Anti-tumor response induced by immunologically modified carbon nanotubes and laser irradiation using rat mammary tumor model

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The ideal treatment modality for metastatic cancer would be a local treatment that can destroy primary tumors while inducing an effective systemic anti-tumor response. To this end, we developed laser immunotherapy, combining photothermal laser application with an immunoadjuvant for the treatment of metastatic cancer. Additionally, to enhance the selective photothermal effect, we integrated light-absorbing nanomaterials into this innovative treatment. Specifically, we developed an immunologically modified carbon nanotube combining single-walled carbon nanotubes (SWNTs) with the immunoadjuvant glycated chitosan (GC). To determine the effectiveness of laser irradiation, a series of experiments were performed using two different irradiation durations — 5 and 10 min. Rats were inoculated with DMBA-4 cancer cells, a metastatic cancer cell line. The treatment group of rats receiving laser irradiation for 10 min had a 50% long-term survival rate without residual primary or metastatic tumors. The treatment group of rats receiving laser irradiation for 5 min had no long-term survivors; all rats died with multiple metastases at several distant sites. Therefore, Laser+SWNT-GC treatment with 10 min of laser

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irradiation proved to be effective at reducing tumor size and inducing long-term anti-tumor immunity.

Keywords: Anti-tumor immune response; laser immunotherapy; single-walled carbon nanotubes; glycated chitosan; laser irradiation.

1. Introduction

Metastases continue to be one of the primary reasons for cancer patient deaths and treatment failures. The systemic and recurrent nature of cancers should be the primary focus when developing new treatment modalities against cancers. Recent studies suggest that the immune system can be activated against cancer cells.^{1–3} In particular, the secretion of cytokines, reduction of the tumor's defense systems, activation of dendritic cells, and proliferation of T and B cells, are of vital importance for inducing a systemic anti-tumor immunological response. However, the method of activating the immune system is one of the many challenges facing current immunotherapies.

Laser immunotherapy was developed for the systemic treatment of metastatic cancers by synergistically combining thermal laser irradiation with immunological stimulation.⁴ Studies have shown that thermal laser irradiation on cancer cells can immediately increase cytokine production and tumor antigen release, which could aid in activation of the immune system.⁵ The immunoadjuvant glycated chitosan (GC) was formulated to enhance the activation of the immune system.⁶ GC is biocompatible, water soluble, and capable of increasing the concentration of cytokines as well as activating immune cells.^{7,8} Additionally, GC can be used as a surfactant. With the synergistic combination of thermal laser irradiation and immunological stimulation, laser immunotherapy is capable of inducing an effective anti-tumor immunological response in late stage metastatic cancer patients.⁹

Recently, nanostructures have become increasingly popular in nearly all aspects of scientific research, particularly in drug delivery. One such nanostructure is the single-walled carbon nanotubes (SWNTs). SWNTs are composed of carbon atoms double bonded together creating a graphene single layered tube.¹⁰ The three-dimensional shape of the SWNTs allow the nanostructure to have an increased absorption of light at specific wavelengths.¹¹ Specifically, SWNTs are chiral and can be

synthesized in an (m, n) fashion, which influences their enhanced light absorption property. Furthermore, due to the polarity of the SWNTs and the increased mitochondrial membrane potential in cancer cells, the nanostructure can cross cell membranes and enter into the mitochondria of cancer cells.¹² Therefore, SWNTs can not only be used as a drug delivery vehicle, but also as a locally selective light absorption enhancing agent.

The goal of this work is to determine the efficacy of this novel nanotechnology-based laser immunotherapy on treating metastatic tumors, and to determine the effectiveness of laser irradiation on treating tumors injected with SWNT–GC. Furthermore, we conjugated the SWNTs to a nonbiological active surfactant, sodium cholate (NaC), to determine how thermal laser application alone induces anti-tumor reaction. Rats with an aggressive tumor model were intratumorally injected with SWNT–GC, and, 2 h later, received topical laser treatment on the tumor for 5 or 10 min.

2. Materials and Methods

2.1. Single-walled carbon nanotube

We used SWNTs produced by the CoMoCAT method obtained from Southwestern Nanotechnologies, Inc. (Norman, Oklahoma). The production of SWNTs using the CoMoCAT method was performed using a silica support and a bimetallic catalyst prepared from cobalt nitrate and ammonium heptamolybdate precursors. Total metallic loading in the catalyst was between 2 and 3 wt.%, with a Co:Mo molar ratio of 1:3. Prior to exposure to the CO feedstock, the catalyst was heated to 500°C and then to 750°C under a constant flow of helium gas. Utilizing a fluidized bed reactor under a flow of pure CO at 5 atmospheres total pressure, a CO disproportionation reaction was used to produce SWNTs. The material grown by this method remained mixed with the spent catalyst, containing silica support and the Co and Mo species. To remove the silica support from

this mixture, the solid product was suspended in a stirred 10–20 wt.% HF solution for 10 min to 3 h at 25°C. This suspension was then filtered through a polytetrafluoroethylene (PTFE) membrane with a pore size of 0.2 μm and washed with deionized water to a neutral pH.^{13–15}

2.2. Immunologically modified carbon nanotube

GC was prepared and obtained as previously described.⁴ 4 mg SWNTs were placed in a 50 mL test tube. Then, 8 mL of 0.5% GC was dispensed in the test tube. The mixture was placed in an ice bath and sonicated for 2 h at 30 min intervals, and then centrifuged at 10,000 rpm for 10 min. The supernatant was then collected and stored at 4°C.

2.3. Determination of chirality, diameter combination, and optical absorbance of SWNTs

Using a solution of SWNT–GC (3 mg SWNTs in 7 mL 0.5% GC), a one-dimensional emission spectra probed at 532 nm, 636 nm, 671 nm, and 783 nm wavelengths for excitation were collected using an NS2 Nanospectralyzer (Applied Nanofluorescence, LLC). These emission spectra were used to fit a model to a two-dimensional photoluminescence map in order to estimate the semiconducting chirality composition and diameter distribution of the SWNTs solution. Using a different SWNT–GC solution (15 mg SWNTs in 7 mL 0.5% GC) and its dilutions (10- or 100-fold), Raman spectra excited at 532 nm and UV-Vis optical absorbance spectra were also acquired using the NS2 equipment.

2.4. SWNT–NaC

About 2 mg of sodium cholate was completely dissolved in 98 mL of deionized H₂O to produce a 2% NaC solution. Then, 10 mL of the 2% NaC solution was dispensed into a 50 mL centrifuge tube containing 5 mg of SWNTs. The SWNT–NaC mixture was placed in an ice bath and sonicated for 2 h at 30 min intervals.

2.5. Animal treatment

A 0.1-mL solution of DMBA-4 cells (1×10^5) were injected into the flank region of 6–8 weeks old

female Wistar Furth rats. The rats were treated 10–14 days after tumor inoculation, when tumor volume was approximately 0.5–1 cm³. Tumor-bearing rats were separated into five different treatment groups: control $n = 12$, laser for 10 min only $n = 8$, SWNT–NaC with 10 min of laser irradiation $n = 12$, as well as Laser+SWNT–GC with 5 min or 10 min of laser irradiation, $n = 8$ and $n = 10$, respectively. 2 h prior to laser irradiation, the hair on the tumor was shaved and a 200 μL solution of SWNT–GC (100–1000 μg) was injected directly into the center of the primary tumor. Laser irradiation encompassed the primary tumor and 0.5–1 cm of the surrounding skin, and was delivered through an optical fiber noninvasively at 980 nm with a power density of 1.0 W/cm² for either 5 min or 10 min. The rats were anesthetized with isoflurane during treatment. The rats were observed and the primary tumor and metastatic tumors were measured twice a week after treatment. Surviving rats were re-challenged 100 days after treatment with an 0.1-mL injection of 1×10^6 DMBA-4 cells.

3. Results

3.1. Size distribution of SWNTs and optical absorbance

As shown in Fig. 1(a), the SWNTs had a (n , m) ratio distribution of: 23% (6,5), 21% (7,3), 9% (10,3), 9% (7,6), 8% (8,4), and 32% (other). The average diameter from fluorescence analysis of the SWNTs was 0.856 nm [Fig. 1(b)]. Due to the (n , m) configuration, SWNTs had an increased absorbance peak near 980 nm, which increased proportionally with the amount of SWNTs (Fig. 2).

3.2. Anti-tumor effects of treatment

SWNT–GC with laser irradiation for 5 min was capable of inducing complete primary tumor regression in one out of eight rats (12.5%) (Fig. 3). However, SWNT–GC with 10 min of laser irradiation was capable of inducing complete primary tumor regression in 7 out of 10 rats (70%) (Fig. 3). In addition, SWNT–GC with 10 min of laser irradiation was capable of inducing complete metastatic tumor regression at the left and right axillae (Fig. 4). Furthermore, rats treated by SWNT–GC with 10 min of laser irradiation had a 50% (5 out of

Fig. 1. Determination of chirality and diameter of SWNTs. Using a SWNT–GC solution (3 mg SWNTs and 7 mL 0.5% GC), one-dimensional emission spectra excited at 532, 636, 671, and 783 nm wavelengths were collected. The emission spectra were used to fit a model to a two-dimensional photoluminescence map (a) in order to estimate the semiconducting chirality composition and diameter distribution of the SWNT solution (b).

10) long-term survival rate, while Laser+SWNT–GC with 5 min of laser irradiation produced no long-term survivors (Fig. 5). Additionally, 10 min of laser irradiation alone was capable of inducing a 12.5% survival rate and SWNT–NaC with 10 min of laser irradiation was capable of inducing 22% survival

rate. A total of 100 days after treatment of all the surviving rats were re-challenged with 10 times the original amount of DMBA-4 tumor cells. All the surviving rats failed the re-challenge except the SWNT–GC with 10 min of laser irradiation, which had a 100% survival rate (figure not shown).

Fig. 2. Optical absorbency of SWNTs. Raman spectra of different SWNT–GC solutions were collected from NS2 equipment at 532 nm and UV-Vis optical absorbance. (a) Raman spectrum of solution with lower SWNT concentration (3 mg SWNT in 7 mL GC, diluted 10 folds); (b) Raman spectrum of solution with lower SWNT concentration (3 mg SWNT in 7 mL GC, diluted 100 folds). (c) Raman spectrum of solution with higher SWNT concentration (15 mg SWNT in 7 mL GC, diluted 10 folds); (d) Raman spectrum of solution with higher SWNT concentration (15 mg SWNT in 7 mL GC, diluted 100 folds).

Fig. 2. (Continued)

Fig. 3. Regression of primary tumors. Tumor-bearing rats were treated 10–14 days after tumor inoculation, when tumor volume was approximately 0.5 cm³. A 200- μ l solution of SWNT-GC (100–1000 μ g) was injected directly into the center of the primary tumor 2 h prior to laser irradiation, with a power density of 1.0 W/cm² for either 5 or 10 min. (a) Volumetric measurements of primary tumors receiving SWNT-GC and 5 min of laser irradiation. (b) Volumetric measurements of primary tumors receiving SWNT-GC and 10 min of laser irradiation.

Fig. 4. Regression of metastatic tumors. Primary tumors in rats were treated 10–14 days after tumor inoculation, when tumor volume was approximately 0.5 cm³. Untreated metastatic tumors were measured twice per week. (a) Left axillary metastases of rats receiving SWNT-GC with 5 min of laser irradiation. (b) Left axillary metastases of rats receiving SWNT-GC with 10 min of laser irradiation. (c) Right axillary metastases of rats receiving SWNT-GC with 5 min of laser irradiation. (d) Right axillary metastases of rats receiving SWNT-GC with 10 min of laser irradiation.

Fig. 4. (Continued)

Fig. 5. Overall survival rates. Primary tumors in rats were treated 10–14 days after tumor inoculation, when tumor volume was approximately 0.5 cm^3 . The treated rats were observed up to 100 days. Rats receiving Laser+SWNT-GC for 10 min had a 50% long-term survival rate, rats receiving Laser+SWNT-GC for 5 min had a 0% survival rate, rats receiving Laser+SWNT-NaC for 10 min had a 22% long-term survival rate, and rats receiving laser only treatment for 10 min had a 12.5% long-term survival rate.

4. Discussion

Immunotherapies are beginning to show promise in the treatment of cancers.^{3,4,7–9} When using laser irradiation as part of a cancer treatment, problems can arise when the tumor is deeply embedded in tissue or under heavily pigmented skin, as the laser light is not effectively absorbed and distributed evenly in the tumor.^{16,17} Treatment modalities utilizing laser irradiation require other methods to overcome such obstacles. There are many potential options, including absorption-enhancing dyes, invasive laser fibers, and nanomaterials. We believe that incorporating nanomaterials into laser immunotherapy could enhance the delivery and distribution of laser light. Other approaches, such as

interstitial laser irradiation, could further enhance laser immunotherapy.

Previously, laser immunotherapy utilized a laser-absorbing dye known as indocyanine green (ICG). A similar experiment utilizing Laser+ICG-GC and DMBA-4 tumor-bearing rats resulted in a 25% survival rate.¹⁸ The current study increased animal survival by taking advantage of the geometrical configuration of the SWNTs to allow better delivery and distribution of the 980 nm laser light. Additionally, this study investigated the anti-tumor effects of SWNTs induced when not conjugated to an immunoadjuvant and laser irradiation was administered. This study concluded that while the SWNTs enhanced the anti-tumor effect, they were not capable of inducing tumor immunity when the surviving rats from the SWNT-NaC with 10 min of laser irradiation were re-challenged with the DMBA-4 tumor cells.

One of the problems facing immunotherapies is the effective activation of the innate and adaptive arms of the immune system. One possible mechanism could be utilization of heat shock proteins (HSP). Studies show that HSP70, harvested from tumor cells, can induce tumor immunity in mice inoculated with identical tumor cells.¹⁹ Furthermore, an HSP gp96-engineered vaccine induced CD8⁺ T-cell anti-tumor response, as well as proliferation of natural killer cells in a large percentage of patients in a previous clinical trial.²⁰ Interestingly, various studies suggest that the mechanism of cell death is paramount to the release of HSP — necrotic cell death releases HSP while cell death via apoptosis may not.^{21,22} We believe that laser irradiation induces HSP release from tumor cells. By optimizing the laser irradiation parameters, we can

induce necrotic cell death, leading to a more robust immunological anti-tumor response. We believe the required time to induce these HSP at the power densities used in these experiments was more than 5 min. Furthermore, we believe this is the reason why anti-tumor effects were seen in all groups treated with a combination of laser irradiation for 10 min.

The effects of different durations of laser irradiation on inducing anti-tumor activities in the SWNT–GC laser immunotherapy system are very important. In this work, we demonstrated that 10 min of laser irradiation, as opposed to 5 min, at 980 nm with a power density of 1.0 W/cm^2 with administration of SWNT–GC as the light-absorbing immunoadjuvant, was capable of inducing regression of primary and metastatic tumors while also inducing long-term tumor immunity. We believe the longer duration of laser irradiation in this study yielded a higher temperature in the tumor, possibly inducing necrotic cell death, allowing for a more effective anti-tumor response. Furthermore, while the results suggest that Laser and Laser+NaC were capable of inducing tumor regression, the anti-tumor response was not strong enough to induce tumor immunity without the aid of an immunoadjuvant.

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