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Culture medium type affects endocytosis of multi-walled carbon nanotubes in BEAS-2B cells and subsequent biological response ☆

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ABSTRACT

We examined the cytotoxicity of multi-walled carbon nanotubes (MWCNTs) and the resulting cytokine secretion in BEAS-2B cells or normal human bronchial epithelial cells (HBEpCs) in two types of culture media (Ham's F12 containing 10% FBS [Ham's F12] and serum-free growth medium [SFGM]). Cellular uptake of MWCNT was observed by fluorescent microscopy and analyzed using flow cytometry. Moreover, we evaluated whether MWCNT uptake was suppressed by 2 types of endocytosis inhibitors. We found that BEAS-2B cells cultured in Ham's F12 and HBEpCs cultured in SFGM showed similar biological responses, but BEAS-2B cells cultured in SFGM did not internalize MWCNTs, and the 50% inhibitory concentration value, i.e., the cytotoxicity, was increased by more than 10-fold. MWCNT uptake was suppressed by a clathrin-mediated endocytosis inhibitor and a caveolae-mediated endocytosis inhibitor in BEAS-2B cells cultured in Ham's F12 and HBEpCs cultured in SFGM. In conclusion, we suggest that BEAS-2B cells cultured in a medium containing serum should be used for the safety evaluation of nanomaterials as a model of normal human bronchial epithelial cells. However, the culture medium composition may affect the proteins that are expressed on the cytoplasmic membrane, which may influence the biological response to MWCNTs.

1. Introduction

Carbon nanotubes (CNTs) are an important type of nanomaterial and have various applications, including those in the biomedical field (Endo et al., 2008; Saito et al., 2009; Usui et al., 2012). However, potential adverse effects of CNTs on human health are of great concern, considering their increasing use in composite biomaterials and also as innovative solutions for biomedical applications or in nanomedicine (Ajayan and Tour, 2007; Boczkowski and Lanone, 2007; Donaldson et al., 2010; Haniu et al., 2012a). Similar to other nanomaterials, the biological response (including inflammation) and toxicity of CNTs were shown to depend on numerous physicochemical factors, including agglomeration, dispersibility in solution, the presence and nature of impurities, and chemical functionalization (Nel et al., 2006; Sayes et al., 2006; Herzog et al., 2007; Wick et al., 2007; Donaldson and Poland, 2009; Shvedova et al., 2009; Kolosnjaj-Tabi et al., 2010; Nagai et al., 2011; Haniu et al., 2012b).

Abbreviations: AB, Alamar blue; CNT, carbon nanotube; DIC, differential interference contrast; FCM, flow cytometry; F-DPBS, Dulbecco's-PBS containing 10% FBS; Ham's F12, Ham's F12 containing 10% fetal bovine serum; H33342, bisbenzimide H33342 fluorochrome trihydrochloride; HBEpC, human bronchial epithelial cell; IL, interleukin; MWCNT, multi-walled carbon nanotube; PBS, phosphate buffered saline; SE, standard error; SFGM, serum-free growth medium; SSC, side scatter.

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We recently reported that the cell type also plays a critical role in the biological response to CNTs (Haniu et al., 2011b). BEAS-2B human bronchial epithelial cells, MESO-1 malignant pleural mesothelioma cells, and THP-1 cells differentiated to macrophage-like cells that, when exposed to MWCNTs, showed cell growth inhibition and increased cytokine secretion. These cells had the potential to internalize MWCNTs into the cytoplasm. Moreover, we showed that the cellular concentration of MWCNTs correlates with cytotoxicity in BEAS-2B and MESO-1 cells (Haniu et al., 2011a).

BEAS-2B is the most popular cell line for the evaluation of the respiratory safety of nanomaterials (Herzog et al., 2007; Park et al., 2008; Eom and Choi, 2009), and it is used in the safety assessment of CNTs (Lindberg et al., 2009; Hirano et al., 2010; He et al., 2011; Tsukahara and Haniu, 2011; Wang et al., 2011). However, even when the different types of CNTs studied are accounted for, the concentrations of CNTs that show cytotoxicity vary greatly. This variability may be caused by the cell culture medium, because cytotoxicity at low CNT concentrations was observed when the cells were cultured in a medium containing serum, whereas cytotoxicity was only observed at very high CNT concentrations when serum was not present in the medium.

In this study, we determined the influence of serum on the cellular responses to MWCNTs and compared the biological response between BEAS-2B cells and HBEpCs. Moreover, we confirmed the effect of endocytosis of MWCNTs.

2. Materials and methods

2.1. Carbon nanotubes

MWCNTs manufactured by a chemical vapor deposition method were provided by Hodogaya Chemical (MWNT-7; Tokyo, Japan). The properties of these MWCNTs were obtained from Hodogaya Chemicals (Table 1). Autoclave sterilization conditions were 121 °C for 15 min. MWNT-7 was dispersed with 0.1% gelatin (Nippi, Tokyo, Japan) in phosphate-buffered saline (PBS) and sonicated for 30 min by using a water-bath sonicator.

2.2. Cell culture

The BEAS-2B human bronchial epithelial cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Normal HBEpCs were purchased from Cell Application (San Diego, CA, USA). BEAS-2B cells were cultured in Ham's nutrient mixture F-12 (Nacalai, Tokyo, Japan) with 10% fetal bovine serum (Ham's F12) and passaged twice a week, or cultured in bronchial/tracheal epithelial cell serum-free growth medium kit with 0.1 μg/ml retinoic acid (SFGM; Cell Application) and passaged every 4 days in SFGM, with the medium exchanged every other day. HBEpCs were cultured in SFGM and passaged every 4 days, with the medium exchanged every other day. HBEpCs were used by passage 4. For each study, the cells were seeded at a density of 2×10^5 cells/cm^2 and allowed to adhere for 24 h.

Table 1
Basic properties of MWNT-7.

Average primary particle diameter size	Diameter (nm)	60
	Length (μm)	10
Purity		>99.5%
Specific surface area (m^2/g)		25–30
Real density (g/cm^3)		0.005–0.01

2.3. Alamar blue (AB) assay

To determine the viability of cells exposed to MWNT-7, we performed an AB assay (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cells were incubated for 24 h at 37 °C in 0.1 ml of culture medium with various concentrations of MWNT-7 in 96-well culture plates. The control cells were cultured in the culture medium containing dispersant. Viable cells metabolized the dye, which resulted in an increase in the fluorescence intensity, as determined by excitation/emission at 550/600 nm on a fluorescence multiplate reader (PowerScan 4, DS Pharma Biomedical, Osaka, Japan). Cytotoxic activity was calculated as follows: percent cytotoxicity = 100 × experimental value/control value. Test media were assayed 6 times.

To determine the effect of endocytosis inhibitors, cells cultured on 96-well culture plates for 24 h were pretreated with chlorpromazine hydrochloride (20 μM; Nacalai) dissolved in PBS or indomethacin (50 μM; SIGMA, St. Louis, MO, USA) dissolved in ethanol for 15 min. The cells were then exposed to MWNT-7 (50 μg/ml) with the inhibitors for 2 h. The cells were washed twice with Dulbecco's PBS (DPBS) at 4 °C and cultured in each medium without MWNT-7 or the inhibitors for 22 h. Thereafter, the cells treated with the AB reagent were assayed.

2.4. Assessment of MWNT-7 uptake by fluorescence microscopy

Cells were cultured on ibiTreat dishes (μ-dish$^{35\ mm\ high}$; ibidi GmbH, Martinsried, Germany) for 24 h in a 5% CO_2 incubator. The cells were then incubated with or without MWNT-7 (1 μg/ml) for 24 h. Prior to observation, the cells were washed twice and stained with bisbenzimide H33342 fluorochrome trihydrochloride (H33342, 1 μg/ml; Nacalai) for 30 min. The cells were visualized using differential interference contrast (DIC) and fluorescence by fluorescence microscopy (AxioObserverZ1, Zeiss, Jena, Germany) in a 5% CO_2 chamber at 37 °C using a 40× objective.

To determine the effect of endocytosis inhibitors, cells cultured on ibiTreat dishes for 24 h were pretreated with 2 types of endocytosis inhibitors for 15 min and then exposed to MWNT-7 (10 μg/ml) and H33342 for 2 h. The cells were washed twice with DPBS at 4 °C and observed in each medium without MWNT-7 or the inhibitors.

2.5. Cytokine measurement

We previously have reported that certain cytokines as secreted as part of the inflammatory response in BEAS-2B cells exposed to MWCNTs (Tsukahara and Haniu, 2011). Although the secretion of interleukin (IL)-6 and IL-8 was shown to increase upon exposure to MWCNTs, other cytokines (IL-12, TNF-α, IL-10, and IL-1β) were not detected. Therefore, we selected IL-6 and IL-8 for evaluation in this study. Cytokines in the culture supernatant were measured using a cytometric bead array flex set system (BD Biosciences, San Jose, CA, USA), according to the manufacturer's protocol. Briefly, cells in a 12-well plate were exposed to various concentrations of MWNT-7 for 24 h; subsequently, cytokine capture beads for IL-6 and IL-8 were added to the samples, or cytokine standards (10–5000 pg/ml) were prepared in flow cytometry (FCM) tubes. The mixtures were vortexed, and antibodies for fluorescence detection were added to each tube. The samples were then incubated at room temperature for 2 h. Following incubation, the beads were washed once and resuspended prior to reading by a FACS Calibur™ apparatus (BD Biosciences). Test media were assayed in triplicate for each treatment condition. The limits of detection in this kit were lower than 1.6 pg/ml (IL-6) and 1.2 pg/ml (IL-8).