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Identification of potential biomarkers from gene expression profiles in rat lungs intratracheally instilled with C_{60} fullerenes

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ABSTRACT

The use of C_{60} fullerenes is expected to increase in various industrial fields. Little is known about the potential toxicological mechanism of action of water-soluble C_{60} fullerenes. In our previous research, gene expression profiling of the rat lung was performed after whole-body inhalation exposure to C_{60} fullerenes to gain insights into the molecular events. These DNA microarray-based data closely matched the pathological findings that C_{60} fullerenes caused no serious adverse pulmonary effects under the inhalation exposure condition. Taking advantage of this, we attempted to characterize time-dependent changes in the gene expression profiles after intratracheal instillation with C_{60} fullerenes at different dosages and to identify the candidate expressed genes as potential biomarkers. The hierarchical cluster analysis revealed that the up- or downregulation of genes after intratracheal instillation with 1.0 mg C_{60} fullerene particles in rat lung tissue was significantly over-represented in the "response to stimulus" and "response to chemical stimulus" categories of biological processes and in the "extracellular space" category of the cellular component. These results were remarkable for 1 week after the instillation with C_{60} fullerenes. In the lung tissues instilled with 1.0 mg C_{60} fullerene particles, many representative genes involved in "inflammatory response," such as the *Cxcl2*, *Cxcl6*, *Orm1*, and *Spp1* genes, and in "matrix metalloproteinase activity," such as the *Mmp7* and *Mmp12* genes, were upregulated for over 6 months. The expression levels of 89 and 21 genes were positively correlated with the C_{60} fullerene dose at 1 week and 6 months after the instillation, respectively. Most of them were involved in "inflammatory response", and the *Ccl17*, *Ctsk*, *Cxcl2*, *Cxcl6*, *Lcn6*, *Orm1*, *Rnase9*, *Slc26a4*, *Spp1*, *Mmp7*, and *Mmp12* genes were overlapped. Meanwhile, the expression levels of 16 and 4 genes were negatively correlated with the C_{60} fullerene dose at 1 week and 6 months after the instillation, respectively. Microarray-based gene expression profiling suggested that the expression of some genes is correlated with the dose of intratracheally instilled C_{60} fullerenes. We propose that these genes are useful for identifying potential biomarkers in acute-phase or persistent responses to C_{60} fullerenes in the lung tissue.

1. Introduction

Recent advancements in nanotechnology have raised concerns regarding the effects of manufactured ultrafine nanoparticles on human health and on the environment. The use of C_{60} fullerenes is expected to increase in diverse industrial fields. Despite interest in the potential toxicological impact of water-soluble C_{60} fullerenes, little is known about its mechanism of action *in vivo*. It has been reported that suspensions of C_{60} fullerenes in water had little or no difference in the lung toxicity effects (Sayes et al., 2007). The assessment of toxicity resulting from inhalation exposure to C_{60} fullerene nanoparticles and microparticles revealed minimal changes in the

Abbreviations: BALF, bronchoalveolar lavage fluid; FC, fold change; ECM, extracellular matrix; GEO, Gene Expression Omnibus; GO, gene ontology; RT-PCR, reverse transcriptase-polymerase chain reaction; TEM, transmission electron microscope; Uf-NiO, ultrafine nickel oxide; LC-UV, liquid chromatography-ultraviolet; ICP-MS, inductively coupled plasma-mass spectrometry.

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toxicological endpoints (Baker et al., 2008). However, the precise underlying mechanism of action is still unknown. In order to discuss the toxicity of C_{60} fullerenes, further information regarding the *in vivo* mechanisms is needed.

We have previously reported the pulmonary effects of the inhalation of C_{60} fullerenes compared with those of ultrafine nickel oxide particles (Uf-NiO) (Fujita et al., 2009a). On exposure to C_{60} fullerenes (0.12 mg/m^3, 4.1×10^4 particles/cm^3, 96 nm diameter) for 6 h a day for 4 weeks, C_{60} fullerenes were found in alveolar epithelial cells at 3 days post-exposure, and they were engulfed by macrophages at both 3 days and 1 month post-exposure. However, conventional methods such as histochemical analysis and bronchoalveolar lavage fluid (BALF) cell analysis demonstrated that C_{60} fullerenes might not have adverse effects on the lung under the inhalation exposure condition.

Assessing cellular responses upon exposure to ultrafine particles could provide new insights into their toxicological behavior. The DNA microarray-based approach has been available to elucidate the toxicological responses of metal oxide nanoparticles in *in vivo* and *in vitro* studies (Chen et al., 2006; Fujita et al., 2009b). We have examined the gene expression profiles in rat lungs after the intratracheal instillation with Uf-NiO particles (Fujita et al., 2009c). The results corresponded well with the results obtained using conventional methods such as immunohistochemical analysis and BALF cell analysis (Nishi et al., 2009; Morimoto et al., 2010a). Gene expression profile in *in vivo* studies would therefore provide invaluable information for assessing the effects of ultrafine particles at the molecular level. In previous studies, gene expression profiles of rat lungs after whole-body inhalation exposure to C_{60} fullerenes revealed that few genes involved in the inflammatory response, oxidative stress, apoptosis, and metalloendopeptidase activity were upregulated at both 3 days and 1 month post-instillation (Fujita et al., 2009a). Meanwhile, these results were significantly different from those of Uf-NiO particles, which induced high expression of genes associated with chemokines, oxidative stress, and matrix metalloproteinase 12 (Mmp12); this suggests that Uf-NiO particles lead to acute inflammation for the exposure period, and the damaged tissues are repaired in the post-instillation period. The expression profiles provide convincing evidence for the pulmonary toxicity of manufactured ultrafine nanoparticles.

In the present study, we performed DNA microarray-based gene expression profiling of the rat lung after intratracheal instillation with C_{60} fullerene suspensions at 0.1 mg, 0.2 mg (as the low dose), and 1.0 mg (as the high dose) injected dose per rat. Using clustering and gene ontology (GO) analysis, time-dependent change (from 3 days to 6 months post-instillation) in gene expression by 1.0 mg C_{60} fullerenes was examined in order to determine the alteration in the representative gene expression. Besides, the expressed genes that were positively or negatively correlated with the dosage of C_{60} fullerenes in the short term (1 week post-instillation) and the long term (6-month post-instillation) after intratracheal instillation were screened in order to propose candidate genes as potential biomarkers. These results are discussed with regard to histopathological changes in the lung tissues after intratracheal instillation with C_{60} fullerenes as shown in our latest reports (Morimoto et al., 2010b). They will provide further insight into the pulmonary toxicity and mechanisms of action of C_{60} fullerenes on a molecular level.

2. Materials and methods

2.1. Particle characterization

Bulk high-purity (>99.5%) C_{60} fullerene was purchased from Frontier Carbon Corporation (Nanom purple, Fukuoka, Japan). The manufacturer's specifications indicated a specific surface area of 0.92 m^2/g. Bulk C_{60} fullerene material dispersed in 0.1 mg/ml polyoxyethylene sorbitan monooleate (Tween-80, Wako Pure Chemical Industries, Ltd., Tokyo, Japan) was milled in an agate mortar for 30 min under a nitrogen atmosphere. The milled C_{60} fullerene material was suspended with zirconium particles (50 μm diameter) using a high-performance dispersion machine (UAM-15, Kotobuki Industries Co., Ltd., Tokyo, Japan) and was centrifuged at 8000 × *g* for 60 min. After the preparations, 0.1 mg, 0.2 mg, or 1.0 mg of C_{60} fullerenes suspended in 0.4 ml distilled water containing 0.1% Tween-80 was used for the intratracheal instillation study. The average geometric diameter of C_{60} fullerene particles was estimated to be 33 nm by using the laser light diffraction method performed with the Microtrac® UPA150 device (Nikkiso Co., Ltd., Tokyo, Japan). The crystal lattice of C_{60} fullerenes was observed, and their diameter was estimated to be approximately 30 nm by a transmission electron microscope (TEM) at 200 kV (Leo, Oberkochen, Germany) (Fujita et al., 2009b). The dispersion status of the C_{60} fullerene suspension was maintained for at least 2 months (Shimada et al., 2009).

2.2. Animals

Nine-week-old male Wistar rats purchased from Kyudo Co., Inc. (Kumamoto, Japan) were divided into groups (n = 4 per group per time point). C_{60} fullerene was intratracheally administered into rats as a single injection (0.1 mg, 0.2 mg, or 1.0 mg C_{60} fullerenes per rat). The vehicle control groups received 0.1% Tween-80 per rat. After intratracheal instillation treatment, rats were housed within polycarbonate cages at a controlled temperature of 22 °C with a chow diet *ad libitum*, and were dissected at 3 days, 1 week, 1 month, 3 months, and 6 months post-instillation. Lungs of anesthetized rats were perfused with physiological saline, excised, and used for DNA microarray analysis (n = 4, right lungs). Animal procedures were approved by the National Institute of Advanced Industrial Science and Technology, Japan Animal Care and Use Committees.

2.3. RNA extraction and DNA microarray

The lungs were homogenized using QIAzol lysis reagent with a TissueRuptor (Qiagen, Tokyo, Japan). Total RNA was extracted from the homogenates using the RNeasy Midi kit (Qiagen, Tokyo, Japan) following the manufacturer's instructions. RNA quality and concentration were determined using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) and a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). cRNA labeled with fluorescent Cyanine 3-CTP was used for hybridization onto the Whole Rat Genome Oligo Multiplex Microarray slides (#G4131F, Agilent Technologies, Santa Clara, CA) containing approximately 41,000 oligonucleotide probes at 65 °C for 17 h. Hybridized microarray slides were washed according to the manufacturer's instructions, and were scanned with an Agilent DNA Microarray Scanner (#G2565BA, Agilent Technologies, Santa Clara, CA) at 5 μm resolution. The scanned images were analyzed numerically using the Agilent Feature Extraction Software version 9.5.3.1.

2.4. Microarray data analysis

Normalized data were analyzed using GeneSpring GX version 10.0.1 software (Agilent Technologies, Santa Clara, CA). Each set of experiments consisted of the intratracheal instillation of lungs with 0.4 ml distilled water including 0.1% Tween-80 in the absence or presence of C_{60} fullerenes for the same time period. In each set, genes with normalized intensity ratios that were 2-fold higher or 0.5-fold lower in C_{60} fullerenes-instilled lungs than in the vehicle control lungs were considered as up- or downregulated, respectively. Fold changes (FCs) represented the average of four independent experiments. Gene expression data for each of the experimental group are provided in Supplementary Table 1, and the data were deposited in the Gene Expression Omnibus (GEO) database (Accession number: GSE17747; http://www.ncbi.nlm.nih.gov/projects/geo/). Differences between the vehicle control and C_{60} fullerenes groups were evaluated with the Student's *t*-test. *P*-values less than or equal to 0.05 were considered as statistically significant. The web-based application GOstat (http://gostat.wehi.edu.au/) was used to identify statistically over-represented GO terms (Beissbarth and Speed, 2004) with the Rat Genome Database (RGD: http://rgd.mcw.edu/). The Gene Ontology Database (http://www.geneontology.org/) was used for the functional categorization of the gene expression profiles.

Expressed genes that were correlated with the dosage of C_{60} fullerenes at 1 week or 6 months post-instillation were selected and classified into groups in a time- and concentration-dependent manner. We considered that genes in which the FC was higher than 1.0 by 1.0 mg fullerenes were potential candidates for positive biomarkers at 1 week (short-term period) or 6 weeks (long-term period) post-instillation, respectively. Further, we considered that genes in which the FC was less than −1.0 by 1.0 mg fullerenes were potential candidates for negative biomarkers at 1 week or 6 weeks post-instillation, respectively. The genes for which the functions were not well known were filtered.