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Airway barrier dysfunction induced by exposure to carbon nanotubes *in vitro*: which role for fiber length?

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Although carbon nanotubes (CNTs) are increasingly used, their biological effects are only incompletely characterized. However, experimental evidence suggests that the intratracheal instillation of CNTs causes the formation of interstitial granulomas and progressive pulmonary fibrosis in rodents. Using human epithelial Calu-3 cells as a model of airway epithelium *in vitro*, we have recently reported that the exposure to commercial multiwalled CNTs (MWCNTs) causes a progressive decrease of the transepithelial electrical resistance (TEER), pointing to a CNT-dependent impairment of the epithelial barrier function. To characterize better this behavior, we compared the effects of two types of MWCNTs and single-walled CNTs (SWCNTs) of different lengths on the TEER of Calu-3 monolayers. All the materials were used at a dose of 100 μg/mL corresponding to an exposure of 73 μg/cm² of cell monolayer. Only the longer MWCNTs and SWCNTs cause a significant decrease in TEER. To elucidate the mechanism underlying the changes in barrier function, the expression of the junction proteins occludin and ZO-1 has been also assessed. No significant decrease in the mRNA for either protein is detectable after the exposure to any type of CNTs. It is concluded that the impairment of barrier function in Calu-3 monolayers is a peculiar effect of CNTs endowed with clear cut fiber properties and is not referable to marked changes in the expression of junction proteins.

Key words: airway epithelial cells; carbon nanotubes; lung–blood barrier; tight junctions

Introduction

Although carbon nanotubes (CNTs) are produced in increasing amounts for several industrial applications, the consequences of the interaction of CNT with biological structures are not completely known and their potentially toxic effects are still object of debate.[1,2] The available information, obtained mostly in rodents, points to lung toxicity as the major consequence of exposure to CNTs through intratracheal instillation or pharyngeal aspiration, with the early formation of fibrosis and granulomas, hypertrophy of epithelial cells, and a progressive functional impairment.[3–6] The pathogenetic mechanisms underlying these changes are incompletely characterized and, in particular, the consequences of exposure to CNTs on airway epithelial cells have been scarcely investigated thus far. A recent contribution shows that multiwalled CNTs (MWCNTs) have genotoxic effects on rat type II pneumocytes,[7] whereas data from our laboratory indicate that the human airway epithelial cell line Calu-3, derived from a lung adenocarcinoma, are quite resistant to the acute cytotoxic effects of either MWCNTs or single-walled CNTs (SWCNTs).[8] However, in the same contribution, we have also showed that MWCNTs impair the barrier function of Calu-3 monolayers, causing a decrease in the transepithelial electrical resistance (TEER) and an increase in the paracellular permeability to mannitol.[8] Less evident changes of TEER are caused by SWCNTs, whereas, interestingly, amorphous carbonaceous nanoparticles (Carbon Black) are without significant effects.[8]

Alterations in airway barrier function may be particularly relevant for CNTs toxicity, since airway epithelial cells are the first body barrier for inhaled

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particles and constitute a major determinant of the interaction of the potential toxicant with other body compartments. To better characterize the effect of CNTs on the airway barrier, we have compared the effects of different preparations of MWCNTs and SWCNTs on the TEER of Calu-3 monolayers. Moreover, the expression of two protein components of the tight junctions, occludin and ZO-1, has been studied under the same conditions.

Methods

Cells and TEER measurements

Calu-3 cells, obtained from a human lung adenocarcinoma and derived from serous cells of proximal bronchial airways,[9] were obtained from the Istituto Zooprofilattico Sperimentale della Lombardia (Brescia, Italy). Cells were routinely cultured in 10-cm diameter dishes in Eagle's Minimum Essential Medium (EMEM), supplemented with 1 mM sodium pyruvate, 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), and penicillin (100 U/mL) in a humidified atmosphere of 5% CO_2 in air. Unless otherwise described, the experiments were performed on Calu-3 cells seeded into cell culture inserts for Falcon 24-well-multitrays endowed with membrane filters (pore size of 0.4 μm, Cat. N° 3095, Becton, Dickinson & Company, Franklin Lakes, New Jersey, USA) at a density of 75×10^3 cells/well. In this culture system, epithelial cells, after reaching confluence, form a tight, low-permeability monolayer that separates an apical from a basolateral compartment. The progressive formation of a tight monolayer can be followed through the increase of TEER. Measurements of TEER were made with an epithelial voltohmmeter (EVOM, World Precision Instruments Inc., Sarasota, Florida, USA) that produces an AC current.

Before the experiments, cells were allowed to grow for 10 d, when they had formed a tight monolayer (TEER > 1000 Ω/cm^2).

Images of cells grown on permeable filters were obtained in phase contrast with a Nikon DS5MC digital camera (Nikon Instruments SpA, Firenze, Italy).

Carbon nanotubes

Details of the commercial CNTs used in this study are shown in Table 1, as declared by the supplier. L-MWCNTs (Long-MWCNTs, Aldrich 659258) are largely made of multiwalled nanotubes (carbon content > 90%), with residual amorphous carbon and metal traces (iron < 0.1%); S-MWCNTs (Short-MWCNTs, Aldrich 636843) have a carbon content > 95%; L-SWCNTs (Long-SWCNTs Aldrich 636797) are >50% SWCNTs and 40% other CNTs; S-SWCNTs (Short-SWCNTs Aldrich 652512) are >90% SWCNTs. Ultrafine Carbon Black (CB) nanoparticles (Printex 90™, 14 nm diameter) were a generous gift of Degussa Italia SpA, Advanced Fillers & Pigments, Ravenna, Italy.

Exposure to nanomaterials

Before the experiments, all the nanomaterials were heated at 220 °C for 3 h to eliminate possible contamination from lipopolysaccharide.[6] After cooling at room temperature, nanomaterials were dispersed at a concentration of 1 mg/mL in sterile phosphate-buffered saline (PBS) to obtain stock suspensions for a series of experiments. Immediately before the single experiments, nanomaterials were extensively vortexed, sonicated three times for 15 min in a Bransonic Ultrasonic ultrasound bath, and then added to the apical chamber of the culture system at a nominal dose of 100 μg/mL, corresponding to an exposure of 73 μg/cm^2 of monolayer. No detergent was used to improve the solubility of nanomaterials in aqueous solutions. Proper volumes of PBS were added to control cultures.

Cell viability

Cell viability was tested with the resazurin method.[10] This method is based on the reduction of the nonfluorescent compound resazurin into the fluorescent resorufin by viable cells. After its production, resorufin accumulates into the medium and can be readily

Table 1 Principal characteristics of CNT preparations

Type	*Dimensions (diam. × length)*	*Density (g/cm^3)*	*Surface area (m^2/g)*	*Preparation method*
L-MWCNT	110–170 nm × 5–9 μm	2.1	1.3×10^2	CVD
S-MWCNT	40–70 nm × 0.5–2 μm	2.1	NC	CVD
L-SWCNT	1.1 nm × 0.5–100 μm	1.7–1.9	1.7×10^3	CVD
S-SWCNT	1.0–2.0 nm × 0.5–2.0 μm	1.7–1.9	4.8×10^2	CVD

CNT, carbon nanotubes; CVD, chemical vapor deposition; NC, not communicated.
L-MWCNTs are Aldrich 659258; S-MWCNTs are Aldrich 636843; L-SWCNTs are Aldrich 636797; S-SWCNTs are Aldrich 652512. The characteristics of the nanomaterials are reported as communicated by the supplier.

determined with a fluorometer. Cells were incubated for 90 min with fresh, serum-free medium supplemented with 44 μM resazurin, added at both the basolateral and the apical compartments. Because the culture inserts did not allow direct fluorescence reading from the wells, the measurement has been performed on the medium of the apical chamber transferred in a clean 96-well dish. Fluorescence was then immediately measured at 572 nm with a fluorometer (Wallac 1420 Victor^2 Multilabel Counter, Perkin Elmer, Wellesley, MA, USA).

Real-time PCR

Cell extracts obtained from three permeable filters were used for each condition. One microgram of total RNA, isolated with RNeasy Mini Kit® (Qiagen S.p.a., Milano, Italy), was reverse transcribed and 40 ng of cDNA amplified as described previously.[11] The following forward and reverse primers (5 pmol each) were used: 5′-AGT CCC TTA CCT TTC GCC TGA-3′ and 5′-TCT CTT AGC ATT ATG TGA GCT GC-3′ for ZO-1, 5′-CTC TCA GCC AGC CTA CTC TTT T-3′ and 5′-CCG TAG CCA TAG CCA TAA CCA-3′ for occludin and 5′-AGC CTC AAG ATC ATC AGC AAT G-3′ and 5′-CAC GAT ACC AAA GTT GTC ATG GA-3′ for GAPDH. Data were expressed as the ratio between proband mRNA and GAPDH mRNA.

Western blot

These experiments were performed on Calu-3 cells seeded into cell culture inserts for Falcon 6-well-multitrays endowed with membrane filters (pore size of 0.4 μm, Cat. N° 3090, Becton, Dickinson & Company) at a density of 600×10^3 cells/well. Monolayers were rinsed twice in PBS, lysated in Sample Buffer (6.3 mM TrisHCl pH 6.8, 1% sodium dodecylsulphate, 10% glycerol), and passed 10 times into 27-G needles. Lysates were then centrifuged at 16,000 *g* for 10 min. Quantification of proteins in the supernatants was performed with a modified Lowry assay,[12] and aliquots of 10 μg (for occludin) or 30 μg (for ZO-1) were loaded on an 8% gel for SDS-PAGE. After the electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Nonspecific binding sites were blocked with an incubation in 5% low-fat dried milk (Amersham Pharmacia Biotech Italia, Milan, Italy) in PBS/0.1% Tween for 2 h at room temperature. The blots were then exposed overnight at 4 °C to antioccludin (1:1000) or antiZO-1 rabbit polyclonal antisera (1:500), both obtained from Zymed Laboratories (San Francisco, California, USA), diluted in blocking solution. After washing, the blots were exposed for 1 h to horseradish peroxidase–conjugated antirabbit IgG (Bio-Rad Laboratories S.r.l., Segrate, Italy) diluted 1:50,000 in blocking solution. For standardization, membranes were exposed to monoclonal antirabbit antiserum against GAPDH (Chemicon International Inc., Temecula, California, USA, 1:1000). The membranes were washed and the immunoreactivity visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech Italia).

Statistical analysis

Values of TEER, viability, and gene expression of control and treated cultures were compared with a *t*-test for unpaired data.

Materials

Unless otherwise indicated, Sigma-Aldrich (Milan, Italy) was the source of all the chemicals.

Results

Changes in cell viability in Calu-3 monolayers after exposure to CNTs

When supplemented to the apical side of a tight Calu-3 monolayer, all the nanomaterials used tend to aggregate forming more or less extended ropes and bundles, which appear as irregular, black masses reaching a size of several tens of micrometers on a roughly spared monolayer (Figure 1).

The effects of the various nanomaterials, all used at a nominal dose of 100 μg/mL, on cell viability are shown in Figure 2. These experiments were performed on Calu-3 monolayers grown on permeable filters so as to adopt the same conditions of TEER measurements. However, as already observed in Calu-3 cells grown on plastic,[8] L-MWCNTs, L-SWCNTs, and CB caused no significant change in cell viability after 7 d of exposure. Also, S-MWCNTs were without effect, whereas S-SWCNTs produced a moderate decrease in cell viability.

Changes in TEER of Calu-3 monolayers after exposure to CNTs

In the experiment shown in Figure 3, the nanomaterials were added to the apical chamber of high-resistance Calu-3 cell monolayers, and the TEER was measured daily up to 7 d of treatment. The results indicated that only L-MWCNTs (Panel A) and L-SWCNTs (Panel C) produced an appreciable and progressive decrease in TEER, whereas the shorter counterparts (S-MWCNTs, Panel B; S-SWCNTs, Panel D) were without any significant

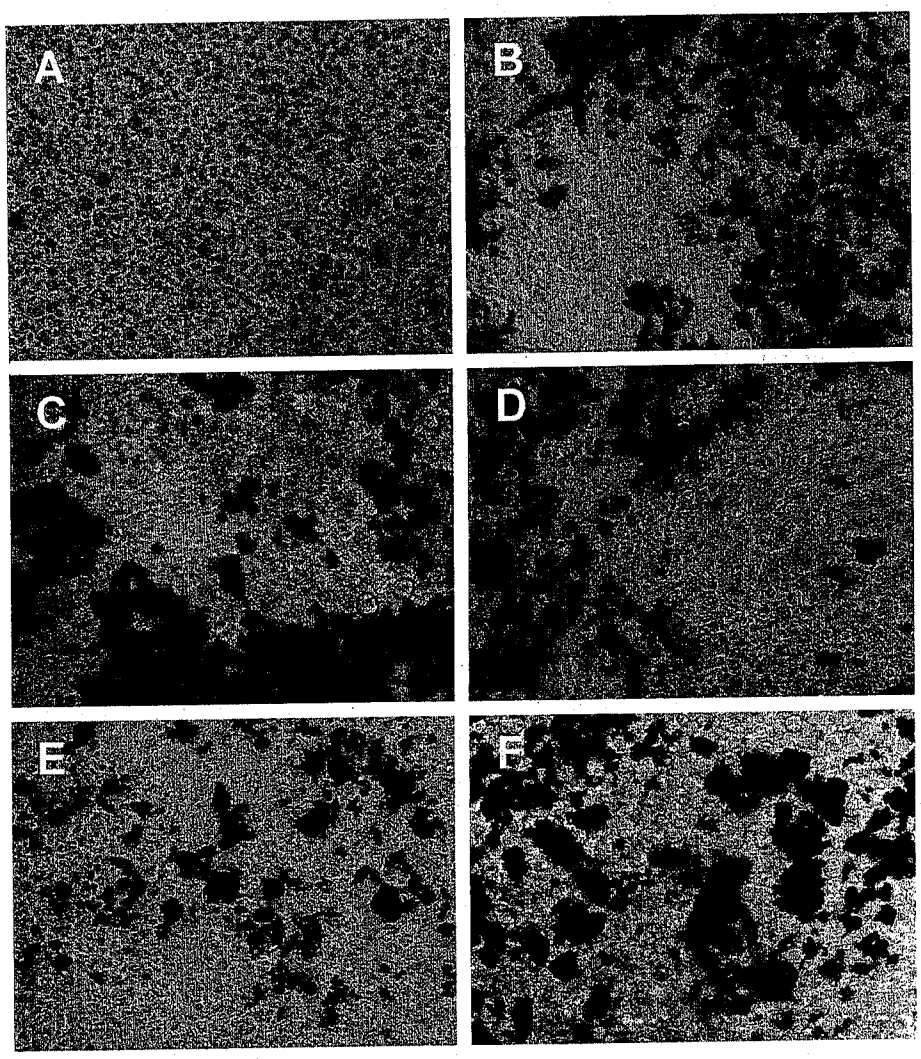

Figure 1 Carbon nanoparticles on Calu-3 cell monolayers. Cells were cultured for 7 d on membrane filters in the absence (Panel A) or in the presence of L-MWCNTs (B), S-MWCNTs (C), L-SWCNTs (D), S-SWCNTs (E), or CB (F). All the nanomaterials were used at a nominal dose of 100 μg/mL. Magnification, ×100.

effect. As expected,[8] CB (Panel E) did not produce significant changes in TEER.

Effect of nanomaterials on the expression of junctional proteins in CNT-treated airway epithelial cells

To verify if the effects of CNTs on TEER were associated to changes in the expression of protein components of the tight junctions, we have measured the expression of mRNA for occludin and ZO-1 with quantitative real-time PCR. mRNA expression was assessed in cell extracts from monolayers grown on permeable filters and treated under the same experimental conditions adopted for TEER and viability experiments. The results, reported in Figure 4, indicate that no overt alteration in the expression of either gene was detectable for any nanomaterial used at mRNA level. In particular, L-MWCNTs do not cause a decrease in occludin and ZO-1 mRNA expression although TEER, measured in the same monolayers just before mRNA extraction, was decreased by more than 40% compared with control values (not shown).

Under the same conditions, we studied the effect of L-MWCNTs on the expression of occludin and ZO-1 proteins through Western blot (Figure 5). Consistent with PCR results, L-MWCNTs did not appreciably change occludin expression (Figure 5) while ZO-1 protein was consistently more abundant in treated than in control untreated cultures although the change was not statistically significant ($P = 0.065$, $n = 3$).

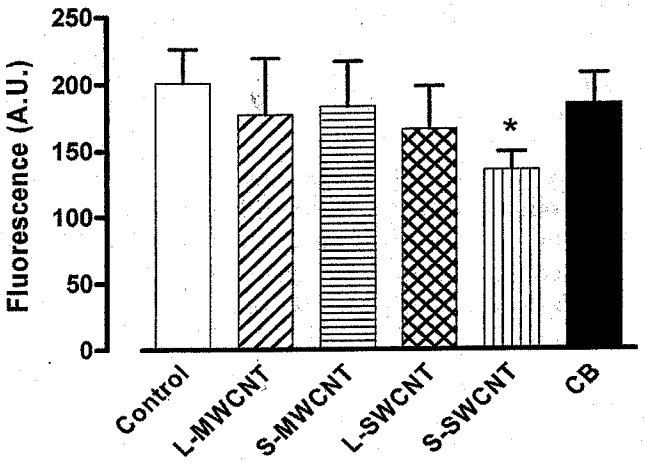

Figure 2 Effect of carbon nanoparticles on cell viability. Calu-3 cell monolayers were cultured for 7 d on membrane filters in the absence or in the presence of L-MWCNTs, S-MWCNTs, L-SWCNTs, S-SWCNTs, or CB, as indicated. All the nanomaterials were used at a nominal dose of 100 μg/mL. At the end of the incubation, cell viability was assessed with resazurin test (see Methods). Data are means ± SD of three independent determinations obtained in a single experiment repeated twice with comparable results. $*P < 0.05$ versus control, untreated cultures.

Discussion

We have recently reported that commercial MWCNTs and SWCNTs impair the barrier function of human airway cell monolayers.[8] The change is time- and dose-dependent and it is not associated with evident viability changes, as evaluated with a standard biochemical method. The present study was undertaken 1) to assess whether the barrier-perturbing effect of CNTs are related to their physicochemical features and 2) to evaluate whether the above effect is associated with changes in the expression of protein components of the junction complexes.

As far as the first issue is concerned, we have compared the nanomaterials used in our recent contribution (i.e., L-MWCNTs – Aldrich 659258 and L-SWCNTs – Aldrich 636797) with two preparations of nanotubes (S-MWCNTs and S-SWCNTs, Aldrich 636843 and 652512, respectively), obtained from the same commercial sources, which are significantly shorter (maximal length of 2 μm) compared with, respectively, 9 and 100 μm. Because the shorter

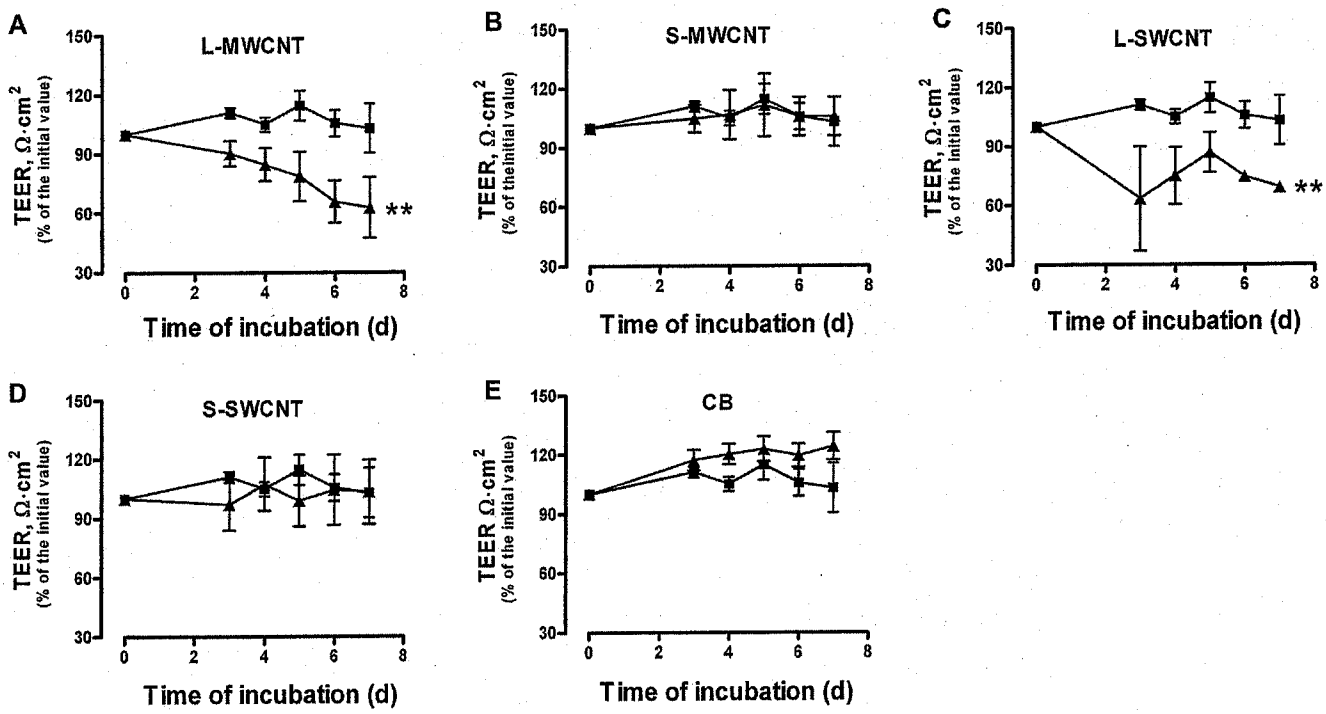

Figure 3 Effect of carbon nanotubes on the transepithelial electrical resistance (TEER) of tight Calu-3 cell monolayers. Calu-3 cells were cultured for 10 d on 0.4 μm membrane filters. At the end of this period (Time 0, average TEER of 1440 Ωcm²), L-MWCNTs, S-MWCNTs, L-SWCNTs, S-SWCNTs, or CB were added to the apical chamber of the culture system at a dose of 100 μg/mL and TEER was determined at the indicated times. For the sake of clarity, the same data from control filters (plain PBS added to the medium) have been shown in all the Panels. Squares, control filters maintained in the absence of nanomaterials. Triangles, filters exposed to the nanomaterials indicated in each panel. For all the panels, three filters were used for each condition. Data are means ± SD of three independent determinations in a representative experiment repeated twice with comparable results. $**P < 0.01$ versus control, untreated cultures measured at the same experimental time (7 d of treatment).

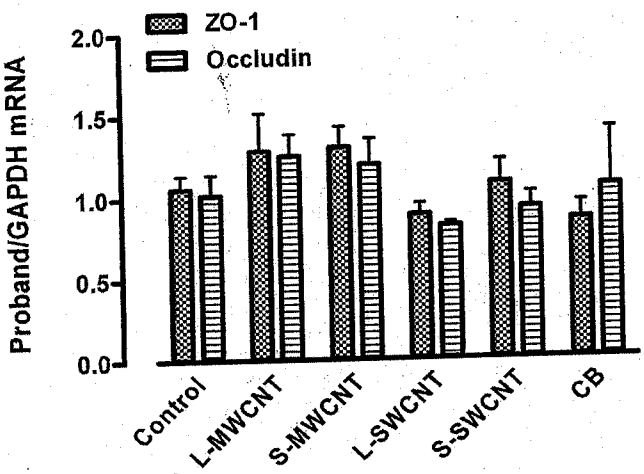

Figure 4 Expression of mRNA for ZO-1 and occludin in Calu-3 monolayers exposed to carbon nanomaterials. Calu-3 monolayers were treated as described in the legend to Figure 3. After 7 d of treatment, the mRNA for the tight-junctional proteins ZO-1 and occludin was determined in qRT-PCR as described in Materials and Methods. Expression data are expressed relative to the housekeeping gene GAPDH and are means with SD of four determinations obtained in two independent experiments.

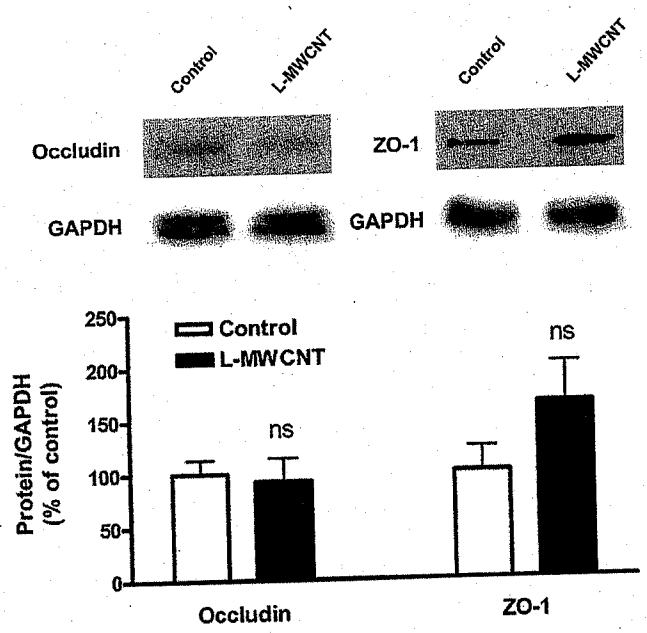

Figure 5 Expression of ZO-1 and occludin proteins in Calu-3 cells. Calu-3 monolayers were exposed for 7 d to MWCNTs (100 μg/mL in PBS) or to PBS alone (control). After this period, proteins were extracted and the expression of occludin and ZO-1 was determined with Western blot, using GAPDH as loading control (Upper). The relative abundance of the two proteins was determined from the densitometric analysis (Lower). Bars indicate means ± SD of three independent experiments with the expression under control conditions considered 100. ns, not significantly different versus the respective control.

CNTs have no effect on the TEER of tight, confluent monolayers, while the longer counterparts confirm their junction-perturbing effect, it is tentative to attribute their divergent biological effects to the different length.

Besides the length, each CNT preparation has many other peculiar features, which may be of relevance to explain the effects observed here. For instance, from data released by the supplier, L-MWCNTs (Aldrich 659258) have traces of Fe contamination (<0.1%), whereas S-MWCNTs (Aldrich 636843) contain 0.1% Fe together with small amounts of Ni (1%), and S-SWCNTs (Aldrich 652512) present 2.9% Co. All these nanomaterials have not been purified before experimental treatments. Therefore, because L-MWCNTs are able to perturb the epithelial barrier while S-MWCNTs and S-SWCNTs are completely ineffective, it is possible that CNT-induced epithelial barrier alteration is not related to the metal contamination of the CNT preparations.

The TEER decrease observed upon treatment with L-MWCNTs or L-SWCNTs should not be attributed to CNT-induced cytotoxicity. Indeed, Calu-3 epithelial cells, grown on standard plasticware, do not seem to be particularly affected by the exposure to "long" CNTs in terms of changes in viability,[8] although these nanomaterials significantly lower TEER, as confirmed by the results presented in Figure 3 of this report. Here, we extend those observations to "short" CNTs and to cultures grown on permeable filters, confirming that cell viability is not significantly affected by L-MWCNTs, S-MWCNTs, L-SWCNTs, or CB. However, a significant decrease in fluorescence was detected with S-SWCNTs, which, in contrast, do not lower TEER. Recent data from our laboratory indicate that S-SWCNTs significantly quench the fluorescence of the viability indicator (from 541788 ± 14477 to 466607 ± 20810 AU, $n = 4$, $P < 0.01$, BM Rotoli and O Bussolati, unpublished results). This effect may therefore justify artifactual low viability data.

The results presented in this report may be linked to a growing body of evidence that suggests that the biological behavior of long, fiber-shaped CNTs presents several analogies with that of mineral fibers, such as asbestos[13] (see also the 2003 Agency for Toxicity Substance and Disease Registry Expert Panel report on toxicity and fiber length at http://www.atsdr.cdc.gov/HAC/asbsestospanel/). Moreover, recent results indicate that MWCNTs, like asbestos, induce mesothelioma when administered intraperitoneally to p53 heterozygous mice.[14] Possible similarities between asbestos and CNTs, as far as their toxic effects are concerned, should prompt

careful investigations, although we must note that the dose used in this study (100 μg/mL, corresponding to an exposure of 73 μg/cm^2) could be regarded as relatively high, if compared with possible exposure levels for workers.

Field studies have investigated the potential exposure to SWCNTs and MWCNTs of workers in small-scale production settings.[15,16] Because the amount of material produced/handled is small, airborne concentrations from trace amount to few thousands micrograms/m^3 have been detected. However, it has been speculated that 20 μg dose of SWCNTs given by pharyngeal aspiration to C57BL/6 mice deposited in the alveolar region corresponds to approximately the same estimated dose for a worker exposed to the OSHA PEL (Permissible Exposure Limit) for graphite – 5 mg/m^3, 8 h workday, 40 h/week – over a period of 20 work days.[15] The same Authors indicated that doses of 10 and 40 μg SWCNTs/mouse given by aspiration are relevant to predicted deposited doses after 2 and 8 years of exposure at peak airborne concentrations measured in National Institute for Occupational Safety and Health (NIOSH) facilities,[15] which is actually lower as compared with the levels potentially occurring in larger facilities and production plants where tonnes are handled. Furthermore, inhalatory doses of CNTs of 5 mg/m^3 for 5 h/day administered for 4 days gave an estimated lung deposition of 5 μg in mice, a burden which is associated with severe acute and subacute effects and that could be achieved by workers in about 12 months following an exposure to airborne peak concentrations shown by field investigations.[17]

Thus, although the dose of 100 μg/mL used *in vitro* to maximize the effects can be apparently high and irrelevant for workers, it should be speculated that owing to its biopersistence, similar amount of CNTs can be accumulated over time by daily/repeated exposures giving rise to unpredictable effects.

Interestingly, experimental work performed several years ago showed that asbestos microfibers raise the paracellular permeability and decrease the TEER of cultured airway epithelial cell monolayers in the absence of obvious changes in occludin expression.[18,19] Consistently, also the preliminary studies reported in the present contribution indicate that the exposure of monolayers to L-MWCNTs does not decrease the expression of two essential protein components of the tight-junctional complexes, the structural protein occludin and the peripheral protein ZO-1, both at mRNA and protein level. Rather, the abundance of ZO-1 protein seems even increased upon exposure of Calu-3 cultures to L-MWCNTs, although the change does not reach statistical significance due to the variability of absolute expression levels in the different experiments (Figure 5). Although this finding awaits confirmation, it is interesting to note that in endothelial cells the increased expression of ZO-1 is caused by mechanical stress,[20] although, in that model, the change is associated to an increase in TEER and not, as in the present report, to its decrease.

However, tight junctions are very complex structures (see Matter and Balda[21] and Cereijido, *et al.*[22] for recent reviews) and, therefore, the expression and distribution of other protein components should be assessed, before concluding that the exposure to CNTs does not affect the junctional complexes. Moreover, if relatively only few cells of the monolayer were damaged by MWCNTs, both overall viability and expression of junctional proteins may be apparently unaffected. The identification of the mechanisms of epithelial barrier dysfunction airway cell monolayers exposed to long CNTs will require, therefore, further investigation.

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