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EVALUATION OF THE INTERACTIONS BETWEEN MULTIWALLED CARBON NANOTUBES AND CACO-2 CELLS

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The aim of this study was to determine whether multiwalled carbon nanotubes (MWNCT) are taken up by and are toxic to human intestinal enterocytes using the Caco-2 cell model. Caco-2 cells were exposed to 50 μg/ml MWCNT (oxidized or pristine) for 24 h, and experiments were repeated in the presence of 2.5 mg/L natural organic matter. Cells displayed many of the properties that characterize enterocytes, such as apical microvilli, basolateral basement membrane, and glycogen. The cell monolayers also displayed tight junctions and electrical resistance. Exposure to pristine and oxidized MWCNT, with or without natural organic matter, did not markedly affect viability, which was assessed by measuring activity of released lactate dehydrogenase (LDH) and staining with propidium iodide. Ultrastructural analysis revealed some damage to microvilli colocalized with the MWCNT; however, neither type of MWCNT was taken up by Caco-2 cells. In contrast, pristine and oxidized MWCNT were taken up by the macrophage RAW 264.7 line. Our study suggests that intestinal enterocytes cells do not take up MWCNT.

Since their identification in the early 1990s, carbon nanotubes (CNT) have been the subject of intense research in many disciplines because of their unique properties and range of anticipated applications. Carbon nanotubes generally exist in two varieties: single-walled and multiwalled carbon nanotubes (SWCNT and MWCNT). Most of the existing and projected applications of CNT take advantage of their strength, electrical and thermal conductivity, or surface functionalization capabilities (Lam et al. 2006). The volume of CNT available on the market is expected to increase rapidly in the near future (UNEP 2007). As production levels and consumer applications of CNT increase, it is likely that humans might be exposed in occupational and/or consumer settings.

The growth in CNT production is accompanied by an increasing need for assessing the risks CNT pose to human health. There is limited information on the toxicity of CNT and even less information on the potential routes and magnitudes of human exposure. In vitro studies in lung and other cell types reported increased oxidative stress, inflammation, cell death, and genotoxicity (Shvedova et al. 2003; Sayes et al. 2006; Kisin et al. 2007; Zhu et al. 2007; Rotoli

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et al. 2008; Murray et al. 2009). In vivo studies found elevated incidence of granulomas, fibrosis, and/or inflammation following pulmonary exposure (Lam et al. 2004; Warheit et al. 2004; Shvedova et al. 2005). Because asbestos and CNT both have fibrous structures, studies compared the effects of CNT to those of amphibole asbestos. Following ip administration, two studies reported lesions in the peritoneal cavity suggestive of mesothelioma (Poland et al. 2008; Takagi et al. 2008), while one did not (Muller et al. 2009). There is also evidence that CNT penetrate the pulmonary pleura of mice (Porter et al. 2009b). In regard to exposure, CNT may be aerosolized and deposited on hands or gloves during manufacture and processing (Maynard et al. 2004; Han et al. 2008).

The potential for toxicity and uptake of CNT and other nanomaterials via the intestine is not well understood, yet ingestion may be a significant source of exposure, both occupationally and environmentally. Occupationally, ingestion might occur from mucociliary clearance of inhaled particles (Whitsett 2002) or from hand-to-mouth transfer (Roels et al. 1982; Far et al. 1993; Chuang et al. 1999). In addition, exposure through ingestion might also occur through contaminated food and water sources and, particularly for children, contaminated soil (Calabrese et al. 1997). Research on intestinal uptake of particles is more often directed toward pharmaceutical applications of nanomaterials. Studies in animals suggested that particles are taken up by the intestine; however, the mechanism of uptake and efficiency of uptake by the human intestine is largely unknown (Jani et al. 1990; Hillyer & Albrecht 2001; Behrens et al. 2002; Smyth et al. 2008).

The aim of this study was to characterize interactions between the human intestine and two types of MWCNT, pristine (p) and oxidized (o). The pristine MWCNT (p-MWCNT) are not functionalized postmanufacture, and are thus hydrophobic and unstable in aqueous solutions. It was hypothesized that their hydrophobic behavior may also increase their ability to interact with cell membranes. The oxidized MWCNT (o-MWCNT) have relatively high surface oxygen content (approximately 12% surface oxygen) and are readily dispersible in water. The influence of natural organic matter (NOM) on the interactions between MWCNT and the intestine was also considered. Although the potential for environmental exposure to CNT is not well defined, it is reasonable to assume that CNT may be released into the environment and interact with NOM present in the environment. Recent studies demonstrated that NOM increases the aqueous stability of CNT, which may translate both into enhanced environmental mobility and decreased agglomeration in biological media (Hyung et al. 2007; Wang et al. 2008; Alpatova et al. 2010).

The Caco-2 cell line was used as a model of human intestinal enterocytes. Caco-2 cells are a human intestinal cancer cell line and are widely used to model human intestinal uptake of various therapeutic agents, nutrients, and environmental contaminants (Glahn et al. 1998; Navarro et al. 2000; Artursson et al. 2001; Bannon et al. 2003). When fully differentiated, the cells exist as a monolayer and express many of the features of human intestinal enterocytes, including tight junctions, microvilli, and many of the same transporters (Hubatsch et al. 2007).

MATERIALS AND METHODS

Cleaning and Processing of MWCNT

p-MWCNT, with diameters of 10 to 20 nm and lengths of 5 to 20 μm, were purchased from Nanolab (Newton, MA). To prepare MWCNT with high levels of surface oxygen, 2 g of p-MWCNT was placed in 120 ml of a 3:1 mixture of H_2SO_4:HNO_3 and maintained at 70°C for 8 h without stirring. After treatment, the newly oxidized o-MWCNT were washed, dried, and ball-milled until they reached a fine powder consistency (Cho et al. 2008). Both types of MWCNT were sonicated and dialyzed against de-ionized water to remove amorphous carbon and free catalyst.

Characterization of p- and o-MWCNT

Following sonication and dialysis, all MWCNT were characterized for surface composition, surface area, and size distribution using x-ray photon spectroscopy, Brunauer Emmet Teller analysis and transmission electron microscopy (TEM), respectively, as described elsewhere (Cho et al. 2008; 2010). The p-MWCNT and o-MWCNT had surface areas of 270 and 134 m^2/g, respectively. The surface oxygen content of the p-MWCNT and o-MWCNT were 1 and 12%, respectively. The sonication did not change the diameter of the MWCNT, but did shorten them considerably. The lengths of the sonicated MWCNT were within a range of 200 to 500 nm (based on measuring 100 randomly selected MWCNT of both types). A representative image of the p-MWCNT is shown in Figure 1. Iron (Fe) content of the MWNCT was approximately 0.4% (w/w) in both types of MWCNT, as determined by inductively coupled plasma mass spectrometry (Agilent ICP-MS 7700).

Culturing Caco-2 and RAW 264.7 Cell Lines

Caco-2 cells and RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA). The Caco-2 cells were cultured as described previously by Bannon et al. (2003). Briefly, both cell lines were used for studies between passage numbers 13 and 25. The cell lines were maintained at 37°C in a humidified 95% air/5% CO_2 atmosphere in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco; Carlsbad, CA) supplemented with the following, also purchased from Gibco: 10 mM *N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid], 10% fetal bovine serum (FBS), and 1 × nonessential amino acids (Cellgro; Manassas, VA). Media for RAW 264.7 was also supplemented with Glutamax (Gibco).

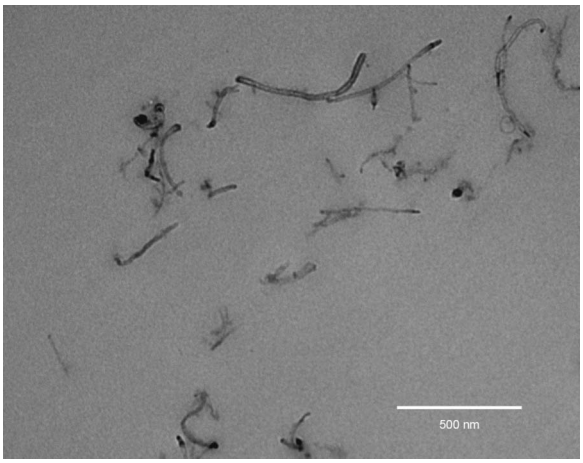

FIGURE 1. Transmission electron micrograph of p-MWNCT. MWCNT were prepared as described in the text and resuspended in media.

Exposure of Caco-2 and RAW 264.7 cells to MWCNT for TEM Analysis

For TEM studies, Caco-2 cells were seeded on 1-μm pore size polyethylene terephthalate filters in 12-well plates (Becton Dickinson; Franklin Lakes, NJ) at a density of 300,000 cells per filter and the medium was changed approximately twice per week. After 21 to 28 d, the transepithelial electrical resistance (TEER), an indicator of monolayer integrity, was assessed. The media surrounding each insert was replaced with prewarmed HBSS (at 37°C), and two TEER measurements were made per well using chopstick electrodes (World Precision Instruments, Sarasota, FL). Filters with TEER $<100\ \Omega \cdot cm^2$ (after correcting for the blank filter insert) were discarded. RAW 264.7 cells were seeded at a density of 500,000 cells per well on glass cover slips in 12-well cell culture plate, and studies commenced 24 h later.

Caco-2 and RAW 264.7 cells were exposed apically for 24 h to 0.5 ml of 50 μg/ml p-MWCNT or o-MWCNT suspended in the media described above less FBS. In preliminary range-finding studies, exposure at the 1 μg/ml level did not yield visible MWCNT by TEM, which was considered important for identifying uptake. Exposures at levels significantly higher than 50 μg/ml resulted in highly agglomerated masses of the MWCNT that quickly settled out in the media. Although agglomeration is unavoidable at any concentration (in the absence of surfactants), it was decided not to study these

higher exposure levels because MWCNT could not be dispersed and cell exposures would have been highly nonuniform. Further, it was of interest to assess uptake, for which it was necessary to minimize the presence of rapidly forming large agglomerates. It was decided not to use artificial surfactants to increase dispersion, as this would not mimic intestinal exposure to MWNCT. However, these studies were repeated in the presence of 2.5 mg/L NOM collected from the Great Dismal Swamp, which is naturally occurring in the environment and has surfactant-like effects on MWNCT. The same exposure media as described earlier was used, except that a 1-g/L NOM solution was added to the culture medium to a give final concentration of 2.5 mg/L, the highest dose found not to be toxic to the cells after 24 h (by the cytoxicity assays noted below and by visual inspection of TEM images). Although 6 h is the approximate duration that food resides in the small intestine, preliminary studies demonstrated little difference between results for 6 and 24 h, so 24 h was used to increase likelihood of observing effects.

Prior to dosing the cells, the MWCNT/media/(NOM) mixture was sonicated briefly to increase the dispersion of MWNCT. For the first 60 min following introduction of exposure media, cells were placed on a rotating plate in a 37°C environment to assist in distributing the MWCNT across the cell surface and then returned to the incubator.

Preparation of Samples for TEM Analysis

Cells were fixed for 1 h at room temperature with 3% paraformaldehyde and 1.5% glutaraldehyde in a 0.1-*M* sodium cacodylate solution, pH 7.4, 2.5% sucrose, supplemented with 1 m*M* $MgCl_2$ and 0.5 m*M* $CaCl_2$. After washing with 100 m*M* sodium cacodylate, pH 7.4, filters were postfixed with Palade's OsO_4, immersed in Kellenberger uranyl acetate overnight, dehydrated in ethanol, and embedded. Samples were sectioned vertically to the knife (basolateral to apical). Sections were examined on either an FEI Technai 12 TWIN with an SIS Megaview III wide-angle camera or a Hitachi 7600 transmission electron microscope.

Cytotoxicity Assays

To measure lactate dehydrogenase (LDH) release, medium from the apical chamber was centrifuged and the enzyme activity was assayed using the Promega Cytotox 96 kit according to the manufacturer's instructions. For cell counting, cells were treated for 24 h after plating on glass coverslips in 12-well plates (1×10^6 per plate). Cells were washed with phosphate-buffered saline (PBS), fixed in cold 70% ethanol for 5 min, washed in PBS again, and then incubated in propidium iodide (PI) solution (4 μg/ml PI/0.1% Triton X-100/0.5 mg/ml RNaseA in PBS) at room temperature for 5 min. The coverslips were then washed twice in PBS and mounted using Prolong with DAPI (Invitrogen; Carlsbad, CA), and the numbers of live cells (PI negative) and total cells (DAPI) were counted on a Zeiss axiovision microscope (100×) by an observer blinded to the experimental conditions. Results are expressed as PI negative cells (live) as the percent of total cells (DAPI); at least 100 cells were counted for each condition.

Statistics

Different treatment groups were compared using one-way of analysis variance with Tukey's post test in the software package GraphPad Prism 4 for Windows. Only p values $\leq .05$ were considered statistically significant.

RESULTS

Toxicity

Two different assays were used to determine cytotoxicity to MWCNT. No statistically significant differences were observed between control Caco-2 cells and cells following 24 h of exposure to 50 μg/ml of either type of MWCNT, with or without NOM, as determined by LDH activity or the number of cells taking up PI (Figure 2). No statistically significant

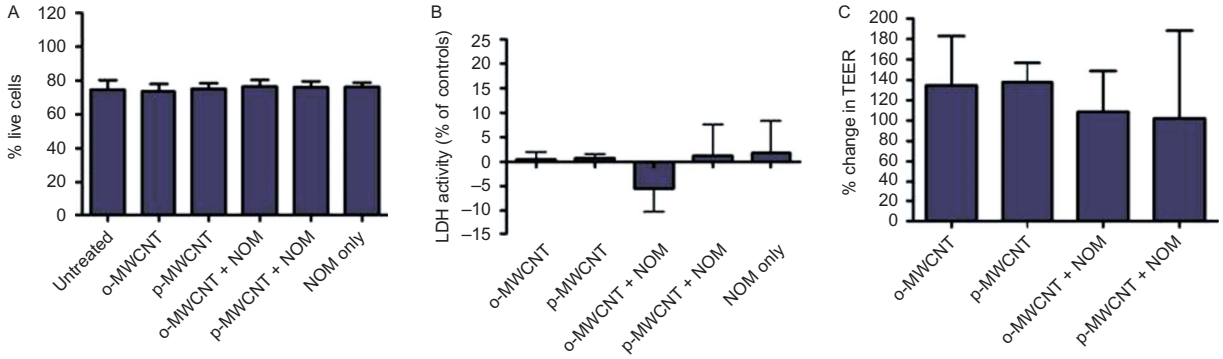

FIGURE 2. Toxicity in Caco-2 cells exposed to pristine and oxidized MWCNT. Dead and live cells were measured by staining nuclei with PI and DAPI, respectively, after treatment with 50 μg/ml pristine (p) or oxidized (o) MWCNT. The effect of natural organic matter (NOM) was also tested (A). Cell death was also assessed following the same treatment by measuring the activity of released LDH (B). The change in TEER was assessed by measuring the initial and final TEER at 24 h after treatment (C). In each assay, the results are displayed as the mean of triplicates ± standard error (color figure available online).

cytotoxicity was observed in the RAW 264.7 cells using the LDH activity assay. Changes in TEER, which is indicative of the strength of the tight barrier, would indicate effects on overall cellular function because maintenance of TEER requires several processes including macromolecule synthesis and ATP production. However, statistical differences were not observed in TEER following a 24-h exposure to 50 μg/ml MWCNT, with or without NOM, after adjusting for changes in TEER among control cells (Figure 2).

Cellular Interactions With MWCNT Analyzed by TEM

Caco-2 cells were well differentiated, as evidenced by well-formed brush borders, the presence of glycogen storage areas, and tight junctions joining cells (Figure 3). The cells line up in columns with the nuclei set towards the basolateral side (near the filter). To examine uptake, sections were initially viewed in which the knife sliced vertical sections from the apical to basolateral side of the monolayer. In the resulting images, it appeared that the knife dragged the MWCNT across the sample, creating artifacts (Figure 4, a and b). When sections were prepared in the opposite direction (i.e., knife traveling from basolateral to apical), it was not possible to locate MWCNT in the any of the cells examined (Figure 4, c and d). Even when MWCNT were visible at the surface of cells, there was no evidence that they penetrated the cell membrane or were engulfed by the cells. The only differences

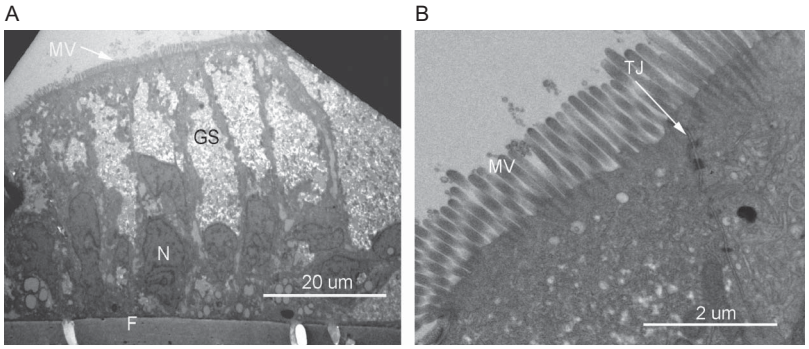

FIGURE 3. Ultrastructural analysis of differentiated Caco-2 cells. Cells were grown for 21–28 d on a filter (F) and processed for TEM. Differentiated cells displayed a nucleus (N) toward the abluminal side (facing the filter), glycogen storage (GS), and microvilli on the apical side (A). At higher magnification, tight junctions (TJ) were detected (B).

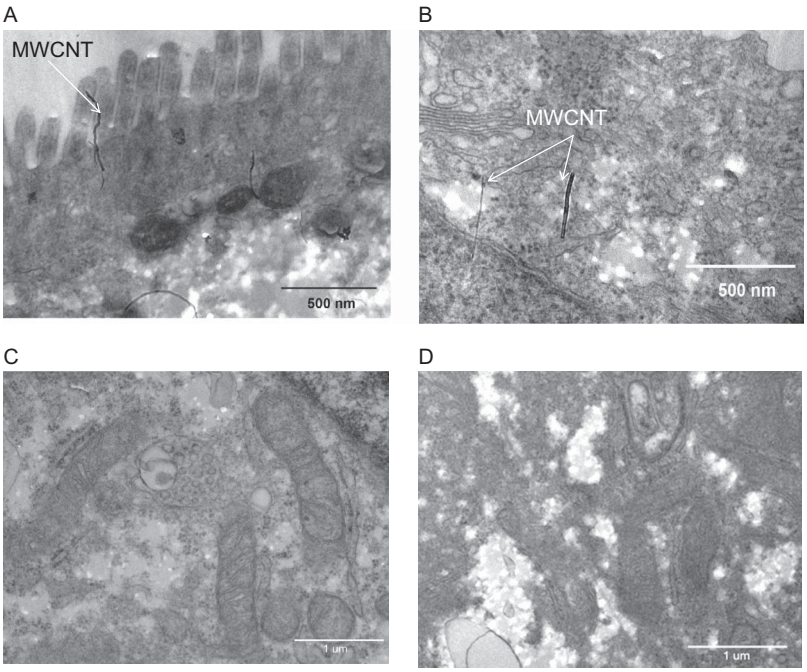

FIGURE 4. Analysis of uptake of MWCNT in Caco-2 cells. Cells were exposed to 50 μg/ml o- or p-MWCNT. Intracellular MWCNT are observed but appeared to be dragged into the cells in the vertical sections, which were sliced in the apical to basolateral direction (arrows point to the MWCNT, A and B). When vertical sections were cut from basolateral to apical, MWCNT were not observed in cells (C and D).

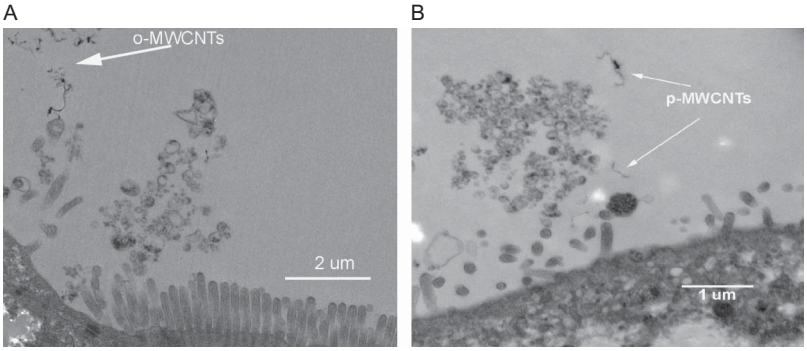

FIGURE 5. Microvilli damage in Caco-2 cells exposed to MWCNT. Sections were analyzed from cells exposed to p- or o-MWCNT at 50 μg/mL for 24 h. Microvilli (MV) were often damaged, or missing, in areas where MWCNT were observed extracellularly.

observed in the MWCNT-exposed cells were that when MWCNT were observed above the cells in the image, the microvilli in the vicinity were typically sloughing off or missing altogether (Figure 5). However, as there was not widespread damage to the microvilli in exposed cells, and abnormal microvilli were observed occasionally in control cells also, this effect is probably minor. This was confirmed by scanning electron microscopy, in which no appreciable differences were observed between control and exposed cells (results not shown).

The possibility that the culture conditions or the TEM preparation and analysis were responsible for the absence of uptake of MWCNT in Caco-2 cells was examined. Because previous studies found MWCNT uptake by macrophages, the RAW 264.7 macrophage cell line was also studied (Cheng et al. 2009; Porter et al. 2009a). TEM images revealed significant uptake of MWCNT by the

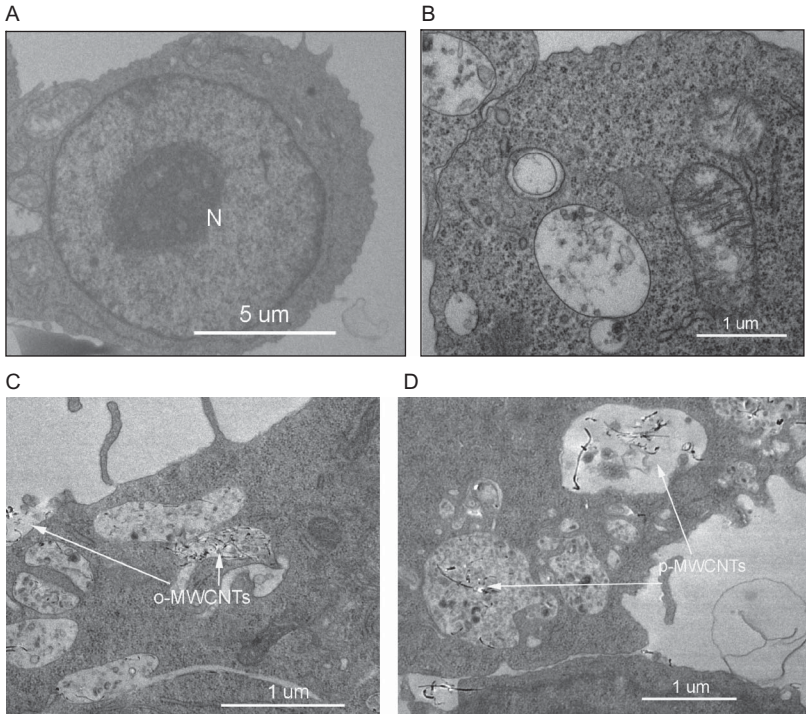

FIGURE 6. Ultrastructural analysis of uptake of MWCNT in RAW 264.7 cells. Untreated cells are shown at two different magnifications (A, B). Cells were exposed to 50 μg/ml of o- (C) or p- (D) MWCNT for 24 h. Both types of MWCNT are visible in membrane-bound vesicles. In some regions, the MWCNT appear to puncture the vesicles and enter the cytoplasm.

RAW 264.7 cells under similar conditions cultures to those of the Caco-2 cells (Figure 6), yet cytotoxicity was not observed. MWCNT were present within membrane-bound vesicles but also appeared to puncture the vesicles and enter the cytoplasm.

DISCUSSION

The aim of this study was to characterize whether MWCNT are taken up by or toxic to the intestine, and how this may change in the presence of noncytotoxic levels of NOM. At an exposure level of 50 μg/ml MWCNT, cytotoxicity was not observed; nor did the MWCNT affect TEER. In other cell types, exposures to CNT (single- and multiwalled) at levels lower than those used here were found to reduce cell viability using similar methods (Walker et al. 2009; Hu et al. 2010). Further, other studies also demonstrated differences in toxicity between oxidized (also termed acid-treated or carboxylated) and pristine CNT in other cell types using doses lower than those used here (Porter et al. 2009a; Vittorio et al. 2009). These results suggest that enterocytes are more resistant than other cell types to the cytotoxic effects of MWCNT, regardless of oxidation level. The addition of 2.5 mg/L NOM exerted no obvious effects on uptake or cytotoxicity; as NOM increases dispersability of MWCNT, this suggests that it was not agglomeration alone that led to the absence of MWCNT toxicity or uptake. A recent study also found no significant changes in toxicity following addition of NOM in rat liver epithelia cells exposed to SWCNT (Alpatova et al. 2010).

A previous study on toxicity of carboxylated SWCNT to Caco-2 cells reported cytotoxicity at concentrations greater than 100 μg/ml in Caco-2 cells (Jos et al. 2009). This study did not assess SWCNT uptake in the cells, and in general it cannot be assumed that MWCNT behave similar to SWCNT. In our preliminary range-finding studies, concentrations significantly higher than 50 μg/ml of

MWCNT formed highly agglomerated clumps that quickly settled in media; it was determined that 50 ug/ml was the highest dose of MWCNT that could be reasonably studied in this system. Therefore, it is not clear if toxicity would have been found in this dose range had it been possible to disperse the MWCNT at these levels.

In the absence of changes in TEER and cytoxicity, the possibility that MWCNT could undergo paracellular transport or increase transport of other nonessential chemicals is less likely, thereby restricting transport to a transcellular pathway. MWCNT, however, were not taken up by Caco-2 cells under any of the conditions studied as assayed by TEM. It was initially unclear whether it would be possible to identify MWCNT in the cells; however, the use of RAW 264.7 cells increased confidence that MWCNT could be detected by our TEM methods. The uptake of MWCNT by RAW 264.7 macrophage cell line confirms results from other studies that showed uptake of MWCNT and SWNCT in macrophages and other cell lines (Cheng et al. 2009; Mu et al. 2009; Porter et al. 2009a).

The TEM images suggested that MWCNT may produce localized damage to the microvilli of the intestine; in locations where MWCNT were visible above the cells, there was often observable damage to microvilli structure in the immediate area. However, the observation of MWCNT (and associated damage to microvill) was infrequent by both TEM and SEM imaging, suggesting such damage was not widespread. The observed effects were probably due to physical interactions or tangling of MWCNT agglomerates with the microvilli.

The primary advantages of the use of TEM are its ability to resolve nano-sized structures and its ability to localize structures within intracellular organelles. However, TEM is limited because (1) it is expensive (limiting the number of samples that can be reasonably analyzed), (2) it is not able to quantify the amount of uptake, and (3) it is use to identify rare events is difficult. Although a considerable number of samples were analyzed, it is possible that MWCNT uptake occurred but was infrequent. Another limitation in our study was the unavoidable agglomeration of MWCNT at the concentration used. Although less than at higher concentrations, aggregated MWCNT were observed following 24 h of exposure (visible to the eye and by TEM). Unagglomerated particles may represent the worst-case scenario in terms of nanomaterial uptake and toxicity, since larger particles are less likely to undergo transport through different pathways. However, doses that are low enough to minimize agglomeration limited the probability of finding CNT in the imaged sample.

Overall, this study suggests that enterocytes do not readily take up MWCNT. A recent study suggested that MWCNT do not cross the gut lumen of daphnia (Edgington et al. 2010). Future studies assessing ingestion as a route of human exposure of CNT may want to also consider M-cells in the Caco-2 cell model as an alternative route of CNT uptake from the intestine. Although present in far lower numbers in the intestine, M-cells are more similar to macrophages than enterocytes and were shown to enhance uptake of nano-sized particles rats (Jani et al. 1990).

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