Role of Caveolin-1 in Carbon Nanotube-Induced Stem-Like Cells and Tumorigenesis

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ABSTRACT

Carbon nanotubes (CNT) hold great promise to create new and better products, but their long-term adverse health effects are a major concern. The objective of this study was to develop an *in vitro* model for mechanism-based risk assessment of CNT carcinogenesis. We tested the ability of single-walled (SW) CNT to induce cancer stem cells (CSC) as the underlying cause of carcinogenesis and determined the role of caveolin-1 (Cav-1) in the process. Our results demonstrated the induction of CSC or stem-like cells from human lung epithelial cells by chronic exposure to SWCNT. The CSC cells were found to overexpress Cav-1 and exhibit aggressive behaviors of enhanced cell invasion and migration. Overexpression of Cav-1 promoted CSC formation, cell motility and *in vivo* tumorigenesis in mice. Together, our findings support the use of CSC as a functional assay for carcinogenicity testing of CNT and indicate Cav-1 as a potential biomarker and drug target for CNT-induced carcinogenesis.

Keywords: carbon nanotubes, cancer stem cells, cell motility, tumorigenesis, caveolin-1

1 INTRODUCTION

Carbon nanotubes (CNT) are engineered high aspect ratio nanomaterials that are being produced on a massive scale for a wide variety of industrial and biomedical applications. Due to their growing usage trend, there is a great concern about their potential health hazards especially long-term carcinogenic effect. The high aspect ratio, mode of exposure, and biopersistence of CNT are similar to asbestos fibers, which is a known human lung carcinogen [1,2]. Although no human data are yet available at present, accumulating experimental data have indicated the potential carcinogenicity of certain nanomaterial such as single-walled (SW) and multi-walled (MW) CNT [3-6]. However, due to the diversity and complexity of CNT, e.g. differences in purity, fiber length, diameter, wall number, functionalization, and surface chemistry, it has been difficult to perform safety evaluation to predict which of these materials would potentially contribute to the disease pathogenesis.

Understanding the biological fate and pathological mechanisms of CNT could aid in their toxicological safety assessment. As evolving research have provided evidence for the existence of cancer stem cells (CSC) in various human solid tumors and their role in tumor initiation and progression [7-9], we postulated that CNT may induce CSC that contributes to carcinogenesis. In this study, we assessed the impact of chronic exposure of SWCNT on the induction of CSC in human lung epithelial cells, the primary cellular target of lung carcinogenesis and CNT exposure. We also investigated the role of caveolin-1 Cav-1), which is a known human lung oncogene [10,11], in the regulation of CSC and tumorigenesis.

Using molecular techniques, we demonstrated for the first time that SWCNT can interact with human lung epithelial cells to induce CSC and that these cells express a high level of Cav-1 that plays an important role in the regulation of CSC and tumorigenesis.

2 METHODS

2.1 Chronic SWCNT Exposure

Non-tumorigenic human lung epithelial BEAS-2B cells (ATCC, Manassas, VA) were continuously exposed to low-dose SWCNT (surface area dose of 0.02 μg/cm^2 or concentration dose of 0.1 μg/mL) in culture for 6 months. These cells, designated as BSW cells, were previously shown to exhibit malignant transformation and induce tumor formation in mice [5]. Parallel culture grown in SWCNT-free medium, designated as BC cells, provided a passage-matched control.

2.2 Detection and Isolation of CSC

CSC properties were assessed by tumor sphere formation and side population (SP) assays. Tumor sphere assay was performed under non-adherent and serum-free conditions as previously described as stem cell-selective conditions [7]. Briefly, 5×10^3 cells were suspended in 0.8% methylcellulose-based serum-free medium supplemented with 20 ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor, and 4 mg/mL insulin, and plated in ultralow adherent 24-well plates. Tumor sphere formation was assessed at two weeks after the culture.

For SP analysis, 1×10^6 cells were labeled with 5 μg/mL of Hoechst 33342 in Dulbecco's Modified Eagle Medium (DMEM)-F12 medium containing 2% fetal bovine serum (FBS) in the presence or absence of 25 μM ABCG2 inhibitor fumitremorgin C (FTC) at 37 °C for 90 minutes. The cells were then centrifuged and resuspended in ice-cold Hank's buffer salt solution (HBSS) and flow cytometry was performed using BD FACSAria (BD Biosciences, San Jose, CA). SP fraction was calculated based on the disappearance of SP cells in the presence of FTC using the formula: SP percentage in the absence of FTC − SP percentage in the presence of FTC.

CSC and non-CSC cells were isolated from BSW cells based on their SP phenotype using FACSAria cell sorter and were designated as SP and non-SP (NSP), respectively.

2.3 Cell Motility Assays

In vitro cell migration and invasion were determined using a 24-well Transwell® unit with polycarbonate (PVDF) filters (8-μm pore size) (Corning, Tewksbury, MA). The membrane was coated with Matrigel® for the invasion assay, while control non-coated inserts were used for the migration assay. Cells at the density of 3×10^4 cells per well (invasion) or 1.5×10^4 cells per well (migration) were seeded into the upper chamber of the Transwell® unit in serum-free medium. The lower chamber of the unit was filled with a normal growth medium containing 5% FBS. After 48 hours, non-migrating or non-invading cells were removed from the inside of the insert with cotton swab. Cells that migrated or invaded to the underside of the membrane were stained with 10 μg/mL Hoechst 33342 for 30 minutes and were counted under a fluorescence microscope. Cell migration and invasion was expressed as the ratio of migrated or invaded cells from the NSP and SP cells (relative level).

2.4 Plasmid and Transfection

Cells were transfected with Cav-1 or GFP control plasmid by nucleofection using Nucleofector® system (Lonza, Basel, Switzerland). Briefly, cells were suspended in 100 μL of nucleofection solution with 2 μg of plasmid and nucleofected using the device program T020. The cells were then resuspended in 500 μL of complete medium and seeded in 60-mm cell culture dishes. Cells were allowed to recover for 48 hours and were cultured for 28 days in G418-containing medium (800 μg/mL). The pooled stable transfectant was identified by Western blotting of Cav-1 and was cultured in the G418-free medium for at least three passages prior to use

2.5 *In Vivo* Tumorigenesis Assay

NOD/SCID gamma (NSG) (Jackson Laboratory, Bar Harbor, ME) mice were subcutaneously injected with 3×10^5 luciferase-labeled cells suspended in 100 μL of ExtraCel® hydrogel. Mice were inspected daily for any signs of distress such as weight loss, hunching, failure to groom, and red discharge from the eyes. Tumor growth of the injected luciferase-labeled cells was monitored weekly using IVIS® *in vivo* imaging system (Perkin Elmer, Waltham, MA). At the end of experiments, mice were euthanized and tumors were dissected for histological and biochemical analyses.

3 RESULTS

3.1 SWCNT Exposure Induces CSC

We have previosuly shown that chronic exposure of human lung epithelial BEAS-2B cells to subcytotoxic concentration of SWCNT induced malignant transformation and tumor formation in mice. However, the fundamental understanding of SWCNT tumorigenesis is unclear. In this study, we determined the existence of CSC in SWCNT-transformed cell population using tumor sphere formation and SP assays, which are the most stringent indicators of CSC. Figure 1A shows that the SWCNT-transformed BSW cells remained viable and formed large floating tumor spheres in high numbers after two weeks of culture under CSC-selective conditions. In contrast, minimal viable cells were observed in passage-control BC cells. Figure 1B similarly shows the difference in SP subpopulation between the SWCNT-transformed BSW and control BC cells (~15% *vs.* 3%). Together, these results indicate the existence of CSC and their derivation from non-tumorigenic BEAS-2B cells by chronic exposure to SWCNT.

3.2 CSC Display Enhanced Cell Motility

To determine whether CSC contribute to the aggressive cancer phenotypes of SWCNT-transformed BSW cells, we isolated CSC based on their SP phenotypes by flow cytometry and evaluated their aggressive behaviors by cell migration and invasion assays. Figure 1C shows that the CSC-like SP cells exhibited a significant increase in cell migratory and invasive activities as compared to non-CSC-like NSP cells. These results indicate that the CSC acquired increased cell motility, which is one of the most important features of aggressive cancer cells.

3.3 Cav-1 Promotes CSC

Cav-1 is a well-known lung cancer oncogene [10,11]. We observed that CSC-like SP cells expressed a high level of Cav-1 (data not shown). To study the potential role of Cav-1 in CSC regulation and tumorigenesis, we ectopically expressed Cav-1 in SWCNT-transformed BSW cells by gene transfection and studied its effects on tumor sphere formation and cell motility. Overexpression of Cav-1 resulted in the formation of large tumor spheres of CSC (Figure 2A). Comparing to vector-transfected BSW cells, the sphere size and number are much greater in the Cav-1-overexpressing cells. These cells also exhibited increased

migratory activity over parenteral BSW cells (Figure 2B). Altogther, these results indicate the involvment of Cav-1 in CSC regulation and cancer cell aggressiveness.

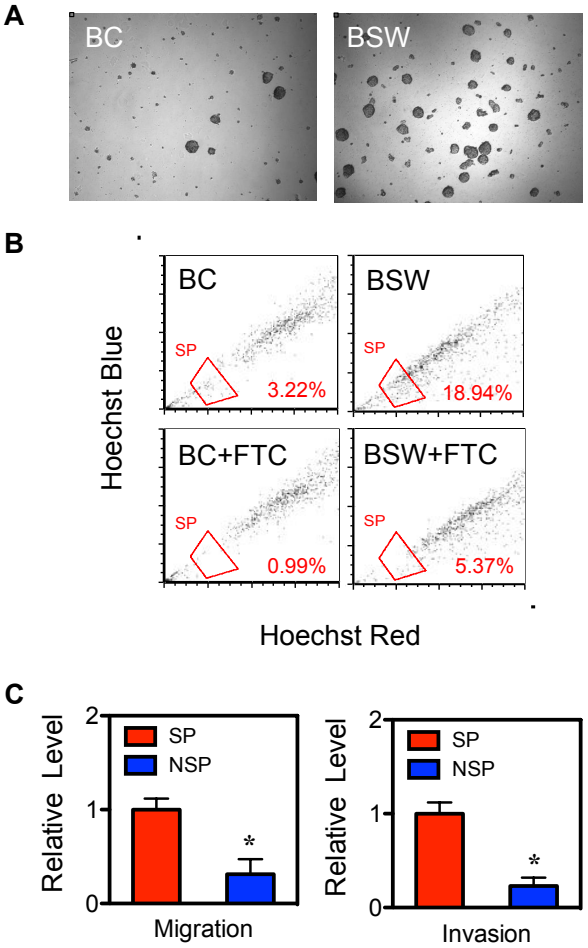

Figure 1: Chronic SWCNT exposure reprograms human lung epithelial BEAS-2B cells into CSC. (A) SWCNT-transformed BSW cells and passage-control BC cells were subjected to tumor sphere formation assay. Tumor spheres were visualized microscopically after two weeks of culture. (B) Analysis of SP in BC and BSW cells in the presence or absence of fumitremorgin c (FTC) using FACS. SP cells (box) were determined by their disappearance in the presence of FTC and were shown as a percentage of pool population. (C) CSC and non-CSC were isolated based on their SP phenotype and were designanted as SP and NSP cells, respectively. Cell migration and invasion were analyzed using Traswell chambers at 48 hours after incubation. Data are mean ± SD (n = 3). *p < 0.05 vs. SP cells.

3.4 Cav-1 Promotes Tumorigenesis

The role of Cav-1 in SWCNT tumorigenesis was assessed using luciferase-labeled xenograft transplantation in NSG immunodeficient mice. Figure 2C shows that overexpression of Cav-1 promoted tumor growth of SWCNT-transformed BSW cells as determined by IVIS® bioluminescence imaging. Tumor signals were significantly higher in mice bearing the Cav-1-overexpressing cells as compared to control mice receiving an equal number of parenteral BSW cells. These results indicate the positive regulatory role of Cav-1 on tumor growth of SWCNT-transformed BSW cells.

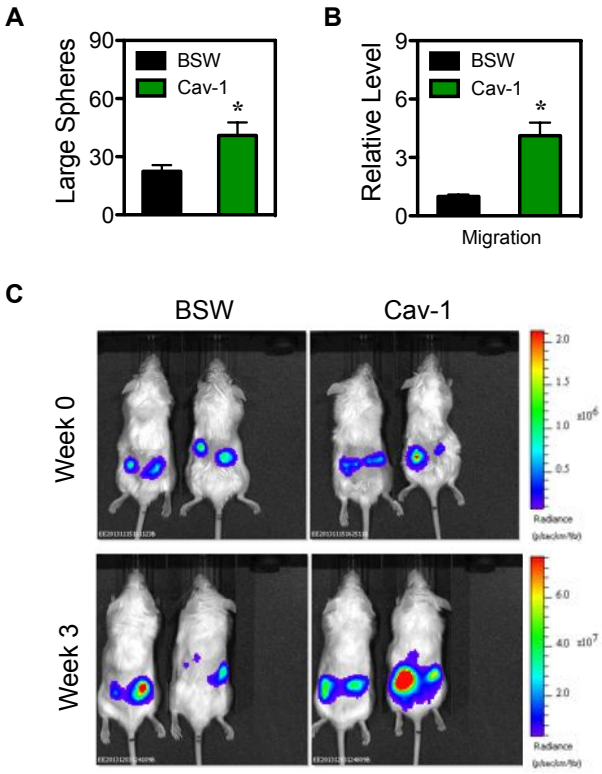

Figure 2: Role of Cav-1 in CSC regulation and SWCNT tumorigenesis. SWCNT-transformed BSW cells were stably transfected with Cav-1 or control plasmid. (A) Transfected cells were subjected to sphere formation assay and tumor spheres were scored after two weeks of cutlure. (B) Transfected cells were evaluated for cell migration at 48 hours after incubation in Transwell® chambers. (C) Transfected ells were genetically labeled with luciferase (Luc2) gene and were subcutaneously injected into the left and right flanks of NSG mice. Tumor growth was monitored by IVIS® bioluminescence imaging at 3 weeks postinjection.

4 CONCLUSION

In conclusion, the present study demonstrated that SWCNT are capable of reprogramming human lung epithelial cells into CSC-like cells and inducing tumorigenesis. We also demonstrated a positive regulatory role of Cav-1 on CSC regulation and tumor formation. Our findings suggest that detection of CSC may provide a valuable tool for early detection of CNT carcinogenesis and that Cav-1 might serve as a biomarker or drug target for CNT carcinogenesis.

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Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the national institute for occupational safety and health.

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