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Nitrative DNA damage induced by multi-walled carbon nanotube via endocytosis in human lung epithelial cells

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ABSTRACT

Carbon nanotube (CNT) has a promising usage in the field of material science for industrial purposes because of its unique physicochemical property. However, intraperitoneal administration of CNT was reported to cause mesothelioma in experimental animals. Chronic inflammation may contribute to carcinogenesis induced by fibrous materials. 8-Nitroguanine is a mutagenic DNA lesion formed during inflammation and may play a role in CNT-induced carcinogenesis. In this study, we examined 8-nitroguanine formation in A549 human lung alveolar epithelial cells treated with multi-walled CNT (MWCNT) by fluorescent immunocytochemistry. Both MWCNTs with diameter of 20–30 nm (CNT20) and 40–70 nm (CNT40) significantly induced 8-nitroguanine formation at 5 and 10 μg/ml ($p<0.05$), which persisted for 24 h, although there was no significant difference in DNA-damaging abilities of these MWCNTs. MWCNTs significantly induced the expression of inducible nitric oxide synthase (iNOS) for 24 h ($p<0.05$). MWCNTs also significantly increased the level of nitrite, a hydrolysis product of oxidized NO, in the culture supernatant at 4 and 8 h ($p<0.05$). MWCNT-induced 8-nitroguanine formation and iNOS expression were largely suppressed by inhibitors of iNOS (1400 W), nuclear factor-κB (Bay11-7082), actin polymerization (cytochalasin D), caveolae-mediated endocytosis (methyl-β-cyclodextrin, MBCD) and clathrin-mediated endocytosis (monodansylcadaverine, MDC). Electron microscopy revealed that MWCNT was mainly located in vesicular structures in the cytoplasm, and its cellular internalization was reduced by MBCD and MDC. These results suggest that MWCNT is internalized into cells via clathrin- and caveolae-mediated endocytosis, leading to inflammatory reactions including iNOS expression and resulting nitrative DNA damage, which may contribute to carcinogenesis.

Introduction

Carbon nanotube (CNT) is one of the most promising nanomaterials in many industrial and medical applications, because of its unique physicochemical properties, such as high electrical conductivity and excellent strength (Medina et al., 2007; Pacurari et al., 2010). However, there is a growing concern that CNT may exert harmful effects on humans, especially carcinogenicity. The fibrous-like shape and durability of CNT suggest that its toxicity and carcinogenicity may be analogous to those observed with fibrous particles, such as asbestos (Oberdörster, 2010; Pacurari et al., 2008; Poland et al., 2008). Recent animal studies have shown that intraperitoneal application of CNT caused mesothelioma in mice (Nagai et al., 2011; Poland et al., 2008; Takagi et al., 2008), and that intrascrotal administration of CNT induced mesothelioma in rats (Sakamoto et al., 2009). However, the mechanism of CNT-induced carcinogenesis has not been well understood. Since CNT is expected to be used in various industrial fields in future, the risk assessment for CNT-induced harmful effects on humans in working places and the environment is of extreme importance.

Chronic inflammation, which is induced by various environmental factors, is postulated to play a substantial part in human carcinogenesis (Balkwill and Mantovani, 2001; Coussens and Werb, 2002; Hussain and Harris, 2007). Inhalation of fibrous particles causes inflammation in respiratory systems that may lead to fibrosis and carcinogenesis. Several studies have demonstrated that administration of CNT induces inflammatory responses in lung tissues of experimental

Abbreviations: CNT, carbon nanotube; ROS, reactive oxygen species; RNS, reactive nitrogen species; NO, nitric oxide; O_2^-, superoxide; $ONOO^-$, peroxynitrite; MWCNT, multi-walled CNT; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; iNOS, inducible NO synthase; Bay, Bay11-7082; NF-κB, nuclear factor-κB; MBCD, methyl-β-cyclodextrin; MDC, monodansylcadaverine; CytoD, cytochalasin D; PBS, phosphate-buffered saline; NO_2^-, nitrite; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester; TEM, transmission electron microscopy; ANOVA, analysis of variance; LM, light microscopy.

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animals (Jacobsen et al., 2009; Porter et al., 2010; Shvedova et al., 2009). *In vitro* studies have shown that CNT is capable of inducing inflammatory reactions, such as cytokine production, in lung epithelial cell lines (Davoren et al., 2007; Kisin et al., 2011; Ye et al., 2009). These findings raise the possibility that CNT directly induces inflammatory reactions within the target cells, which may contribute to carcinogenesis.

Under the inflammatory conditions, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in inflammatory and epithelial cells. These reactive species interact with DNA bases to form oxidative and nitrative DNA lesions (Hussain et al., 2003; Ohshima et al., 2003). 8-Nitroguanine is a mutagenic nitrative DNA lesion formed during chronic inflammation. Nitric oxide (NO) reacts with superoxide (O_2^-) to form highly reactive peroxynitrite ($ONOO^-$), which interacts with guanine to form 8-nitroguanine (Yermilov et al., 1995a). We first demonstrated that 8-nitroguanine was formed at the sites of carcinogenesis in various animal models and clinical specimens of cancer-prone inflammatory diseases, and proposed this DNA lesion as a potential biomarker of inflammation-related carcinogenesis (Hiraku et al., 2010; Kawanishi et al., 2006). We have recently reported that asbestos exposure induces 8-nitroguanine formation in bronchial epithelial cells of mice (Hiraku et al., 2010). Although *in vitro* experiments have been carried out to explore CNT-induced DNA damage, which was detected by comet assay or micronucleus test (Karlsson et al., 2008; Kisin et al., 2011; Lindberg et al., 2009; Yamashita et al., 2010), and the expression of DNA repair enzymes (Zhu et al., 2007), the precise mechanism has not been elucidated.

In this study, to investigate the mechanism of CNT-induced carcinogenesis, we performed immunocytochemical analysis to examine the formation of 8-nitroguanine in A549 human alveolar carcinoma epithelial cells treated with multi-walled CNT (MWCNT). Although phagocytic cells, including alveolar macrophages, are known to take up exogenous particles by phagocytosis and exert inflammatory reactions (Costantini et al., 2011), even non-phagocytic cells can internalize particles via endocytosis mediated by caveolae and clathrin (Gratton et al., 2008; Rejman et al., 2004). Therefore, we also investigated the role of endocytosis in MWCNT-induced DNA damage.

Materials and methods

Preparation of MWCNT. Two different types of MWCNTs with diameter of 20–30 nm (CNT20) and 40–70 nm (CNT40) were obtained from Wako Pure Chemical Industries, Ltd. (Purity >95%, Osaka, Japan). The length of these MWCNTs was 0.5–2 μm (disclosed by Wako). MWCNT was suspended in phenol-red free high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL, New York, NY, USA) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS, Cambrex Bio Science Walkersville, Walkersville, MD, USA) and 100 mg/l kanamycin, and then sonicated for 4 h with a ultrasonic water bath (Model 5210, Branson Ultrasonics, Danbury, CT, USA) to disperse agglomerates of MWCNT. Then the suspensions were centrifuged at 79 x *g* for 3 min to precipitate coarse particles, and the supernatant was used for experiments. Size distribution of MWCNT agglomerates in the supernatant was measured with a Zetasizer Nano particle size analyzer (Malvern, Worcestershire, UK). The supernatant was stored at −80 °C until use.

MTT assay. To evaluate a cytotoxic effect of MWCNT, we performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as reported previously (Kobayashi et al., 2009). A549 cells (2×10^4 cells/well) were cultured in a 96-well plate overnight and treated with 10 μg/ml MWCNT for 24 h at 37 °C in DMEM containing 5% (v/v) FBS and 100 mg/l kanamycin. Then the cells were incubated with 0.5 mg/ml MTT in the medium for 4 h at 37 °C, followed by the treatment with dimethylsulfoxide for 10 min at room temperature to dissolve formazan crystals. The absorbance of each well was measured at 570 nm using a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Immunocytochemistry. A549 cells (5×10^5 cells/ml) were cultured in DMEM containing 5% (v/v) FBS and 100 mg/l kanamycin overnight on culture slides (BD Falcon, Franklin Lakes, NJ, USA) and then treated with MWCNT for indicated durations at 37 °C. In certain experiments, 1 μM 1400 W [an inhibitor of inducible NO synthase (iNOS)], 10 μM Bay11-7082 [Bay, an inhibitor of nuclear factor-κB (NF-κB) activation], 2 mM methyl-β-cyclodextrin (MBCD, an inhibitor of caveolae-mediated endocytosis), 50 μM monodansylcadaverine (MDC, an inhibitor of clathrin-mediated endocytosis) or 1 μM cytochalasin D (CytoD, an inhibitor of actin polymerization) was added. These inhibitors were purchased from Sigma-Aldrich (St. Louis, MO, USA).

After the treatment with MWCNT, the cells were fixed with 4% (v/v) formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature and washed with PBS 3 times. The cells were treated with 0.5% (v/v) Triton X100 for 3 min and then incubated with 1% (w/v) skim milk for 30 min at room temperature. To detect 8-nitroguanine, the cells were incubated with rabbit polyclonal anti-8-nitroguanine antibody (1 μg/ml) produced by our group (Hiraku and Kawanishi, 2009; Pinlaor et al., 2004) overnight at room temperature. To detect iNOS, rabbit polyclonal antibody (1:1000, Calbiochem, Darmstadt, Germany) was used instead. Then the cells were incubated with fluorescent secondary antibody Alexa 594-labeled goat antibody against rabbit IgG (1:400, Molecular Probes, Eugene, OR, USA) for 3 h. The nuclei were stained with 5 μM Hoechst 33258 (Polysciences Inc., Warrington, PA, USA). The stained cells were examined under a florescent microscope (BX53, Olympus, Tokyo, Japan). The staining intensity per cell of 8-nitroguanine or iNOS was evaluated by analyzing three separate fields containing approximately 670 cells in average for each sample with an ImageJ software.

To confirm the specificity of the anti-8-nitroguanine antibody, MWCNT-treated cells were incubated with 8-nitroguanine-adsorbed antibody produced as described previously (Hiraku et al., 2007; Pinlaor et al., 2004). Then, the cells were incubated with Alexa 594-labeled secondary antibody and examined as described above. We compared the immunoreactivity of these samples with those treated with anti-8-nitroguanine antibody.

Analysis of NO products. We analyzed NO generation from MWCNT-treated cells by measuring the concentration of nitrite (NO_2^-), a hydrolysis product of oxidized NO, in the culture supernatant using the Griess method (Tinti et al., 2011). A549 cells (5×10^5 cells/ml) were treated with 10 μg/ml of MWCNT for indicated durations at 37 °C in phenol red-free DMEM (Gibco/BRL) containing 5% (v/v) FBS and 100 mg/l kanamycin. Then the culture medium was centrifuged at 40,000 x *g* for 10 min at 4 °C to precipitate MWCNT. The supernatant was incubated with 0.5% (w/v) sulfanilamide (Griess reagent I, Wako) and 0.05% (w/v) naphthylethylenediamine (Griess reagent II, Sigma-Aldrich) in 1.25% (v/v) phosphoric acid for 10 min at room temperature. The absorbance at 540 nm of a colored azo dye product of the Griess reaction was measured with a Model 680 microplate reader (Bio-Rad Laboratories), and NO_2^- concentration was determined by comparison with a standard curve generated with sodium nitrite ($NaNO_2$, Wako).

Flow cytometry. To examine ROS generation in MWCNT-treated cells, we measured intracellular peroxide levels by flow cytometry as reported previously (Mizutani et al., 2010). A549 cells (5×10^5 cells/ml) were treated with 10 μg/ml of MWCNT for indicated durations at 37 °C in DMEM containing 5% (v/v) FBS and 100 mg/l kanamycin. To measure peroxide levels, 5 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA), a fluorescent probe, was added 30 min before the end of the incubation.