with 24 oligo-dT plus T7 promoter as primers (Proligo LLC, Boulder, CO) and the Superscript Choice System (Life Technologies, Invitrogen, Milan, Italy). After double-stranded cDNA synthesis, the products were purified by phenol-chloroform extraction, and then biotinylated antisense complimentary RNA (cRNA) was generated through *in vitro* transcription using a BioArray RNA High-Yield Transcript Labeling Kit (ENZO Life Sciences Inc., Farmingdale, NY). The biotinylated labeled cRNA was then fragmented, and 10 g of the total fragmented cRNA was hybridized to the Affymetrix Mouse 430 2.0 GeneChip array (P/N900470, Affymetrix Inc., USA). The Affimetrix Fluidics Station 400 was then used to wash and stain the chips, after which the non-hybridized target was removed. Next, the samples were incubated with a streptavidin-phycoerythrin

conjugate to stain the biotinylated cRNA. The staining was then amplified using goat IgG as blocking reagent and biotinylated antistreptavidin antibody (goat), followed by a second staining step using a streptavidin-phycoerythrin conjugate. The fluorescence was detected using the Genechip System Confocal Scanner (Hewlett-Packard), and analysis of the data contained on each GeneChip was conducted using the GeneChip 3.1 software produced by Affymetrix, using the standard default settings. To compare different chips, global scaling was used, with all probe sets being scaled to a user-defined target intensity of 150.

Data Analysis

The MAS5 algorithm was used to evaluate the expression signals generated by the Affymetrix Mouse 430 2.0 array. Global scaling normalization was then performed and the normalized data were log-transformed with base 2. Next, Fold change was applied to select the differentially expressed genes (DEGs) using a fold change threshold of 1.5-fold and a $P < 0.05$ to indicate significance. Each probe set used in the Affymetrix GeneChip produces a detection call, with P (present call) indicating good quality, M (marginal call) indicating intermediate quality and A (absent call) indicating relatively low reliability. Therefore, probe sets that resulted in A calls in the compared groups were removed to filter false positives. The 1.5 -fold DEGs were clustered using the GenPlex™ v2.3 software (ISTECH Inc., Korea using hierarchical clustering with Pearson correlation as a similarity measure and complete linkage as the linkage method). In addition, gene ontology significance analysis was conducted to investigate the functional relationships among the 1.5-fold DEGs using high-throughput GoMiner. The 1.5-fold DEGs were then mapped to relevant pathways using GenPlex™ v2.4 software (ISTECH Inc., Korea). The pathway resources were provided by the KEGG database.

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