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Multi-walled carbon nanotubes (NM401) induce ROS-mediated *HPRT* mutations in Chinese hamster lung fibroblasts

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ABSTRACT

Although there is an important set of data showing potential genotoxic effects of nanomaterials (NMs) at the DNA (comet assay) and chromosome (micronucleus test) levels, few studies have been conducted to analyze their potential mutagenic effects at gene level. We have determined the ability of multi-walled carbon nanotubes (MWCNT, NM401), to induce mutations in the *HPRT* gene in Chinese hamster lung (V79) fibroblasts. NM401, characterized in the EU NanoGenotox project, were further studied within the EU Framework Programme Seven (FP7) project NANoREG. From the proliferation assay data we selected a dose-range of 0.12 to 12 μg/cm^2 At these range we have been able to observe significant cellular uptake of MWCNT by using transmission electron microscopy (TEM), as well as a concentration-dependent induction of intracellular reactive oxygen species. In addition, a clear concentration-dependent increase in the induction of *HPRT* mutations was also observed. Data support a potential genotoxic/ carcinogenic risk associated with MWCNT exposure.

1. Introduction

Multi-walled (MW) carbon nanotubes (CNT) consist of several concentric graphene sheets with high length *vs* thickness ratio. In general, both single- (SWCNT) and multi-walled carbon nanotubes (MWCNT) possess unique physico-chemical properties with potential use in many fields (Mundra et al., 2014; Zhang et al., 2014; Titirici et al., 2015).The large number of MWCNT applications implies that their presence in the environment is dramatically increasing. This has caused new environmental and health concerns, particularly since CNT exposure has been compared to asbestos, a well-known agent inducing mesothelioma and lung cancer (Takagi et al., 2012).

Among the potential health risks associated with MWCNT exposure, those involving damage to DNA are among the most critical. Different *in vitro* studies have shown that exposure to MWCNT can be associated with the induction of primary DNA damage (Ghosh et al., 2011; Cavallo et al., 2012, Ursini et al., 2012; Linderberg et al., 2013), as well as chromosome damage (Sargent et al., 2012; Kato et al., 2013; Siegrist et al., 2014).

Due to the existence of different mechanisms inducing genotoxicity, a battery of assays testing for different genetic endpoints is required. The current test guidelines include, among the assays to be considered, an *in vitro* mammalian gene mutation test such as the forward gene mutation assay in the X chromosome-linked hypoxanthine phosphoribosyl transferase locus *HPRT* (OECD, Guideline 476, 1997). In this assay mutant cells are selected by incubation with a purine analogue that is toxic to normal cells but not to mutants. The *HPRT* mutation assay has been already successfully applied to the evaluation of different nanomaterials (NMs) (Doak et al., 2012). Thus, positive effects were obtained using TiO_2 NMs (Wang et al., 2007; Chen et al., 2014) and silver NMs (Huk et al., 2014), but negative findings were reported for synthetic amorphous silica NMs (Guichard et al., 2015). Interestingly, negative results were obtained with TiO_2 NMs in a long-term (60 days) exposure experiment. In this case CHO cells appear to adapt to the chronic exposure to nano-TiO_2 and detoxify an excess of reactive oxygen species (ROS), possibly through up-regulation of

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super oxide dismutase (SOD), in addition to reducing particle uptake (Wang et al., 2011). With regard to studies using MWCNT to induce *HPRT* mutations, a previous study was reported (Asakura et al., 2010) where no effects were observed in the CHL/IU cell line; however, a positive effect was observed in lymphoblastoid cells, but using SWCNT (Manshian et al., 2013). It is perhaps relevant to point out that germ-line *HPRT* mutations in humans lead to a sex-linked human neurological disorder called the Lesch–Nyhan syndrome, and that biomonitoring of human populations has shown that somatic mutations at the *HPRT* gene are linked to genotoxic/carcinogenic risk (Albertini, 2001).

In this context our work aims to investigate the ability of MWCNT to induce mutations in the *HPRT* gene. Cellular uptake of MWCNT was assessed by transmission electronic microscopy (TEM) and the induction of ROS, as a potential mechanism of action, was also determined.

2. Material and methods

2.1. Cell cultures

Chinese hamster lung fibroblast cells (V79) were cultured in 75 cm^2 flasks in DMEM low glucose medium (Sigma), with 10% FBS, 1% penicillin–streptomycin and L-glutamine (Sigma) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Nanomaterial characterization, dispersion and cell exposure

The selected MWCNT (NM401) was obtained from the EU Joint Research Centre (Ispra, Italy). This material was well characterized in the EU Nanogenotox (2012), with transmission electron microscopy (TEM) showing diameter 64.2 ± 34.5 nm and length 4048 ± 2371 nm with a DLS (Dynamic Light Scattering) Zeta size in 0.05% BSA medium of 710 ± 17 nm. It also contains a small amount of impurities accounting for 0.11% of the total weight including Al, Mg, Na, Cr, Fe and Co traces (Nanogenotox, 2014). Further confirmatory studies were carried out by us using TEM, to obtain size and morphology, on a JEOL JEM-2011 instrument.

For dispersion procedures the Nanogenotox (2011) was used. Briefly, MWCNT were pre-wetted in 0.5% absolute ethanol and afterwards dispersed in 0.05% bovine serum albumin (BSA) in MilliQ water and sonicated for 16 min to obtain a stock dispersion of 2.56 mg/mL.

2.3. Cellular uptake detection by TEM

V79 cells, unexposed and exposed to 12 $\mu g/cm^2$ NM401 for 24 h, were fixed in 2.5% (v/v) glutaraldehyde (EM grade, Merck, Darmstadt, Germany) and 2% (w/v) paraformaldehyde (EMS, Hatfield, PA) in 0.1 M cacodylate buffer (PB, Sigma-Aldrich, Steinheim, Germany), pH 7.4. Samples were first post-fixed with osmium, dehydrated in acetone, later embedded in Epon, and finally polymerized at 60 °C and cut with an ultramicrotome. Ultrathin sections were placed on copper grids and contrasted with uranyl acetate and Reynolds lead citrate solutions and then observed using a Jeol 1400 (Jeol LTD, Tokyo, Japan) TEM equipped with a CCD GATAN ES1000W Erlangshen camera (Annangi et al., 2015). The presence of MWCNT inside the cells was determined by visualizing different TEM images.

2.4. Intracellular ROS analysis by flow cytometry

The generation of intracellular reactive ROS was determined by flow cytometry using the 6-carboxy-2,7'-dichlorodihydro-fluorescein diacetate (DCFH-DA) assay (Toduka et al., 2012). Treated and untreated cells were seeded in triplicate in 6-well plates at a density of 1×10^5 cells/well. Treatments were carried out 24 h after seeding. After 24 h of NM401 exposure, cells were washed twice with PBS and incubated in 5 μM DCFH-DA in serum-free DMEM medium for 30 min at 37 °C. The presence of intracellular ROS converts the non-fluorescent DCFH-DA to its fluorescent form, measured using fluorescence-activated cell sorting (FACS; Calibur). Flow cytometry used excitation and emission wavelengths of 480 nm and 530 nm, respectively. Data were analyzed with the software Flowjo Ver. 7.6.5.

2.5. Proliferation assay-relative cell growth activity (RGA)

V79 cells were seeded on 12-well plates (1×10^5 cells per well) and incubated at 37 °C. After 24 h, cells were exposed to NM401 for 24, 48 and 72 h at concentrations ranging from 0.12 to 75 $\mu g/cm^2$. After exposure, medium was removed; cells were washed with PBS, trypsinized and re-suspended in 1 mL medium. Ten μL of the cell suspension was mixed with 10 μL 0.4% trypan blue (Invitrogen) and the percentages of living cells (unstained) and stained cells with damaged membranes were measured using a Countess™ Automated Cell Counter (Invitrogen). RGA was calculated as already published (Huk et al., 2014).

2.6. Plating efficiency (PE)

To determine cytotoxicity, V79 cells exposed to NM401 for 24 h were washed, trypsinised and counted as described above. Fifty cells per well were inoculated in 6-well plates (1 plate for each concentration tested) and left in an incubator at 37 °C for 7 days. Cells were then stained with 1% methylene blue (Sigma) and the number of colonies was counted manually. PE was calculated according to the follow formula:

$$PE(\%) = \frac{numberofcoloniesinexposedcultures}{numberofcoloniesinunexposedcultures} \times 100\%$$

2.7. HPRT gene mutation test

The mammalian *in vitro HPRT* gene mutation test was carried out according to OECD guidelines (OECD 476, 1997). V79 cells were seeded on 6-well plates (1×10^5 cells per well) and incubated at 37 °C. After 24 h, the cells were exposed to NM401 for 24 h, at concentrations ranging from 0.12–12 $\mu g/cm^2$. In each culture at least 2 million of cells were exposed. After exposure, the medium was removed, and cells were washed, trypsinized and re-suspended in medium. Cells were seeded in 100 mm Petri dishes (3×10^5 cells/Petri dish, 3 dishes per sample to achieve approximately 10^6 cells per sample), and cultivated in culture medium for an additional 8 days period. Cells were harvested twice for detecting mutants at days 6 and 8 after the treatment. Cells were inoculated in 100 mm Petri dishes and grown in selective medium containing 6-thioguanine (5 μg/mL, Sigma) for 10 days to form colonies. For each sample two replicas were used. Mutant colonies were stained with 1% methylene blue and counted manually. Only colonies with a minimum of 50 cells were considered.

The number of surviving cells was assessed by PE assay as described above in each of the two harvests: treated and untreated cells were plated into 6-well plates (50 cells per well, 1 plate for each sample) and incubated at 37 °C for 7 days to form colonies. Viability was determined at the time of each mutation harvest (i.e. 6 and 8 days after exposure) and calculated on the basis of the number of colonies versus the number of inoculated cells and mutant frequency was determined as described previously (Huk et al., 2014). Methyl methanesulfonate (MMS, 0.1 mM, 3 h) (Sigma)