

The Role of Carbon Nanotubes in Enhancing the Effects of Chemotherapeutic Agents in Cancer Treatment

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ABSTRACT

Nanotoxicology is an important field of study that fills the knowledge gap concerning the introduction of nanomaterials into human cells, their interactions with biological systems, and the assessment of their risks to living organisms. In this study apoptosis and topoisomerase II-mediated DNA breakages in human cervical cancer cells (HeLa cells) were effectively induced by the commonly used chemotherapeutic agent etoposide at a concentration of (75×10^{-6} M) during 6 hrs of exposure. The cytotoxic effect was significantly increased by the addition of (20 $\mu\text{g/ml}$) single wall carbon nanotubes (SW- CNTs) during the incubation time. The apoptosis of the cells was observed by analyzing intracellular active caspase-3 and the DNA fragmentation by combining the nanomaterials with the Etoposide. It was found that single walled carbon nanotubes significantly increased the apoptotic efficacy of chemotherapeutic drugs such as etoposide in the treatment of malignancies. Such a combination could lead to the development of novel hybrid drugs that contain both nanostructural materials as well as apoptotic substances for the treatment of chemo resistant cancers.

Keywords: cancer, carbon nanotubes, antitumor drug, combined chemotherapy.

INTRODUCTION

Carbon nanotubes (CNTs) with their unique physical and chemical properties hold great hopes for cancer targeting, imaging and drug delivery [1,2]. Nevertheless, besides precise targeting tumor and toxicity concerns, drug resistance remains a major obstacle for the treatment of advanced cancerous tumors. To illustrate this problem, we selected etoposide, as one of the most widely used chemotherapeutic drugs, which is a derivative of podophyllotoxin with apoptotic action, due to its ability to inhibit the topoisomerase II enzyme [3].

We propose here a new treatment strategy of cancer based on the combination of conventional drugs and nanomaterials, assuming that these nanostructures can act as bioactive molecules and promote drug action through specific interaction with the cellular structures. To verify this hypothesis, we studied the action of etoposide and CNTs on HeLa cancer cells as single and combined therapy. We observed highly increased anti-tumor activity of Etoposide-CNTs combination compared to the single administration of each agent, which suggests that the CNTs can alter the mechanisms of chemo-resistance in malign cells. We believe that this finding could lead to new treatment approaches and improvement of current cancer therapies by using both cytostatic and nanostructural materials (such as CNTs), which synergistically are more aggressive and possess greater curative rates in the treatment of cancer.

2 MATERIALS & METHODS

2.1 Nanoparticles Synthesis

Single wall CNTs were grown by Radio-Frequency Chemical Vapor Deposition on a Fe:Mo/MgO catalyst with methane as the carbon source. CNTs were freshly sonicated with the cell growth medium just before introducing them to the cell cultures. The concentration of the CNTs was determined by UV-Vis- NIR spectroscopy and Atomic Force Microscopy (AFM).

2.2 Cell Cultures & Treatment

Human cervix adenocarcinoma, HeLa, cells were obtained from the American Type Culture Collection. Cells were normally grown in 75 cm² flasks (density of 10⁶) with F-12K medium containing 10% fetal bovine serum (FBS), 1% penicillin (500 units/ml) and streptomycin (500 units/ml) at 37°C in 5% CO₂ atmosphere, the media was changed every 48 hours. For treatment the cells were seeded at a density of 1x10⁴ cells/well in 48 well plates; incubated with CNTs (20 µg/ml) for 24 hrs., then treated with (75 X 10⁻⁶ M) of etoposide and a vehicle as a control for 6 hours.

2.4 Cell Assessment

Cells were grown on 35 mm plates at a density of 25 X 10⁴ cells/dish; following incubation methods above, washed thoroughly with 10 mM phosphate buffered saline (PBS, pH 7.4) 3 times and then fixed with 10 % formaldehyde solution for 10 min, washed 3 times with PBS and stained with Methyl Green dye for 10 min. Monitored by light transmission microscopy using an Olympus BX 51 microscope.

2.5 Cell Viability Analysis

The percentage cell viability was measured by Trypan blue dye. The cells were cultured as described above. The cells were then dissociated from the bottom of the plate by trypsinization and transferred to 1.5 eppendorf tubes and centrifuged. Finally, 2 µL of 1 X Trypan blue dye was added to each sample and incubated for less than 5 min. The number of viable cells was counted using a hemocytometer.

2.6 Caspase-3 Assay

Biovision GaspGLOW Red Active Caspase-3 staining kit was used for the caspase-3 assay. The HeLa cells were incubated with and without the CNTs overnight. The assay agent was then added and incubation continued for additional 6 hrs. The cells were collected by scrapping and were transferred to 1.5 eppendorf tubes, incubated with 1 µl of the Red-DEVD-FMK for 1 hour at 37 °C with 5% CO₂, and then centrifuged for 5 minutes at 3000 rpm. The supernatant was removed and the cells were resuspended in 50 µl of buffer and centrifuged again. Finally, the cells were resuspended in 100 µl of the washing buffer and a few drops of the cell suspension were transferred to the microscopic slides in order for the brightness of the red stain to be measured and analyzed.

2.7 Flow Cytometry

Flow cytometry was performed by staining the cells with YO-PRO-I and PI dyes from a Vybrant kit # 4 (V-13243, Molecular Probes). The cells were seeded at a density of 1 x 10⁶ cells/ 25 cm² culturing flask. DMSO was used as vehicle control while etoposide was used as the positive control. After 6 hrs of additional treatment, cells were trypsinized and washed twice with cold PBS, pH 7.4. Aliquots of 1µl YO-PRO-I stock solution (component A) and 1ml PI stock solution (component B) were mixed per ml of cell suspension. After 30 min incubation at 4°C, the cells were analyzed using a BD FACS Calibur flow cytometer. Fluorescence emissions were measured at 515-545 nm for FITC using FL-1 PMT detector and 564-606 nm for PI using FL-2 PMT detector.

2.8 Statistical Analysis

All data were expressed as mean ± SD. Independent sample *t*-test was performed for 2 group comparisons. *P* value ≤ 0.05.

3 RESULT & DISSCUSION

The trypan blue assays revealed (Fig. 1) a relatively low percentage of dead cells in culture medium at level $2.26 \pm 0.45\%$, administration of CNTs alone led to the increase of the cellular death rate to $14.1 \pm 2.1 \%$. Compared to the control experiment, the CNTs increased the relative rate of the dead cells by $11.84 \pm 1.4 \%$. The death rate of the HeLa cells after the etoposide administration was $7.8 \pm 1.87 \%$ and therefore etoposide increased the death rate of HeLa cells by $5.54 \pm 1.34 \%$ over controls. The incubation of the HeLa cells with CNTs and etoposide at identical doses as when incubated with each of the two agents individually showed a significantly higher cell death rate of $49.1 \pm 1.5 \%$, resulting in an increase by $46.84 \pm 2.3 \%$ rate over controls (Fig. 1). Thus the combined effect was approximately 2.7-fold higher ($p < 0.05$) than the sum of individual effects of CNTs and etoposide ($17.38 \pm 1.2 \%$). The optical images of single HeLa cells in (Figure 2) illustrate that the administration of CNTs alone do not change the cell morphology compared to the control cells while the combined action led to a significant change of cellular morphology.

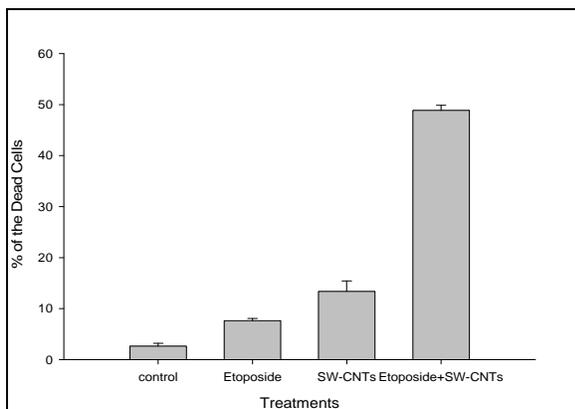


Figure 1: Percentage of dead HeLa cells after exposure to various agents (etoposide, CNTs, and their combination) as a control. The cells were incubated for 24 hours with CNT (20 mg/ml) and an additional 6 hours with etoposide ($75 \times 10^{-6} \text{M}$).

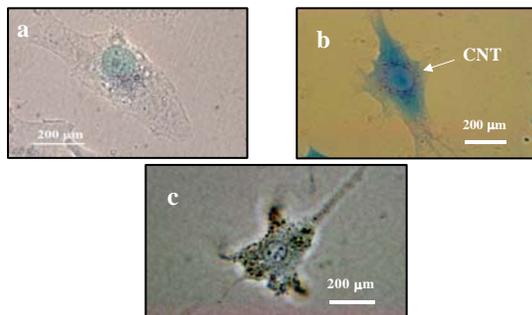


Figure 2: Optical image of a single HeLa cell morphology before (a), after (b) the delivery of CNTs alone ($20 \mu\text{g/ml}$, 24 hours). (c) Image of a HeLa cell that was exposed for 24 hours to CNT (5mg/ml) and 6 hours to etoposide ($75 \times 10^{-6} \text{M}$).

Figure 3 shows that the treatment of HeLa cells with etoposide and CNTs alone enhanced apoptosis as compared with DMSO (vehicle control) with more profound effects for etoposide. Both agents induced slight levels of necrosis, which is more significant for etoposide. However, the combined treatment of CNTs with etoposide increased the population of dead cells undergoing late stages of apoptosis or necrosis (upper left and upper right quadrants). Apparently CNTs enhance etoposide treatment by shifting early apoptotic cells into late apoptotic cells that further undergo necrosis. These results were confirmed by Caspase-3 activity (Method section). It became significantly brighter for the cells incubated with the CNTs alone (Figure 4) compared to the control samples.

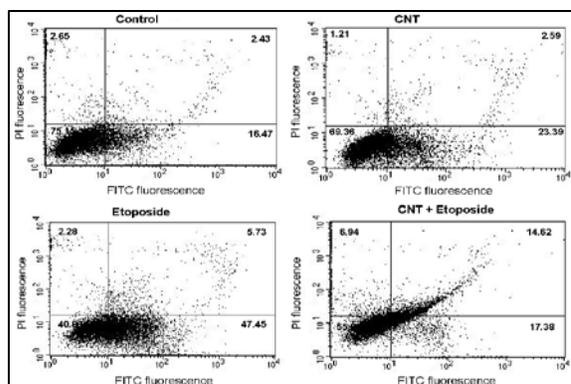


Figure 3: Flowcytometry data indicating the effect of DMSO (as vehicle control) ($75 \times 10^{-6} \text{M}$), CNTs ($20 \mu\text{g/ml}$, 24 hours) and etoposide ($75 \times 10^{-6} \text{M}$, 6 hours) as mono- and combined therapy of the HeLa cells.

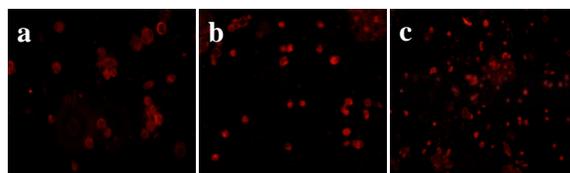


Figure 4: Caspase3 activity in HeLa cell cultures: (a) Control sample (unexposed to any agents); (b) samples exposed to CNTs ($20 \mu\text{g/ml}$, 24 hours), and (c) samples exposed to a combination of CNTs and Etoposide ($75 \times 10^{-6} \text{M}$, 6 hours).

DNA damaging agent can trigger cell death through activation of p53-mediated caspase cell death signaling cascade [4]. In the present study, the death rate of the untreated HeLa cells was 2.26%, this low response rate of etoposide alone is caused by the chemo-resistance mechanisms activated inside the cells [5]. It has been shown that resistance to etoposide is due to molecular changes that affect apoptotic cascade at different levels [6]. It has been shown that etoposide administration leads to the up-regulation of pro-apoptotic proteins such as Bax proteins [7]. These protein families translocate from the cytosol to the mitochondria being preceded by the release of the cytochrom-c that contributes to the induction of the mitochondrial permeability transition (MPT). This process is accomplished by the coupling of the DNA damages and leads to the necrosis of the cells. The action of some cofactors in the cytoplasm like Apaf-1 (apoptotic protease activating factor-1), ATP/dATP, as well as the presence pro-caspase-9, lead to the formation of the apoptosome complex in the cytoplasm, which activates caspase-9 that directs the activation of the caspase-3 proteins. Caspase-3 is one of 13 aspartate-specific cystein proteases that plays an important role in the execution of the apoptosis program and is primarily responsible for the cleavage of PARP (poly ADP ribose protease) during cell death and leads to the degradation and the fragmentation of the chromosomal DNA inside the nucleus and apoptosis inducement of the cell.

The first response of the cell in contact with CNTs is represented by the induction of antioxidant and detoxification enzymes followed by inflammation, which is a result of the activation of pro-inflammatory signaling cascade. Based on our funding some hypothetical processes with focus on apoptosis are depicted in Figure 5.

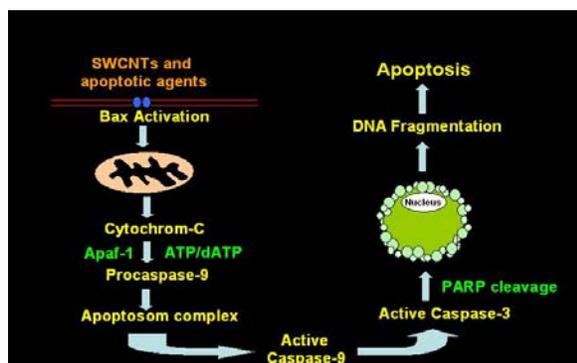


Figure 5: The proposed hypothetical mechanisms for Etoposide- CNT combined action for the induction of apoptosis, which leads to cellular death.

4 CONCLUSION

To our knowledge this is the first demonstration of the impressive synergistic therapeutic potential of the combination Etoposide - CNTs compared to the poor efficacy of etoposide as an apoptotic agent used individually. Based on these results, we believe that the CNTs initiate the apoptotic cascade via caspase pathways and interfere with resistance mechanisms at this level enhancing the apoptotic affect of etoposide. Eventually rapid shifting from early to late apoptosis and undergoing necrosis lead to cell death. Such a combined treatment could lead to the development of novel hybrid drugs that contain both nanostructural materials as well as apoptotic substances for the treatment of cancer.

5 REFERENCES

- [1] Liu Z, Chen K, Davis C, Sherlock S, Cao Q, Chen X, Dai H. Drug delivery with carbon nanotubes for in vivo cancer treatment. *Cancer Res.* 2008 Aug 15;68(16):6652-60.
- [2] Prato M, Kostarelos K, Bianco A. Functionalized carbon nanotubes in drug design and discovery. *Acc Chem Res.* 2008 Jan;41(1):60-8.
- [3] Kenneth D. Bromberg, Alex B. Burgin, and Neil Osheroff. A Two-drug Model for Etoposide Action against Human Topoisomerase II. *J. Biol. Chem.*, Vol. 278, Issue 9, 7406-7412, February 28, 2003.
- [4] Li Jia, Srinivasa M. Srinivasula, Feng-Ting Liu, Adrian C. Newland, Teresa Fernandes-Alnemri, Emad S. Alnemri, and Stephen M. Kelsey. Apaf-1 protein deficiency confers resistance to cytochrome c-dependent apoptosis in human leukemic cells. *Blood*, July 2001, vol. (98), no.2.
- [5] Reddy RK, Mao C, Baumeister P, Austin RC, Kaufman RJ, Lee AS. Endoplasmic Reticulum Chaperone Protein GRP78 Protects Cells from Apoptosis Induced by Topoisomerase Inhibitors. *Biol. Chem.*, Vol. 278, Issue 23, 20915-20924, June 6, 2003
- [6] J.I.-Hwan Hwang, J.U-Young Kim, M.I.-Ran CHA, I.N.-Ja Ryoo, Soo-J.I.N Choo, Sung-Min Cho, Yoshinori Tsukomu, Akihiro Tomida, Kazuo Shin-Ya, Yong-I.L. Hwang, I.C.K.-Dong Yoo, and Hae-Ryong Park. Etoposide-Resistant HT-29 Human Colon Carcinoma Cells During Glucose Deprivation Are Sensitive to Piericidin A, a GRP78 Down-Regulator. *J. Cell. Physiol.*, 215: 243-250, 2008.
- [7] Theocharis Panaretakis, Katja Pokrovskaja, Maria C. Shoshan, and Dan Grandér, *Activation of Bak, Bax, and BH3-only Proteins in the Apoptotic Response to Doxorubicin. J. Biol. Chem.*, 2002.