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Toxicology

The role of the iron catalyst in the toxicity of multi-walled carbon nanotubes (MWCNTs)

Giuseppa Visalli[a], Alessio Facciolà[a], Daniela Iannazzo[b], Anna Piperno[c], Alessandro Pistone[b], Angela Di Pietro[a,*]

[a] Department of Biomedical and Dental Sciences and Morphofunctional Imaging, University of Messina, Italy
[b] Department of Electronic Engineering, Industrial Chemistry and Engineering, University of Messina, Italy
[c] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Italy

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ABSTRACT

This study aimed to investigate the role of iron, used as a catalyst, in the biological response to pristine and functionalized multi-walled carbon nanotubes (p/fMWCNTs) with an iron content of 2.5–2.8%. Preliminarily, we assessed the pro-oxidant activity of MWCNTs-associated iron by an abiotic test. To evaluate iron bioavailability, we measured intracellular redox-active iron in A549 cells exposed to both MWCNT suspensions and to the cell medium preconditioned by MWCNTs, in order to assess the iron dissolution rate under physiological conditions. Moreover, in exposed cells, we detected ROS levels, 8-oxo-dG and mitochondrial function. The results clearly highlighted that MWCNTs- associated iron was not redox-active and that iron leakage did not occur under physiological conditions, including the oxidative burst of specialized cells. Despite this, in MWCNTs exposed cells, higher level of intracellular redox-active iron was measured in comparison to control and a significant time-dependent ROS increase was observed ($P < 0.01$). Higher levels of 8-oxo-dG, a marker of oxidative DNA damage, and decreased mitochondrial function, confirmed the oxidative stress induced by MWCNTs. Based on the results we believe that oxidative damage could be attributable to the release of endogenous redox-active iron. This was due to the damage of acidic vacuolar compartment caused by endocytosis–mediated MWCNT internalization.

1. Introduction

The impact of carbon nanotubes (CNTs) on human health has not yet been clarified, although they seem to elicit toxicity in the respiratory system following inhalation. Inhaled single and multi-walled carbon nanotubes (SWCNTs and MWCNTs, respectively), penetrate deeply, inducing pulmonary inflammation, cytotoxicity and carcinogenesis [1]. Numerous *in vitro* and *in vivo* studies have shown the acute and chronic inflammatory responses to CNTs [2–8]. CNTs, especially MWCNTs, have strong similarities with asbestos fibres, including a needle-like shape, pro-oxidant capability and biopersistence [9,10]. Similar to asbestos, CNTs are also able to cause mesothelioma [11] as well as alveolitis, pulmonary fibrosis, granuloma and bronchogenic carcinoma [12,13].

According to several authors [14,15], the induction of the carcinogenic asbestos-like effects of MWCNTs is driven by the presence of transition metals, especially iron, used as a catalyst in nanotube synthesis. In a previous study, we observed in A549 cells a strong pro-oxidant effect by using MWCNTs synthesized in our laboratory by the catalytic chemical vapor deposition (CCVD) method [16]. In our experiments, the role of iron apparently was confirmed by using the chelator deferoxamine mesylate (DFX); the redox imbalance was completely neutralized in cells treated with DFX-MWCNTs mixtures. However, the iron content of the tested MWCNTs was largely trapped inside the nanotubes; thus, it did not fully explain the observed redox imbalance. All this led us to

Abbreviations: CNT, carbon nanotubes; pMWCNT, pristine multi-walled carbon nanotubes; fMWCNT, acid-treated multi-walled carbon nanotubes; CCVD, catalytic chemical vapor deposition; FCS, fetal calf serum; PBS, phosphate buffered saline; FACS, Fluorescence-activated cell sorting; DFX, deferoxamine mesylate; FAS, $(NH_4)_2Fe(SO_4)_2$; NTA, nitrilotriacetic acid; calcein-AM, calcein-acetoxymethyl ester; Δy_m, mitochondrial transmembrane potential; DCF-DA, 2′,7′-dichlorofluorescein-diacetate; FAU, fluorescence arbitrary units; 8-oxo-dG, 8-Oxo-2′-deoxyguanosine.

* Corresponding author at: Department of Biomedical and Dental Sciences and Morphofunctional Imaging, University of Messina, Via C. Valeria n°1, Gazzi, 98100, Messina, Italy.

E-mail addresses: gvisalli@unime.it (G. Visalli), alessiof1976.af@gmail.com (A. Facciolà), diannazzo@unime.it (D. Iannazzo), apiperno@unime.it (A. Pistone), adipietr@unime.it, pistone@unime.it (A. Di Pietro).

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hypothesize that oxidative damage was mainly attributable to the release of endogenous redox-active iron, due to cell damage caused by endocytosis–mediated MWCNT internalization. In order to test this hypothesis, and thus to explain the true role of the iron catalyst in the toxicity of MWCNTs, in the present study we performed further experiments using the same homemade nanomaterials.

2. Materials and methods

2.1. Pristine and functionalized MWCNTs

Since the biological effects of MWCNTs are strongly linked to their physicochemical properties, which are in turn related to postsynthetic modifications, we examined both pristine and functionalized MWCNTs, designated pMWCNT and fMWCNT, respectively. pMWCNT were synthesized by catalytic chemical vapor deposition (CCVD) using Fe/Al_2O_3 as the catalyst and they were successively purified as previously reported (16). fMWCNT, i.e. MWCNT-COOH, were prepared by the oxidation of purified pMWCNT using a mixture (1:1 vol ratio) of sulfuric acid and nitric acid [17]. As extensively reported in our previous study, MWCNTs were characterized by thermogravimetric analysis (TGA), UV spectra, scanning electron microscopy and high-resolution transmission electron microscopy. In pMWCNTs, the inorganic fraction, assessed by oxidative TGA analysis, was equal to 3.5-4%, almost all comprised of Fe_2O_3, showing the high quality (>95% purity) of the MWCNTs. As confirmed by atomic absorption spectroscopy analysis, the percentage of metallic iron was 2.5-2.8%. The percentages of inorganic fraction and metallic iron present in the fMWCNTs were evaluated following the same procedure and were found to be equal to 3.1–3.5% for Fe_2O_3 and 2.0–2.2% for the metallic iron. Based on the synthesis method used, the iron was localized within the MWCNTs and, sometimes, at one end. This was evident by observing the images of pMWCNTs obtained by HRTEM. Fig. 1 clearly shows iron catalyst clusters encapsulated within the multi-walled nanostructure (Fig. 1a and b). These were formed of 15–20 layers and the outer surfaces were smooth and well-graphitized. Sometimes, point defects among graphitic carbon planes were observed. Other nanotubes appeared to have been formed using as a base the catalyst that, remaining at one end of the nanostructure (Fig. 1c), was largely exposed to the external environment. As previously reported, pMWCNT had an average length of 10–20 μm and a diameter close to 15–30 nm, while fMWCNT were by far shorter (average length between 200 and 1000 nm) and showed an external layer eroded at many points, due to the oxidative insertion of terminal functional groups.

Due to their hydrophobicity common to all CNTs, our MWCNTs, in particular pMWCNT, had a strong propensity to agglomerate in water. In order to minimize this feature, which makes MWCNT/cell interactions highly variable, we made concentrated MWCNT suspensions (100 x in PBS) that were sonicated for 20 min in an ice bath. Since proteins stabilize MWCNT suspensions [18], just before each experiment, the concentrated suspensions were diluted in cell culture medium (containing 2% FCS) and again subjected to a fast sonication (3 min).

To assess the dissolution ratio of iron under physiological conditions, additional experiments were performed. A first set of tests consisted in treating cells with the supernatant obtained from cell culture medium (containing 2% FCS) preconditioned with nanotubes. Briefly, the MWCNT suspensions were incubated at 37 °C for 48 h and then centrifuged (3000*g* for 10 min). Considering the leaning of nanotubes to the sedimentation, this treatment allowed to obtain the MWCNT-free supernatants that were used for the experiments. AAS analysis was performed on the preconditioned media and on the untreated cell medium. No relevant difference in terms of iron content were observed. The values were 50, 52 and 51 mg/L^{-1} for the untreated, pMWCNT- and fMWCNT-preconditioned media respectively ($P > 0.05$). To assess the possible iron leakage from the nanotubes subjected to the action of reactive oxygen species, we performed further abiotic experiments. Briefly, the MWCNTs were placed in contact with the ROS generated *in situ* by treating peroxides in the presence of Cu^{2+} catalyst. Both *p*-MWCNT and f-MWCNT samples (50 μg mL^{-1}) were separately treated with hydrogen peroxide or peroxyacetic acid (400 μM), in deionized water at 37 °C, in the presence of a catalytic amount of $CuSO_4$ (50 μM) and incubated for 6 h, as in *in vitro* experiments. Then, the mixtures were centrifuged (3500*g* for 10 min) and the supernatants were analyzed by AAS spectroscopy while MWCNTs were washed by PBS and analyzed to assess the presence of redox active iron.

2.1. Cells *and exposure conditions*

As an *in vitro* cell model, we used the human alveolar cell line A549, derived from a lung carcinoma (ATCC, Rockville, USA). Cells were cultured in RPMI medium with 2 mM L-glutamine, 10% (v/v) fetal calf serum (FCS), 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. All reagents for cell cultures were purchased from Gibco (Invitrogen Gibco, Milan, Italy). For all experiments, we used subconfluent monolayers grown in 6-well cell plates. In parallel, A549 cells were exposed to pMWCNT and fMWCNT (50 μg mL^{-1}), to their respective supernatants, as reported above, and to an iron solution at the same concentration present in pMWCNTs. This was obtained from $(NH_4)_2Fe(SO_4)_2$ (final concentration 54.4 μM) and used as a positive control. Monolayers treated with PBS were included in each experiment as a negative control (i.e. control cells). To confirm MWCNT uptake, previously quantified in the same cell model [16], we performed a qualitative analysis by microscopic observation of A549 semiconfluent monolayers. The cells were grown on cell slides (Invitrogen Gibco) and treated as reported above for 180 min.

2.2. Assessment of iron bioavailability

To study the role of iron in the toxicological effect of MWCNTs, we used two different analyses aimed to assess the bioavailability of MWCNTs-associated iron and to measure the intracellular concentration of redox-active iron in exposed cells. The first analysis was performed by using a fluorimetric abiotic assay, based on the method devised by Esposito et al. [19].Briefly, in MWCNT suspensions (50 μg mL^{-1}), both as such and subjected to the action of ROS, and in the supernatant of MWCNT pre-treated cell culture medium, we measured the oxidation of the non-fluorescent probe dihydrorhodamine (DHR) to its fluorescent form rhodamine, a reaction catalyzed by redox-active iron. To exclude other DHR oxidation mechanisms not caused by the presence of redox-active iron, the samples were analyzed in parallel by the addition of the iron chelator deferoxamine mesylate (DFX),which quenches only iron-induced fluorescence. Briefly, samples were assayed in quadruplicate in 96-well plates by adding the DHR (50 μM) in reagent solution (pH 7.3) containing 40 μM of ascorbate to regenerate Fe(II) after its oxidation to Fe(III). DFX (50 μM) was added to this solution in two of the wells. A Fe:NTA (nitrilotriacetic acid) (1:7 mM) complex, starting from freshly prepared FAS $(NH_4)_2Fe(SO_4)_2$ and NTA (pH 7.0), was used to build a calibration curve (1–54.4 μM). To assess the kinetics of the reaction, emitted fluorescence was recorded every 2 min, starting from 15 min up to 40 min, by using 485/535 nm excitation/emission filters (plate reader Tecan, Brescia, Italia). The differences between the samples with and without DFX, due to redox-active iron, were used to calculate fluorescence units

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