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Effect of different carbon nanotubes on cell viability and proliferation

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Abstract

Carbon nanotubes (CNTs) are a focus of intense research for their potential applications in multiple diverse applications, including innovative biomedical applications. Due to their very recent discovery, little information is available about the biocompatibility and toxicity of this new class of nanoparticle, and a systematic study on biological interference is lacking. Thus, we decided to explore the toxicity of three different types of carbon nanotube, differing in preparation (arc discharge versus catalysed chemical vapour deposition); size (10–50 versus 100–150 nm wide $\times$ 1–10 μm long); contaminants (amorphous C, graphite, fullerenes or iron) and morphological type (multi-walled, MW, or single-walled, SW) on human leukemic U937 cells. We found that these carbon nanotubes exert a strong effect on the proliferation of the reporter U937 monocytic cell. However, these CNTs did not significantly affect the cell viability. These results show that CNTs, though not directly exerting a direct cytotoxic effect, are nonetheless able to deeply alter cell behaviour, and thus we recommend thorough analyses to limit health risk due to uncontrolled exposure.

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1. Introduction

Multi-walled CNTs (MWCNTs) [1] and single-walled carbon nanotubes (SWCNTs) are constituted by a unique graphitic sheet or two or more sheets nested together in a tubular multilayer structure, respectively. CNTs can be synthesized by three main methods: laser ablation, arc discharge (both these methods are based on the evaporation of graphite due to high temperatures) and chemical vapour deposition (CVD); evaporated carbon condenses successively in the form of CNTs and other structures, producing longer and less graphitized forms than the material obtained by the other methods. The arc discharge method has proved to be an interesting method for the mass production of CNTs. In particular it was demonstrated how MWCNTs are also present in the cathode deposit if the discharge is ignited under a liquid environment [2].

Due to their interesting electrical, mechanical and thermal properties, mass production of CNTs is expected in the next few years. In fact future applications include energy storage devices [3], high-resistance composites [4], electronic devices [5] and biomedical applications [6, 7]. As far as the last is concerned, the attractiveness of CNTs comes from the possibility of functionalizing them with peptides, proteins, or in general specific target molecules, to develop delivery systems that can move through the body to target cells [8, 9] or to build tissue scaffolds to promote cell proliferation and differentiation [10, 11]. These potential applications call for thorough studies on biocompatibility. Besides, toxicological studies are required to prevent possible health hazards among workers involved in the research and manufacture of these materials, and in future of the general public [12].

The literature about the health effects of CNTs suggests a potential toxicity of this new material [13]. In particular, the first studies, focused mainly on inhalation and dermal exposure, revealed a pulmonary [14–16] and dermal [17–19] toxicity. We selected three different types of CNT, synthesized by different methods, including: (a) MWCNTs synthesized by an electric arc discharge in deionized water [2], with diameter 10–50 nm, 10 μm long, containing graphite as contaminant; (b) a sample of CNTs synthesized by an electric arc discharge in helium [20] composed of 50% MWCNTs (10–40 nm diameter, 1–5 μm long) + 30% SWCNTs (1–2 nm), the residual 20% containing graphite, fullerene and amorphous C as contaminants; (c) MWCNTs produced by the CVD method, of larger size (110–170 nm diameter and 5–9 μm long), which contain 0.1% iron as chemical contaminant.

We analysed the effects of these different CNTs on human monocytic U937 cells, a well-known model of toxicological analysis. Since nanoparticles can enter the blood stream, blood cells may be potential targets of CNTs, and the effects must be analysed [13, 21–23].

The surprising developments of cell toxicology in recent years led to the recognition of two different type of cell death: apoptosis is a cell-intrinsic mechanism that leads cells with mild damage to choose self-elimination [24, 25], whereas severe damage leads cells to passively die by necrosis [26, 27].

In the present study we provide evidence that the three different types of CNT are not directly cytotoxic, but they exert deep cytostatic effects.

2. Materials and methods

2.1. Preparation of carbon nanotubes

Three different samples containing multi-walled CNTs were used (table 1). The first type of CNT used was MWCNTs synthesized by an electric arc discharge in deionized water. A specially designed experimental device was realized to stabilize and homogenize the electric arc. The equipment is not connected to any vacuum system, so it can operate both in a liquid phase and in gaseous environments at atmospheric pressure. A 6 mm diameter anode and a

Table 1. Description of the three types of carbon nanotube used in the present study.

Material	Synthesis	Dimensions	Contaminants
MWCNTs	Synthesized by an electric arc discharge in deionized water	The diameter is in the range 10–50 nm and the length is up to 10 μm	Graphite
50% MWCNTs + 30% SWCNTs	Synthesized by an electric arc discharge in helium	The diameter of the MWNTs is in the range 10–40 nm and the length is 1–5 μm	20% fullerene and amorphous C
MWCNTs	Produced by CVD	The diameter is in the range 110–170 nm and the length is between 5 and 9 μm	0.1% of Fe

30 mm diameter cathode, both made of pure graphite (99.99%), were used, and we applied 26 V and 65 A. The experiments were carried out with the anode approaching the cathode at a constant speed of 11 mm min^{-1}. The diameter of these MWCNTs is in the range 10–50 nm and the length is up to 10 μm [2].

X-ray analysis confirmed that the powder used does not contain metals. This is important because every effect observed on cells is therefore completely due to the CNTs.

The second type of CNT was synthesized by an arc discharge between two pure graphite electrodes at 24 V and 110 A, under a pressure of 600 Torr of helium. Typical duration times were 3 min. This sample of CNTs is constituted of 50% of MWCNTs, 30% of SWCNTs (with a diameter 1–2 nm) and 20% of amorphous C and fullerene. These CNTs form bundles with a diameter in the range 10–40 nm, and their lengths are in the range 1–5 μm [20].

The third type was MWCNTs produced by CVD, purchased from Sigma-Aldrich. The diameter is in the range 110–170 nm while their length is comprised between 5 and 9 μm. 0.1% of Fe is present in this sample.

2.2. Cell culture and treatments

U937 cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 IU ml^{-1} penicillin and streptomycin, and kept in a controlled atmosphere (5% CO_2) incubated at 37 °C. The experiments were performed on cells in the logarithmic phase of growth, in conditions of excellent viability, as assessed by trypan blue exclusion, $\geqslant$98%.

The CNTs were dispersed in tissue culture complete medium, vortexed (4–5 min) and then sonicated (3–4 min). The cells used for this study grow in suspension cultures: CNTs are added to the medium containing the floating cells, and CNTs and cells left to sediment.

25 μg ml^{-1} of each of the three CNT types was added to the cell culture.

2.3. Analysis of cell proliferation

The number of viable cells was estimated in a hemocytometer at the times indicated.

2.4. Analysis of apoptosis

For the detection of apoptosis, cells were stained with the DNA-specific, cell-permeable dye Hoechst 33342 (Calbiochem), at a concentration of 10 μg ml^{-1}. Apoptotic cells

were recognized according to their nuclear morphology (different stages of nuclear fragmentation) [28, 29].

Apoptosis was quantified as previously described [29]. Briefly, the fraction of U937 cells with fragmented nuclei among the total cell population is calculated in the Hoechst 33342 stained cells, counting at least 300 cells in at least ten random selected fields.

2.5. Analysis of necrosis

For the detection of necrosis, cells were stained with the cell-impermeable dye propidium iodide (PI) at a concentration of 5 μg ml^{-1} (Sigma-Aldrich). Necrotic cells were recognized according to their inability to exclude PI.

Necrosis was quantified as the fraction of U937 cells that uptake propidium iodide (PI) among the total cell population, counting at least 300 cells in at least ten random selected fields.

2.6. Statistical analysis

Statistical analyses were performed using Student's t-test for unpaired data and p-values <0.05 were considered significant. Data are presented as mean $\pm$ SD (standard deviation).

3. Results

3.1. Lack of cytotoxic effects of carbon nanotubes

First of all, we observed that CNTs did not induce any morphological alterations detectable by phase contrast microscopic analysis in cells exposed to the different CNTs (not shown). We next analysed whether incubation with CNTs at different incubation times might reduce the cell viability. Thus, we measured the extent of the apoptotic or necrotic cells.

In figure 1 we report the fraction of apoptotic and necrotic cells found in the cultures exposed to 25 μg ml^{-1} of each of the three CNT types. Independently of the type, these CNTs, with their different properties, did not induce apoptosis over the basal level (around 2%, always found in U937 cultures) up to 48 h of exposure.

3.2. Carbon nanotubes affect cell proliferation

Next, we analysed the effect of CNT exposure on the proliferation of U937 cells.

Cells were incubated with 25 μg ml^{-1} of each of the three CNT types, and the cell number was evaluated by means of hemocytometric analysis, and compared with control cells. Exposure to CNT reduces the rate of cell growth with respect to untreated cells, as evidenced by figure 2. Since these reduced increments were not due to cell death (as we showed in figure 1), one must conclude that the CNTs affect (reduce) the rate of cell proliferation.

4. Discussion

In this study we show that the three different types of CNT studied did not exert a significant direct cytotoxic effect on human monocytic U937 cells. It was previously shown that CNTs do not affect the cell viability of macrophages [21]. Instead, CNTs were shown to induce apoptosis on the T lymphocytic cells Jurkat [23]. What might be the reason for such opposite effects? We might speculate that the lack of cytotoxicity of CNTs on the monocyte/macrophage lineage cells might be a tissue-specific characteristic. We have preliminary data which confirm that

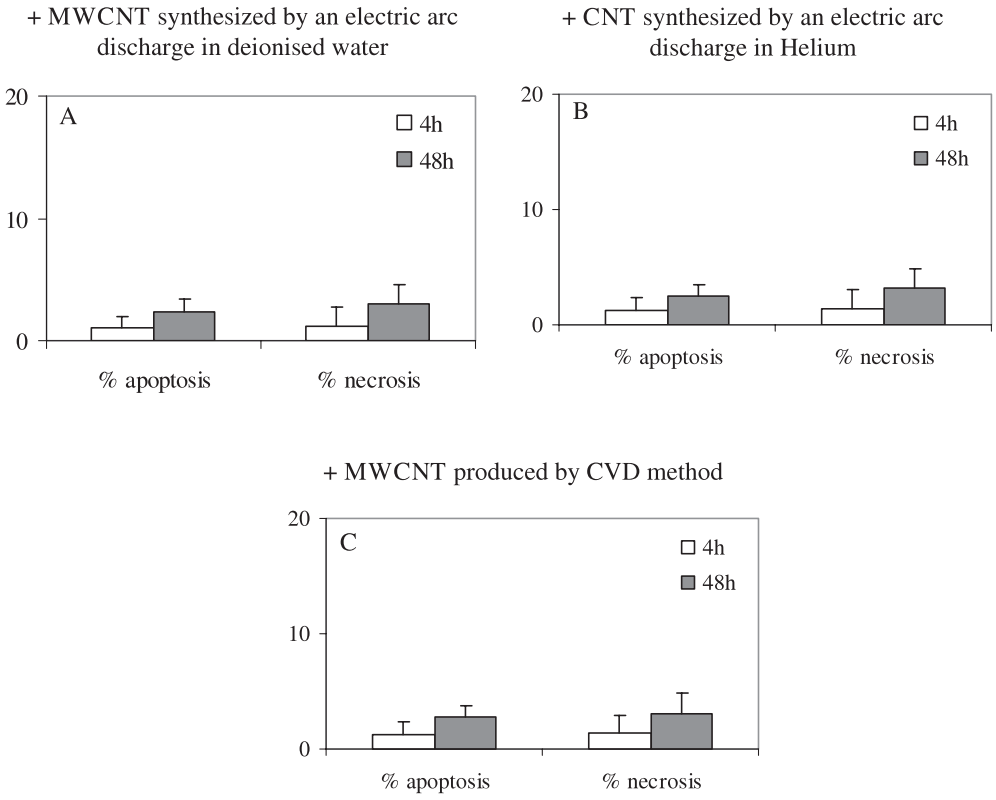

Figure 1. Effect of CNTs on apoptosis of U937 cells. U937 cells were incubated with 25 μg ml^{-1} of each of the three CNT types and the apoptosis was quantified at 4 and 48 h. (A) Cells incubated with MWCNTs synthesized by an electric arc discharge in deionized water. (B) Cells incubated with CNTs synthesized by an electric arc discharge in helium. (C) Cells incubated with MWCNTs produced by the CVD method. All values are the average of three measurements ±SD.

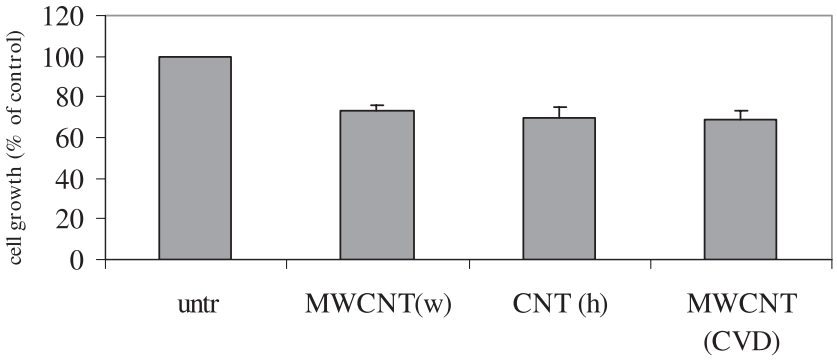

Figure 2. Effect of CNTs on U937 cell growth. U937 cells were incubated with 25 μg ml^{-1} of CNTs, and the cell number was quantified at 48 h. Values are expressed as per cent of control. The x-axis indicates the type of nanotube used: MWCNT(w) indicates MWCNTs synthesized by an electric arc discharge in deionized water; CNT(h), CNTs synthesized by an electric arc discharge in helium; and MWCNT(CVD), MWCNTs produced by the CVD method. All values are the average of three measurements for each CNT ± SEM.

the same preparations of CNTs used in this study are cytotoxic on a clone of Jurkat cells [31]; interestingly, the type of cell death elicited is of both apoptotic and necrotic nature, indicating that it is not the result of a specific signal transduction (which would only solicit apoptosis), but of a mild cell damage. It will be most interesting to explore if the cell parameters of oxidative stress and cytosolic Ca2+ levels, usual mediators of damage-induced cell death, are equally affected by CNTs on U937 versus Jurkat cells. In order to speculate on a possible physiological meaning of the different behaviour of U937 versus Jurkat in terms of cell death, it must be kept in mind that monocytes/macrophages, unlike lymphocytes, are programmed to be present and functional in areas of inflammation, where the environment is highly toxic, thus possessing more efficient cell defence apparatuses.

One should also keep in mind an additional effect that is recently receiving much attention, i.e., the spontaneous functionalization of CNTs in biological fluids. In culture media, especially in the presence of fetal calf serum, electrophilic biomolecules (amino acids, vitamins, etc) bind to CNTs, sheltering them from the environment. This may exert two important consequences: (a) pauperize the media of necessary nutrients, or (b) mask possible toxic features of CNTs. Both these effects may differently affect different cells.

CNTs exert a strong effect on U937 cell proliferation. Since this is not due to cell loss (apoptosis or necrosis), it must be concluded that CNTs directly affect the cell cycle. Indeed, it was reported that SWCNTs can inhibit human HEK293 cells and induce a cell cycle arrest in the G1 phase [30].

The main message from this study is that CNTs, independently of the type of preparation and presence of contaminants, deeply affect cell behaviour in terms of control of cell proliferation.

With this study we mean to contribute to the important toxicological screening strategy necessary to identify the potential toxic effect of CNTs, and thus to prevent expositive risk and develop safe biomedical applications.

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