Gene Expression Analysis of Lung Injury in Rats Induced by Exposure to MMA-SS Welding Fume for 30 Days

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Abstract

The welding fume has been implicated as a causal agent in respiratory disease such as pneumoconiosis. The molecular mechanism by which welding fume induces toxicity in the lung is still unknown, but studies have focused on histological structure and indirect approach measuring the pulmonary damage markers. In the present study, gene expression profiles were analyzed in the lung of rats exposed by manual metal-arc stainless-steel (MMA-SS) welding fume for 30 days using Affymetrix GeneChip®. Totally, 379 genes were identified as being either up- or down-regulated over 2-fold changes ($P < 0.01$) in the lung of low- or high-dose group and were analyzed by using hierarchical clustering. We focused on genes involved in immune/inflammation responses were differentially regulated during lung injury induced by welding fume exposure. The information of these deregulated genes may contribute in elucidation of the inflammation mechanism during lung injury such as lung fibrosis.

Keywords: Welding fume, Long term exposure, Inflammation, Gene expression, Lung fibrosis

The chronic inhalation of welding fume causes the respiratory impairment including pneumoconiosis[1], bronchitis and airway irritation[2] and these respiratory effects were often observed in full-time welders. The most common type of welding, manual metal arc welding (MMA), combined with stainless steel (MMA-SS) is known to highly emit the toxic compound. Studies have shown that well-established MMA-SS welding fume generation system can cause the pneumotoxic effect such as fibrosis in the lung of Sprague-Dawley (SD) rats[3-5]. The toxic components of welding fume have been shown to be mutagenic to mammalian cells[6,7] and to cause significant lung macrophage toxicity by generating the free radicals[8,9]. And also, the welding fume exposure has been linked to an increase of cancer risk as reported in some studies[10,11].

Analysis of cellular component of normal lung suggested that the lung contains large amount of inflammatory mediators and endogenous cytokines that regulate the homeostasis of the lung[12,13]. However, how this homeostasis is strictly regulated in the normal lung and the detailed mechanisms of lung injury are not well understood. In particular, inflammation likely plays a role in disease genesis and progression in human idiopathic fibrosis (IPF) and murine models of lung fibrosis. Many of cytokines and immune cells have been implicated in the lung disease, but their exact role and molecular mechanism remains intangible. To date, five hypotheses for the pulmonary fibrosis were proposed as follows; 1) the direct inflammation hypothesis suggests that inflammatory cells directly damage the lung cells *via* releasing enzyme like elastases[14], 2) the matrix hypothesis suggests that inflammatory mediators in the extracellular matrix leads to a prolonged wound-repair mechanism that results in the fibrosis[15], 3) some cell types with various growth factor receptors activate and amplify the inflammatory cascade as interacting with the corresponding ligands[16], 4) the plasticity hypothesis suggests that many of cells can differentiate into various cell types; for example, neutrophils and monocyte can differentiate to macrophage. The complex interaction of inflammatory mediators and other unidentified factors cause this differentiation and then activated cells can mediate the fibrotic phenotype[17], 5) the vascular hypothesis suggests that inflammation with autoimmune antibodies deposited in the vascular endothelium was activated by initial endothelial injury and result in fibrosis[18]. By using high-throughput techniques like microarray, we anticipate a better understanding of molecular mechanism during early response of lung injury.

Recently, the genomic approaches using microarray in the field of toxicity have been expanded to characterize how genes responds to toxicants and to understand the molecular mechanism in disease and dys-

function[19-22]. For the lung fibrosis induced by welding fume exposure, microarray analyses have been limited although some investigations were performed using whole blood sample to monitor the toxicity and discover the pulmonary damage markers[23,24]. We describe the global gene expression patterns in the lung to understand the molecular mechanism of early response of lung injury induced by welding fume exposure. Using Affymetrix GeneChip system, the gene expression profile at low- or high-dose group has been analyzed after inhalation of welding fume, for 2 hours per day during 30 days. Genes that are differentially expressed in the exposure group of welding fume were analyzed and genes involved in inflammation response were further analyzed in detail. In brief, we report on the gene expression profiling of early response of lung injury induced by welding fume exposure.

Lung Injury Induced by Welding Fume Exposure

To assess the lung injury induced by low- or high-
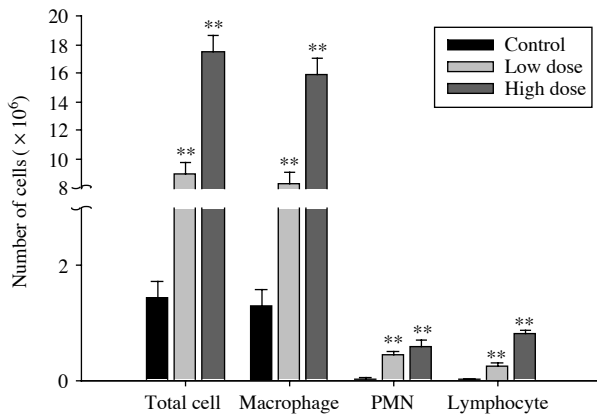
Figure 1. Changes of cell profiles in bronchoalveolar lavage fluid after 30 days of welding fume exposure. The error bars indicate standard error: **$P < 0.01$ (low and high dose group vs. control group).

dose of welding fume exposure, cell distribution in branchoalveolar lavage fluid (BALF), inflammation parameter, and histopathological change were analyzed. As shown in Figure 1, the inhalation of welding fumes induced remarkable increase of cell population in BALF. In both low- and high-dose group comparing to control group, it was observed that significant elevation of total cells, macrophage, polymorphonuclear cell (PMN), and lymphocyte ($P < 0.01$). These significant increases of inflammatory cells indicate that inflammation response was triggered and significant cells infiltrate into lungs by welding fume exposure. Biochemical value of inflammatory parameters of lung injury such as β-n-acetyl glucosaminidase (β-NAG) and lactate dehydrogenase (LDH) was statistically significantly increased over 4 to 10 times in the low- and high-dose group (Table 1). The increase of β-NAG and LDH indicates that phagocytes were activated and lung injury was induced. As shown in Figure 2, histopathological observation of lung showed that the pleural surfaces of the rats exposed by welding fume appeared mottled and black foci were evident on the lung surfaces. The particle-laden macrophage, lymphocyte and foamy giant macrophage were observed in the small bronchioles and the gas exchange regions such as alveolar ducts, alveolar sacs, and alveoli in the both of low- and high-dose group. The histopathological change of low- and high-dose group was almost similar and no distinct fibrosis in the bronchioles or lung parenchyma was not observed

Table 1. BALF biochemical values on 30 days of welding fume exposure.

Exposure group	Inflammatory parameters	
	β-NAG (IU/L)	LDH (U/L)
Control	1.00 ± 0.47	26.50 ± 5.91
Low dose	4.40 ± 0.46**	155.00 ± 39.51**
High dose	6.25 ± 2.30**	362.72 ± 141.02**

Values are means ± S.D. *indicates the P values; **, $P < 0.01$ (low or high dose group vs. control).
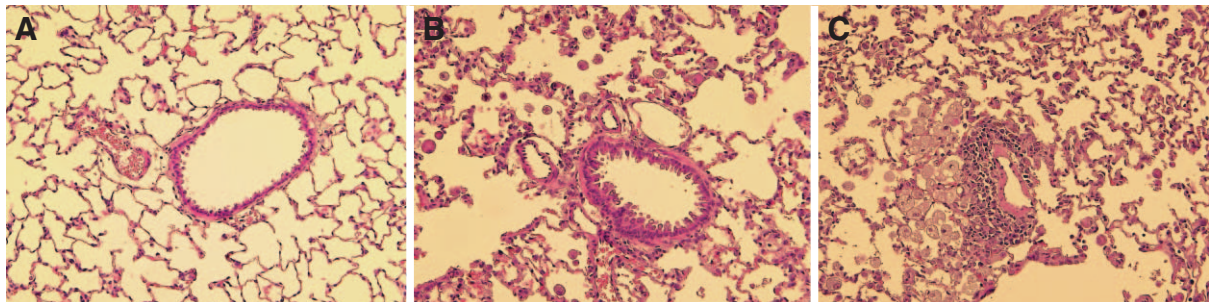
Figure 2. Light micrograph of lung from rats after 30 days of welding fume exposure. (A) Control, (B) Low dose, (C) High dose.
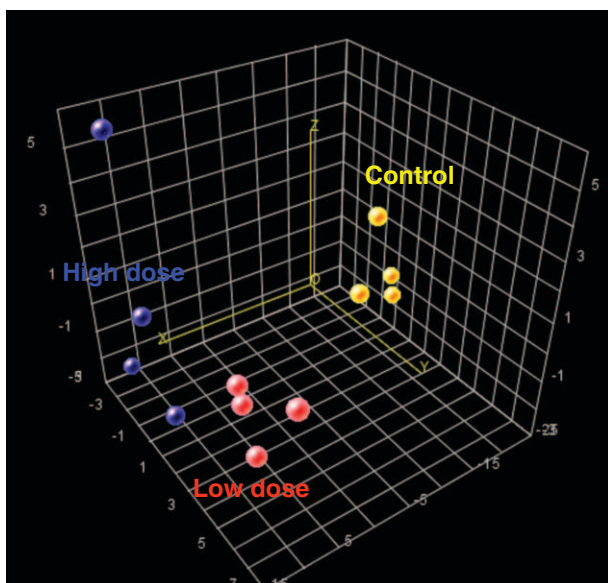
Figure 3. Principal component analysis for 379 differentially expressed genes. The colored circles represent tested groups as follows: control, red; low dose, blue; high dose, orange.

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Microarray Analysis

To elucidate the molecular mechanism during the lung injury induced by welding fume exposure, gene expression profiling in the lung of welding fume exposed rats was analyzed using Affymetrix GeneChip system. The analysis of samples was performed according to the instructions of the manufacturer, as described in the Methods section.

The differentially expressed genes were compared between low- or high-dose group and control group. Each data set consisted of an expression array from each of four animals. Three hundred and seventy nine genes including 246 and 297 genes corresponding to low- and high-group, respectively, were differentially expressed with over 2-fold modulation ($P < 0.01$, two-tailed, unpaired Welch's t test) comparing to control group. These deregulated 379 genes were analyzed using principal component analysis (PCA, Figure 3) and hierarchical clustering (Figure 4). PCA result showed that two major discrepancies between exposed groups and controls were observed and high-dose group and low-dose group could be distinguished in detail. This observation is consistent with results of histopathological change. Hierarchical clustering showed that samples corresponding to control and exposed group were clustered in a dose dependent manner and many of genes were up-regulated in welding fume exposed group comparing to control.
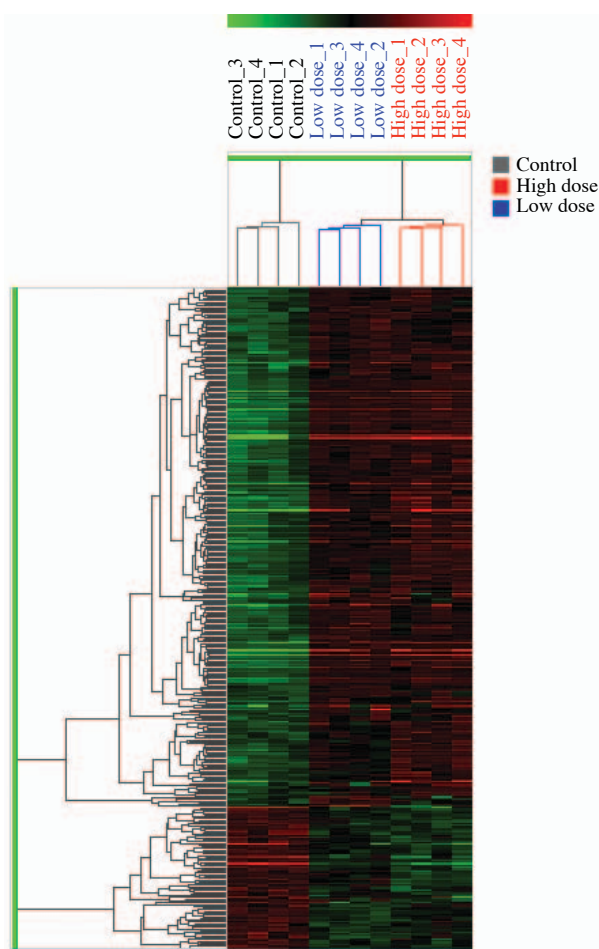
Figure 4. Hierarchical clustering of differentially expressed genes in the lung of welding fume exposed rats.

Differentially Expressed Genes in the Lung of Welding Fume Exposed Rats

Based on the hierarchical clustering, 297 genes were up-regulated and 82 genes were down-regulated in the lung of welding fume exposed group. Among these genes, significantly up- or down-regulated top 20 genes were selected as shown in Table 2 and Table 3, respectively. The up- or down-regulated genes in low- or high-dose group showed that many of genes involved in immune response (*Trem2a*, *Mmp12*, *Spp1*, *Cxcl5*, *Ccl2*, *Fabp4*, *Arg1*, and *Msr1* for up-regulated; *Pla2g2d*, *Epha4*, *Alox15*, and *F13a1* for down-regulated). Among these genes, *Trem2a* and *Mmp12* are most highly up-regulated genes in the welding fume exposed group. *Trem2a* belongs to *Ig* superfamily and myeloid cells seem to express the TREM2A receptor responding to stimuli such as welding fume exposure. *Mmp12* is a macrophage elastase and plays roles in invasion, proliferation, cell movement, and invasive-

Table 2. Up-regulated genes in the lung of welding fume exposed rats.

Gene symbol/ID	Gene title	Acc. No	Fold change	
			Low dose	High dose
Up-regulated genes				
Trem2a	Triggering receptor expressed on myeloid cells 2 (predicted)	XM_001062602	5.78	6.21
Mmp12	Matrix metallopeptidase 12	NM_053963	5.24	5.97
Cd5l	CD5 antigen-like	NM_212507	4.18	4.86
Gtf2f2	General transcription factor IIF, polypeptide 2	−	4.43	4.81
RGD1305807	Hypothetical LOC298077	−	4.19	4.09
Slc26a4	Solute carrier family 26, member 4	NM_019214	3.51	3.99
Trem2a	Triggering receptor expressed on myeloid cells 2 (predicted)	−	3.86	3.93
Spp1	Secreted phosphoprotein 1	NM_012881	2.28	3.77
Cxcl5	Chemokine (C-X-C motif) ligand 5	XM_001081752	3.33	3.71
Ccl2	Chemokine (C-C motif) ligand 2	NM_031530	2.88	3.67
1386049_at	−	XM_001079703	3.26	3.60
Fabp4	Fatty acid binding protein 4, adipocyte	NM_053365	3.46	3.42
IgG-2a	Gamma-2a immunoglobulin heavy chain	−	3.15	2.94
RGD1561521_predicted	Similar to 1110014F24Rik protein (predicted)	XM_001079275	2.68	2.89
1379376_at	Transcribed locus	NM_031010	2.41	2.79
Arg1	Arginase 1	NM_017134	2.42	2.68
LOC500736	Immunoglobulin heavy chain (alpha polypeptide) (mapped)	−	3.41	2.61
Slamf9_predicted	SLAM family member 9 (predicted)	NM_017350	1.90	2.58
Msr1	Similar to scavenger receptor type A SR-A (predicted)	XM_574708	2.46	2.55
1398566_at	Transcribed locus	−	2.59	2.49

Table 3. Down-regulated genes in the lung of welding fume exposed rats.

Gene symbol/ID	Gene title	Acc. No.	Fold change	
			Low dose	High dose
Down-regulated genes				
Cdh22	Cadherin 22	NM_019161	−3.08	−3.95
Serpinb10	Serine peptidase inhibitor, clade B (ovalbumin), member 10	−	−2.86	−3.94
1397219_at	Transcribed locus	−	−2.10	−2.92
Pla2g2d	Phospholipase A2, group IID	NM_213629	−1.76	−2.43
1393624_at	Moderately similar to XP_576460.1 PREDICTED	XM_001079234	−1.19	−1.81
1385061_at	−	−	−0.88	−1.63
Alpk3_predicted	Alpha-kinase 3 (predicted)	XM_001066959	−1.18	−1.60
Epha4	Similar to Eph receptor A4 (predicted)	XM_244186	−1.08	−1.46
Alox15	Arachidonate 15-lipoxygenase	XM_001065526	−0.92	−1.46
1380689_at	−	NM_001013086	−1.41	−1.45
1381800_at	Transcribed locus	NM_022617	−1.34	−1.44
Arhgap20	Rho GTPase activating protein 20	−	−1.14	−1.43
Avil	Advillin	NM_024401	−1.11	−1.43
Palmd	Palmdelphin	NM_001025688	−1.27	−1.41
F13a1	Coagulation factor XIII, A1 subunit	NM_021698	−0.31	−1.39
Col12a1	Procollagen, type XII, alpha 1	−	−0.38	−1.36
1385978_at	Transcribed locus	NM_031349	−0.84	−1.33
Clca2_predicted	Chloride channel, calcium activated, family member 2 (predicted)	−	−0.72	−1.31
F13a1	Coagulation factor XIII, A1 subunit	NM_021698	−0.65	−1.31
1389706_at	Transcribed locus	NM_031088	−1.39	−1.31

ness of immune cells. Except the genes involved in immune response, *Cd5l* for cellular defense response, *Gtf2f2* for transcriptional regulation, *Slc26a4* for transport, and several functionally unknown genes were up-regulated in welding fume exposed group. On the other hand, genes representing a down-regulated expression included genes involved in cell adhesion and cytoskeleton organization (*Cdh22, Avil*, and

Table 4. Gene expression changes related to inflammation response in the lung after welding fume exposure.

Gene symbol/ID	Gene title	Acc. No.	Fold change	
			Low dose	High dose
Up-regulated genes involved in inflammation response				
Tnf	Tumor necrosis factor (TNF superfamily, member 2)	–	1.46	1.62
Spp1	Secreted phosphoprotein 1	NM_012881	2.28	3.77
Cybb	Cytochrome b-245, beta polypeptide	NM_023965	1.30	1.21
Cxcl1	Chemokine (C-X-C motif) ligand 1	NM_053546	1.70	1.59
Cxcl5	Chemokine (C-X-C motif) ligand 5	XM_001081752	3.33	3.71
Cxcl11	Chemokine (C-X-C motif) ligand 11	NM_012724	1.30	1.30
Ccl2	Chemokine (C-C motif) ligand 2	NM_031530	2.88	3.67
Ccl3	Chemokine (C-C motif) ligand 3	NM_013025	1.66	1.84
Ccl17	Chemokine (C-C motif) ligand 17	NM_057151	1.02	1.13
Ccl20	Chemokine (C-C motif) ligand 20	NM_019233	1.50	1.22
Ccl22	Chemokine (C-C motif) ligand 22	NM_057203	1.18	1.40
LOC498335	Similar to Small inducible cytokine B13 precursor (CXCL13)	XM_001070245	1.70	1.89
Gm1960	Gene model 1960, (NCBI)	–	0.47	1.08
C5r1	Complement component 5, receptor 1	NM_053619	1.22	1.17
Igf1	Insulin-like growth factor 1	NM_178866	1.29	1.13
Cd14	CD14 antigen	NM_021744	0.76	1.09
Cd200	Cd200 antigen	XM_001072138	0.85	1.00
Blnk	B-cell linker	NM_001024745	0.45	1.05
Fabp4	Fatty acid binding protein 4, adipocyte	NM_053365	3.46	3.42
Fcgr2b	Fc receptor, IgG, low affinity IIb	NM_175756	1.65	1.99
Fcer1a	Fc receptor, IgE, high affinity I, alpha polypeptide	NM_001014790	0.87	1.36
Fcgr3	Fc receptor, IgG, low affinity III; Fc gamma receptor II beta	–	1.21	1.32
Rgs1	Regulator of G-protein signaling 1	XM_001068689	1.55	1.67
Prdx5	Peroxiredoxin 5	NM_053610	1.31	1.46
Gpr68_predicted	G protein-coupled receptor 68 (predicted)	NM_198760	0.81	1.24
Plaur	Plasminogen activator, urokinase receptor	–	0.60	1.07
Slc39a6	Solute carrier family 39 (metal ion transporter), member 6	XM_001072431	1.12	1.01
Adora3	Adenosine A3 receptor	NM_012896	1.09	0.70
Down-regulated genes involved in inflammation response				
Ccl11	Chemokine (C-C motif) ligand 11	XM_001064103	−1.28	−0.67
Pla2g2d	Phospholipase A2, group IID	NM_213629	−1.76	−2.43

Col12a1) and *Serpinb10*, which contains the elastase binding activity and regulated by *Tnf* or *Tgfb1*, important growth factors associated with inflammation.

Gene Expression Change of Inflammatory Response Genes

To analyze the gene set associated with inflammatory response, we selected a total of 30 differentially expressed genes over 2-fold change ($P < 0.01$) in the welding fume exposed lung by using public database such as KEGG pathway and GO and by surveying the reported literature. As shown in Table 4, *Tnf* and *Spp1* and many of cytokines related to chemotaxis, Fc receptor such as *Fcgr2b*, *Fcer1a*, and *Fcgr3*, and genes related to antigen presenting activity were up-regulated in lung injury. Meanwhile, *Ccl11* and *Pla2g2d* were down-regulated in welding fume exposed lung. In particular, *Spp1*, *Ccl2*, and *Fabp4* were significantly up-regulated over 10-fold in high dose group. The

induction of early fibrosis by welding fume exposure leads the recruit of macrophages and mediates the inflammatory response by secreting many inflammatory factors. Several studies reported that albumin and LDH were continuously increased but not TNF-α and IL-1β during the 30 days of welding fumes exposure[25]. In the selected 30 inflammatory response genes, there are no previously reported genes related to pulmonary damage except TNF-α. We also analyze the gene expression level of previously reported pulmonary damage markers such as β-NAG and LDH and several inflammatory response genes in the extended differentially expressed genes (data not shown). *Alb* (albumin), *Ldh* subfamilies (LDH), and *Hexb* (β-NAG) were up-regulated over 1.2-fold in both of low- and high-dose group. On the other hand, *Il1b* (IL-1β) is down-regulated in the low dose group but becomes up-regulated in the high dose group. This result suggests that the gene expression level of *Il1b*

is less sensitive comparing to other pulmonary damage markers.

Discussion

In recent decades, toxicogenomic approaches including transcriptomics, proteomics, and metabonomics have been carried out to investigate the toxicological mechanism and assess the drug safety[26,27]. However, the toxicogenomic approaches about pulmonary disease such as lung fibrosis is still lacking. Several studies on inhalation and instillation of welding fume have been conducted to induce lung fibrosis and lung fibrosis model have been successfully established by welding fume generation system that expose the welding fume to SD rats for 30 days[3-5,28,29]. Welding fumes include many components, such as heavy metals, gases, and particulate matters (PM) such as Fe, Mn, Ni, ozone, Cr (VI), and SiO_2. These welding fumes were known to be cytotoxic to macrophages and induce a release of reactive oxygen species[8,25]. Yu, I. J. et al.[5] showed that interstitial lung fibrosis was induced after MMA-SS welding fumes were repeatedly exposed to rats for 30 days. However, the molecular mechanism of lung fibrosis induced by welding fume exposure has not yet been completely understood.

Here, microarray analysis showed that gene expression change in the lung of MMA-SS welding fume exposed rats. Among the deregulated genes in welding fume exposed group, many of genes were related to immune or inflammatory response. To investigate the inflammatory response in more detail, we selected the inflammatory response genes by using public database and surveying the literature. The selected genes include a set of cytokine and chemokines such as *Cxcl1*, *Cxcl5*, *Cxcl11*, *Ccl2*, *Ccl3*, *Ccl17*, *Ccl20*, *Ccl22*, *LOC498335*, and *Gm1960*. Cytokines and chemokines that are important in inflammatory responses were all significantly up-regulated in the welding fume exposed group. Interestingly, no effect of gene expression change associated with T cell immunity such as Th2 cytokines was observed in this study except *Il6* and *Il10* with over 1.2-fold and 1.9-fold change, respectively. It has been reported that Th2 cytokines such as IL-4, IL-5, and IL-13 are significantly higher in cellular cultures from human pulmonary fibrosis patients[30]. In the case of rodent model of short term exposure of welding fume, it was shown that IL-6, IL-10, and IL-12 were increased in the welding fume exposed group[31]. This result was consistent with our gene expression change for those except IL-12, although our study is designed based on long-term exposure. It is thought that these variations are cause by different mechanism of inflammation between human and rodent model. Gene expression profiles of inflammatory response genes showed that complex interaction between numerous cytokines and interacting proteins drives the inflammation during lung injury and the delicate transcriptional regulation of these inflammatory mediators leads to the lung fibrosis.

Using the microarray analysis, we examined the difference in gene expression profiles in the lung of welding fume exposed rats. The information on differentially expressed genes in the lung could help to understand the genetic events in lung injury, especially lung fibrosis, induced by welding fume exposure.

Methods

Generation and Analysis of MMA-SS Welding Fumes

The MMA-SS welding fumes were generated by using a modification of previously reported method[3]. The welding fumes were generated using a rotating stainless disc (SUS 304, 2.5 cm thickness) as the base metal and a welding rod (KST 308, 26×300 mm, Korea Welding Electrode, Korea). The welding fumes move into low or high exposure chamber (whole body type, 1.5 m³, Dusturbo, Korea). The welding fumes were sampled using a personal sampler (Escort ELF pump, USA) with a flow rate for 2 liter per minutes, and then welding fume particles were captured on the PVC membrane filters (pre size 5 μm, 37 mm diameter, PALL GLA-5000, Pall, USA). The analysis of welding fume particles were performed by atomic absorption spectrophotometer or inductive coupling plasma analyzer (Thermojeralash, USA) using the NIOS 7300 method.

Animals

Approximately 6-week-old SD rats, purchased form the Charles River laboratory (Japan), were acclimated up to three weeks prior to exposure of welding fumes. The rats were kept in a 12-h light/dark cycle, under controlled temperature and humidity and were fed with standard food pellets. Rats weighing 164.08 ± 6.18 g were randomly assigned to three group; control, low dose, and high dose. Rats for low- and high-dose group were exposed with 51.4 ± 2.89 mg/m³ and 84.63 ± 2.87 mg/m³, respectively, for 2 hours per day up to 30 days. Seven rats were used in each test group and necropsies were then performed after 30 days-exposure. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC)

and conducted in accordance with Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) international guidelines.

Bronchoalveolar Lavage and Inflammation Parameters

BALs were obtained and analyzed by previously reported Antonini methods[18]. The rats were deeply anesthetized with an overdose of isoflurane, and then exsanguinated by severing the abdominal aorta. The left bronchus was clamped off and BAL was performed on the right lung of rats at each group. The right lung was first lavage with two separate 3 mL aliquots of warm, calcium- and magnesium-free phosphate buffer solution (pH 7.4). These two BAL samples were centrifuged at $500 \times g$ for 10 min and the acellular fluid from the first and second fraction was used for the determination of lung damage. Total cell numbers recovered by BAL were determined using coulter counter multisizer 3 (Berkman Coulter, USA). BAL cells were centrifuged at 800 rpm for 5 min and pelleted onto a slide using a Shandon cytospin 4 centrifuge (Thermo Scientific, USA) and cells (300/rat) were differentially counted as macrophages, and neutrophils (PMN) and lymphocyte after staining with Wright-Giemsa Sure stain. The activity of two inflammatory parameters such as β-NAG and LDH were measured to detect the release of enzymes from activated phagocytes and to detect general cytotoxicity, respectively. LDH was analyzed using automated clinical chemistry analyzer (Fujifilm, Japan), β-NAG were measured using TBA 200FR (Toshiba, Japan).

Histopathology

Lung samples collected from exposed and control rats were fixed in 10% neutral buffered formalin, and embedded in paraffin. The sections (4 μm thick) were cut using RM2165 microtome (Leica, Germany), stained with hematoxylin and eosin (H & E), and examined under a light microscope (Nikon E400, Japan).

Isolation of RNA

The part of left lung samples were homogenized in Trizol reagent (Invitrogen, USA) and total RNA was isolated using Trizol reagent and purified using RNeasy mini kit (Qiagen, Germany) according to manufacturer's instruction. Total RNA was quantified using NanoDrop (NanoDrop, USA) and the quality of RNA was evaluated using 2100 Bioanalyzer (Agilent Technologies, USA).

Microarray Analysis

Sample labeling, microarray hybridization, washing, and scanning were performed according to the manufacturer's protocol (Affymetrix, USA) as described previously[32]. The preprocessing procedure of resultant cell intensity files (CEL) and following microarray analysis were performed using GenPlex software (Istech, Korea). Data normalization was performed using global scale normalization. The differentially expressed genes in the welding fume exposure group were selected based on fold-change and *t*-test (over 2-fold and $P < 0.01$). The selected deregulated genes were analyzed by principal component analysis and hierarchical clustering algorithm. The genes involved in inflammation response were selected using public database such as KEGG and GO database and by surveying the literature. The selected genes were annotated based on NetAffx, linked at http://www.affymetrix. com.

Acknowledgements

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