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The effect of hyaluronic acid functionalized carbon nanotubes loaded with salinomycin on gastric cancer stem cells

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ABSTRACT

Gastric cancer stem cells (CSCs) play a crucial role in the initiation, development, relapse and metastasis of gastric cancer because they are resistant to a standard chemotherapy and the residual CSCs are able to proliferate indefinitely. Therefore, eradication of this cell population is a primary objective in gastric cancer therapy. Here, we report a gastric CSCs-specifically targeting drug delivery system (SAL-SWNT-CHI-HA complexes) based on chitosan(CHI) coated single wall carbon nanotubes (SWNTs) loaded with salinomycin (SAL) functionalized with hyaluronic acid (HA) can selectively eliminate gastric CSCs. Gastric CSCs were identified as CD44+ cells and cultured in serum-free medium. SAL-SWNT-CHI-HA complexes were capable of inhibiting the self-renewal capacity of CD44+ population, and decrease mammosphere- and colon-formation of CSCs. In addition, the migration and invasion of gastric CSCs were significantly blocked by SAL-SWNT-CHI-HA complexes. Quantitative and qualitative analysis of cellular uptake demonstrated that HA functionalization facilitated the uptake of SWNTs in gastric CSCs while free HA competitively inhibited cellular uptake of SAL-SWNT-CHI-HA delivery system, revealing the mechanism of CD44 receptor-mediated endocytosis. The SAL-SWNT-CHI-HA complexes showed the strongest anti-tumor efficacy in gastric CSCs by inducing apoptosis, and in CSCs mammospheres by penetrating deeply into the core. Taken altogether, our studies demonstrated that this gastric CSCs-targeted SAL-SWNT-CHI-HA complexes would provide a potential strategy to selectively target and efficiently eradicate gastric CSCs, which is promising to overcome the recurrence and metastasis of gastric cancer and improve gastric cancer treatment.

1. Introduction

Gastric cancer (GC) remains the fourth most common cancer and the second leading cause of cancer-related mortality all over the world. There are about one million new cases per year worldwide and 850,000 deaths from the disease. Despite the development of surgery and chemotherapy, the overall 5-year survival rate of GC patients is only about 20% due to recurrence and metastasis [1].

Recently, cancer stem cells (CSCs) hypothesis has obtained increasing attention for its better explanation of the initiation of relapse and metastasis in several types of carcinomas including gastric carcinoma. CSCs are a subpopulation of cells with differentiation potential within a tumor that possesses the capability to sustain self-renewal [2]. Recent studies demonstrated that CSCs are responsible for tumor initiation, invasion, metastasis, recurrence and resistance to chemotherapy and radiotherapy. These CSCs provide a reservoir that can cause tumor recurrence and metastasis after therapy. Moreover, emerging clinical trial data show that the existence of CSCs is positively correlated with a high risk for recurrence and poor prognosis for survival [3]. Chemotherapy and radiotherapy are able to kill differentiated cancer cells but not undifferentiated CSCs, which remain in quiescence, have the ability of intrinsic detoxification, and locate within hypoxic niches or other mechanisms to help them escape these treatments [4]. CSCs have emerged as the new targets for anti-cancer therapy. In light of this, development of CSCs-targeting therapeutic strategy to eliminate CSCs has important clinical implications in cancer therapy.

It is therefore important to quickly and accurately identify gastric CSCs for targeted treatment. Several studies have identified CD44 as a cell surface marker of gastric CSCs, because only CD44+ cells from gastric cancer cell lines, tumor tissues and peripheral blood of GC patients possess cancer stem cell-like properties such

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as higher sphere-forming capacity in vitro, migratory and invasive potential, drug resistance capability, and tumorigenic ability in vivo [5–8]. Recent reports further indicated that CD44 expression in human gastric carcinoma specimens could serve as an independent prognostic indicator for tumor progression, metastasis and patient survival [9–11].Therefore, CD44+ subpopulation of gastric tumor cells can be targeted for the treatment of gastric CSCs.

A naturally occurring polysaccharide hyaluronic acid (HA) can specifically recognize its receptors CD44 and has been identified as a potent targeting ligand of tumors possessing CD44-overexpressing cells [12]. In recent years, hyaluronic acid (HA) has been extensively used as a targeting moiety in HA-drug conjugates [13], nanogels [14], microparticles [15], nanoparticles [16,17], liposomes [18], micelles [19], nanoclusters [20] and polymersomes [21] for cancer targeting drug delivery systems. Based on the fact that the CD44 receptor is expressed exclusively within gastric CSCs, we selected HA as a targeting moiety for gastric CSCs-targeting. Further, the HA coating provides a hydrophilic shield, similar to polyethylene glycol (PEG), for promotion of long blood circulation [22]. HA was also selected because of its favorable properties such as good biocompatibility, biodegradability, non-toxicity, and non-immunotoxicity [23].

Salinomycin, a polyether antibiotic isolated from *Streptomyces albus*, was recently identified as a selective inhibitor of human breast cancer stem cells, owning to that it can reduce the proportion of breast CSCs by more than 100-fold compared with paclitaxel, a commonly used breast cancer chemotherapeutic drug [24]. Salinomycin has been proven to be able to selectively deplete human breast CSCs from tumorspheres and inhibit breast cancer growth and metastasis in mice [24]. Furthermore, salinomycin has been reported as an effective agent for elimination of leukemia stem cells [25], endometrial CSCs [26], lung CSCs [27] and colorectal CSCs [28]. These findings strongly suggested that SAL may represent a class of agents for targeting CSCs. However, SAL was administered only by intraperitoneal injection in vivo with the aid of ethanol owing to its poor aqueous solubility [24], which limited its application. Nanoscale drug delivery system loaded with drug for CSCs was found to be more efficient to solid tumors compared to free drug [29]. Therefore, nanoscale drug delivery system can be developed to overcome the poor solubility as well as improve biodistribution of anti-cancer stem cell agent, being able to yield superior drug encapsulation and accumulation in tumors.

In recent years, functionalized single-walled carbon nanotubes (SWNTs) are emerging as promising drug delivery systems for cancer therapies due to their unique properties, including, water-solubility, remarkable cell membrane penetratability, high drug-carrying capacities, prolonged circulating times, selective retention in the tumor, reduced toxic effects and intrinsic fluorescent, photothermal, photoacoustic and Raman properties [30]. In spite of the prominence of SWNTs studies in the nanomedical areas, few papers have addressed the use of SWNTs as a CSCs-targeting vector to delivery drugs in anti-CSC therapy.

In the present study, we have constructed a gastric CSCs-targeting drug delivery system (SAL-SWNT-CHI-HA complexes) employing oxidized single-walled carbon nanotubes (O-SWNTs) as drug carriers, salinimycin (SAL) as anti-CSC agent and hyaluronic acid (HA) as targeting ligand for treatment of gastric CSCs. By utilization of high surface area of SWNTs, SAL was loaded on them with a high drug-loading content by non-covalent interactions. Then, the SAL-SWCNT complexes were non-covalently wrapped by chitosan (CHI) to improve aqueous solubility and biocompatibility. HA was also bounded to the outer CHI layer to realize selective killing of gastric CSCs expressing CD44. The objectives of the present study were to characterize the SAL-SWNT-CHI-HA complexes, to evaluate their CSCs-targeting and inhibitory effect on the CSCs and mammospheres, and to assess their effect on biologic characteristic in gastric CSCs.

2. Materials and methods

2.1. Preparation of SAL-SWNT-CHI-HA

2.1.1. Loading of SAL onto O-SWNTs

Purification, cutting and oxidation of SWNTs was carried out using a modified literature procedure [31]. Pristine SWNTs (95% in purity, 5–20 μm in length, 1–2 nm in diameter, obtained from Shenzhen Nanotech Port Co., Ltd., China) were purified via refluxing in HNO_3 (2.6 M, 200 mL) for 24 h in an oil bath in order to break down amorphous carbon, remove metal-containing residual catalysts and introduce acidic groups at defect sites along the sidewalls of the nanotubes. The purified SWNTs were dispersed in a mixture of 98% H_2SO_4 and 65% HNO_3 (3:1, V:V) and exposed to ultrasonication at 40 °C for 8 h. Afterward, the oxidized SWNTs solution was diluted with deionized water and filtrated with cellulose membrane (0.22 μm pore size, Shanghai ANPEL Instrument Co. Ltd), and washed with deionized water for several times until the pH value of filtrate reached neutral. The obtained O-SWNTs were then dried under vacuum at 60 °C for further use.

For the loading of SAL (Sigma–Aldrich, Beijing local agent, China) onto O-SWNTs, 50 mg of O-SWNTs was mixed with SAL 150 mg in anhydrous ethanol 3 mL under ultrasonication for 6 h. The solid was collected by centrifugation filtration at 9000 rpm for 30 min using membranes with a molecular weight cutoff of 100 K, and then dried under vacuum at room temperature for 30 min. The dried solid was dispersed in 10 ml of deionized water under ultrasonication for 6 h, and the solution obtained was filtrated through a membrane with pores of 5 μm diameter to remove free SAL aggregates. Finally SAL-loaded O-SWNTs (SAL-SWNTs) were collected by lyophilization of the filtrate.

In order to investigate whether the aggregates of free SAL in the aqueous solution can be removed by filtration through membranes with pores of 5 μm diameter, 10 mg of SAL solid from dry SAL solution in ethanol was dispersed in 10 ml of deionized water under ultrasonication (6 h). The solution was filtrated through membranes with pores of 5 μm diameter. The filtrate was lyophilized, and the solid obtained was determined to be negligible. This indicates that the aggregates of free SAL in aqueous solution could be removed by the filtration.

2.1.2. Preparation of SAL-SWNTs-CHI

The obtained SAL-SWNTs were non-covalently functionalized by chitosan according to the previous method [32]. Briefly, the SAL-SWNTs (10 mg) were stirred by ultrasonication in 10 ml ultrapure water for 30 min, then CHI (MW: 3000–5000 Da, deacetylated 75–85%, viscosity 50–100 m Pa s, obtained from TCI, Japan) solution (20 mg/ml in 0.1 M pH 6.0 sodium acetate buffer, 10 ml) was added and stirred for 24 h at room temperature. The supernatant containing SAL-SWNTs-CHI was collected by centrifugation for 3 h (20,000 rpm). Then, the solution was washed with 0.1 M sodium acetate buffer (pH 6.0) and filtered through an Amicon centrifugal filter device (100 kDa MWCO, Millipore) at 10,000 rpm for 30 min, repeated three times to remove unbound CHI. The recovered material was stored in ultrapure water. Finally, freeze-drying was performed to obtain solid SAL-SWNTs-CHI.

2.1.3. Preparation of SAL-SWNTs-CHI-HA

The SAL-SWNTs-CHI (20 mg) were suspended with HA (Sigma–Aldrich, Beijing local agent, China) solution (2 mg/ml in pH 7.4 PBS buffer solution, 20 mL) by sonication for 1 h. After stirring the reaction mixture at room temperature for 16 h, the product was washed with ultrapure water several times by repeated ultracentrifugation to remove unbound HA, then collected and dried at room temperature to obtain SAL-SWNTs-CHI-HA.

2.1.4. Preparation of FITC-labeled SWNTs-CHI-HA

FITC (Sigma–Aldrich, Beijing local agent, China) was used as the fluorescent probes to label SWNTs-CHI-HA. FITC (0.5 mg dissolved in 1 ml acetone) was added to O-SWNTs and stirred overnight at 4 °C. The reaction solution was centrifuged with an Amicon centrifugal ultrafiltration tube at 15,000 rpm for 5 min, and unbound FITC was removed by filtration and washed thoroughly with water (over 10 times). The unbounded FITC was collected and determined by UV-VIS absorbance spectra using an UV2800 spectrophotometer at 520 nm. Then the FITC-labeled SWNTs-CHI-HA (FITC-SWNTs-CHI-HA) complexes were prepared as the same procedures with those of SAL-SWNTs-CHI-HA.

2.2. Characterization of SAL-SWNTs-CHI-HA

The particle sizes, polydispersity indexes (PDI) and zeta potential values of all SWNTs were determined by dynamic light scattering (DLS) analysis using a Malvern Zetasizer Nano ZS (Malvern, U.K.).

The size, structures and morphology of various SWNTs, including pristine SWNTs, O-SWNTs, SAL-SWNTs, SAL-SWNTs-CHI, SAL-SWNTs-CHI-HA were evaluated using transmission electron microscopy (TEM; Hitachi H7650, Japan). Each sample (0.5 mg/ml) was resuspended in water and mixed by ultrasonication for 30 s. One drop of this suspension was placed over a carbon-coated copper TEM grid