The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Letter

mRNA expression profile of cytokines in rat primary alveolar macrophages treated with multiwalled carbon nanotube (MWCNT)

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(Received January 20, 2023; Accepted January 27, 2023)

ABSTRACT — Multiwalled carbon nanotubes (MWCNT) are fiber-shaped nanomaterials that have a potential risk for cancer due to properties that are similar to asbestos. One type of nanotube called MWCNT-7 was categorized in Group 2B as possibly carcinogenic to humans by the International Agency for Research on Cancer. MWCNT-N, which is similar to MWCNT-7, is carcinogenic to the lung and pleura when administered to rats via the respiratory tract using intra-tracheal intra-pulmonary spraying. Macrophages have an important role in the MWCNT induced pulmonary carcinogenicity. In this study, rat primary alveolar macrophages were employed to examine possible mechanism of carcinogenic effects of the MWCNT-N. MWCNT-N was fractionated into flow-through and retained fraction by passing it through a sieve with a pore size of 25 μm. Microarray analysis showed up-regulation of various cytokines in macrophages exposed to MWCNT-N. MWCNT-N strongly induced the expression of cytokines such as interleukin-6 (IL-6) in macrophages. In contrast, few cytokines were prominently down-regulated in macrophages treated with MWCNT-N. The sieve fractions did not have a significant effect on mRNA expression of cytokines in macrophages. These results provide useful information for understanding MWCNT-induced carcinogenicity via cytokine expression by macrophages.

Key words: Carbon nanotube, Cytokine, Microarray, Macrophage

INTRODUCTION

Multiwalled carbon nanotubes (MWCNT) are engineered nanomaterials that have a fibrous structure. The similarity between the physical characteristics of MWCNT and asbestos fibers was concerned about their harmful effects on human health (Poland *et al*., 2008). Exposure to asbestos causes asbestosis, pleural fibrosis, pleural plaques, bronchogenic carcinoma, and mesothelioma in humans. It was demonstrated that direct injection of MWCNT-7 into the scrotum or the peritoneal cavity induced malignant mesotheliomas in rodents (Takagi *et al*., 2008; Sakamoto *et al*., 2009; Nagai *et al*., 2011). MWCNT-7 administered via the respiratory tract induced the development of malignant pleural mesothelioma or lung tumor in rats (Numano *et al*., 2019; Hojo *et al*., 2022). A whole-body inhalation study demonstrated that inhaled MWCNT-7 was a complete pulmonary carcinogen in the rat (Kasai *et al*., 2016). MWCNT-7 is classified by the International Agency for Research on Cancer as group 2B, possibly carcinogenic

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to humans (IARC, 2017).

MWCNT-N, which is similar to MWCNT-7, was shown to induce both malignant pleural mesothelioma and lung tumors in rats when administered via the respiratory tract using intra-tracheal intra-pulmonary spraying (Suzui *et al*., 2016). After administration of MWCNT-N, they were mostly found in the lung alveoli. In the alveoli, MWCNT-N was found in macrophages. Macrophages have an important role in the MWCNT induced pulmonary toxicity (Wang *et al*., 2020). Several studies have shown that macrophages are the principal infiltrating cells after MWCNT enter the lung tissue. A large part of the infiltrating cells in pleural cavity of MWCNT-administered rats are CD68, a macrophage marker, positive cells, indicating that macrophages are major infiltrating cell-type, and most MWCNT fibers in the pleural cavity of these rats are within the macrophages (Xu *et al*., 2012). Phagocytosis of MWCNT by macrophages triggers inflammation, fibrosis, and other reactions by releasing oxidants and cytokines. Accumulation of macrophages that retained MWCNT is observed on the surface of the visceral pleura (Donaldson *et al*., 2013). As the long MWCNT cannot be completely enclosed by the macrophages, leading to frustrated phagocytosis, there is persistent interaction of macrophages in the pleural cavity with these MWCNT. This results in chronic inflammatory reactions, fibrosis, and stimulation of lung epithelial and/or mesothelial cells. Thus, macrophages in the pleural cavity promote pleural injury (Donaldson *et al*., 2013; Wang *et al*., 2020).

Our previous studies demonstrated that MWCNT administered to the lung translocated to the pleural cavity and caused accumulations of macrophages phagocytosing the MWCNT fibers, and hyperplastic proliferation of the visceral mesothelium (Xu *et al*., 2012, 2014). Notably, pleural translocation and induction of lesions in the lung and pleura by MWCNT administered to the lung was size- and shape-dependent (Xu *et al*., 2014). In this study, we performed microarray analysis of mRNA derived from rat alveolar macrophages exposed to MWCNT-N. We also fractionated the MWCNT-N by passing it through a sieve with a pore size of 25 μm to examine whether there were any size-dependent effects on induction of mRNA expression associated with different fiber lengths.

MATERIALS AND METHODS

Preparation of the multiwalled carbon nanotube fractions

MWCNT-N (Nikkiso, Tokyo, Japan) was suspended and fractionated as reported previously (Suzui *et al*., 2016). A portion of the MWCNT-N was fractionated by passing it through a sieve with a pore size of 25 μm to obtain fractions with different length of MWCNT-N.

Isolation of primary alveolar macrophages

Primary alveolar macrophages were isolated from 10 male F344/Crj rats (10-week-old) (Charles River Laboratories Japan, Yokohama, Japan) as described previously (Xu *et al*., 2010, 2012). Animal experiments were performed with the approval of the Animal Ethics Committee of the Nagoya City University (approval no. H25M-16) and according to the guidelines of the committee. Primary alveolar macrophages were treated with fractionated MWCNT-N at a final concentration of 10 μg/mL and then incubated for 24 hr in a 37°C, 5% CO_2 incubator.

Microarray analysis

Total RNA was extracted from the primary macrophages by using TRIzol Reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quality was analyzed by Agilent 2100 bioanalyzer. Microarray analysis was performed by TORAY Industries (Kanagawa, Japan) using 3D-Gene Rat Oligo chip 20k (TORAY Industries).

RESULTS AND DISCUSSION

Three preparations (unfiltered, flow-through, and retained fraction) of fractionated MWCNT-N were obtained. The estimated mean lengths of the fractionated MWCNT-N were essentially identical to previous results (the unfiltered, 4.2 μm; the flow-through, 2.6 μm; the retained, 2.6 μm <) (Suzui *et al*., 2016). Primary alveolar macrophages were isolated from the lungs of F344 rats, and were confirmed to be macrophages by morphology and CD68 staining, as described in a previous report (data not shown) (Xu *et al*., 2010).

The alveolar macrophages were treated with each of fractionated MWCNT-N (the unfiltered, the flow-through, and the retained fraction). Transcriptional data for over 20,000 genes in macrophages were analyzed by the microarray. The mRNA expression levels of 676, 813, and 562 genes were increased more than 2.0 fold, and those of 884, 1189, and 855 genes were decreased less than 0.5 fold in the macrophages treated with the unfiltered, the flow-through, and the retained fraction, respectively.

The differentially expressed genes were categorized according to their functional annotation. As a result, it was revealed that administration of MWCNT-N significantly up-regulated the expression of cytokines in macrophages (Table 1). In contrast, few cytokines were prominently down-regulated in macrophages treated with

Table 1. Microarray data: list of top 10 up-regulated cytokine genes.

MWCNT preparations	Gene	Description	Fold change (MWCNT-N/vehicle)
Flow-through	Il6	Interleukin-6 precursor	2411
	Il17f	Interleukin 17F	756
	Csf3	Colony stimulating factor 3	710
	Csf2	Granulocyte-macrophage colony-stimulating factor	277
	Cxcl2	Macrophage inflammatory protein 2 precursor (MIP2) (C-X-C motif chemokine 2)	135
	Il10	Interleukin-10 precursor	125
	Inhba	Inhibin β A chain precursor (Activin β-A chain)	121
	Ccl4	C-C motif chemokine 4 precursor (MIP-1-β)	70
	Cxcl1	Growth-regulated α protein precursor (C-X-C motif chemokine 1)	48
	Il1b	Interleukin-1 β precursor	32
Retained	Il6	Interleukin-6 precursor	402
	Csf3	Colony stimulating factor 3	121
	Il17f	Interleukin 17F	81
	Csf2	Granulocyte-macrophage colony-stimulating factor	64
	Cxcl2	Macrophage inflammatory protein 2 precursor (MIP2) (C-X-C motif chemokine 2)	62
	Ccl4	C-C motif chemokine 4 precursor (MIP-1-β)	35
	Il1b	Interleukin-1 β precursor	25
	Cxcl1	Growth-regulated α protein precursor (C-X-C motif chemokine 1)	19
	Inhba	Inhibin β A chain precursor (Activin β-A chain)	19
	Il10	Interleukin-10 precursor	19
Unfiltered	Il6	Interleukin-6 precursor	532
	Csf3	Colony stimulating factor 3	158
	Il17f	Interleukin 17F	157
	Cxcl2	Macrophage inflammatory protein 2 precursor (MIP2) (C-X-C motif chemokine 2)	97
	Csf2	Granulocyte-macrophage colony-stimulating factor	72
	Ccl4	C-C motif chemokine 4 precursor (MIP-1-β)	49
	Inhba	Inhibin β A chain precursor (Activin β-A chain)	49
	Il1b	Interleukin-1 β precursor	35
	Il10	Interleukin-10 precursor	29
	Cxcl1	Growth-regulated α protein precursor (C-X-C motif chemokine 1)	28

Table 2. Microarray data: list of cytokine genes down-regulated less than 0.1 fold.

MWCNT preparations	Gene	Description	Fold change (MWCNT-N/vehicle)
Flow-through	Il16	Interleukin-16	0.033
Retained	Il16	Interleukin-16	0.026
Unfiltered	Il16	Interleukin-16	0.032

MWCNT-N (Table 2). Table 1 lists the top 10 up-regulated genes encoding cytokines. Microarray analysis revealed that the MWCNT-N-treated macrophages exhibited significantly higher expression of interleukin (IL)-6, IL-17F, colony stimulating factor 3 (CSF3), granulocyte-macrophage colony-stimulating factor (CSF2), macrophage inflammatory protein 2 (Mip2/Cxcl2), IL-10, inhibin β A chain (Inhba), C-C motif chemokine 4 (Ccl4/Mip1β), growth regulated α protein (GROα/C-X-C motif chemokine 1, Cxcl1), and IL-1β than those in the vehicle

treatment. Among them, mRNA expression level of IL-6 expression was commonly highest in macrophages treated with the three fractionated MWCNT-N. IL-6 is a major proinflammatory cytokine released during acute inflammation, and a marked increase in expression level would reflect a significant inflammatory response in macrophages exposed to MWCNT-N. The results of microarray analysis showed that mRNA expression profile of cytokines was not much different among the macrophages treated with any fraction. Although the order of gene expression level of cytokines was different, the top 10 cytokines were commonly up-regulated in the macrophages treated with each fraction. In the present study, we could not find any relationship between the mRNA expression level of cytokines and the length of MWCNT-N. In line with these results, our previous study showed that no significant difference in incidence of lung tumors or total tumor burden was found among the three animal groups administered the different MWCNT-N sieve fractions (Suzui *et al*., 2016).

Our present study identified several cytokines that may be responsible for MWCNT's toxicity in macrophages. The current mRNA expression profile provides useful information for understanding MWCNT-induced alterations in alveolar macrophages.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Sachi Sri Kantha (formerly at Gifu University) for comments and editing the manuscript. This study was supported by Health and Labor Sciences Research Grants of Japan (H21-kagaku-ippan-008, H22-kagaku-ippan-005, H25-kagaku-ippan-004, H24-kagaku-sitei-009).

Conflict of interest---- The authors declare that there is no conflict of interest.

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