

Toxicity of Carbon Nanotubes to *p21* and *hus1* Gene Deficient Mammalian Cells

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Carbon nanotubes including single wall and multi wall carbon nanotubes (SWNT and MWNT) are attractive nanomaterials with great promise in industrial and medical applications. However, little is known about the role of *p21* and *hus1* gene in the toxic response of SWNT and MWNT to mammalian cells. The aim of this study is to investigate the role of the *p21* and *hus1* genes in the toxicity of carbon nanotubes. Comparison of micronucleus fraction between the wild type and *p21*^{-/-}, *hus1*^{+/+} mouse embryo fibroblast (MEF) cells was performed experimentally. Our results show that the yield of the micronucleus ratio in *p21* gene knock-out MEF cells is lower than that in the wild type counterpart, indicating that *p21* may play as anti-apoptosis factor during the signal transduction of DNA damage caused by carbon nanotubes in mammalian cells.

Keywords: SWNT, MWNT, Genotoxicity, *p21* Gene, Antiapoptosis.

1. INTRODUCTION

Carbon nanotubes (CNTs) including single wall and multi wall carbon nanotubes (SWNT and MWNT) have shown great promise as an important new class of multifunctional building blocks and innovative tools in a large variety of applications ranging from nanocomposite materials to biomedical devices. Production of carbon nanotubes has significantly increased during the past decade and they constitute a new potential source of airborne nanosized particles. The general public, especially industrial workers and researchers who work with pristine CNTs, may be exposed to CNTs through inhalation, ingestion and skin uptake.¹

On account of the unique physicochemical properties such as large aspect ratio and high reactivity,^{2,3} the safety of CNTs has aroused attention in recent years. There is evidence that functionalized CNTs can be taken up and intracellularly transported by various types of mammalian cells.⁴ Massimo Bottini et al., found that MWNT induced T lymphocyte apoptosis.⁵ Lin Zhu and Dong Wook Chang et al. reported that MWNT was accumulated in mouse ES cells, induced apoptosis and mutation, and activated the tumor suppressor protein P53 within 2 h of exposure.⁶

RESEARCH ARTICLE

SWNTs have also been shown to inhibit cell proliferation, decrease cell adhesive ability, and increase the ROS in a dose- and time-dependent manner.^{7,8} Although the cytotoxicity and genotoxicity of functional SWNTs or MWNTs have been investigated by quite a few groups, the toxicity of pristine CNTs at the gene level and the involved DNA damage repair pathway are largely unknown.

Eukaryotic cells have developed several mechanisms to protect genomic integrity under adverse conditions. For example, the cell cycle checkpoints are activated by *p53*, *p21* and *hus1* gene allowing repair of the damaged DNA.⁹ To investigate the role of *p21* and *hus1* gene in the genotoxicity of pristine SWNTs and MWNTs, wild type, *p21* single knockout (*p21*^{-/-}, *hus1*^{+/+}), and *p21*, *hus1* double knockout (*p21*^{-/-}, *hus1*^{-/-}) mouse embryo fibroblast (MEF) cells are used in the present study. The activity of mitochondrial dehydrogenase and the formation of micronucleus are also determined.

2. MATERIALS AND METHODS

2.1. Preparation of SWNTs and MWNTs

The SWNTs and MWNTs were purchased from SES Research (Cat no. 900-1301 and 900-1199). The SWNTs have a diameter of approximately 2 nm and a mean length

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of about 5–15 μm . The outer diameter of the MWNTs is less than 10 nm and the length is about 5–15 μm . These carbon nanotubes were dispersed in absolute ethanol with concentrations of 500, 50, 5 and 0.5 $\mu\text{g}/\text{ml}$. All the stocked solution was stored at 4 $^{\circ}\text{C}$.

The stock solutions were ultrasonicated for 3 h before using. The stock solutions were diluted with fresh cell culture medium to final concentrations of 20, 10, 1, 10^{-1} , 10^{-2} , and 10^{-3} $\mu\text{g}/\text{ml}$ immediately.

2.2. Cell Culture

Wild type, (*p21*^{-/-} *hus1*^{+/+}) and (*p21*^{-/-} *hus1*^{-/-}) MEF cells (provided by Institute of Biophysics, CAS) were maintained in a Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Cat no. 12200-046) with 15% Fetal Calf Serum (FCS, Hyclone Cat no. SV 30087.02) and 1% pen strep Glutamine (Gibco 10378). The cultures were maintained at 37 $^{\circ}\text{C}$ under 95% air and 5% CO_2 . The culture medium was changed every 3 days until the cells reached 80–100% confluence.

2.3. Determination of Cytotoxicity

The relative cytotoxicity of pristine CNTs was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) viability assay against MEF cells. The cells were seeded in the 24-well plates at 50,000 cells per well in 1 ml complete DMEM complete medium at 37 $^{\circ}\text{C}$ and in a 5% CO_2 atmosphere for 24 h. Different doses (10, 1.0, 0.1 and 0.01 $\mu\text{g}/\text{ml}$) of CNTs were added to the cultured medium and incubated for another 24 h. At the end of the treatment period, the medium was removed, 150 μl of MTT stock solution (5 g L^{-1} in PBS) was added to each well to achieve a final concentration of 1 g L^{-1} , and the cultures were incubated for another 4 h. The supernatant was removed and 1 ml acidic isopropanol was added to dissolve the formazan crystals which were converted by the catalysis of mitochondrial dehydrogenase from a water-soluble tetrazolium salt. The absorbance at 570 nm was determined by a Microplate reader (SpectraMax M2, USA).

2.4. Induction of Micronucleus

Wild type and gene knockout (*p21*^{-/-} *hus1*^{+/+} and *p21*^{-/-} *hus1*^{-/-}) MEF cells were employed to determine the genotoxicity of SWNTs and MWNTs. The frequency of micronucleus formation was measured by the cytokinesis block technique.¹⁰ Cells growing exponentially on 60 mm petri dishes (Falcon, BD) were treated with graded doses of CNTs for 24 h and then trypsinized and inoculated on 35 mm petri dishes. Cytochalasin B (CB, Sigma, St. Louis, MO, USA) was added to the culture medium to the final concentration of 2.5 $\mu\text{g}/\text{ml}$ at 6 h post seeding. After further incubation for 60 h, the cells were

rinsed in PBS and fixed in methanol/acetic [9:1(v/v)] for 20 min, stained with 0.01% acridine orange for 5 min, and examined by a fluorescence microscope (Olympus, Tokyo, Japan). The micronucleus frequency was scored as the ratio of number of MN to the number of binucleated cells and at least 1000 cells were examined in each sample.

3. RESULTS

3.1. Uptake of CNTs by MEF Cells

The cells were treated with SWNTs at a final concentration of 1.0 $\mu\text{g}/\text{ml}$ for 24 h, then observed under the microscope (Olympus, Japan) and the results are shown in Figure 1. The black particles dispersed in the medium and on the Petri dish were the aggregates of SWNTs and MWNTs because of the large aspect ratio and high reactivity. The white arrow indicates a black cycle formed around the nucleus (Fig. 1(a)) and the cell membrane (Fig. 1(b)), indicating that SWNTs were uptook by cells and located in

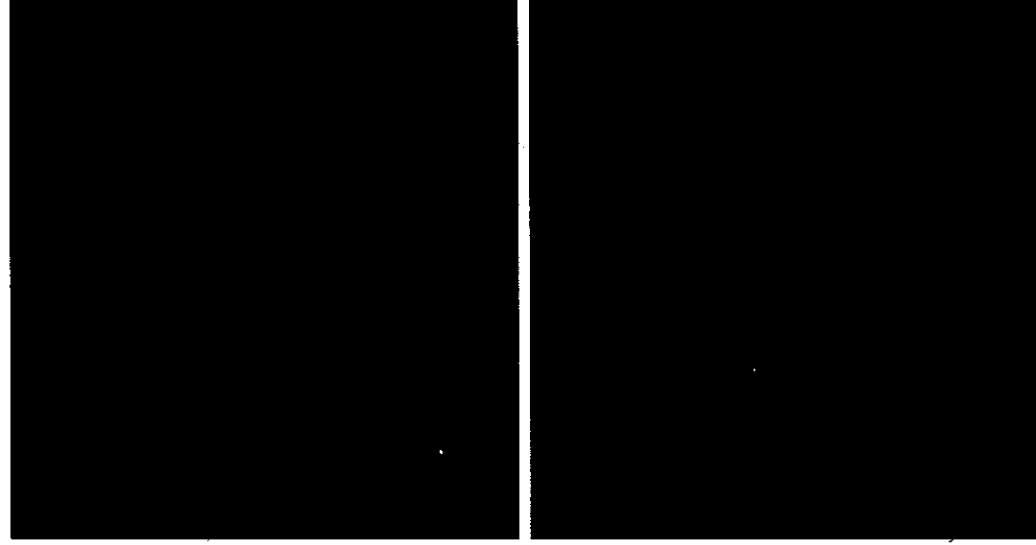


Fig. 1. Uptake of SWNTs (a) and MWNTs (b) by MEF cells.

the cytoplasm. This phenomenon provides proof of cellular uptake of SWNTs.

3.2. CNTs Induced Dose-Dependent Cytotoxicity in MEF Cells

The cytotoxicity of CNTs in the MEF cells is evaluated using MTT method. As shown in Figure 2, exposure of MEF cells to graded doses of either SWNTs or MWNTs ranging from 0.001 to 20 $\mu\text{g/ml}$ indicates a dose-dependent decrease in the cellular viability. The viabilities of MEF cells are reduced to 67.79, 60.63, 48.84 and 42.83% when the concentrations of SWNTs are 0.1, 1.0, 10 and 20 $\mu\text{g/ml}$, respectively. In contrast, the cytotoxicity of MWNTs is slightly lower. The viabilities of MEF cells exposed to MWNTs at concentrations of 0.1, 1.0, 10 and 20 $\mu\text{g/ml}$, are reduced to 85.59, 81.50, 68.19 and 60.52%, respectively.

3.3. MN Formation Induced by CNTs

To evaluate the genotoxicity of MEF cells induced by CNTs, MEF cells were exposed to graded doses of either SWNTs or MWNTs from 0.1 to 10 $\mu\text{g/ml}$. The MN ratio of the MEF treated with ethanol only was defined as a background. All data were normalized for comparison. There is an increasing tendency in the MN ratio in the cells treated with SWNTs. A significant increase is observed at the concentration of 1.0 $\mu\text{g/ml}$, which is 1.63 times higher than that of the control group ($p < 0.05$). The average values of the MN ratio in the MWNT treated group are higher than that of the control, but this difference is not significant. These data indicate that SWNTs are genotoxic to MEF cells, while the genotoxicity of MWNT cannot be determined from the MN level.

3.4. Comparison of Micronucleus Formation in Wild Type and Gene Knockout MEF Cells

In order to determine the roles of *p21* and *hus1* genes in the genotoxicity of SWNT, the yields of micronucleus induction by 1 $\mu\text{g/ml}$ SWNTs in the wild type, *p21*^{-/-}, *hus1*^{+/+} and *p21*^{-/-}, *hus1*^{-/-} MEF cells are determined and the results are displayed in Figure 4. The yield of MN in *p21* knockout MEF cells exposed to SWNTs is lower than that in wild type. This increase of the MN ratio is significantly different indicating that *p21* may be involved in the DNA damage signal transduction caused by SWNTs.

The yields of micronucleus in *p21*^{-/-}, *hus1*^{+/+} and *p21*^{-/-}, and *hus1*^{-/-} MEF cells treated by SWNTs are also shown in Figure 4. Compared to the *p21* single knockout counterpart, the increase is not significant. It is

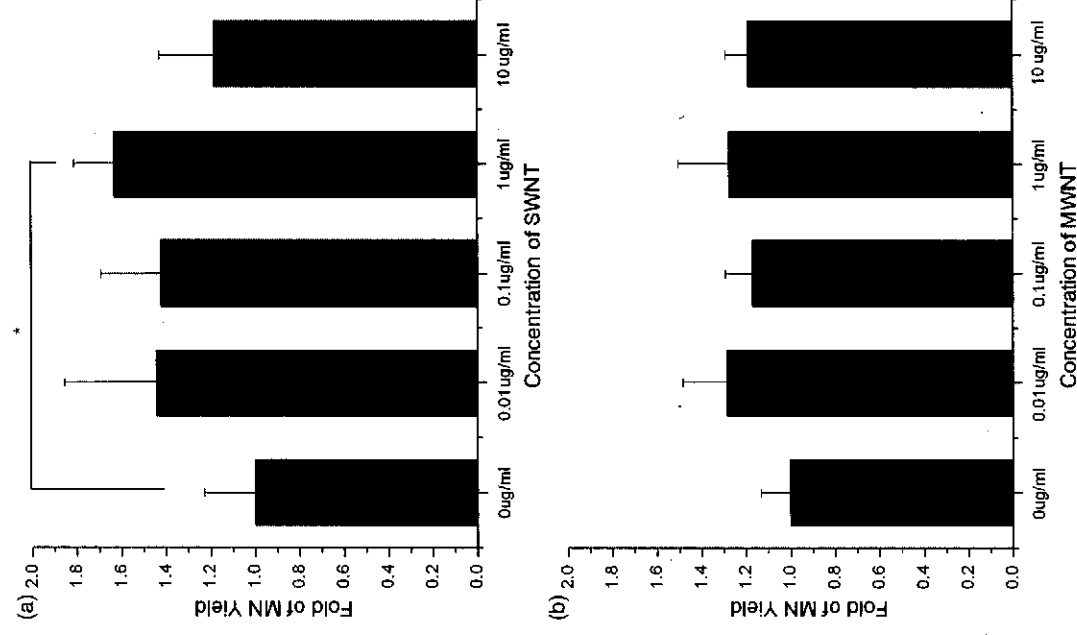


Fig. 3. Increase of micronucleus in MEF cells after 24 h treatment of SWNTs (a) and MWNTs (b). Data are average at least three independent experiments. * $P < 0.05$.

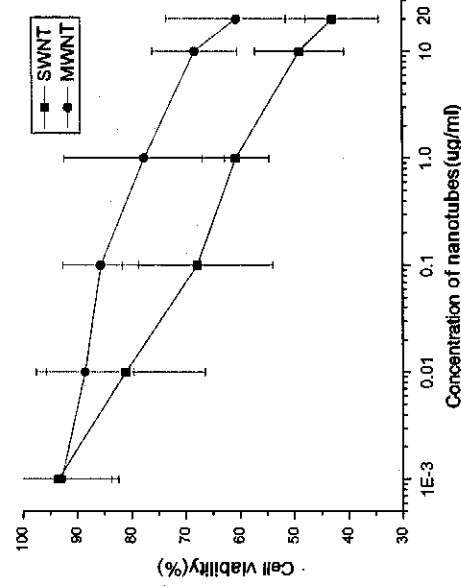


Fig. 2. Cell viability of MEF cells treated with graded doses of CNTs for 24 h. Data are the average of at least three independent experiments. Error bars indicate \pm SD.

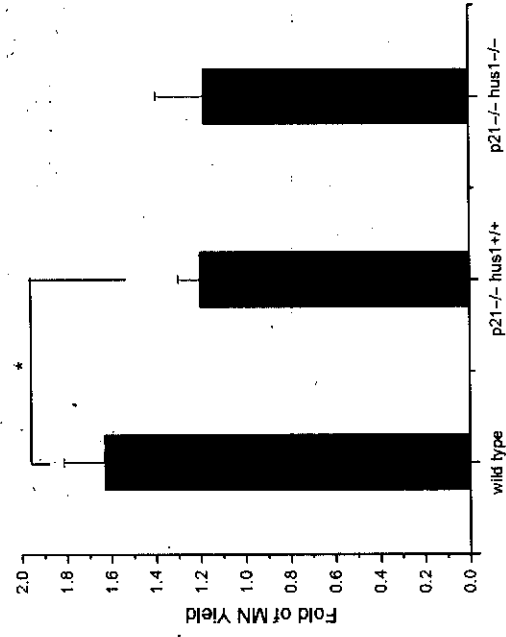


Fig. 4. Comparing the increase of Micronucleus (MN) yield induced by 1 $\mu\text{g/ml}$ SWNTs in wild type, *p21*^{-/-}, *hus1*^{+/+} and *p21*^{-/-}, *hus1*^{-/-} MEF cells. All the experiments are repeated at least three times. **P* < 0.05.

considered that the *hus1* gene may not be related to DNA damage or repair signal transduction.

4. DISCUSSION

SWNTs and MWNTs have been reported to be absorbed by mammalian cells in a way similar to asbestos. These materials can induce cytotoxicity and genotoxicity after uptake and the cytotoxicity of SWNTs is more profound than that of MWNTs at the same dose.¹¹ Our results are concordant with previous work. Both the cell viabilities and formation of micronucleus in the MEF cells caused by SWNTs are higher than those caused by MWNTs at the same dose, suggesting that the cytotoxicity and genotoxicity of SWNTs may be higher than those of MWNTs.

Protein P21, which is a DNA-damage-inducible cell-cycle inhibitor, acts as an inhibitor of the SAPK group of mammalian MAP kinases and can participate in regulating signaling cascades that are activated by cellular stresses such as DNA damage.¹² The P21 expression will increase to arrest the cell-cycle in the checkpoint if DNA damage is examined. P21 can also play as an anti-apoptosis role on the other hand. Stefania Mattiussi, Paolo Turrini et al., have reported that P21 expression in vascular cells is important to the inhibition of apoptosis *in vitro*.¹³ It means that cell apoptosis can be suppressed in the presence of P21 protein. In other words, the number of apoptosis cells will increase if P21 protein is absent.

In our experiments, *p21* gene knock-out MEF cells are chosen. This kind of gene defective cells cannot express P21 protein normally. The MN formation frequency is lower in the *p21* gene knock-out group compared to the wild type MEF cells. Hence, *p21* may be acts as an anti-apoptosis factor during the signal transduction of DNA damage caused by SWNTs. In other words, more cells

undergo apoptosis after *p21* knockout, leading to decreased micronucleus yield. Further work is required to determine the apoptosis effect of wild type and *p21* knockout MEF cells induced by SWNTs.

Hus1 protein is one participator that works together with ATR and other additional proteins to generate the checkpoint signal.¹⁴ It is hypothesized that they can assemble into a heteromeric protein complex and load the checkpoint-sliding clamp onto chromatin at sites of DNA damage,¹⁵ but our results show that the *hus1* gene may not play an important role in the SWNTs induced MEF cells genotoxicity.

Surfactant, such as SDS is a popular dispersion medium to disperse CNTs in current conditions. However, most surfactants are harmful to cells although this way can disperse CNTs effectively. In order to get pristine CNTs without modification of the outerwall and to exclude the negative effect of surfactants, absolute ethanol is chosen as the dispersion medium. After ultrasonication for 3 h, the CNTs can be dispersed but the stability is poor. The CNTs can aggregate after quiescence for several minutes. The dimensions of the aggregated particles vary from several hundred nanometers to several micrometers (Data not shown here). The large variation of the particle dimension leads to bad biological stability directly and so a more effective method to disperse CNTs is need to be investigated.

5. CONCLUSION

The toxicity of carbon nanotubes including single wall and multi wall carbon nanotubes (SWNT and MWNT) is evaluated. Comparison of micronucleus fraction between the wild type and *p21*^{-/-}, *hus1*^{+/+} mouse embryo fibroblast (MEF) cells shows that the yield of the micronucleus ratio in *p21* gene knock-out MEF cells is lower than that in the wild type counterpart, indicating that *p21* may play an important role in the toxicity caused by single wall carbon nanotubes in mammalian cells.

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