Acta Biomaterialia xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Manipulation of VEGF-induced angiogenesis by *2-N, 6-O*-sulfated chitosan

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ARTICLE INFO

Article history:
Received 12 November 2017
Received in revised form 17 February 2018
Accepted 26 February 2018
Available online xxxx

Keywords:
Sulfated chitosan
Vascular endothelial growth factor
Affinity
Angiogenesis

ABSTRACT

Emerging evidence suggests that vascular endothelial growth factor (VEGF) is important in the treatment of various ischemic and cardiovascular diseases. However, it often suffers from high cost and easy deactivation with a short half-life. Here, we describe a synthetic *2-N, 6-O*-sulfated chitosan (26SCS) with a high affinity to VEGF promoting the binding of the signaling protein to its VEGF receptor 2 (VEGFR2), activating receptor phosphorylation and pro-angiogenic related genes expression, and further stimulating downstream VEGF-dependent endothelial cell viability, migration, tube formation and rat aortic rings outgrowth. Interestingly, the obvious recruitment of mural cells were occurred to stabilize the sprouted microvessels. In addition, the pro-angiogenic potential of 26SCS composited VEGF was confirmed *in vivo* using the chick embryo chorioallantoic membrane (CAM) assay with an extensive perfusable vascular network. A longer monitoring was administered subcutaneously to mice in a biocompatible gelatin sponge and showed that VEGF with 26SCS had the capability to efficiently enhance neovascularization. These findings highlight that 26SCS, the semi-synthetic natural polymer, may be a promising coagent with VEGF for vascular therapy.

Statement of significance

Vascular endothelial growth factor (VEGF) is crucial for facilitating angiogenesis to supply oxygen and nutrient during wound healing and tissue regeneration. However, appropriate use of VEGF is an ongoing challenge due to its rapidly clearance and severe side effects at higher dosage. In this study, we described a synthetic *2-N, 6-O*-sulfated chitosan (26SCS) with a high affinity to VEGF, which could significantly promote its binding capacity to VEGF receptor 2 and further stimulate the angiogenic behavior of endothelial cells. We further confirmed that 26SCS was spatially combined with VEGF in a "lying manner", and this spatial arrangement was more conducive to exposure of the receptor binding domain of VEGF. Additionally, it also promoted *in vivo* angiogenesis in a chicken chorioallantoic membrane assay and mouse subcutaneous implant model. This strategy may afford a new avenue to enhance pro-angiogenic capacity of VEGF.

1. Introduction

The promotion of vascular network reconstruction to re-establish adequate perfusion in ischemic tissues remains a major challenge so far for tissue engineering and regeneration strategies, which is critical for preventing tissue necrosis and for survival of tissue damaged by hypoxia [1,2]. As the most potent angiogenic factor, VEGF is associated with stimulating endothelial cells proliferation, migration and sprouting to enhance new blood vessels formation [3,4]. However, an excess dose of VEGF was commonly adopted for treatment on account of its susceptibility to enzymatic and chemical denaturation in physiological environment, which often associated with severe side effects such as vasodilation and inappropriate blood vessel growth with the non-sequestered growth factor diffusing into the lymphatic system [5,6]. Therefore,

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https://doi.org/10.1016/j.actbio.2018.02.031
1742-7061/

focusing on improving the efficiency of VEGF with reduced dosage is imperative.

Nowadays, there is a general consensus in the literature regarding the effect of heparin on enhancing VEGF responsible for cell proliferation and differentiation, which mediate the formation of blood vessels [7–9]. However, the use of heparin as a therapy to potentiate the bioactivity of VEGF has been hindered by its limited availability and chemical heterogeneity, and often associated with anticoagulation and osteoporosis [10,11]. To address these issues, some studies have evolved to utilize sulfonated monosaccharide modified nanostructures with capacity to bind growth factors for the purpose of optimizing their function [12,13]. Unfortunately, the bioactivity of monosaccharide is low, and different sites of sulfonation and type of saccharide unit may lead to distinct results [14–16]. Recently, we designed a neo heparin-like polysaccharide (*2-N, 6-O*-sulfated chitosan, 26SCS) with alleviated side effects to induce VEGF-dependent pro-angiogenesis. This bioactive substitute has attracted popular interests for several advantages: (1) similar structure with heparin but more homogeneous, and higher sulfonation degree which represents a higher binding activity with VEGF [15,17], (2) cheap and abundant raw materials [18], (3) convenient synthesis and purification, and adjustable molecular weight [19]. Notably, our previous studies have demonstrated that 26SCS modified hierarchical scaffolds could efficiently promote vascularization during bone regeneration and enhance VEGF-mediated angiogenesis *in vitro* [17,20]. These observations raised interesting questions regarding the factors that enriched sulfate groups on 26SCS might have favorable effects on VEGF. However, the synergetic role is still vague; and up to now, few investigations have focused on the mechanism of 26SCS interacted with VEGF for pro-angiogenesis.

The present work is to aim at exploiting the affinity of 26SCS with VEGF, and evaluating its potential of pro-angiogenic activity. Cell-based angiogenic assays *in vitro* were carried out to evaluate the biological activity of VEGF influenced by 26SCS, such as cell viability, migration, sprouting, and aortic rings assay. Specifically, in order to elucidate whether the promotion of VEGF is owing to the integration of sulfated polysaccharide moiety, circular dichroism (CD) analysis was performed to measure the conformational change of VEGF, along with quartz crystal microbalance (QCM-D) to inspect the binding affinity between 26SCS and VEGF. Furthermore, we also demonstrated the conceivable mechanism of 26SCS on VEGF-mediated activation of receptors and expression of intracellular angiogenic-related genes. Finally, a chicken embryo chorioallantoic membrane examination and mouse subcutaneous implantation experiment were implemented to verify the potency of 26SCS synergized with VEGF-induced angiogenesis *in vivo*. We hypothesize this sulfated polysaccharide-binding approach may offer an effective strategy for manipulating the VEGF-induced angiogenic cascade and prove as a promising alternative for the treatment of ischemia disease.

2. Materials and methods

2.1. Materials

Chitosan (Mw: $\sim 30 \times 10^4$ Da, 92% deacetylated) was from Shenzhen Zhongfayuan Biological technology co., LTD. Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection. Recombinant human vascular endothelial growth factor (VEGF) was provided by PeproTech. Fluorescein isothiocyanate-phalloidin (FITC-phalloidin), and 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Growth factor reduced Matrigel™ Matrix and rat tail collagen type I were obtained from Corning Inc. (Corning, NY, USA). Trizol reagent, PrimeScript RT reagent kit and SYBR Premix Ex Taq™ were from Takara Biotechnology Co., Ltd. (Dalian, China). Gelatin sponge was purchased from Xiang'en Medical Technology Development Co., Ltd (Jiangxi, China). All cell-culture-related reagents were available from Gibco (Grand Island, NY, USA).

2.2. Cell viability assay

HUVECs were seeded at a density of 5×10^3 cells/well in 96-well plate, attached overnight in Dulbecco's modified Eagle's medium (DMEM, 10% FBS, 1% penicillin–streptomycin) at 37 °C, 5% CO_2, in a humidified environment. Subsequently, the medium was replaced with DMEM in the presence of VEGF (2 ng/mL) or VEGF plus a series of concentration of 26SCS for 3 days. After the incubation period, the cell viability was evaluated with a MTS assay according to the manufacturer's procedure. All samples were tested in triplicate and expressed as mean ± SD (n = 6).

2.3. Scratch wound assay

A scratch wound healing assay was performed to assess the migratory capacity of HUVECs induced by VEGF and 26SCS. VEGF (2 ng/mL) pre-incubated with various concentrations of 26SCS (0, 40, 160 ng/mL) were prepared and denoted as V2S0, V2S40 and V2S160, respectively. Pure culture medium without VEGF and 26SCS (denoted as V0S0) served as a negative control. Briefly, HUVECs were seeded in 96-well plates at a density of 1×10^4 cells/well and cultured to confluence in the complete medium. The scratch wounds were created by scraping the cell monolayer vertically with a sterile pipet tip. After washing twice with PBS to remove cell debris, experimental medium (V0S0, V2S0, V2S40, V2S160) containing 2% FBS were replaced to eliminate the effect of cell proliferation on cell movement. Afterwards, images were captured at 0 h and 18 h post-scratching under a light inverted microscope (TE2000U, Nikon, Japan). The infiltration area of migrated HUVECs was quantified by ImageJ software (n = 6).

2.4. In vitro sprouting analysis

In vitro sprouting analysis was conducted as previously reported protocol [20]. In brief, growth factor reduced Matrigel (100 μL) was pipetted into each well of 20 mm glass bottom culture dishes on ice, and then transferred to a cell culture incubator at 37 °C for 30 min to allow gelation. HUVECs incubated with different experimental medium (V0S0, V2S0, V2S40, V2S160) for 7 days in advance were detached by trypsin, and then seeded on the gel substrate at a density of 1×10^5 cells/well. Branching formation of tube-like structures labeled with FITC-phalloidin and DAPI were captured by confocal laser scanning microscopy (CLSM; Nikon, Japan) after incubation for another 4 h. Total capillary tube lengths and number of branch points per field were quantified using the ImageJ software. Sprouts were defined as a linear extension containing more than one cell.

2.5. Rat aortic ring angiogenesis assay

A rat aortic ring assay was conducted to test the effects of 26SCS enhanced VEGF-mediated angiogenesis *in vitro* as described previously [21]. Aortas isolated from 8 to 10 weeks old Sprague Dawley rats (Slac, Inc. Shanghai, China) were cleaned off periadventitial fat and sectioned into 1–1.5 mm long rings. The aortic rings were then embedded in 100 μL neutral rat tail collagen type I and incubated at 37 °C for 30 min to allow gelation. Afterward, the aortic rings were cultured with diverse experimental medium (V0S0, V2S0, V2S40 and V2S160) for 7 days. The outgrowthed cells of microves-

Please cite this article in press as: Y. Yu et al., Manipulation of VEGF-induced angiogenesis by *2-N, 6-O*-sulfated chitosan, Acta Biomater. (2018), https://doi.org/10.1016/j.actbio.2018.02.031