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Original Article

mRNA expression levels of CCL4, IL6, and CXCL2 in multiwalled carbon nanotube induced lung tumors in rats

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ABSTRACT — Some types of multiwalled carbon nanotube (MWCNT) are similar to asbestos in length and diameter. When these fibers are introduced into the respiratory tract, MWCNTs can induce pulmonary lesions including inflammation, fibrosis, and hyperplasia. By using an intrapulmonary spraying method, we demonstrate that MWCNT causes lung cancer in a 2-year experimental protocol (Suzui *et al*., 2016). In samples of the 5 histologically diagnosed archival lung cancer tissues and 4 control normal lung tissues, we examined the mRNA expression level of specific cytokines such as CCL4, IL6, and CXCL2. These cytokines were chosen for the analysis since their mRNA expression levels are upregulated in MWCNT-treating macrophages (Sultana *et al*., 2023). The level of expression of CCL4 markedly decreased in adenocarcinoma compared to that of the control normal lung tissue. In several adenocarcinoma samples, the level of expression of IL6/CXCL2 was higher than that of the control normal lung tissue. In the granuloma sample, the level of IL6 was higher than that of the control normal lung tissue and the level of CCL4/CXCL2 was lower than that of the control normal lung tissue. Taken together, histology specific expression profile of these cytokines may provide additional insights into lung tumorigenesis induced by MWCNT.

Key words: Adenocarcinoma, Cytokine, Multiwalled carbon nanotubes, Tumorigenesis

INTRODUCTION

Carbon nanotube (CNT)-based technology has been promoted with rapid growth and production in diverse industrial and biomedical applications due to its unique physicochemical properties (Abdalla *et al*., 2015; Jacobsen *et al*., 2017). However, some types of CNT are similar to asbestos in length and diameter (Donaldson *et al*., 2011; Fraser *et al*., 2020). When these fibers are inhaled, CNT can induce pulmonary lesions such as inflammation, fibrosis, and hyperplasia (Arnoldussen *et al*., 2018). High length to diameter aspect ratio, a characteristic shared with asbestos fibers has led to concern that environmental or occupational exposure to these types of CNT may cause asbestos-like pulmonary diseases including cancer. Indeed, multiwalled CNT-7 (MWCNT-7) is classified as a Group 2B agent (possibly carcinogenic to humans) by the International Agency for Research on Cancer (IARC, 2017). Several studies reported that MWCNTs induce pulmonary inflammation,

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fibrosis, and granuloma in the experimental animal models (Muller *et al*., 2005; Elgrabli *et al*., 2008; Porter *et al*., 2010; Sager *et al*., 2022) and that malignant mesothelioma occurs at 11-52 weeks after the intraperitoneal or intrascrotal administration of MWCNT-7 in p53+/- mice or F344 rats (Takagi *et al*., 2008; Sakamoto *et al*., 2009; Takagi *et al*., 2012). We have recently reported that MWCNT-N induces pericardial mesothelioma and pulmonary adenocarcinoma in rats at 64-109 weeks when it was administered by the intra-tracheal intrapulmonary spraying (TIPS) method (Suzui *et al*., 2016). Furthermore, MWCNT-7 administered to the lung by the TIPS method induces pleural mesothelioma and pulmonary tumors (Numano *et al*., 2019; Hojo *et al*., 2022).

Overexpression of specific cytokines are associated with the initiation and progression of various types of cancer through the alteration of the antitumor immune response (Zamarron and Chen, 2011). Some of these cytokines are also related to the occurrence of pulmonary inflammation and fibrosis (Dong and Ma, 2019). We have recently found that CCL4, IL6, and CXCL2 were upregulated in rat alveolar macrophages treated with MWCNT-N, providing useful information for understanding MWCNT-induced tumorigenicity via expression of cytokines (Sultana *et al*., 2023). Therefore, the current study analyzes the mRNA expression levels of cytokines including CCL4, IL6, and CXCL2 to obtain insights into their contribution to tumorigenesis of MWCNT-induced lung adenocarcinoma.

MATERIALS AND METHODS

Tissue samples

This study was performed by using the archival samples of nodules and control tissues derived from lungs of 2-year-old F344 male rats treated with MWCNT-N (NIKKISO, Tokyo, Japan) (Suzui *et al*., 2016). The nodules or lung tissues were excised after 104 weeks of exposure of MWCNT-N. Lung tissues of the untreated control group were noted as N1 and N2, these of the vehicle (0.5% Pluronic F68) control group were also noted as V1 and V2. Nodules of the rat no. 3 were noted as R3T1, R3T2, R3T3, and R3T4. Nodules of the rat no. 5 were noted as R5T1 and R5T2. Identified nodules were carefully removed and cut into two pieces. One piece of the nodule was immediately frozen in liquid nitrogen for mRNA expression analysis, and the second piece was processed for histological examination with hematoxylin and eosin (HE) staining. Tissues derived from five adenocarcinomas and one granuloma were examined.

Extraction of total RNA samples

Total RNA was extracted from the frozen nodule or control tissue samples by using Trizol Reagent (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) as described previously (Sultana *et al*., 2023). Concentration of total RNA was measured by NanoDropOne Microvolume UV Spectrophotometer (Thermo Fisher Scientific), and its quality was assessed by the 1.2% agarose-denaturing formaldehyde gel electrophoresis.

Quantitative reverse transcription PCR (qRT-PCR)

Reverse transcription reaction was performed by using the high-capacity cDNA RT kit with RNase Inhibitor (Applied Biosystems/Thermo Fisher Scientific). Two micrograms of total RNA samples were used to synthesize cDNA using Takara PCR thermal cycler SP (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions under the following thermal conditions: 10 min at 25ºC, 120 min at 37ºC, 5 min at 85ºC. cDNA samples were stored at -20ºC until use. qRT-PCR was conducted to measure mRNA expression levels of CCL4, IL6, and CXCL2 in the nodule or control tissue samples with PowerUp SYBER Green Master Mix (A25742, Applied Biosystems/Thermo Fisher Scientific). Primers used for amplification were as follows: Ccl4 (primer set ID: RA071973), Il6 (primer set ID: RA060834), Cxcl2 (primer set ID: RA058935), and Actb (primer set ID: RA015375) (Perfect Real Time support system: Takara Bio Inc.). 10 ng of cDNA sample was used to perform qRT-PCR using 7900HT Fast Real-Time PCR System and SDS software version 2.4 (Applied Biosystems/Thermo Fisher Scientific). PCR amplification was conducted according to the manufacturer's instructions as follows: UDG activation at 50ºC for 2 min, Dual-Lock DNA polymerase at 95ºC for 2 min, followed by 40 cycles of 95ºC for 3 sec, 60ºC for 30 sec. Dissociation curves were used to determine the specificity of the amplified products, and the relative expression level of the molecule was normalized with that of β-actin.

Statistical analysis

Data were analyzed using IBM SPSS Statistics version 24. Comparisons among the untreated control group, vehicle control group, and MWCNT-N treated group were performed by one-way ANOVA, and then statistical significance was evaluated by using Tukey (for equal variance data) or Games-Howell (for unequal variance data) test. Data were presented as the mean ± SE. $P < 0.05$ was considered statistically significant.

RESULTS

Nodules formed after 104 weeks of exposure of MWCNT-N in the experimental group were macroscopically sessile in shape. These nodules were histologically either adenocarcinoma (R3T1, R3T2, R3T3, R3T4, and R5T2) or granuloma (R5T1) (Fig. 1). Representative histological features of the nodule are shown in Fig. 1E-J. Adenocarcinoma shows a glandular appearance with cytological and architectural abnormalities. In a granuloma, the epithelioid cells have a pale pink granular cytoplasm with indistinct cell boundaries. No histological abnormalities were seen in any sample of the untreated control and vehicle control groups. Significant lower expression levels of CCL4 mRNA were observed in the adenocarcinoma than those of the control (Fig. 2A). When combining expression data, expression level of the adenocarcinoma significantly decreased compared to that of the control and granuloma (Fig. 2B). The expression levels of IL6 mRNA were most elevated in R3T4 (adenocarcinoma), followed by other tumor samples including R5T1 (granuloma) (Fig. 2C). Significant higher expression level of IL6 mRNA was observed in the adenocarcinoma and granuloma compared to that of the control (Fig. 2D). The expression levels of CXCL2 mRNA markedly increased in 2 of 6 tumor samples (Fig. 2E), and that of the adenocarcinoma was also increased compared to that of the granuloma but not to that of the control (Fig. 2F).

DISCUSSION

As stated in the "Introduction", specific cytokines are of considerable interest because they have been shown to be linked not only to inflammation but tumorigenesis as well. A wide range of documentation compatible with our findings is available. The elevated mRNA and protein expression level of IL6 was found in non-small cell lung cancer (NSCLC) tissues in humans (Yeh *et al*., 2006; Gao *et al*., 2007; Dutkowska *et al*., 2021). The mRNA expression level of IL6 increased in cells obtained by bronchoalveolar lavage fluids in patients with sarcoid granuloma (Ishioka *et al*., 1996). Also, IL6 has been shown to play a critical role in the formation of granuloma via a mechanism involving the activation of several types of murine immune cells (Tristão *et al*., 2017). CCL4 interacts with various types of immune cells through its specific receptor, CCR5. The CCL4/CCR5 axis functions in the development of human lung cancer and metastasis (Lee *et al*., 2012). We infer that the use of our animal model system may be helpful to examine the involvement of CCL4 in the WNT/β-catenin signaling pathway; this is because the activation of this pathway in lung cancer cells suppresses the CCL4 production, leading to the potential treatment application of immune checkpoint inhibitors (Takeuchi *et al*., 2021). The mRNA expression levels of CXCL2 are not consistent in either normal human lung tissue or ade-

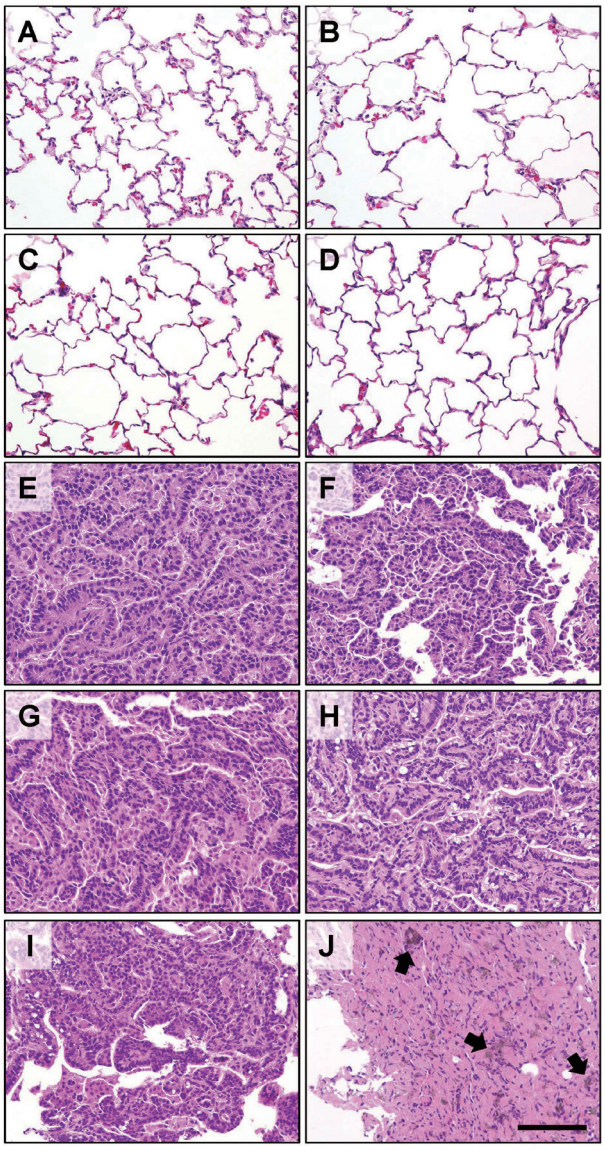

Fig. 1. Representative histological features of the lung tumors. (A, B) N1 and N2, untreated control; (C, D) V1 and V2, vehicle control (0.5% Pluronic F68); (E) R3T1, adenocarcinoma; (F) R3T2, adenocarcinoma; (G) R3T3, adenocarcinoma; (H) R3T4, adenocarcinoma; (I) R5T2, adenocarcinoma; (J) R5T1; granuloma, Note the deposition of MWCNT-N fibers (arrows). Bar = 100 μm. Original magnification x200.

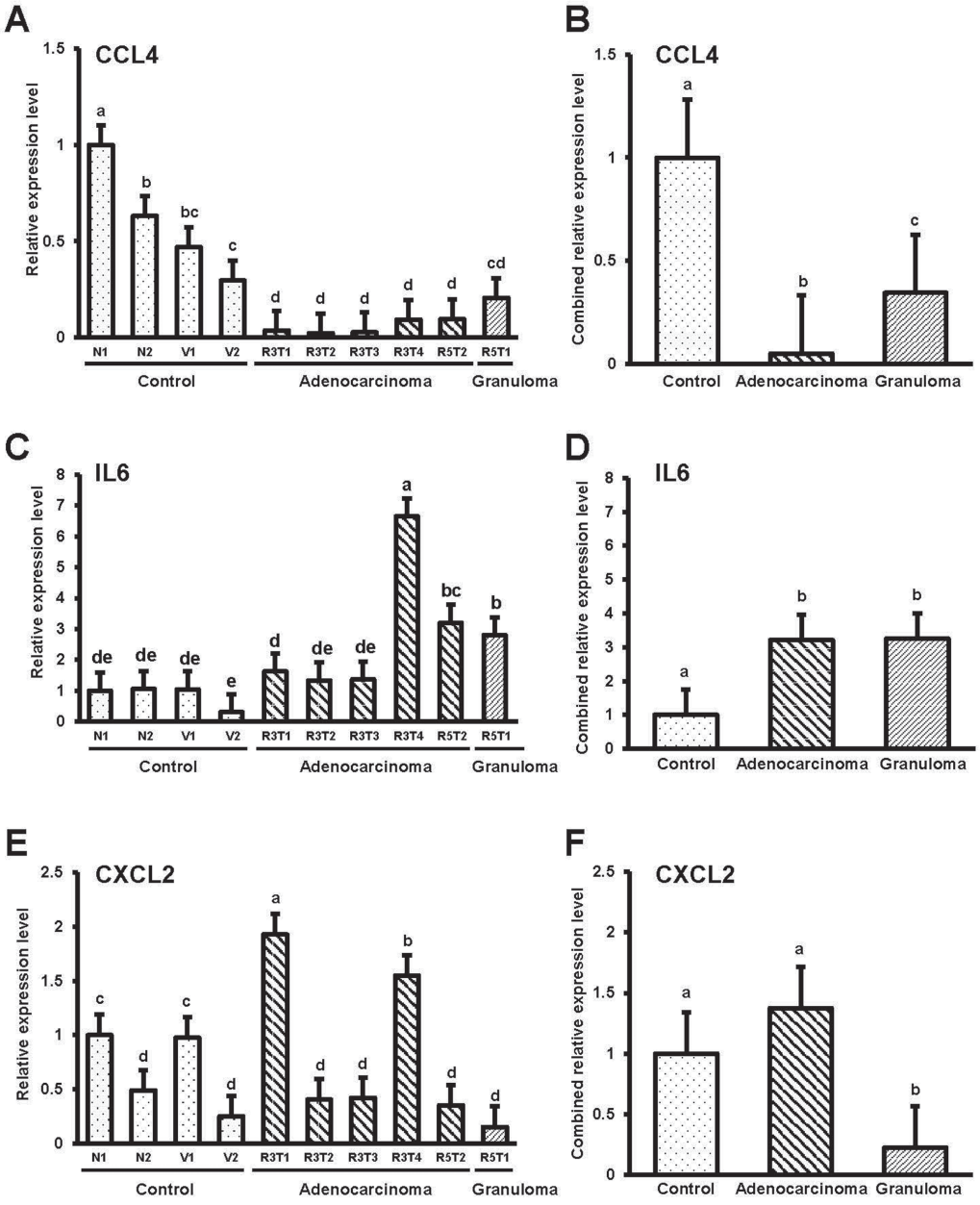

Fig. 2. mRNA expression levels of CCL4, IL6, and CXCL2. (A, B) CCL4; (C, D) IL6; (E, F) CXCL2. (A, C, E) mRNA expression levels in individual controls and tumor samples. (B, D, F) Combined mRNA expression levels in samples of 5 adenocarcinomas, one granuloma, and 4 controls. The normalized expression is presented relative to the control. In case of the same alphabets (a-e), the difference is not statistically significant. Different alphabet letters indicate that the difference is statistically significant. Statistical significance represents $P < 0.05$. Each assay was performed in triplicate to confirm the results.

nocarcinoma tissues (Kim *et al*., 2021). However, surgically resected specimens reveal that a high expression level of CXCL2 is associated with poor prognosis in NSCLC patients (Gu *et al*., 2021; Kim *et al*., 2021). Thus, we conclude that the mRNA expression profile as shown here provides additional information for comprehending MWCNT-induced lung tumorigenesis.

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

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