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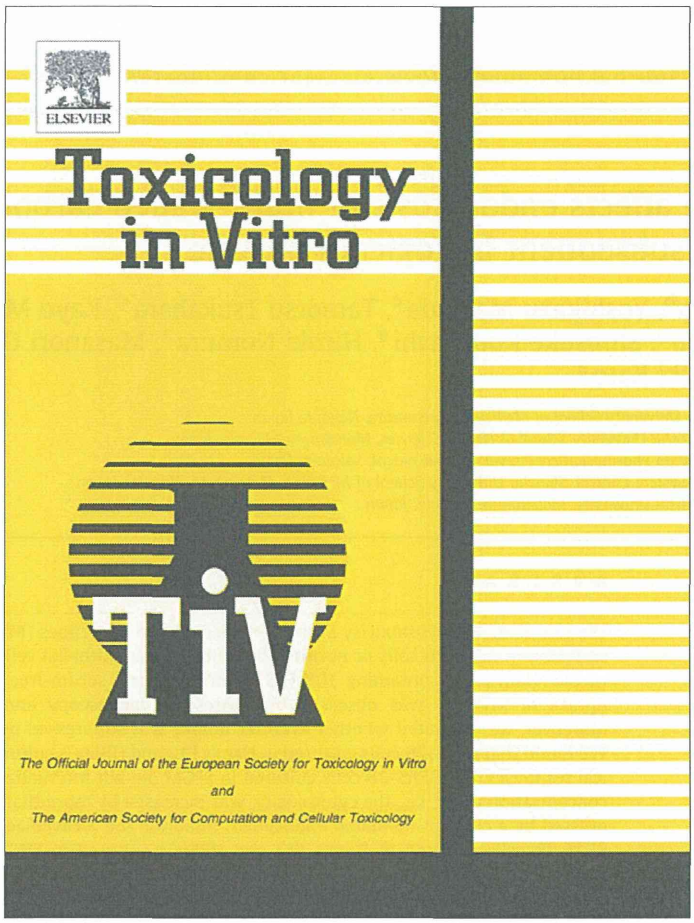

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Culture medium type affects endocytosis of multi-walled carbon nanotubes in BEAS-2B cells and subsequent biological response ☆

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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).

2.6. Assessment of MWNT-7 uptake by FCM

MWNT-7 uptake was determined by FCM using our previous methods with slight modifications (Haniu et al., 2011a). Briefly, the cells were grown on 12-well plates for 24 h and were incubated for 2 h at 37 °C in the presence or absence of MWNT-7 (50 μg/ml). For the endocytosis inhibitor tests, the inhibitors were pre-treated for 15 min prior to MWNT-7 exposure. The cells were washed with DPBS at 4 °C, harvested with trypsin, and centrifuged. The precipitated cells were suspended in DPBS containing 10% FBS and filtered through a nylon mesh (67-μm pore size). Side scatter (SSC) in more than 8000 events was immediately measured by light-scattering analysis using an FACS Calibur™ apparatus. The SSC relative ratio was calculated as follows: SSC relative ratio = SSC value of the cells in the presence of MWNT-7/SSC value of the cells in the absence of MWNT-7. The suspended cells were assayed in triplicate for each treatment condition.

2.7. Statistical analysis

Data are presented as the mean ± standard error (SE). Student's *t*-test was used for data analysis, and $p < 0.05$ was defined as statistically significant.

3. Results

3.1. Cytotoxicity

We compared the cytotoxicity of MWNT-7 under the same conditions in HBEpCs, which are normal human bronchial epithelial cells, and BEAS-2B cells, which are immortalized normal human bronchial epithelial cells (Fig. 1). Although the cell growth of HBEpCs was suppressed by approximately 50% at an MWNT-7 concentration of 10 μg/ml, the growth of BEAS-2B cells was suppressed by less than 30%, even at an MWNT-7 concentration of 50 μg/ml.

Therefore, we evaluated the effect of different culture media on BEAS-2B cells. The cytotoxicity of MWNT-7 in BEAS-2B cells in different media determined using the AB assay is shown in Fig. 2. The viability of BEAS-2B cells incubated in Ham's F-12 during the assay significantly decreased upon treatment with 1 μg/ml MWNT-7, regardless of the culture medium used during passage. However, BEAS-2B cells that were incubated in SFGM during exposure to

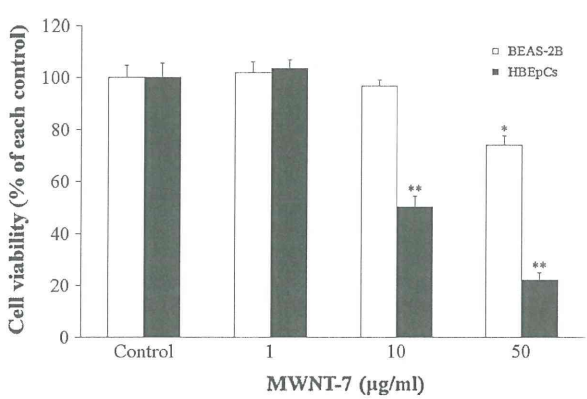

Fig. 1. Cytotoxicity of MWNT-7 in BEAS-2B cells and HBEpCs cultured in SFGM. Cells were exposed to varying concentrations of MWNT-7 or dispersant for 24 h. The cytotoxicity is indicated as a percentage of the control, and the data were statistically compared to the data for each control (mean ± SE, $n = 6$, $^{*}p < 0.01$, $^{**}p < 0.001$).

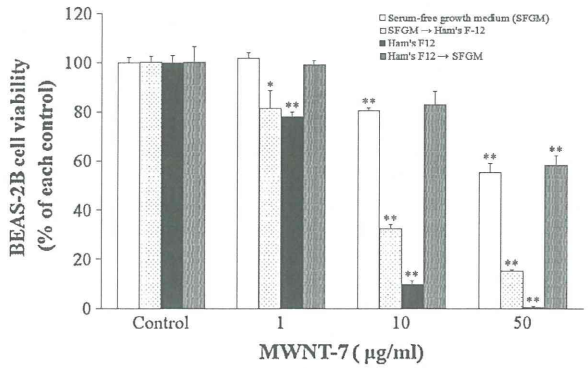

Fig. 2. Cytotoxicity of MWNT-7 in BEAS-2B cells that were cultured in different media. BEAS-2B cells were passaged in SFGM and Ham's F12. In the experiment, trypsinized cells were separated into 2 groups that were cultured in the same medium or a different medium and allowed to adhere for 24 h. The cells were exposed to varying concentrations of MWNT-7 or dispersant for 24 h. The cytotoxicity is indicated as a percentage of the control, and the data were statistically compared to the data for each control (mean ± SE, $n = 6$, $^{*}p < 0.05$, $^{**}p < 0.001$).

MWNT-7 did not show growth inhibition upon exposure to 1 μg/ml MWNT-7; they only showed inhibition of cell growth without accompanying cell death, even upon exposure to 50 μg/ml MWNT-7 and even when they were cultured in Ham's F12 during passage. Because the doubling time of BEAS-2B cells is approximately 26 h and the exposure time of MWNT-7 was 24 h, we speculate that BEAS-2B cells that were exposed to 50 μg/ml MWNT-7 in Ham's F12 and showed less than 50% viability underwent growth suppression but not cell death.

Images of BEAS-2B cells and HBEpCs exposed to MWNT-7 are shown in Fig. 3. MWNT-7 was observed near the nuclei and cytoplasm in BEAS-2B cells in Ham's F12 and HBEpCs in SFGM. However, BEAS-2B cells in SFGM showed low internalization of MWNT-7, and some MWNT-7 adhered to the cell surface.

3.2. Cytokine secretion

We evaluated cytokine secretion by BEAS-2B cells incubated in Ham's F12 and SFGM as well as HBEpCs incubated in SFGM in response to MWNT-7. Although IL-6 secretion by untreated BEAS-2B cells in Ham's F12 and untreated HBEpCs was sufficient for detection (33.8 ± 5.0 and 5.1 ± 0.5 pg/ml, respectively), secretion of IL-6 by BEAS-2B cells in SFGM was not detected (under 1.6 pg/ml). Exposure to MWNT-7 increased IL-6 secretion by BEAS-2B cells in Ham's F12 and HBEpCs (Fig. 4a). However, the degree of the increase and the MWNT-7 concentration that stimulated the maximal increase were different: BEAS-2B cells in Ham's F12 and HBEpCs showed a 20-fold and 2-fold upregulation in response to 10 μg/ml and 1 μg/ml MWNT-7, respectively. Moreover, IL-6 secretion in response to 50 μg/ml MWNT-7 was the same as that in response to 10 μg/ml MWNT-7 in BEAS-2B cells in Ham's F12, but decreased to the level of the control in HBEpCs. IL-6 secretion by BEAS-2B cells in SFGM was lower than the detectable limit when the cells were exposed to MWNT-7, even at the maximum concentration. IL-8 was secreted by both cell types under the untreated condition, and the concentration was on the order of HBEpC > - BEAS-2B in SFGM > BEAS-2B in Ham's F12 (814.1 ± 78.9, 260.2 ± 18.6 and 169.3 ± 22.0, respectively), (Fig. 4b). Upon exposure to 10 μg/ml MWNT-7, BEAS-2B cells in SFGM did not demonstrate a change in secretion, whereas other cell conditions produced increased IL-8 secretion. However, secretion in response to 50 μg/ml MWNT-7 did not show a further increase. The increase

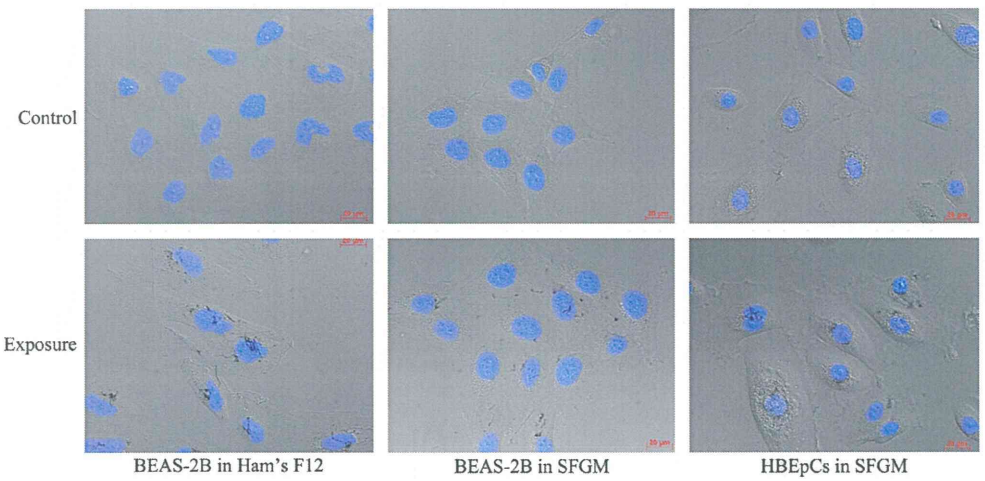

Fig. 3. Imaging of MWNT-7 uptake in BEAS-2B cells and HBEpCs. BEAS-2B cells and HBEpCs were exposed to 1 μg/ml MWNT-7 or dispersant for 24 h. DIC and fluorescence images were merged. Nuclei were stained blue by H33342. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

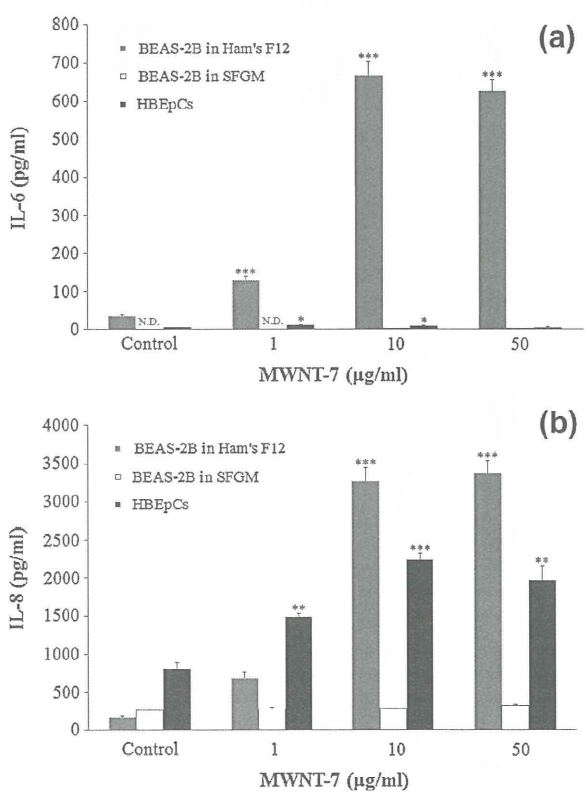

Fig. 4. Cytokine secretion by BEAS-2B cells and HBEpCs in SFGM or Ham's F12 in response to MWNT-7. Cells were exposed to varying concentrations of MWNT-7 or dispersant for 24 h. Secretion of IL-6 (a) and IL-8 (b) was compared to that in each control (mean ± SE, $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). N.D. = not detected.

was more pronounced in BEAS-2B cells in Ham's F12 than in HBEpCs.

3.3. Inhibition of endocytosis

Internalization of MWNT-7 by BEAS-2B cells in Ham's F12 and HBEpCs in SFGM was suppressed by chlorpromazine, which is a clathrin-mediated endocytosis inhibitor, and indomethacin, which is a caveolae-mediated endocytosis inhibitor. The cells showed extensive internalization of MWNT-7 for 2 h without the inhibitors, whereas cells pre-treated with the inhibitors showed little internalization of MWNT-7 and some MWNT-7 on the plasma membrane, as determined using fluorescence microscopy (Fig. 5a). The amount of internalized MWNT-7 was determined using the SSC relative ratio in BEAS-2B cells in Ham's F12 and HBEpCs in SFGM treated with or without the inhibitors after exposure to MWNT-7 for 2 h, as shown in Fig. 5b and c. The SSC relative ratio for BEAS-2B cells that internalized MWNT-7 in SFGM is also shown in Fig. 5b. The amount of MWNTs internalized by BEAS-2B cells was significantly lower in SFGM medium than in F12 (Fig. 5b). HBEpCs exposed to MWNT-7 in SFGM internalized them to the same extent as BEAS-2B cells exposed to MWNT-7 in Ham's F12 (Fig. 5b and c). Significant 21.6% and 31.8% reductions of internalization were observed in the presence of chlorpromazine in BEAS-2B cells in Ham's F12 and HBEpCs in SFGM, respectively, and 50.1% and 28.0% reductions were observed in the presence of indomethacin. Moreover, we assayed cell growth inhibition by using the AB assay to confirm the influence of the endocytosis inhibitors. Both endocytosis inhibitors suppressed the cell growth inhibition mediated by MWNT-7 in BEAS-2B cells in Ham's F12 and HBEpCs in SFGM (Fig. 5d). Chlorpromazine suppressed MWNT-7 internalization and cell growth inhibition to a higher degree than did indomethacin in BEAS-2B cells in Ham's F12, and the reverse pattern was observed for HBEpC in SFGM.

4. Discussions

BEAS-2B cells were originally established by infection of normal human bronchial epithelial cells with an adenovirus 12-SV40 hybrid virus (Reddel et al., 1988). Ke et al. reported that in BEAS-2B cells, most cells at clonal density undergo squamous differentiation when incubated in media containing more than 4% serum (Ke et al., 1988). In this study, BEAS-2B cells in Ham's F12 internalized MWNT-7 and demonstrated a 50% inhibitory concentration that was approximately 10-fold lower than that of BEAS-2B in SFGM, as shown in Fig. 2. This result supports our hypothesis that the culture medium affects cytotoxicity in BEAS-2B cells. Cellular uptake of MWNT-7 by differentiated BEAS-2B cells observed in the presence of fetal bovine serum was lost when the MWNT-7 treatment

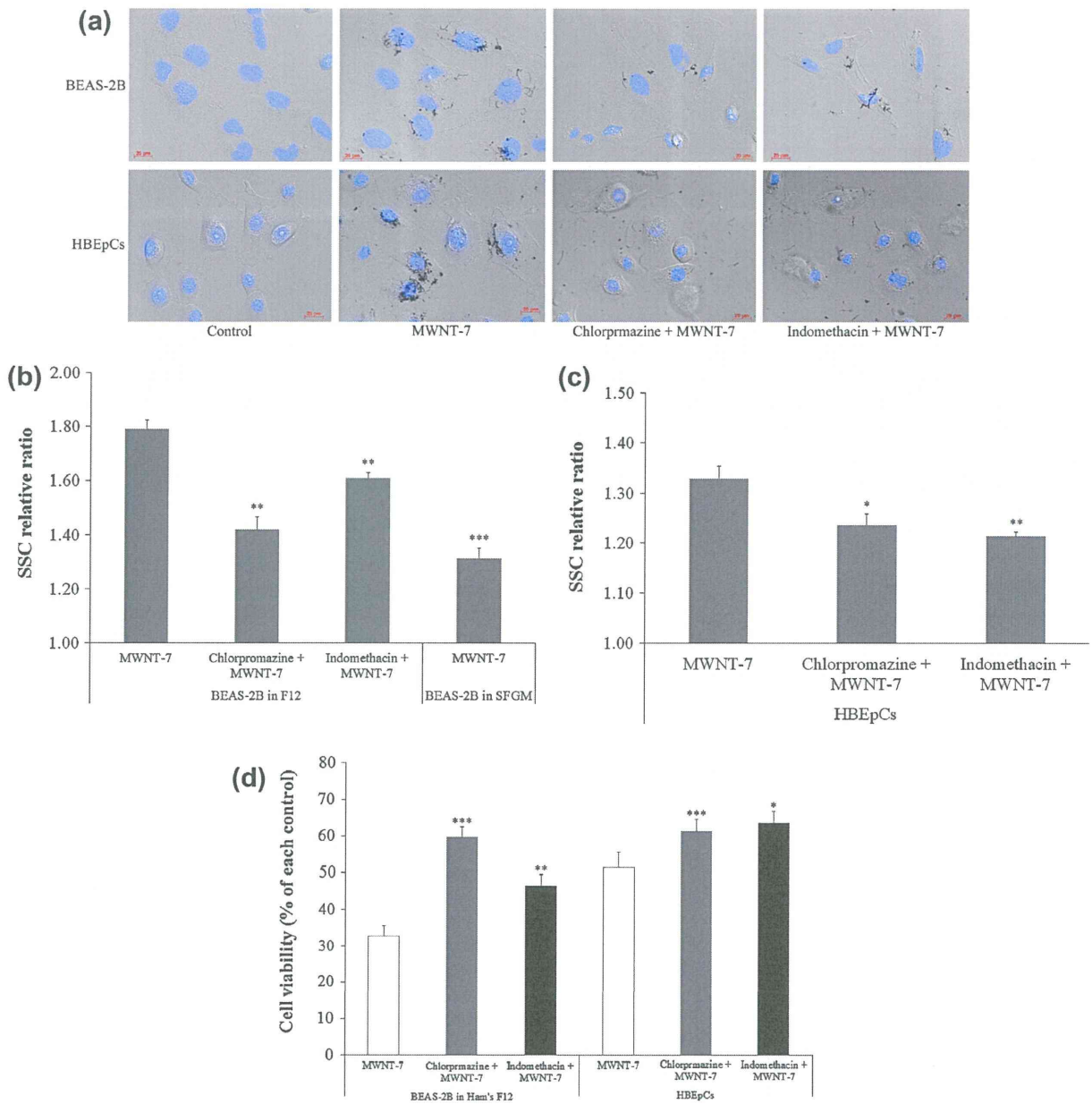

Fig. 5. Effect of endocytosis inhibitors on biological responses to MWNT-7 in BEAS-2B cells and HBEpCs. (a) BEAS-2B cells in Ham's F12 and HBEpCs were pre-treated with or without chlorpromazine (clathrin-mediated endocytosis inhibitor) or indomethacin (caveolae-mediated endocytosis inhibitor) for 15 min and exposed to 10 μg/ml MWNT-7 or dispersant for 2 h. The cells were washed, and the nuclei were stained blue by H33342. DIC and fluorescence images were merged. (b) BEAS-2B cells in Ham's F12 were pre-treated with or without chlorpromazine for 15 min and exposed to 50 μg/ml MWNT-7 or dispersant for 2 h. BEAS-2B cells in SFGM were exposed to 50 μg/ml MWNT-7 or dispersant for 2 h. The cells were suspended in F-DPBS, and the SSC value was measured using a flow cytometer. The SSC relative ratio was compared to that in BEAS-2B cells exposed to MWNT-7 in Ham's F12 (mean ± SE, $n = 3$, $^{**}p < 0.01$, $^{***}p < 0.001$). (c) HBEpCs in SFGM were pre-treated with or without indomethacin for 15 min and exposed to 50 μg/ml MWNT-7 or dispersant for 2 h. The cells were suspended in F-DPBS, and the SSC value was measured using a flow cytometer. The SSC relative ratio was compared to that of HBEpCs exposed to MWNT-7 (mean ± SE, $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$). (d) BEAS-2B cells in Ham's F12 and HBEpCs were pre-treated with or without chlorpromazine or indomethacin for 15 min and exposed to 50 μg/ml MWNT-7 or dispersant for 2 h. Subsequently, the cells were subjected to AB assay after washing and culture for an additional 22 h. The cytotoxicity is presented as a percentage of the control, and the data were statistically compared to the data for cells exposed to MWNT-7 without the inhibitors (mean ± SE, $n = 6$, $^{*}p < 0.01$, $^{**}p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was performed in SFGM, which indicates that CNT uptake by BEAS-2B cells is not an original property and is induced by FBS (Fig. 2). Moreover, MWNT-7 was again internalized when BEAS-2B cells that had been cultured in SFGM and had thus lost their capacity for MWNT-7 uptake were again cultured in Ham's F12. Normal HBEpCs in SFGM showed MWNT-7 internalization and growth inhibition identical to the observations in BEAS-2B cells in Ham's F12 (Figs. 1 and 3). We also used another line of HBEpCs purchased from a different company and obtained the same result (data not shown). These cells had an ellipsoid phenotype, although the HBEpCs appeared to be cuboidal, and BEAS-2B cells in Ham's F12 were squamous. In contrast, BEAS-2B cells in SFGM displayed a

spindle shape that is typically observed when normal human bronchial epithelial cells differentiate (Zhang et al., 2011). These results cannot be attributed to the increased solubility of CNTs in serum; rather, they are based on functional changes with resulting morphological changes that occur in the presence of serum (Fig. 3).

Cytokine secretion also showed a similar pattern in response to CNT internalization. BEAS-2B cells in Ham's F12 and HBEpC showed increased secretion of IL-6 and IL-8 upon exposure to CNTs, although there was a large difference in IL-6 secretion between cell types. We did not detect secretion of IL-6 in untreated BEAS-2B cells in SFGM (Fig. 4a). IL-8 secretion was not increased by exposure of BEAS-2B cells in SFGM to MWNT-7, although BEAS-2B cells in both types of culture medium secreted the same amount of IL-8 without MWNT-7 exposure (Fig. 4b). In the 1990s, it was reported that BEAS-2B cells cultured in SFCM produced cytokines, including IL-6 and IL-8, when stimulated by bioactive substances such as tumor necrosis factor α or histamine (Nakamura et al., 1991; Noah et al., 1991; Levine et al., 1993). BEAS-2B cells used for the safety evaluation of nanomaterials are cultured in a medium in which serum is present or absent. Some previous studies detected IL-6 or IL-8 secretion by untreated BEAS-2B cells cultured in a medium containing serum, and showed that such secretion was increased by nanomaterials (Hirano et al., 2010; Heng et al., 2011; Zhao et al., 2012). However, few researchers have assayed the cytokines secreted by BEAS-2B cells exposed to nanomaterials in SFCM (Ovrevik et al., 2009). Our findings of growth inhibition and cytokine secretion, in conjunction with the previous studies described above, indicate that the biological response to nanomaterials in BEAS-2B cells varies depending on the bioactive substances present, and BEAS-2B cells cultured in a medium containing serum seem to better reflect the biological response of normal human bronchial cells than BEAS-2B cells cultured in a serum-free medium. Moreover, it is suggested that internalization of MWNT-7 is important for the induction of IL-6 and IL-8 secretion.

We previously reported that CNT internalization was suppressed by cytochalasin D, which is an endocytosis inhibitor, in 3 types of cells (Haniu et al., 2011b). In this study, we used 2 types of endocytosis inhibitors. One was chlorpromazine, which is a clathrin-mediated endocytosis inhibitor, and the other was indomethacin, which is a caveolae-mediated endocytosis inhibitor (Yumoto et al., 2012). CNT internalization was suppressed by both types of endocytosis inhibitors (Fig. 5a–d). Kostarelos et al. (2007) reported that the cellular uptake of functionalized carbon nanotubes is independent of cell type and not inhibited by sodium azide, which is an endocytosis inhibitor. However, our present study and previous findings indicate that cellular uptake changes in response to cell differentiation and is inhibited by endocytosis inhibitors (Haniu et al., 2011b). The MWCNTs that we used in this study were not functionalized or labeled with fluorescein isothiocyanate. The mechanism of MWCNT uptake may depend on whether the MWCNT is modified (Tabet et al., 2011). Additionally, the recognition mechanism may vary depending on the proteins expressed on the cytoplasmic membrane (Shi et al., 2011; Vácha et al., 2011). Further study is necessary to identify the proteins on the cytoplasmic membrane that are affected by the medium composition to explain the exact mechanism of endocytosis.

In conclusion, we found that the differentiation of the cells affected MWCNT uptake and the biological responses to MWCNTs in BEAS-2B cells cultured in a medium containing serum or not. Furthermore, we found that BEAS-2B cells cultured in a medium containing serum show biological responses that are very similar to those of normal human bronchial epithelial cells, as determined by comparison with HBEpCs. These results reveal the importance of appropriate usage of cell lines and culture conditions when performing safety assessment of nanomaterials for humans *in vitro*. It is necessary to determine not only the pharmacokinetics of the nanomaterial but also the mechanism of its cellular internalization.

Conflict of interests

The authors declare that they have no competing financial or non-financial interests.

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ORIGINAL RESEARCH

Biological responses according to the shape and size of carbon nanotubes in BEAS-2B and MESO-1 cells

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Abstract: This study aimed to investigate the influence of the shape and size of multi-walled carbon nanotubes (MWCNTs) and cup-stacked carbon nanotubes (CSCNTs) on biological responses in vitro. Three types of MWCNTs – VGCF®-X, VGCF®-S, and VGCF® (vapor grown carbon fibers; with diameters of 15, 80, and 150 nm, respectively) – and three CSCNTs of different lengths (CS-L, 20–80 μm; CS-S, 0.5–20 μm; and CS-M, of intermediate length) were tested. Human bronchial epithelial (BEAS-2B) and malignant pleural mesothelioma cells were exposed to the CNTs (1–50 μg/mL), and cell viability, permeability, uptake, total reactive oxygen species/superoxide production, and intracellular acidity were measured. CSCNTs were less toxic than MWCNTs in both cell types over a 24-hour exposure period. The cytotoxicity of endocytosed MWCNTs varied according to cell type/size, while that of CSCNTs depended on tube length irrespective of cell type. CNT diameter and length influenced cell aggregation and injury extent. Intracellular acidity increased independently of lysosomal activity along with the number of vacuoles in BEAS-2B cells exposed for 24 hours to either CNT (concentration, 10 μg/mL). However, total reactive oxygen species/superoxide generation did not contribute to cytotoxicity. The results demonstrate that CSCNTs could be suitable for biological applications and that CNT shape and size can have differential effects depending on cell type, which can be exploited in the development of highly specialized, biocompatible CNTs.

Keywords: multi-walled carbon nanotube, cup-stacked carbon nanotube, cytotoxicity, in vitro, intracellular acidity

Introduction

Due to their unique physicochemical properties, carbon nanotubes (CNTs) have applications in a wide variety of industries. One major area of application is in the manufacture of biomaterials and devices, which include biosensors and drug and vaccine delivery vehicles.[1,2] CNTs have the advantage of superior mechanical strength, and carbon materials in general are considered inert and therefore biocompatible.[3,4] However, before CNTs can be incorporated into new and existing biomedical devices, their toxicity and biocompatibility need to be thoroughly investigated. Mice injected intraperitoneally with CNTs exhibited toxicological changes similar to those induced by exposure to asbestos,[5,6] and CNTs have been linked to the induction of mesotheliomas.[7,8] Although some in vivo studies have been conducted on the safety of CNT exposure by inhalation or intratracheal administration, their findings have been indeterminate.[9–13] Results from in vitro studies have also been ambiguous, with some studies reporting that CNTs induce cytotoxicity and cytokine production,[14–18] and others showing that no significant biological responses are elicited.[19,20]

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