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Effects on human bronchial epithelial cells following low-dose chronic exposure to nanomaterials: A 6-month transformation study

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ABSTRACT

The most plausible exposure route to manufactured nanomaterials (MNM) remains pulmonary inhalation. Yet, few studies have attempted to assess carcinogenic properties *in vitro* following long-term exposure of human pulmonary cells to low and occupationally relevant doses. The most advanced *in vitro* tests for carcinogenicity, the cell transformation assay (CTA), rely mostly on rodent cells and short-term exposure. We hypothesized that long-term exposure of human bronchial epithelial cells with a normal phenotype could be a valuable assay for testing carcinogenicity of nanomaterials. Therefore, this study (performed within the framework of the FP7-NANoREG project) assessed carcinogenic potential of chronic exposure (up to 6 months) to low doses of multi-walled carbon nanotubes (MWCNT, NM-400 and NM-401) and TiO_2 materials (NM62002 and KC7000). In order to harmonize and standardize the experiments, standard operating protocols of MNM dispersion (NANOGENOTOX) were used by three different NANoREG project partners. All nanomaterials showed low cytotoxicity in short-term tests for the tested doses (0.96 and 1.92 μg/cm^2). During long-term exposure, however, NM-401 clearly affected cell proliferation. In contrast, no cell transformation was observed for NM-401 by any of the partners. NM-400 and NM62002 formed some colonies after 3 months. We conclude that agglomerated NM-401 in low doses affect cell proliferation but do not cause cell transformation in the CTA assay used.

1. Introduction

In the ever-growing repertoire of manufactured nanomaterials (MNMs), carbon nanotubes (CNTs) and titanium dioxide (TiO_2) have attracted a wide interest. CNTs, because of their fibrous structure and high aspect ratio, bear resemblance to asbestos and are hypothesized to elicit effects similar to asbestos (Donaldson et al., 2013). The severity of toxic effects of CNTs has been attributed to depend on physicochemical properties such as diameter, length, functionalization and surface area (Lanone et al., 2013; Poulsen et al., 2016). In fact, administration of multi-walled carbon nanotubes (MWCNTs) in animals was reported to induce pulmonary inflammation, fibrotic lesions and development of mesothelioma (Muller et al., 2005; Poland et al., 2008; Poulsen et al., 2016; Rittinghausen et al., 2014; Sargent et al., 2014; Xu et al., 2012), and promote lung adenocarcinoma (Sargent et al., 2014). Based on data from several animal and *in vitro* studies, the International Agency for Research on Cancer (IARC) has classified Mitsui MWCNT-7 as possibly carcinogenic to humans (2B) (Grosse et al., 2014). Additionally, apoptotic cell death, cell membrane and DNA damage, activation of inflammasome and mitogen-activated protein kinases, disruption of cell-cell communication have been observed in *in vitro* settings following CNT exposure (Arnoldussen et al., 2016, 2015; Ghosh et al., 2011; Hamilton et al., 2013; Lindberg et al., 2013, 2009; Pacurari et al., 2008; Ursini et al., 2014). Interestingly, chronic exposure to single wall carbon nanotubes caused malignant transformation of human epithelial lung cells BEAS-2B (Wang et al., 2011a). When the same cell line was exposed to MWCNT NM-403 for four weeks, chromosome damage and reduced inflammatory signaling was found to correlate with an increase in attachment-independent growth (Vales et al., 2016).

TiO_2 MNMs, often regarded as nontoxic to humans, are featured in a wide range of consumer products, even though IARC has classified TiO_2 in class 2B (Baan et al., 2006; Baan, 2007). They can penetrate biological barriers (Elgrabli et al., 2015; Suzuki et al., 2016; Wang et al., 2007) and lead to undesired biological effects such as lung

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inflammation, mitochondrial dysfunction, DNA damage, ROS generation and apoptosis (Freyre-Fonseca et al., 2011; Park et al., 2008; Shi et al., 2013, 2010; Trouiller et al., 2009; Zhao et al., 2009). It has also been reported that food-grade TiO_2 exacerbates pre-existent intestinal diseases *in vivo*, but does not induce tumor formation *per se* (Urrutia-Ortega et al., 2016). In contrast, no genotoxic effects of TiO_2 MNMs were observed in *in vivo* studies (Lindberg et al., 2012; Suzuki et al., 2016), or in *in vitro* studies using TK6 (Woodruff et al., 2012), Caco-2 (Fisichella et al., 2012) and CHO-K1 (Wang et al., 2011b) and BEAS-2B (Vales et al., 2015) cells. Interestingly, BEAS-2B cells exposed to TiO_2 nanoparticles for four weeks showed significant increase in the number of clones growing in an anchorage-independent way without the induction of genotoxic damage (Vales et al., 2015). This finding would indicate a potential carcinogenic risk associated to nano-TiO_2 exposure, not mediated by a genotoxic mechanism.

In-depth understanding of genotoxic and carcinogenic effects of MNM is still lacking. Much of our knowledge of possible risk or health hazards associated with exposure to MNMs is derived from acute or short-term *in vitro* and *in vivo* studies. Furthermore, use of high doses of MNMs in toxicological studies has made observations and conclusions associated with MNMs exposure to human health challenging (Bhattacharya et al., 2011). For example, at low doses TiO_2 did not induce inflammatory reactions in mice in contrast to high doses (Husain et al., 2013). Similarly, another study reported that inhalation of low doses of MWCNTs did not cause lung inflammation or tissue damage, but altered systemic immune function (Mitchell et al., 2007). Thus, there is lack of data addressing possible adverse biological effects of long-term, low-dose chronic exposure to MNMs. Such studies are warranted to better evaluate and assess potential negative consequences resulting from exposure to MNMs.

In this study, we evaluated and compared the carcinogenic potential of chronic low-doseTiO_2 and MWCNTs exposures over a period of 26-weeks. We developed an *in vitro* cell exposure model wherein normal human bronchial epithelial (HBEC) cells were continuously exposed *in vitro* with occupationally relevant doses. We selected HBEC cells since they are non-tumorigenic, have intact TP53 and low spontaneous mutation rate (Ramirez et al., 2004). Another strength of this study is chronic low-dose long-term exposure that allowed us to monitor subtle changes over time, and avoid the global cellular stress responses often resulting from high-dose exposure (Klaper et al., 2014). The specific aim of this study was therefore to develop an *in vitro* cellular transformation assay to assess carcinogenic potential of different types of MNMs.

2. Materials and methods

2.1. Nanomaterial dispersion and characterization

MWCNTs (NM-400 and NM-401), and TiO_2 NM62002 (previously designated as NM-104) were obtained from JRC Nanomaterials Repository (Ispra, Italy). TiO_2 KC7000 was from KRONOS worldwide Inc. (Dallas, TX). These MNMs were dispersed according to the NANOGENOTOX dispersion protocol (Jensen et al., 2011). Briefly, MNMs were first pre-wetted in 30 µl absolute EtOH and sonicated for 16 min with 10% amplitude (Branson Sonifier S-450D) in 5.97 ml dispersion medium, which was a mixture of 0.05% (v/v) BSA (Sigma-Aldrich) and MilliQ-H_2O. This yielded a stock dispersion of approximately 2.56 mg/ml that was used to prepare dilution for *in vitro* studies.

Physico-chemical properties of MNMs used in this study have been extensively characterized and reported for the MWCNTs NM400 and NM401 (Rasmussen et al., 2014a), NM62002 (Rasmussen et al., 2014b) and KC7000 (Kete et al., 2014). In addition, morphology of dispersed MNMs was inspected with a scanning (Hitachi) electron microscopy. Furthermore, hydrodynamic diameter was measured by dynamic light scattering (Zetasizer Nano, Malvern Instruments Ltd., UK). Dynamic Light Scattering (DLS) method was used to determine size, stability and agglomeration states of particles in suspension. The procedure used for DLS measurements are as follows: 1 ml of the dispersed MNMs in dispersion media (10 min after sonication) was pipetted into the Zetasizer measurement cuvettes as instructed by the manufacturer (Malvern Instruments, Ltd., UK), at 25 °C, medium viscosity of 0.99 cP, equilibration time of 120 s, automatic option used for all measurement conditions and 10 repeated measurements were performed without pause in between. The results were analyzed by calculating the zeta average (z-average) from the average values of the 10 repeated measurements.

2.2. The human lung cells, cell culture conditions and exposure doses

The immortalized human bronchial epithelial cell line HBEC-3KT (Ramirez et al., 2004) was purchased from American Type Culture Collection (ATCC). HBEC-3KT cells were maintained under standard growth conditions of 5% CO_2 and 37 °C in a 1:1 mixture of LHC-9 (Gibco) and RPMI-1640 (Thermo Scientific) medium, supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin (Biowest). The cells were exposed to two concentrations of MNMs: 1.96 (designated as C1) and 0.96 (designated as C2) µg/cm^2 in all experiments. These doses were chosen because of their relevance for occupational exposure situations of the investigated MNMs. MNM-dilutions for cell exposures were made from freshly prepared MNMs stock dispersions.

2.3. Investigation of the cytotoxicity of MNMs and cell viability

Prior to setting up the long-term chronic exposure experiments, in initial cytotoxicity experiments, cytotoxic effects of the four MNMs on the HBEC-3KT cells were investigated. For cytotoxicity experiments, cells were seeded in 24-well plates and exposed to two doses (C1 and C2) of the MNMs. Control cells were exposed to equal volume of dispersion medium (DM). After 72 h of exposure, cell viability was assayed by Cell Counting Kit-8 (CCK-8) from Sigma-Aldrich, and LDH-release assay (Lactate Dehydrogenase Activity Assay Kit) from Thermo Scientific following the protocols provided with the kit. Both assays measure cell viability albeit by measuring different endpoints. CCK-8 assay determines the viability of cells by measuring the amount of the formazan dye generated by the activities of dehydrogenases in cells, which is directly proportional to the number of living cells. LDH assay determines toxicity by determining presence of membrane damage. We used two assays to be sure that the chosen MNM doses were not cytotoxic. In addition, cell proliferation and growth patterns (indicated as number of cells) were measured using trypan blue exclusion assay every week.

2.4. Long-term chronic exposure

For long-term chronic exposure studies cells were exposed to C1 and C2 doses of MNMs or only to the vehicle DM (unexposed control cells).

The long-term exposure experimental set up is outlined schematically in the flow chart and diagram below. On day 1, HBEC-3KT cells were seeded in two 15-cm cell culture dishes (growth area: 152 cm^2, 2.5×10^5 cells/dish). On day 3, cell culture media were removed and freshly prepared MNMs dilutions were added to the exposed cells and DM to control cells. On day 5, the MNMs and DM were removed and cells were washed with Ca^{2+}, Mg^{2+} free PBS. Again, freshly prepared MNMs dilutions and DM were added to cells and incubated for another 72 h at standard growth conditions. At the end of the 72 h of incubation (by this time cells had been cultured for 7 days), media were removed and cells were trypsinized, cell growth was assessed by trypan blue assay, and subcultured for new round of exposure as in day 1. This procedure was repeated for 26 weeks. Meanwhile, every fourth week, 1.8×10^5 cells from each 15 cm dish were transferred to three soft agar wells in 6-well plates (6×10^4 cells/well) for colony formation in soft agar assays (see below).