Chapter 29

Genotoxic Assessment of Carbon Nanotubes

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Abstract

Carbon nanotubes are unique one-dimensional macromolecules with promising application in biology and medicine. Since their toxicity is still under debate, here we describe an investigation of genotoxic properties of purified single-walled carbon nanotubes (SWCNT), multiwall carbon nanotubes (MWCNT), and amide-functionalized purified SWCNT. We used two different cell systems: cultured human lymphocytes where we employed cytokinesis-block micronucleus test and human fibroblasts where we investigate the induction of DNA double-strand breaks (DSBs) employing H2AX phosphorylation assay.

Key words Carbon nanotubes, Genotoxicity, Human cells, Micronuclei, γ-H2AX foci

1 Introduction

Carbon nanotubes (CNT) are unique, one-dimensional macromolecules, whose outstanding properties have sparked an abundance of research since their discovery in 1991 (1). Single-walled carbon nanotubes (SWCNT) are constructed of a single sheet of graphite (diameter 0.4–10 nm), while multiwall carbon nanotubes (MWCNT) consist of multiple concentric graphite cylinders of increasing diameter (10–100 nm) (2). Both SWCNT and MWCNT possess high tensile strengths, are ultralight weight, and have excellent thermal and chemical stability. In combination with their metallic and semiconductive electronic properties, this remarkable array of features has seen a plethora of applications proposed.

One of the major areas of CNT research is the field of biomedical materials and devices. Many applications for CNT have been proposed including biosensors, drug and vaccine delivery vehicles, and novel biomaterials (2). CNT can be used as nanofillers in existing polymeric materials to both dramatically improve mechanical properties and create highly anisotropic nanocomposites

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(3, 4). They can also be used to create electrically conductive polymers and tissue engineering constructs with the capacity to provide controlled electrical stimulation (5–7). However, before such materials can be incorporated into new and existing biomedical devices, the toxicity and biocompatibility of CNT needs to be thoroughly investigated. Within the realm of biotechnology, carbon nanotubes, a major class of carbon-based tubular nanostructures, have been utilized as platforms for ultrasensitive recognition of antibodies (8) as nucleic acids sequencers (9) and as bioseperators, biocatalysts (10), and ion channel blockers (11) for facilitating biochemical reactions and biological processes. Towards nanomedicine, an emerging field of utilizing nanomaterials for novel and alternative diagnostics and therapeutics has been developed. CNT have been utilized as scaffolds for neuronal and ligamentous tissue growth for regenerative interventions of the central nervous system and orthopedic sites (12), substrates for detecting antibodies associated with human autoimmune diseases with high specificity (13), and carriers of contrast agent aquated Gd^{3+}-ion clusters for enhanced magnetic resonance imaging (14). When coated with nucleic acids (DNA or RNA) or proteins, CNT have been shown as effective substrates for gene sequencing and as gene and drug delivery vectors to challenge conventional viral and particulate delivery systems (15–19). Consequently, efforts to take advantage of the physical and chemical properties of CNT in biological settings must first circumvent the hydrophobicity of these nanomaterials.

Research over the past decade has shown that CNT and fullerenes can be readily modified, either covalently or non-covalently, by incorporating chemical and biological functional groups for much enhanced solubility and bioavailability. The covalent modification of single-walled carbon nanotubes, for example, normally involves esterification or amidation of acid-oxidized nanotubes and sidewall covalent attachment of functional groups (20–24). However, these covalent schemes are often marred by undesirable modifications to the physical and chemical properties of SWCNT (25). Furthermore, such functionalized SWCNT often have dangling bonds at the defective sites and are prone to generating free radicals. In comparison, the non-covalent modifications of SWCNT employ adsorption of proteins, biopolymers and synthetic polymers (DNA, RNA, polyvinyl pyrrolidone, polystyrene sulfonate), and surfactants (sodium dodecyl sulfate or SDS) to form supramolecular assemblies (8, 26–34). For both covalent and non-covalent solubilization schemes, the introduction of surfactants, surface charges, organic solvents, and residues may induce additional genotoxicity. Developing well-characterized solubilization schemes on the base of functionalized nanotubes is thus crucial for facilitating the full range biological and biomedicinal applications of nanotubes and their derivatives (35).

Structural characterization is necessary while investigating the influence of carbon nanotubes on the living systems, since previous investigations showed that the genotoxicity level is different depending of the length, diameter, total surface, irregularities in the lattice organization, purity and presence of the functional groups, and the solubility of carbon nanotubes (36).

Considering numerous possible interactions between CNT and biomolecules, as well as their existing and potential applications in biological sciences and other fields of science and technology, it is of great interest to determine their influence on living systems.

Here we present a study investigating genotoxic properties of carbon nanotubes: purified SWCNT, MWCNT, and amide-functionalized purified SWCNT on cultured human lymphocytes employing cytokinesis-block micronucleus test and enumeration of γ-H2AX foci in human fibroblast cell line.

2 Materials

2.1 Laboratory Equipment

1. Laminar flow cabinet.
2. Ultrasonic bath.
3. Incubator.
4. Water bath.
5. Centrifuge.
6. Vortex mixer.
7. Micropipette.
8. Microscope.
9. Microscope slide staining jar.
10. Microscope with illuminator for fluorescence microscopy.

2.2 Solutions

1. Carbon nanotubes dispersion: Disperse 5 mg of each carbon nanotube sample (SWCNT, MWCNT, and amide-functionalized purified SWCNT) into 1 ml of 96% ethanol. Sterilize in an autoclave. Sonicate prior to use for 30 min.
2. Cytochalasin B stock solution: Dissolve 10 mg cytochalasin B into 33.3 ml DMSO in laminar hood. Filter DMSO with single-use syringe filter with pore sizes 0.20 μm. Aliquot in 2 ml safeseal micro tubes (see Note 1).
3. Hypotonic solution: Mix equal volumes of 0.56% KCl and 0.9% NaCl water solutions. Calculate final volume according to number of specimens. Keep in wash bottle in water bath at 37°C (see Note 2).

4. Fixative solution: In 3× volume of methanol (CH_3OH), add 1× volume of acetic acid (CH_3COOH).
5. SØRENSEN'S phosphate buffer: (Stock solutions: (a) 0.2 M Na_2HPO_4 and (b) 0.2 M NaH_2PO_4) To prepare 100 ml of working buffer (0.1 M, pH 6.8), mix 24.5 ml of (a) with 25.5 ml of (b). Dilute to 100 ml with ddH_2O.
6. 10% Giemsa in SØRENSEN'S buffer: To prepare 100 ml, add 10 ml Giemsa stain to 90 ml of SØRENSEN'S buffer.
7. 1% HCl ethanol solution: To prepare 1 l, add 10 ml 37% HCl to 990 ml 95% ethanol.
8. 90% ethanol solution: To prepare 100 ml, add 10 ml ddH_2O to 90 ml 100% ethanol.
9. 70% ethanol solution: To prepare 100 ml, add 30 ml ddH_2O to 70 ml 100% ethanol.

2.3 Cell Culture Components

1. Human dermal fibroblasts HDMEC (PromoCell GmbH, Heidelberg, Germany).
2. T25 tissue culture flasks.
3. Dulbecco's modified Eagle's medium (DMEM).
4. Fetal bovine serum.
5. 0.05% tripsin-EDTA.
6. Polyprep glass slides.
7. Disposable Petri dishes (100 mm).
8. Lithium heparin BD Vacutainer® tubes with BD Hemogard® closure (Becton-Dickinson, Franklin Lakes, NJ, USA).
9. PB-MAX karyotyping medium (Invitrogen-Gibco, Paisley, UK).

2.4 Immunofluorescence (γ-H2AX Assay) Components

1. 1× phosphate-buffered saline (PBS): To prepare 1 l, add 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 to 800 ml deionized H_2O. Adjust pH to 7.4. Adjust volume to 1 l with deionized H_2O. Sterilize by autoclaving and store at room temperature.
2. 4% formaldehyde: 10 ml 37% formaldehyde dilute in 90 ml PBS. Prepare fresh (see Note 3).
3. 0.2% Triton-X: Add 200 μl Triton-X (Sigma-Aldrich Co., Steinheim, Germany) to 100 ml deionized PBS. Store at 4°C.
4. 1× TBS-T (Tris-buffered saline Tween-20) buffer: To prepare 1 l, add 8.8 g NaCl, 0.2 g KCl, 3 g Tris-base, and 500 μl Tween 20–800 ml ddH_2O. Adjust pH to 7.4. Adjust volume to 1 l with ddH_2O. Sterilize by autoclaving. Store at 4°C (see Note 4).
5. 0.5% BSA: Dissolve 0.05 g bovine serum albumin (BSA, Sigma-Aldrich) into 10 ml deionized PBS.

6. Primary antibody: Anti-phospho-H2AX (Ser 139) mouse monoclonal antibody (Upstate Cell Signaling Solutions), dilute in 0.5% BSA (v/v = 1:500).
7. Secondary antibody: Antimouse fluorescein isothiocyanate (FITC) antibody, dilute in 0.5% BSA (v/v = 1:400).
8. 4,6-Diamidino-2-phenylindole (DAPI)-containing antifade solution (Vector Laboratories Inc., Burlingame, CA, USA).
9. Coverslips (22 × 50 × 0.13 mm).
10. Clear, nonfluorescent nail varnish.

3 Methods

3.1 Cell Culture-Human Blood Cells

1. Collect fresh blood by venepuncture in 6 ml lithium heparin BD Vacutainer® tubes with BD Hemogard® closure. Store blood at 4°C prior to procedure.
2. Aliquot PB-max karyotyping medium (Invitrogen-Gibco, Paisley, UK), 4.5 ml in each 10 ml sterile tissue/culture test tube in laminar flow hood. Add 0.5 ml of whole blood. Close the cap of the test tube and put into the incubator.
3. Keep cell culture in an incubator at 37°C. One hour after the stimulation of cells, add the agent of interest to cultures: different volumes of carbon nanotubes dispersion (25, 50, 100, and 150 μl per 5 ml of total cell culture volume) and one untreated specimen as a control.

3.2 Micronuclei Preparation: Micronucleus Assay

1. Add 0.1 ml of the cytochalasin B solution after 44 h of culture, and then incubate for next 28 h.
2. Centrifuge cell suspension the next day (after 72 h of cell harvesting) at 500 × *g* for 10 min.
3. Remove the supernatant.
4. Resuspend pellet on vortex mixer and add pre-warmed hypotonic solution.
5. Keep in water bath for 5 min at 37°C.
6. Centrifuge at 500 × *g* for 10 min.
7. Remove supernatant up to 1 ml and add fixative solution on a vortex mixer to 12 ml.
8. Leave samples at room temperature for 30 min.
9. Repeat steps 6 and 7 until the suspension is clear.
10. After last centrifugation, aspirate up to 0.5 ml. Resuspend pellet with Pasteur pipette and prepare slides (37).

3.3 Preparing Slides for Micronucleus Assay

1. Degrease slides with detergent, wash thoroughly with distilled water, and keep over night in 1% HCl ethanol solution. Prior to use, wash slides with distilled water and bi-distilled water.
2. Onto clean, dry slides, put three drops of the cell suspension.
3. Air-dry the slides.
4. Stain slides (in staining jar) with 10% Giemsa in SØRENSEN's buffer (pH 6.8) for 10 min.

3.4 Slide Scoring for Micronucleus Assay

1. Score at least 1,000 binuclear (BN) cells per sample. Analyze slides with a microscope using magnification 400× or 1,000× when necessary.
2. Score a minimum of 1,000 binucleated cells to evaluate the percentage of cells with one, two, three, four, or more than four micronuclei.
3. Calculate a cytokinesis-block proliferation index (CBPI) as follows: $CBPI = MI + 2MII + 3(MIII + MIV)/N$, where MI–MIV represents the number of cells with one to four nuclei, respectively, and N is the number of cells scored (38).

3.5 Cell Culture-Human Fibroblasts

1. Incubate normal human dermal fibroblasts HDMEC in tissue culture flask for 48 h under standard tissue culture conditions in DMEM, supplemented with 10% of fetal bovine serum at 37°C and in the atmosphere of 10% CO_2.
2. When reach 80–90% confluence, remove growth medium from the flask by aspiration.
3. Incubate the flask with 1 ml 0.05% Trypsin-EDTA at 37°C for 4–7 min. Examine the flasks microscopically to make sure the cells begin to round. The cells should detach from the flask surface after 7 min (see Note 5).
4. Tighten cap and lightly tap the side of the flask to lift the remaining cells from the flask. Wash the sides of the flask with growth medium to inactivate the trypsin (see Note 6). Gently mix cells and medium. Pipette the cell suspension up and down so as to obtain a suspension of individual cells.

3.6 Immunofluorescence (γ-H2AX Assay)

1. Distribute 1 ml aliquots of the cell suspension to polyprep slides (see Note 7).
2. Transfer polyprep slides into Petri dishes (see Note 8).
3. Add appropriate dose of carbon nanotubes (0.5–30 μl per/ml) into cell suspension seeded on the polyprep slide. Close the dish and incubate at 37°C in a humid atmosphere for the next 24 h (see Note 9).
4. Wash slides in PBS for 5 min.

5. Fix cells in fresh 4% formaldehyde (see Note 3) for 15 min.
6. Permeabilize cells in 0.2% Triton-X at 4°C for 10 min.
7. Block reaction by transferring slides in 0.5% BSA/PBS for 30 min, at room temperature.
8. While blocking, prepare primary antibody.
9. Aspirate blocking solution and apply 100 μl diluted primary antibody to each slide. Incubate for 1 h in a light-tight damp container.
10. Wash slides three times in TBS-T for 3 min each.
11. Incubate slides with 100 μl secondary anti-goat antibody conjugated with Fluorescein isothiocyanate (FITC) for 2 h in a light-tight damp container.
12. Wash slides three times in TBS-T for 3 min each.
13. Fix cells with 70, 90, and 100% ethanol, 5 min each, and air-dry in the dark.
14. Counter stain cells with 15 μl 4′,6′-diamidino-2-phenylindole (DAPI)-containing antifade solution and cover with coverslips. Apply nail varnish to seal the samples.

    For the best results, examine specimens immediately. For long-term storage (several weeks), store slides at 4°C protected from light.
15. At least 200 cells should be analyzed to evaluate the number of γ-H2AX positive foci by using the microscope with illuminator for fluorescence microscopy and the computer software ImageJ.

4 Notes

1. Store at –20°C.
2. Make it prior to use. Use double distilled water.
3. Formaldehyde is toxic; use only in fume hood.
4. Adjustment of pH should be performed with 1 N HCl.
5. If the cells do not become detached after 7 min, incubate an additional 1–2 min.
6. Add growth medium at volume equal to or greater than volume of trypsin added.
7. Sterile pipette must be used.
8. One polyprep slide per Petri dish.
9. All subsequent incubations should be carried out at room temperature unless otherwise noted.

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