Gene Expression Analysis of Murine Lungs Following Pulmonary Exposure to Asian Sand Dust Particles

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The respiratory health impact of Asian sand dust events originating in the deserts of China has become a concern within China and in its neighboring countries. We examined the effects of Asian sand dust particles (ASDPs) on gene expression in the murine lung using microarray analysis and elucidated the components responsible for lung inflammation. Male ICR mice were intratracheally administrated ASDPs, heat-treated ASDPs (ASDP-F, lipopolysaccaride [LPS], or β-glucan free), or kaolin particles. We performed microarray analysis for murine lungs, the results of which were confirmed by quantitative reverse transcription–polymerase chain reaction (RT-PCR). We also assessed the protein expression and histologic changes. Exposure to ASDP, ASDP-F, or kaolin upregulated (>2-fold) 112, 36, or 9 genes, respectively, compared with vehicle exposure. In particular, ASDP exposure markedly enhanced inflammatory response–related genes, including chemokine (C-X-C motif) ligand 1/keratinocyte-derived chemokine, chemokine (C-X-C motif) ligand 2/macrophage inflammatory protein-2, chemokine (C-C motif) ligand 3/macrophage inflammatory protein-1α, and chemokine (C-X-C motif) ligand 10/interferon-gamma–inducible protein-10 (>6-fold). The results were correlated with those of the quantitative RT-PCR and the protein expression analyses in overall trend. In contrast, exposure to ASDP-F attenuated the enhanced expression of these proinflammatory molecules. Kaolin exposure increased the expression of genes and proteins for the chemokines. In histopathologic changes, exposure to ASDP prominently enhanced pulmonary neutrophilic inflammation, followed by kaolin and ASDP-F exposure in the order. Taken together, exposure to ASDP causes pulmonary inflammation *via* the expression of proinflammatory molecules, which can be attributed to LPS and β-glucan absorbed in ASDPs. Furthermore, microarray analysis should be effective for identifying potentially novel genes, sensitive biomarkers, and pathways involved in the health effects of the exposure to environmental particles (e.g., ASDPs).
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Key words: Asian sand dust particles; microarray analysis; chemokines; neutrophilic inflammation

Introduction

Epidemiologic studies have provided evidence for an association between particulate matters (PMs) and daily mortality (1). Most of these studies have been conducted in urban areas, where fine particles (aerodynamic diameters equal to or less than 2.5 μm: PM2.5) are the major type of soot, acid condensates, sulfate, and nitrate particles (i.e., artificial particles; Ref. 2). In fact, diesel exhaust particles (DEPs), the main constituents in PM2.5, have various immunotoxicologic effects *in vitro* and *in vivo*.

Alternatively, coarse particles (between 2.5 and 10 μm in diameter: PM10) originate primarily from geologic sources (soil and other crystal materials). Episodes of high levels of coarse particles are usually associated with high wind speeds that may tend to diminish the concentrations of fine particles (3). Wind erosion in arid and semiarid areas of middle and northwestern China forms the Asian sand dust particles (ASDPs). ASDPs spread over large areas, including East China, the Korean peninsula, and Japan. Recent epidemiologic studies have shown that sand dust events are associated with an increase in daily mortality in Seoul,

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Korea, and Taipei, Taiwan (4), and that ASDPs cause cardiovascular and respiratory dysfunction in Taipei (5). The characteristic of ASDPs changes during its long-range transport; thus, the degenerated/modified particles may enhance the toxic effects. In our recent study, we have detected lipopolysaccaride (LPS) and β-glucan in ASDPs (6). We have also demonstrated that repeated airway exposure of mice to ASDPs induces lung inflammation in the presence or the absence of allergen (6–8), which is concomitant with enhanced lung expression of proinflammatory molecules. However, these studies have been limited to the analysis of only a few molecules, and therefore the mechanisms underlying their proinflammatory properties remain poorly defined. Additionally, from the point of view of ASDP-attributable health assessment, the sensitive biomarkers at the early stage are desired. Global analytic tools elucidating the alteration on intracellular molecules following ASDP exposure can specify the sensitive biomarkers and shed light on the pathogenesis of ASDP toxicity.

Microarray technology (e.g., cDNA microarray or DNA chip) provides rapid analysis and abundance of information on expressed genes. Our recent report using cDNA microarray analysis has demonstrated that pulmonary exposure to DEP and/or LPS significantly upregulates the gene expression of stress responses or proinflammatory molecules. These results have been concomitant with quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis in overall trend (9). Thus, microarray analysis can be effective in identifying the genes involved in ASDP-induced lung inflammation and even in clarifying biologic pathways. The present study investigated the effects of ASDPs on the global pattern of gene expression in the murine lung using microarray analysis and elucidated the responsible components in ASDPs, which are important for lung inflammation.

Materials and Methods

Animals. Five-week-old ICR male mice (28–32 g) were purchased from CLEA Japan Inc. (Kanagawa, Japan) and maintained in conventional conditions for 1 week. They were fed commercial diet CE-2 (CLEA Japan) and water *ad libitum*. Mice were housed in an animal facility that was maintained at 22°C–26°C with 40% to 69% humidity and a 12:12-hr light:dark cycle. The study adhered to the National Institute for Environmental Studies guidelines for the use of experimental animals. The Institutional Review Board approved all animal studies. Thus, the animals were treated humanely and with regard for alleviation of suffering.

Preparation of Particle Samples. ASDPs were collected from desert surface soils at Shapotou Desert on the southern fringe of the Tengger Desert in north central China, where dust storms occur frequently. Kaolin (ASP product no. 400; Engelhard Corp., Iselin, NJ), a white crystal used as an adhesive, was selected because its diameter is to that of ASDP. ASDPs and kaolin were sieved as previously described (7). The mean distribution peak of ASDP or kaolin diameter was observed at 5.5 or 4.8 μm, respectively. In some cases, LPS and β-glucan contained in ASDPs were inactivated by heat sterilization at 350°C for 30 mins before use (ASDP-F).

Analysis of Elements, LPS, and β-Glucan in Particles. The content of elements in ASDPs was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES; 61E Trace and ICP-750; Thermo Jarrell-Ash, Grand Junction, CO). Elements in kaolin were described in the manufacturer's data sheet. The contents of LPS and β-glucan in each particle sample were measured by the kinetic assay using Endospec ES test MK (Seikagaku Corp., Tokyo, Japan) for LPS activity and Fungitec G test MK (Seikagaku) for β-glucan activity, according to the manufacturer's protocol. In brief, approximately 2.5 mg of each particle sample was suspended in 1 ml water (LPS and β-glucan free; Otsuka Co., Kyoto, Japan) for 1 hr and was placed on the bench top at room temperature for 2 hrs. The supernatants then were recovered and tested for LPS and β-glucan concentrations. The detection limits for LPS and β-glucan were <0.001 EU/ml and <2 pg/ml, respectively.

Study Protocol. The animals were randomly allocated to four experimental groups. The vehicle group received phosphate-buffered saline (PBS) at pH 7.4 (Invitrogen Co., Carlsbad, CA). The ASDP-F group, the ASDP group, and the kaolin group received 250 μg ASDP-F, ASDP, or kaolin in the same vehicle, respectively. In each group, mice were anesthetized with 4% halothane (Takeda Chemical Industries Ltd., Osaka, Japan), and then intratracheally inoculated 100-μl aliquots *via* a polyethylene tube as previously described (10).

Microarray Analysis. Total RNA from lungs was extracted with ISOGEN (Nippon Gene, Tokyo, Japan) 4 hrs after the intratracheal inoculation and then was purified using RNeasy mini kit (Qiagen Ltd., Clifton, Australia) according to the manufacturer's instructions. Total RNA was converted to cDNA with Superscript choice for cDNA synthesis (Invitrogen) and was subsequently converted to biotinylated cRNA with an Enzo High-Yield RNA Transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). Microarray hybridization was performed by MouseExpression Array 430A (Affymetrix, Santa Clara, CA). After hybridization, the gene chips were automatically washed and stained with streptavidin-phycocrythrin using a fluidics system. The chips were scanned with a Hewlett Packard GeneArray Scanner (Loveland, CO). From data image files, gene transcript levels were determined using algorithms in the Microarray Analysis Suite Version 5 software (Affymetrix). The microarray analysis was performed with four mice combined in each group, which was confirmed by two separate series of experiments using a total of eight microarrays. Differences between vehicle- and particle-treated mice and those between ASDP-F– and ASDP-treated mice also were determined using GeneSpring software

Table 1. The Contents of Elements in Particles

Components	Element fraction (%) ASDPs	Kaolin
SiO_2	60.0	45.4
Al_2O_3	11.1	38.8
Fe_2O_3	4.1	0.3
Na_2O	1.8	0.1
CaO	9.0	0.1
MgO	2.5	None
TiO_2	0.7	1.5
K_2O	2.2	Trace
Loss on ignition	8.6	13.8

(Silicon Genetics, Redwood City, CA). Genes that were significantly upregulated by more than 2-fold or downregulated by more than 0.5-fold in two separate series of experiments under the same conditions were averaged. The genes were categorized by the biologic process using NetAffyx Analysis (Affymetrix; http://www.affymetrix.com/analysis/index.affx).

Quantitative RT-PCR Analysis. Total RNA was treated with DNase I (TaKaRa BIO Inc., Osaka, Japan) and then was purified with phenol/chloroform/isoamyl alcohol. This RNA was reverse transcribed to cDNA using MuLV reverse transcriptase according to the manufacturer's instructions (Perkin-Elmer Corp., Foster City, CA). The quantitation of mRNA expression was carried out using the ABI Prism 7000 Sequence Detection System (Perkin-Elmer). The PCR amplification was performed as previously described (9). The quantitation of gene expression was derived using the standard curve method according to the manufacturer's protocol. The relative intensity was normalized to an endogenous control gene (18S rRNA). TaqMan probes and pairs for chemokine (C-X-C motif) ligand 1/keratinocyte-derived chemokine (CXCL1/KC), chemokine (C-X-C motif) ligand 2/macrophage inflammatory protein-2 (CXCL2/MIP-2), chemokine (C-C motif) ligand 3/macrophage inflammatory protein-1alpha (CCL3/MIP-1α), chemokine (C-X-C motif) ligand 10/interferon-gamma–inducible protein-10 (CXCL10/IP-10), and 18S rRNA were designed and purchased from Perkin-Elmer, which did not disclose these sequences.

Enzyme-Linked Immunoabsorbent Assay (ELISA). Murine lungs were removed 24 hrs after the intratracheal instillation. They were homogenized and centrifuged, and then the supernatants were recovered as previously described (10). ELISAs for CXCL1/KC, CXCL2/MIP-2, CCL3/MIP-1α, and CXCL10/IP-10 (all from R&D Systems, Minneapolis, MN) in the lung tissue supernatants were conducted according to the manufacturer's instruction. The detection limits of CXCL1/KC, CXCL2/MIP-2, CCL3/MIP-1α, and CXCL10/IP-10 were <2 pg/ml, <1.5 pg/ml, <1.5 pg/ml, and <2.2 pg/ml, respectively.

Table 2. The Contents of LPS and β-glucan in Particles[a]

	LPS (EU/mg particles)	β-glucan (pg/mg particles)
ASDP-F	3.70×10^{-4}	0.14
ASDP	3.66	15.20
Kaolin	3.26×10^{-4}	0.13

[a] LPS and β-glucan contained in ASDPs were inactivated by heat sterilization at 350°C for 30 mins (ASDP-F).

Histologic Evaluation. Murine lungs were removed 24 hrs after the intratracheal instillation. The lungs were fixed by intratracheal instillation of 10% neutral phosphate-buffered formalin (pH 7.2) at a pressure of 20 cm H_2O. After separation of the lobe, 2-mm–thick blocks were taken for paraffin embedding. Sections 3 μm thick were stained with hematoxylin-eosin. Histologic analyses were performed using a microscope (AX80; Olympus, Tokyo, Japan).

Statistical Analysis. Data were reported as mean ± SEM. Differences among groups were determined using ANOVA, followed by Games-Howell multiple comparisons (Stat view version 5.0; Abacus Concepts Inc., Berkeley, CA). *P* values less than 0.05 were considered significant.

Results

The Contents of Elements in Particles. The contents of elements in ASDPs and kaolin were 60.0% and 45.4% for SiO_2, 11.1% and 38.8% for Al_2O_3, 4.1% and 0.3% for Fe_2O_3, 1.8% and 0.1% for Na_2O, 9.0% and 0.1% for CaO, 2.5% and 0 for MgO, 0.7% and 1.5% for TiO_2, and 2.2% and trace for K_2O, respectively (Table 1).

The Contents of LPS and β-Glucan in Particles. We evaluated the contents of LPS and β-glucan in ASDPs before and after inactivation by heat treatment, as well as in kaolin (Table 2). The highest levels of LPS and β-glucan were observed in ASDPs (LPS: 3.66 EU/mg particles; β-glucan: 15.20 pg/mg particles). On the other hand, ASDP-F and kaolin showed very low levels of LPS and β-glucan.

Table 3. Number of Upregulated or Downregulated Genes[a]

Group	>2.0	<0.5
ASDP-F versus vehicle	36	0
ASDP versus vehicle	112	3
Kaolin versus vehicle	9	0
ASDP versus ASDP-F	82	1

[a] We performed microarray analysis using MouseExpression Array 430A to evaluate the gene expression of four experimental groups 4 hrs after intratracheal instillation in two separate series of experiments. We assessed the number of genes that showed a fold-change of >2.0 or <0.5 (average of two series of experiments) compared with the expression in the vehicle group or the ASDP-F group.

Table 4. List of 6-fold Upregulated Genes[a]

Group	Gene name	Average ratio	SD
ASDP-F versus vehicle (14 genes)	Natural killer tumor recognition sequence	12.06	7.20
	Serine/arginine-rich protein specific kinase 2	9.46	7.73
	Pantothenate kinase 3	8.98	8.41
	SMC6 structural maintenance of chromosomes 6-like 1 (yeast)	8.50	6.36
	Metastasis-associated lung adenocarcinoma transcript 1 (noncoding RNA)	7.65	7.57
	Jumonji, AT rich interactive domain 1A (Rbp2 like)	7.48	6.57
	Zinc finger homeobox 1b	7.13	7.06
	Rho GTPase activating protein 5	6.98	6.13
	Runt-related transcription factor 1	6.89	6.26
	Translocated promoter region	6.79	5.32
	Fetal Alzheimer antigen	6.66	6.59
	Eukaryotic translation initiation factor 3, subunit 8	6.51	5.72
	Heat shock protein 1, alpha /// similar to heat shock protein 1, alpha /// similar to heat shock protein HSP 90-alpha (HSP 86) (tumor-specific transplantation 86-kDa antigen)	6.35	5.95
	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6	6.14	2.63
ASDP versus vehicle (21 genes)	**Chemokine (C-X-C motif) ligand 1**	29.91	26.30
	Chemokine (C-C motif) ligand 3	24.31	2.38
	Chemokine (C-X-C motif) ligand 2	17.92	11.41
	CEA-related cell adhesion molecule 1	14.42	13.90
	Colony stimulating factor 2 (granulocyte-macrophage)	14.11	6.03
	Suppressor of cytokine signaling 3	11.72	11.29
	Serum amyloid A 3	8.92	1.31
	Nuclear factor of kappa light polypeptide gene enhancer in B-cells	8.78	8.69
	Jumonji, AT rich interactive domain 1A (Rbp2-like)	8.48	7.71
	ras homolog gene family, member U	8.12	6.89
	Kruppel-like factor 6	8.12	6.89
	Pantothenate kinase 3	7.91	7.19
	Interleukin 4-induced 1	7.21	0.35
	Chemokine (C-X-C motif) ligand 10	7.06	3.02
	Translocated promoter region	6.98	6.13
	Glutamate-cysteine ligase, catalytic	6.79	5.32
	Bobby sox homolog (Drosophila)	6.73	6.48
	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	6.66	6.59
	Interleukin 6	6.58	2.81
	Heat shock protein 1, beta	6.35	5.95
	Zinc finger homeobox 1b	6.07	5.34
Kaolin versus vehicle (3 genes)	**Chemokine (C-X-C motif) ligand 1**	13.85	8.27
	Histocompatibility 2, D region locus 1	6.60	5.60
	Chemokine (C-X-C motif) ligand 2	6.46	2.17
ASDP versus ASDP-F (13 genes)	**Chemokine (C-C motif) ligand 3**	28.13	5.48
	Immunoresponsive gene 1	27.22	25.50
	Interleukin 4-induced 1	19.04	0.93
	Chemokine (C-X-C motif) ligand 1	16.41	11.09
	Chemokine (C-X-C motif) ligand 2	14.06	2.74
	Serum amyloid A 1	13.78	12.52
	Chemokine (C-X-C motif) ligand 10	13.19	7.34
	Colony-stimulating factor 2 (granulocyte-macrophage)	12.06	7.20
	Serum amyloid A 3	11.78	1.73
	Chemokine (C-X-C motif) ligand 5	9.55	3.65
	C-type lectin domain family 4, member	8.36	6.55
	Interleukin 6	7.77	1.14
	Interleukin 1 beta	6.83	1.66

[a] Names of genes upregulated by more than 6-fold in two separate experiments are listed. The genes were categorized by the biologic process using NetAffyx Analysis (http://www.affymetrix.com/analysis/index.affx). Chemokines are denoted by boldface type.

Table 4. Extended.

Group	Accession no.	Biologic process description
ASDP-F versus vehicle (14 genes)	BB317504	Protein folding
	NM_009274	Cell differentiation
	BC027089	Coenzyme A biosynthesis
	AU022584	Chromosome organization
		Chromosome biogenesis
	AW012617	Unknown
	BB376407	Regulation of transcription, DNA-dependent
	NM_015753	Regulation of transcription, DNA dependent
	BM248774	Signal transduction
	NM_009821	Regulation of transcription, DNA dependent
	AW554765	Unknown
	BB380312	Regulation of transcription, DNA dependent
	BB858329	Translational initiation /// translational initiation
	C77384	Response to heat
	BC021452	Unknown
ASDP versus vehicle (21 genes)	NM_008176	Inflammatory response
	NM_011337	Inflammatory response
	NM_009140	Inflammatory response
	M77196	Unknown
	X03019	Immune response
	NM_007707	Regulation of cell growth
	NM_011315	Acute-phase response
	AF155372	Signal transduction
	BB376407	Regulation of transcription, DNA dependent
	AF378088	Signal transduction
	AF072403	Regulation of transcription, DNA dependent
	BC027089	Coenzyme A biosynthesis
	NM_010215	Electron transport
	NM-021274	Inflammatory response
	AW554765	Unknown
	BC019374	Glutathione biosynthesis
	BC024449	Regulation of transcription, DNA-dependent
	AW701251	Regulation of transcription, DNA-dependent
	NM_031168	Immune response
	BI154147	Response to heat
	NM_015753	Regulation of transcription, DNA dependent
Kaolin versus vehicle (3 genes)	NM_008176	Inflammatory response
	M83244	Immune response
	NM_009140	Inflammatory response
ASDP versus ASDP-F (13 genes)	NM_011337	Inflammatory response
	L38281	Unknown
	NM_010215	Electron transport
	NM_008176	Inflammatory response
	NM_009140	Inflammatory response
	NM_009117	Acute-phase response
	NM_021274	Inflammatory response
	X03019	Immune response
	NM_011315	Acute-phase response
	NM_009141	Inflammatory response
	NM_019948	Immune response
	NM_031168	Immune response
	BC011437	Inflammatory response

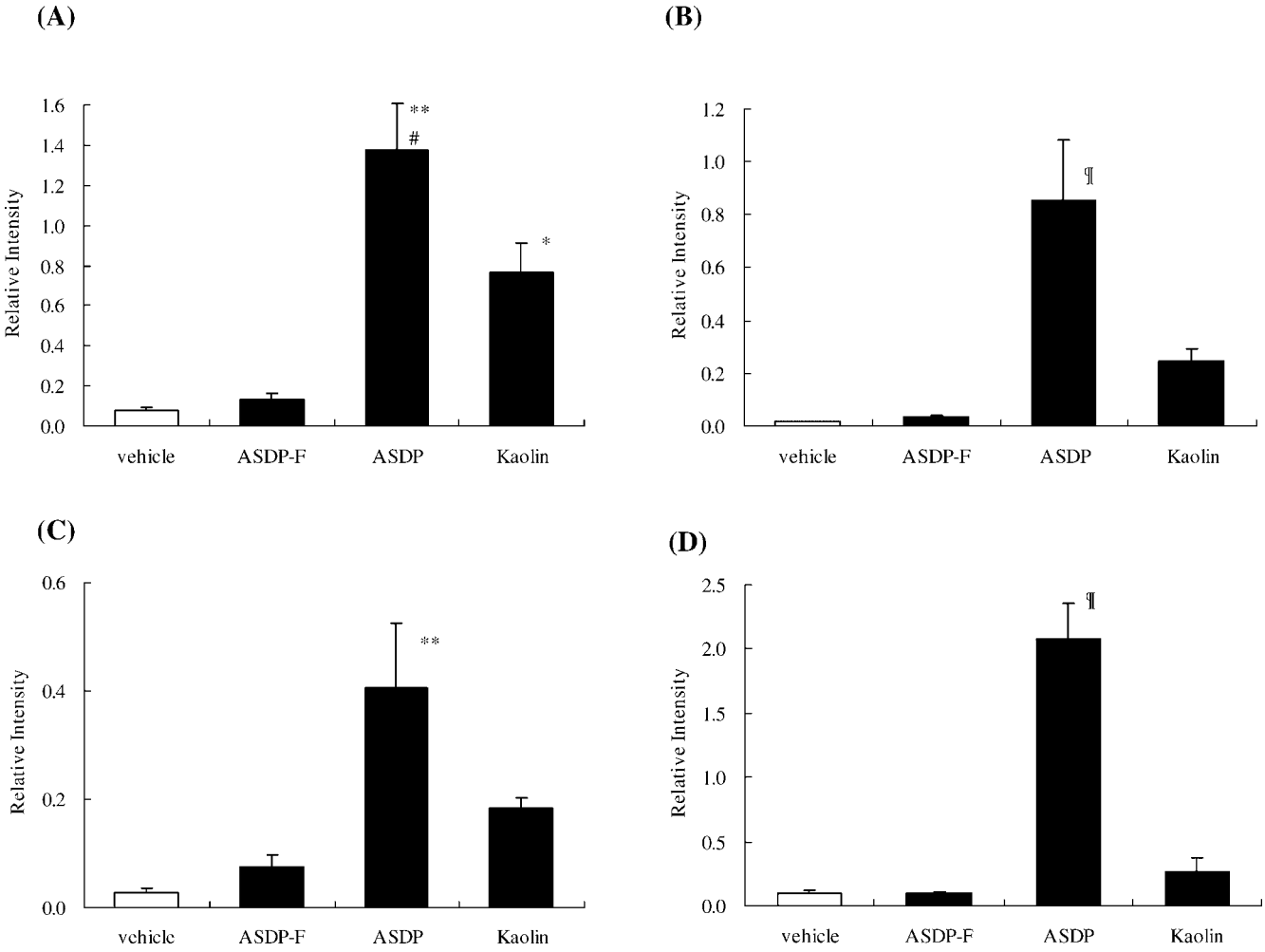

Figure 1. Quantitative RT-PCR analysis revealed increased gene expression of (A) CXCL1/KC, (B) CXCL2/MIP-2, (C) CCL3/MIP-1α, and (D) CXCL10/IP-10 at 4 hours after intratracheal administration of vehicle or particles (250 μg). The relative intensity of gene expression was quantitated by the standard method according to the manufacturer's protocol, and was normalized to 18S rRNA. *$P < 0.05$ versus vehicle group and ASDP-F group; **$P < 0.01$ versus vehicle group and ASDP-F group; #$P < 0.05$ versus kaolin group; ¶$P < 0.01$ versus other groups. Values are mean (SE) of eight animals.

Global Gene Expression Analysis Using Microarray Following Exposure to Particles. We performed microarray analysis using MouseExpression Array 430A to characterize the effects of pulmonary exposure to particles on gene expression in murine lungs 4 hrs after intratracheal instillation. We assessed the number of genes that showed a fold change of >2 or <0.5 (average of two experiments) in each group compared with the expression in the vehicle group (Table 3). Regarding the number of the genes showing 2-fold increased expression, 112 genes were upregulated after exposure to ASDP, which was the most prominent change among the experimental groups. In contrast, ASDP-F and kaolin exposure upregulated only 36 genes and 9 genes, respectively. On the other hand, <0.5-fold downregulated genes were scarcely detected (three genes by ASDP vs. vehicle). When compared to ASDP-F, ASDPs upregulated 82 genes. On the other hand, administration of ASDPs downregulated (<0.5-fold) only one gene compared with ASDP-F administration.

Modulation of Biologic Process Following Exposure to Particles. To elucidate the character of the enhanced genes in the biologic process, we defined the genes in each group that showed 6-fold upregulation compared with that in the vehicle group (Table 4). A total of 21 genes were upregulated by ASDP instillation, whereas 14 and 3 genes were upregulated by ASDP-F and kaolin instillation, respectively. In particular, administration of ASDP induced dramatic upregulation of inflammatory response–related genes, including CXCL1/KC, CXCL2/MIP-2, CCL3/MIP-1α, and CXCL10/IP-10 compared with vehicle administration, and even compared with ASDP-F administration. In contrast, exposure to ASDP-F attenuated the enhanced gene expression of inflammatory response. Administration of kaolin upregulated only the gene for CXCL1/KC and CXCL2/MIP-2 compared with vehicle

Table 5. Protein Expression Analysis in the Lungs[a]

	Pg/total lung supernatants			
Group	CCL3/MIP-1α	CXCLI/KC	CXCL2/MIP-2	CXCL10/IP-10
Vehicle	0.0	0.0	0.2 ± 0.2	47.1 ± 3.25
ASDP-F	5.92 ± 2.62	11.9 ± 3.89	4.94 ± 1.54	40.1 ± 1.31
ASDP	180 ± 22.7**,##	379 ± 34.6**,##	285 ± 49.9**,##	684 ± 161**,#
Kaolin	131 ± 17.7**	355 ± 59.0**	125 ± 30.0*	128 ± 28.4**

[a] Four groups of mice were intratracheally instillated with vehicle, ASDP-F, ASDP, or kaolin. Lungs were removed 24 hrs after instillation. Protein levels of inflammatory molecules in the lung tissue supernatants were analyzed using ELISA. Data are the means ± SEM of 8 animals per group.
*$P < 0.05$ versus vehicle group; **$P < 0.01$ versus vehicle group; #$P < 0.05$ versus ASDP-F group; ##$P < 0.01$ versus ASDP-F group.

administration. Furthermore, ASDP exposure showed a prominent increase in the gene expression involved in signal transduction and regulation of transcription. Interestingly, the upregulation of transcriptional regulation–related genes was shown equally both in the ASDP-F and in the ASDP groups.

Correlation Between Microarray and Quantitative RT-PCR Analyses. We also analyzed the gene expression of the four molecules (CXCL1/KC, CXCL2/MIP-2, CCL3/MIP-1α, and CXCL10/IP-10) by quantitative RT-PCR to confirm the results obtained from the DNA microarray analysis. Administration of ASDPs induced dramatic upregulation of CXCL1/KC, CXCL2/MIP-2, CCL3/MIP-1α, and CXCL10/IP-10 compared with vehicle or ASDP-F administration ($P < 0.01$; Fig. 1). In contrast, exposure to ASDP-F attenuated the gene expression of inflammatory response. Exposure to kaolin significantly upregulated the gene for CXCL1/KC compared with vehicle exposure and ASDP-F ($P < 0.05$). Additionally, the gene expression of CXCL2/MIP-2, CCL3/MIP-1α, and CXCL10/IP-10 also tended to increase in the kaolin group compared with the vehicle group. These results from the quantitative RT-PCR analysis were concomitant with those from the DNA microarray analysis in overall trend.

Changes in Protein Expression of Proinflammatory Molecules in the Murine Lungs Following Exposure to Particles. We further investigated the protein expression of proinflammatory molecules CXCL1/KC, CXCL2/MIP-2, CCL3/MIP-1α, and CXCL10/IP-10 in the lung tissue supernatants 24 hrs after intratracheal instillation (Table 5). Exposure to ASDP or kaolin enhanced the protein levels of four proinflammatory molecules compared with that to vehicle ($P < 0.05$). The most prominent increase was caused by ASDP exposure ($P < 0.01$ vs. vehicle group, $P < 0.05$ vs. ASDP-F group). ASDP-F exposure did not show a significant change in protein levels compared with vehicle exposure. The results from the protein expression analysis in the lungs were paralleled by those from the gene expression analyses.

Pathologic Changes in the Murine Lungs Following Exposure to Particles. Proinflammatory molecules, including chemokines, are mediators to attract leukocytes, which are associated with pulmonary inflammation. To determine the effects of particles on lung histology, we evaluated the lung specimens stained with hematoxylin-eosin 24 hrs after intratracheal instillation (Fig. 2). No pathologic alterations were found in the lung of the vehicle group (Fig. 2A). Recruitment of neutrophils was slight in the ASDP-F group (Fig. 2B). On the other hand, treatment with kaolin (Fig. 2C) or ASDPs (Fig. 2D and E) caused more remarkable infiltration of neutrophils and lymphocytes around the alveoli and terminal bronchioles (i.e., bronchopneumonia) than that with vehicle or ASDP-F. Furthermore, the lung inflammation was most prominent in the ASDP group.

Discussion

The present study has used microarray analysis to elucidate the effects of pulmonary exposure to ASDPs on the global gene expression in the murine lungs. The results have shown that intratracheal administration of ASDP elevates (>2-fold) the expression of various genes (112 genes) compared with vehicle administration. The enhancing effect following exposure to ASDP is characterized by upregulation of inflammatory response–related genes, such as CXCL1/KC, CXCL2/MIP-2, CCL3/MIP-1α, and CXCL10/IP-10. The results are paralleled by those from the quantitative RT-PCR and the protein expression analyses in overall trend. In contrast, exposure to ASDP-F upregulates 36 genes, which is accompanied by neither the gene expression nor the protein expression related inflammatory response such as chemokines. On the other hand, kaolin administration increases the expression of several proinflammatory genes (CXCL1/KC and CXCL2/MIP-2) and proteins (CXCL1/KC, CXCL2/MIP-2, CCL3/MIP-1α, and CXCL10/IP-10), whereas it upregulates only nine genes. In histopathological analyses, exposure to ASDP remarkably enhances neutrophilic inflammation in the murine lungs, which is followed by kaolin and ASDP-F exposure in the order.

The health impact of sand dust events from the desert area of China has become a concern within China and in its neighboring countries. Our previous study has demonstrated that repeated intratracheal instillation of ASDPs enhances

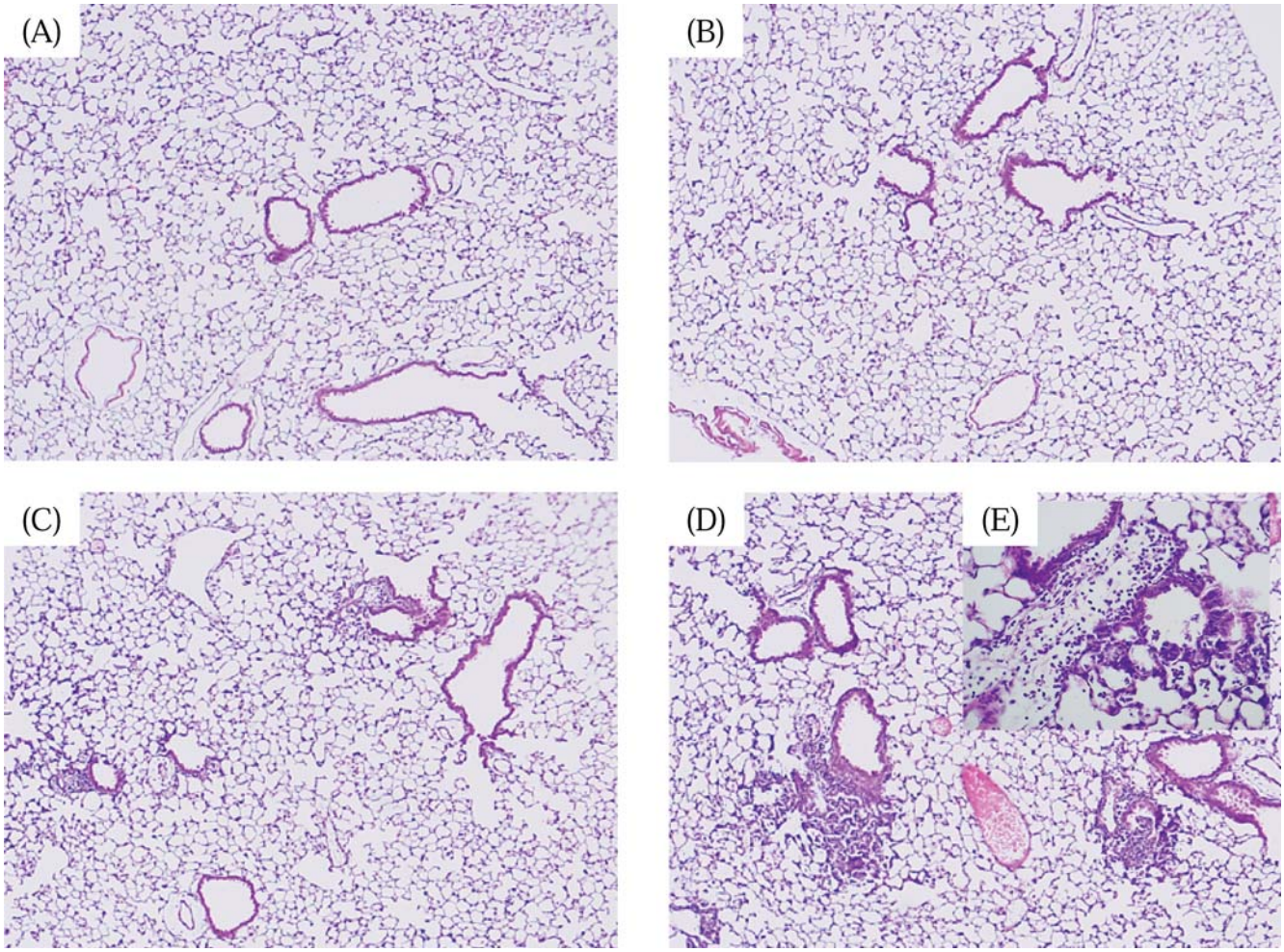

Figure 2. Histologic changes in the murine lung 24 hrs after the intratracheal instillation. The sections were stained with hematoxylin-eosin. (A) Vehicle group. (B) ASDP-F group. (C) Kaolin group. (D and E) ASDP group. Sections were observed at a magnification of ×100 (A–D) or ×400 (E).

airway inflammation in mice (6). This enhancement has been concomitant with the increased protein expression of granulocyte/macrophage colony-stimulating factor, interleukin-1β (IL-1β), IL-12, CXCL1/KC, and CCL3/MIP-1α. The study is, however, limited to the analysis of only a few genes.

In the present study, global gene analysis using DNA microarray showed that exposure to ASDPs prominently upregulated the expression of inflammatory response–related genes, including CXCL1/KC, CXCL2/MIP-2, CCL3/MIP-1α, and CXCL10/IP-10, in the lungs compared with the other exposure, which was consistent with the results from the quantitative RT-PCR and the protein expression analyses. In addition, histologic examination showed that neutrophilic lung inflammation was far more prominent in the ASDP group than in the ASDP-F group. In contrast, exposure to ASDP-F attenuated the enhanced gene and protein expression involved in inflammatory response, including CXCL1/KC, CXCL2/MIP-2, CCL3/MIP-1α, and CXCL10/IP-10, and even the pulmonary neutrophilic inflammation. It has been reported that neutrophil infiltration into the alveolar space is mainly mediated by CXC chemokines. Neutralization of CXCL2/MIP-2 and CXCL10/IP-10 has significantly reduced the accumulation of neutrophils and restrained lung inflammation *in vivo* (11, 12). Administration of anti-CXC chemokine receptor-2, a receptor of CXCL1/KC and CXCL2/MIP-2, has prevented neutrophil migration into the lung (13). CCL3/MIP-1α, a CC chemokine, has also been shown to contribute to leukocyte recruitment and lung inflammation. CCL3/MIP-1α is involved in neutrophil accumulation and lung permeability by LPS (14). Thus, neutrophilic lung inflammation following exposure to ASDP can be related, at least partly, to the gene and the protein expressions of proinflammatory molecules represented by these chemokines. In addition, exposure to ASDP also upregulated the other proinflammatory molecules, such as IL-6 and serum amyloid A3, in the present study (Table 4).

The present study demonstrated that exposure to inactivated ASDP (ASDP-F; i.e., LPS and β-glucan–free

particles) caused a lower number and magnitude of gene expression than ASDP exposure. The results were concomitant with those of protein expression and those of lung histology. LPS and β-glucan are the major structural component of cell walls of Gram-negative bacteria (15) and fungi (16), respectively. LPS induces or aggravates a variety of respiratory diseases, including asthma (17) and acute lung injury (18). On the other hand, β-glucan induces the production of proinflammatory molecules *in vitro* (19, 20). Our previous study has also suggested that LPS and β-glucan in ASDPs relate to neutrophilic lung inflammation (6, 21, 22). Taken together, LPS and β-glucan absorbed in ASDPs may upregulate the gene and the protein expressions for proinflammatory molecules and thereby play a role in the aggravation of lung inflammation.

While our previous microarray analysis system was different from the present one, exposure to LPS dramatically upregulated inflammatory response–related genes, such as serum amyloid A3, lipocalin 2, S100 calcium-binding protein A9, metallothionein 1, and metallothionein 2 (9). The effects caused by LPS exposure in our previous study were similar to those caused by ASDPs (containing LPS and β-glucan) exposure in the present study; however, the magnitude of gene expression was not identical between the two analyses. On the other hand, the gene expression pattern caused by DEP (another environmental particle) rather than ASDP exposure was characterized by the upregulation of oxidative stress response–related genes, such as hemeoxygenase-1 and cytochrome P450 1a1, which were not apparently induced by ASDP exposure.

In the present study, kaolin administration upregulated the expression of several proinflammatory genes (CXCL1/KC and CXCL2/MIP-2) and proteins (CXCL1/KC, CXCL2/MIP-2, CCL3/MIP-1α, and CXCL10/IP-10), in spite of the absence of LPS or β-glucan. In addition, kaolin exposure also induced neutrophilic lung inflammation. These results also suggest the importance of these chemokines in the lung inflammation, whereas the gene expression of the chemokines 4 hrs after the intratracheal instillation was slightly different from their protein expression 24 hrs after the instillation. In our previous studies, LPS and/or DEP significantly upregulated gene expression in murine lungs 4 hrs after intratracheal administration, which was paralleled by the protein levels 24 hrs after administration (21, 22). On the other hand, interestingly, the structure of kaolin is characterized by being multilayered and highly porous, which is different from ASDP, in spite of no dramatic changes between ASDP and kaolin in element analyses. Take together, the different structures of kaolin and ASDP might be responsible for the different process or time course for gene expression. Thus, we should at least investigate the time course of the gene expression in the next stage.

In conclusion, microarray analysis showed that exposure to ASDPs elevated a variety of genes in the murine lung. In particular, the gene expression markedly upregulated >6-fold included proinflammatory molecules, such as CXCL1/KC, CXCL2/MIP-2, CCL3/MIP-1α, and CXCL10/IP-10. The results from microarray analysis were concomitant with those from the quantitative RT-PCR and the protein expression analyses in overall trend. In histopathologic changes, exposure to ASDPs enhanced pulmonary neutrophilic inflammation. The enhancing effects could be attributed to LPS and β-glucan absorbed in ASDP. Furthermore, microarray analysis should be effective for identifying potentially novel genes, sensitive biomarkers, and pathways involved in the health effects of the exposure to environmental particles (e.g., ASDP).

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1. Dockery DW, Pope CA 3rd. Acute respiratory effects of particulate air pollution. Annu Rev Public Health 15:107–132, 1994.
2. Yang CY, Chen YS, Chiu HF, Goggins WB. Effects of Asian dust storm events on daily stroke admissions in Taipei, Taiwan. Environ Res 99:79–84, 2005.
3. Schwartz J, Norris G, Larson T, Sheppard L, Claiborne C, Koenig J. Episodes of high coarse particle concentrations are not associated with increased mortality. Environ Health Perspect 107:339–342, 1999.
4. Kwon HJ, Cho SH, Chun Y, Lagarde F, Pershagen G. Effects of the Asian dust events on daily mortality in Seoul, Korea. Environ Res 90: 1–5, 2002.
5. Chan CC. Adverse health effects of China sandstroms in Taipei [EPA-91-FA11-03-D036]. Taipei, Taiwan: Environmental Protection Administration, 2002.
6. Ichinose T, Nishikawa M, Takano H, Sera N, Sadakane K, Mori I, Yanagisawa R, Oda T, Tamura H, Hiyoshi K, Quan H, Tomura S, Shibamoto T. Pulmonary toxicity induced by intratracheal instillation of Asian yellow dust (Kosa) in mice. Environ Toxicol Pharmacol 20: 48–56, 2005.
7. Hiyoshi K, Ichinose T, Sadakane K, Takano H, Nishikawa M, Mori I, Yanagisawa R, Yoshida S, Kumagai Y, Tomura S, Shibamoto T. Asian sand dust enhances ovalbumin-induced eosinophil recruitment in the alveoli and airway of mice. Environ Res 99:361–368, 2005.
8. Ichinose T, Sadakane K, Takano H, Yanagisawa R, Nishikawa M, Mori I, Kawazato H, Yasuda A, Hiyoshi K, Shibamoto T. Enhancement of mite allergen-induced eosinophil infiltration in the murine airway and local cytokine/chemokine expression by Asian sand dust. J Toxicol Environ Health A 69:1571–1585, 2006.
9. Yanagisawa R, Takano H, Inoue K, Ichinose T, Yoshida S, Sadakane K, Takeda K, Yoshino S, Yamaki K, Kumagai Y, Yoshikawa T. Complementary DNA microarray analysis in acute lung injury induced by lipopolysaccharide and diesel exhaust particles. Exp Biol Med (Maywood) 229:1081–1087, 2004.
10. Takano H, Yoshikawa T, Ichinose T, Miyabara Y, Imaoka K, Sagai M. Diesel exhaust particles enhance antigen-induced airway inflammation and local cytokine expression in mice. Am J Respir Crit Care Med 156: 36–42, 1997.
11. Schmal H, Shanley TP, Jones ML, Friedl HP, Ward PA. Role for macrophage inflammatory protein-2 in lipopolysaccharide-induced lung injury in rats. J Immunol 156:1963–1972, 1996.
12. Zeng X, Moore TA, Newstead MW, Deng JC, Lukacs NW, Standiford TJ. IP-10 mediates selective mononuclear cell accumulation and activation in response to intrapulmonary transgenic expression and during adenovirus-induced pulmonary inflammation. J Interferon Cytokine Res 25:103–112, 2005.

13. Moore TA, Newstead MW, Strieter RM, Mehrad B, Beaman BL, Standiford TJ. Bacterial clearance and survival are dependent on CXC chemokine receptor-2 ligands in a murine model of pulmonary Nocardia asteroides infection. J Immunol 164:908–915, 2000.
14. Standiford TJ, Kunkel SL, Lukacs NW, Greenberger MJ, Danforth JM, Kunkel RG, Strieter RM. Macrophage inflammatory protein-1 alpha mediates lung leukocyte recruitment, lung capillary leak, and early mortality in murine endotoxemia. J Immunol 155:1515–1524, 1995.
15. Nikaido H. Structure of cell wall lipopolysaccharide from Salmonella typhimurium. I. Linkage between o side chains and R core. J Biol Chem 244: 2835–2845, 1969.
16. Hearn VM, Sietsma JH. Chemical and immunological analysis of the Aspergillus fumigatus cell wall. Microbiology 140:789–795, 1994.
17. Tulic MK, Wale JL, Holt PG, Sly PD. Modification of the inflammatory response to allergen challenge after exposure to bacterial lipopolysaccharide. Am J Respir Cell Mol Biol 22:604–612, 2000.
18. Wu Y, Singer M, Thouron F, Alaoui-El-Azher M, Touqui L. Effect of surfactant on pulmonary expression of type IIA PLA(2) in an animal model of acute lung injury. Am J Physiol Lung Cell Mol Physiol 282: L743–L750, 2002.
19. Hahn PY, Evans SE, Kottom TJ, Standing JE, Pagano RE, Limper AH. Pneumocystis carinii cell wall beta-glucan induces release of macrophage inflammatory protein-2 from alveolar epithelial cells via a lactosylceramide-mediated mechanism. J Biol Chem 278:2043–2050, 2003.
20. Vassallo R, Standing JE, Limper AH. Isolated Pneumocystis carinii cell wall glucan provokes lower respiratory tract inflammatory responses. J Immunol 164:3755–3763, 2000.
21. Takano H, Yanagisawa R, Ichinose T, Sadakane K, Yoshino S, Yoshikawa T, Morita M. Diesel exhaust particles enhance lung injury related to bacterial endotoxin through expression of proinflammatory cytokines, chemokines, and intercellular adhesion molecule-1. Am J Respir Crit Care Med 165:1329–1335, 2002.
22. Yanagisawa R, Takano H, Inoue K, Ichinose T, Sadakane K, Yoshino S, Yamaki K, Kumagai Y, Uchiyama K, Yoshikawa T, Morita M. Enhancement of acute lung injury related to bacterial endotoxin by components of diesel exhaust particles. Thorax 58:605–612, 2003.