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Study on glyco-modification of endostatin-derived synthetic peptide endostatin2 (ES2) by soluble chitooligosaccharide

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ABSTRACT

Soluble *O*-(2-hydroxyl)propyl-3-trimethyl ammonium chitooligosaccharide chloride (HTCOSC) was covalently conjugated to the 11-amino-acid peptide derived from amino terminus of endostatin (endostatin2, ES2, IVRRADRAAVP) to overcome its poor stability, low cell affinity and instable activity. Nuclear magnetic resonance spectroscopy and mass spectrometry was used to study the structure and molecular weight information. The anti-angiogenic activities were evaluated using cell counting Kit-8 assay, flow cytometry assay, wounding migration assay, transwell migration assay, chicken chorioallantoic membrane (CAM) assay and zebra fish angiogenesis assay. In contrast with ES2, the novel carbohydrate-polymer HTCOSC-ES2 displayed improved heat stability, higher cell affinity, better inhibition on endothelial cell proliferation, tube formation, 2-dimensional and 3-dimensional migration *in vitro*. According to the evaluation in CAM and zebra fish, HTCOSC-ES2 also displayed better anti-angiogenic activity than ES2 *in vivo*. These results indicate that HTCOSC has good properties as potential candidate for protein/peptide modifier and HTCOSC-ES2 has good potential in angiogenesis related diseases treatment.

1. Introduction

The growth of solid tumors beyond a few millimeters in diameter requires neovascularization (Folkman, 1972 O'Reilly et al., 1997) and one of the most potent negative regulators of angiogenesis is endostatin (Huang et al., 2001; Jung et al., 2002; Lim et al., 2013). It can effectively inhibit endothelial cell proliferation, migration and induce cell cycle arresting and promote cell apoptosis (Dhanabal et al., 1999; Dixelius et al., 2000; Hanai et al., 2002), thus endostatin could inhibit tumor growth and metastasis. As a broad spectrum antitumor drug, it has been used in clinical treatment, but several obstacles like poor stability, short half-time and instable biological activity *in vivo* affect its clinical effects (Folkman, 2006; Tan et al., 2012).

Several groups have reported that one 11-amino-acid peptide (endostatin2, ES2, IVRRADRAAVP) derived from the amino terminus of endostatin could dramatically inhibit angiogenesis, and its RGD peptide modified derivative could inhibit tumor growth in mouse model (Pu et al., 2012; Tjin et al., 2005; Wickström, Alitalo, & Keski-Oja, 2004; Xu, et al., 2008a; Xu, et al., 2008b; Zhou et al., 2009). But its poor stability, low cell affinity and instable activity limit its wide application. After development for decades, chemical modification has became a good way to tackle these disadvantages. Polyethylene glycol (PEG) was widely used as protein or peptide modifier because of its low toxicity and biocompatibility. But the conjugation of PEG could not bring additional bioactivity to the modified protein and the long-term use safety of PEG was also full of controversy because of its non-degradability *in vivo*.

Chitosan, the *N*-deacetylated derivative of chitin, has been widely used in medical field, because of its innocuity, biocompatibility, hemocompatibility and biodegradability (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Muzzarelli, 1993). Chitosan and its derivatives has also been reported to have anti-angiogenesis and anti-tumor activity by repressing endothelial cell proliferation and migration (Harish Prashanth & Tharanathan, 2005; Jiang, Han, Li, Yang, & Liu, 2015; Wu, Yao, Bai, Du, & Lin, 2008; Wu et al., 2012; Xiong et al., 2009; Xu, et al., 2008a; Xu, et al., 2008b), repressing tumor cell proliferation, inducing tumor cell apoptosis and enhancing immune systems (Maeda & Kimura, 2004; Pae et al., 2001; Yu, Zhao, & Ke, 2004). In this work, one soluble chitooligosaccharide, *O*-(2-hydroxyl)propyl-3-trimethyl ammonium chitooligosaccharide chloride (HTCOSC), was synthesized and was then used to mod-

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ify ES2 for the first time. After the conjugation of HTCOSC to ES2, better heat stability, higher cell affinity and anti-angiogenesis activity both *in vitro* and *in vivo* were expected. To study the glyco-modification of ES2 by HTCOSC, nuclear magnetic resonance (NMR) spectroscopy, matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS), cell counting Kit-8 (CCK-8) assay, wounding migration assay, transwell migration assay, chicken chorioallantoic membrane (CAM) angiogenesis assay and zebra fish angiogenesis assay were all used.

2. Experimental

2.1. Materials

Synthetic short ES2 peptide was purchased from China Peptides Co. Ltd (Shanghai, China). Chitooligosaccharide (the degree of deacetylation is 85.85%, the average molecular weight is 3000, and molecular weight polydispersity index is 1.17) was purchased from Jinan Haidebei Marine bioengineering Co. Ltd (Jinan, China). Fluorescein isothiocyanate (FITC), fibronectin, CCK-8, glycidyl trimethylammonium chloride (GTMAC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide sodium (NHS), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was purchased from Beijing solar bioscience&technology Co. Ltd (Beijing, China). Fetal bovine serum (FBS) was purchased from Hangzhou sijiqing biological engineering Co. Ltd (Hangzhou, China). Dulbecco modified Eagle medium (DMEM) was purchased from Gibco®, Life Technologies (Carlsbad, CA, USA). Matrigel matrix was purchased from BD (New Jersey, USA). Transwell Chambers, 96 well plates, 25 mL cell culture bottle and 15 mL centrifuge tube were purchased from Corning INC. (New York, USA). EAhy926 endothelial cells (ATCC Number: CRL-2922) were obtained from Shanghai Cell Bank, the Institute of Cell Biology, China Academy of Sciences (Shanghai, China). All other chemicals and reagents were of the highest commercial grade available.

2.2. Synthesis and characterization of HTCOSC and HTCOSC-ES2

2.2.1. Preparation and characterization of HTCOSC

HTCOSC was prepared according to a previously established method with small modification (Wan, Xu, Sun, & Li, 2013; Xu et al., 2016). Chitooligosaccharide (3 g) was dissolved in 120 mL of 10% (v/v) acetic acid aqueous solution, then 60 mL of anhydrous ethanol was added to the solution with stirring at room temperature for 30 min. After complete swelling, benzaldehyde (15.8 g, 15 mL) was added dropwise and then the solution was stirred for 1 h, till the reaction media became milky and colloidal. Then, reaction product was drying at 60 °C for 20 h, and the pH of reaction product was adjust to 7.0. After filtration, reactant was washed with methanol and then get dried. Then the rectant (2.75 g) was dissolved in isopropyl alcohol (50 mL). After that the solution was stirred for 30 min, and then GTMAC (9 g) was added in for a 16 h reaction at 70 °C temperature. After that, filtration, precipitation collecting, methanol and acetone washing, drying were carried out in sequence. Then the reactant(2.70 g) was collected and hydrochloric acid ethanol solution (50 mL, 0.25 mol/L) was added with stirring for 24 h at room temperature. Most solvents were removed using rotary evaporator. After excessive acetone was added in, HTCOSC was preliminarily obtained via filtering, precipitation collecting and drying and was finally purified by dialyzing against plenty of water, and lyophilized for two days.

The free amino content of HTCOSC was determined using the ninhydrin colorimetric method (Curotto & Aros, 1993; Xu et al., 2016). ^{1}H NMR spectrum was used to confirm the identity of HTCOSC using Bruker avance 600 MHz NMR spectroscopy.

2.2.2. Synthesis and characterization of HTCOSC-ES2

ES2 (25 mg) was dissolved in phosphate buffer solution (PBS) (25 mL, pH5.0, 10 mM), after EDC (80 mg) and NHS (20 mg) was added, the reaction was stirred moderately at room temperature for 30 min. HTCOSC (25 mg) was dissolved in PBS buffer (25 mL, pH 8.0, 10 mM). Then, the HTCOSC solution at the concentration of 1.0 mg/mL and the ES2 solution at the concentration of 1.0 mg/mL were mixed at room temperature for 6–8 h. Finally, the resulting product was purified using SephadexG-25 column, eluted with phosphate buffer (pH 6.0, 25 mM), at the flow rate of 1.0 mL/min and at detection wavelength of 254 nm. The elution was collected, dialyzed and lyophilized. MALDI-TOF-MS was used to study the molecular weight of HTCOSC-ES2.

2.2.3. Heat stability assay of HTCOSC-ES2

Polymer protein conjugates could obtain improved stability against heat (Pelegri-O'Day, Lin, & Maynard, 2014; Tan et al., 2008; Tao, Liu, & Davis, 2009; Tao, Liu, Xu, & Davis, 2009). In this study, to compare the heat stability of ES2 and HTCOSC-ES2, ES2 and its derivative were dissolved in 0.1 M phosphate buffer (pH 7.4) and incubated at 25 °C and 37 °C, respectively. During the incubation, samples were taken at 0 min, 15 min, 30 min, 45 min, 60 min, 75 min and 90 min and their inhibitory effects on endothelial cell proliferation were studied using CCK-8 assay. The concentrations of all groups were all 125 μg/mL. The percentage of retained activity at 0 min was set to be 100%, and the percentages of retained activity of other time points was calculated, respectively. All experiments were repeated three times.

2.3. Anti-endothelial cell proliferation assay and cell affinity assay

2.3.1. Inhibition of HTCOSC-ES2 on endothelial cell proliferation

The inhibitory activity of ES2 and its modified derivatives on endothelial cells were analyzed by testing the ability in inhibiting EAhy926 proliferation *in vitro* using CCK-8 colorimetric assay (Liu et al., 2015; Zhang, Zou, Yang, & Fu, 2016). EAhy926 cells in exponential phase were seeded in 96-well plate (1.0×10^4 cells/well), then incubated at 37 °C, 5% CO_2 for 24 h. ES2 group, HTCOSC-ES2 group and the mixture of HTCOSC and ES2 of different concentrations (concentrations of the ES2 part in three groups were all 25 μg/mL, 50 μg/mL, 75 μg/mL, 100 μg/mL and 125 μg/mL, and the mixture group was a 1:1 mixture of HTCOSC and ES2) were used to treat cells respectively, and then cells were further incubated for another 48 h. After CCK-8 reagent was added, absorbance at 450 nm were assessed. Each assay was repeated three times and all experiments were performed in five wells. The inhibition rate (IR) was calculated according to the formula below:

$$\mathrm{IR}(\%) = \left(1 - \frac{\text{Absorbance of treated group} - \text{Absorbance of blank well}}{\text{Absorbance of control group} - \text{Absorbance of blank well}}\right) \times 100\%$$

2.3.2. Cell affinity assay of HTCOSC-ES2

Flow cytometry was used to study the endothelial cell affinity of ES2 and its derivative. Firstly, peptides were labeled with FITC as previously described (Ishimoto, Nemoto, Nagayoshi, Yamashita, & Hashida, 2006; Liu et al., 2009). Mixed FITC (1 mg) and dimethylsulfoxide (DMSO, 1 mL) was added into ES2 or HTCOSC-ES2 solution dropwise with stirring at room temperature for 2 h. Sephadex G10 column was used to remove the free fluorescein. Secondly, EAhy926 cells were cultured in 12-well plate (3.0×10^4 cells/well), and 1 mL of serum free medium containing FITC labeled ES2 or HTCOSC-ES2 was added in. The concentrations of all groups were set to be 25 μg/mL, 75 μg/mL and 125 μg/mL. After an incubation of 2 h at