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Protective effect of *Astragalus* polysaccharide on endothelial progenitor cells injured by thrombin☆

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ABSTRACT

Several studies have demonstrated that Astragalus polysaccharide (APS) has a protective effect on endothelial cells damaged by various factors. To examine the role of APS in the endothelial inflammatory response, rat bone marrow endothelial progenitor cells (EPCs) were isolated by density gradient centrifugation and identified by immunohistochemistry, then we established a model of inflammatory injury induced by thrombin and measured the effects of APS on EPC viability and proliferation by MTT assays. We also assayed the effect APS had on the inflammatory response, by examining the nuclear factor kappa B (NF-κB) signaling pathway, as well as the expression of intercellular adhesion molecule-1 (ICAM-1), vascular endothelial growth factor (VEGF) and its receptors Flt-1 and KDR. Results demonstrated that EPCs were damaged by thrombin, and APS appeared to inhibit this damage. APS suppressed thrombin-induced ICAM-1 expression by blocking NF-κB signaling in rat bone marrow EPCs, and up-regulating expression of VEGF and its receptors. We believed that APS may be used to treat and prevent EPC injury-related diseases.

1. Introduction

Vascular endothelium integrity plays an important role in the maintenance of vascular function. Endothelial dysfunction and injury contribute to vascular damage by inducing an inflammatory response. Inflammatory responses are the common pathological basis for most ischemic diseases and in groups at high-risk for ischemic disease [1]. High-risk factors for ischemic diseases include smoking, hypoxia, hypertension, coronary heart disease, and diabetes. Endothelial progenitor cells (EPCs) primarily reside in the bone marrow with small populations present in peripheral blood. EPCs are not yet mature, but can proliferate and differentiate into vascular endothelial cells and form blood vessels. The main phenotypes of EPCs are characterized by the presence of CD31, CD34, eNOS, vWF, FLT-1 and KDR. In addition to the role of EPCs in microvascular neogenesis, this cell population is also a critical component used to repair damaged blood vessels and promote the functional recovery of ischemic-injured tissue [2].

A number of studies have revealed that most ischemia diseases exhibit a numerical and functional impairment of EPCs that is characterized by adhesion and proliferation of inflammatory factors, which results in deficient blood vessel formation and impaired angiogenesis [3,4]. Vascular endothelial growth factor (VEGF) acts as a strong mitogen for endothelial cells. VEGF stimulates division, migration, and invasion of the surrounding microenvironment. VEGF binds to the cell surface receptors – Flt-1 and KDR to activate the tyrosine kinase signaling pathway involved in vascular formation and maintenance of cell function [5]. Nuclear factor kappa B (NF-κB) is a ubiquitous transcription factor that binds and activates different NF-κB receptors in various tissues and cells [6]. Activation of the NF-κB signaling pathway stimulates endothelial cells to secrete a number of inflammatory factors including intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), interleukin-1 (IL-1), interleukin-8 (IL-8), P-selectin, and E-selectin [7]. These highly expressed adhesive molecules trigger an inflammatory cascade that aggravates endothelial damage [8]. Thrombin (TM) is one of the most important factors involved in thrombosis [9], and has a dual effect on the permeability of endothelial cells. At low concentrations, thrombin prevents thrombosis and maintains the integrity of the vascular endothelium. However, at high concentrations, thrombin may cause damage to

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the endothelial barrier, which can induce endothelial inflammatory lesions [10]. In addition, we have previously observed that thrombin promotes EPCs proliferation at concentrations lower than 25 U/ml, but significantly inhibits cell viability with higher doses [11]. For these reasons, there is an urgent need for drugs that promote EPC proliferation, activation, and subsequent repair of damaged vascular to prevent and treat ischemic diseases.

Several studies have demonstrated that Astragalus polysaccharide (APS) has a protective effect on endothelial cells damaged by various factors [12]. However, it is not understood whether APS can protect EPCs to ease the clinical symptoms and improve the prognoses of patients with ischemic diseases. To examine the role of APS in the endothelial inflammatory response, we established a model of inflammatory injury induced by thrombin and then measured the effects of APS on EPCs, which were isolated and identified similarly as discribed in our previous study [13]. We also assayed the effect APS had on the inflammatory response by examining the NF-κB signaling pathway and the expression of ICAM-1, VEGF and its receptors Flt-1 and KDR.

2. Materials and methods

2.1. Animals

4–6 week old specific pathogen-free Sprague-Dawley rats were purchased from the Shanghai laboratory animal center. The animals were housed in temperature-controlled environments, and fed with standard chow *ad libitum*. All experimental protocols involving animals were conducted in accordance with the guidelines approved by the ethics committee for the use of experimental animals in Zhejiang Chinese Medical University.

2.2. Isolation and culture of EPCs

Sprague-Dawley rats were sacrificed by cervical dislocation, and then immersed in 75% ethanol for 10 min. Their femurs and tibias were separated under sterile conditions, and the bone marrow cavities were flushed with IMDM (Jienuo, Hangzhou, China) to collect the cells. The individual cell suspensions were added to an equal volume of lymphocyte separation medium, and underwent centrifugation at 20 °C for 20 min at ×400 *g*. From here, the mononuclear cell (MNC) fractions were collected and washed three times with PBS (Jienuo, Hangzhou, China). The MNCs were resuspended in EGM-2MV culture medium (Lonza, MD, USA), adjusted to 1–2 × 10^6 cells/cm^2, and then seeded in 24-well plates that were coated with human fibronectin (Miilipore, USA) prior to the addition of the MNCs. The cells were then incubated at 37 °C with 5% CO_2 in a saturated humidity environment. After 72 h permitted for cell cultivation, the medium was changed for the first time, and then it was refreshed every 2 days thereafter. Primary EPCs were passaged at ratios of 1:2 or 1:3 using a 0.25% (w/v) trypsin–0.53 mM EDTA solution, and grown in IMDM supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA).

2.3. Identification of EPCs by SABC immunocytochemistry

3rd passage cells were washed three times with PBS, and fixed in 4% paraformaldehyde. The cells were then stained with rabbit anti-rat CD31 and CD34 polyclonal antibodies (1:100, Boster, Wuhan, China), followed by biotin-labeled goat anti-rabbit IgG, and lastly with SABC (Boster, Wuhan, China) for development. Primary antibodies were omitted for the negative controls. Cellular images were acquired under an inverted microscope (COIC, XPS-18, China). The imaged cells were counted in 10 random fields to calculate the proportion of positive cells, which displayed brown or brownish yellow membranes and cytoplasms.

2.4. Testing methods and dose groupings

Confluent EPCs were divided into five groups: a control group, an untreated thrombin-damaged group, or groups pre-incubated with APS (Haoyang, Tianjin, China) at high, intermediate, and low doses. According to the preliminary results and literature [14,15], we used 25 U/ml thrombin and 800 (high), 400 (intermediate), and 200 (low) μg/ml APS, all diluted with complete medium. Before injury with thrombin, EPCs were incubated with complete medium or various doses of APS for 24 h. Thrombin was added, and cells were cultivated for 8 h. Cell morphology was observed and photographed under the inverted phase contrast microscope coupled with a digital camera (OLYMPUS, BX20, Japan).

2.5. Determination of cell viability/proliferation

EPC viability and proliferation were determined by MTT assays. Cells were suspended at a final concentration of 1 × 10^4 cells/ml in 100 μl of complete medium, and cultured in 96-well flat-bottomed microplates. After, they were randomly divided into the five experimental and control groups in quintuplicate on three separate occasions. After exposure to APS and thrombin at the indicated concentrations for the indicated time courses, MTT (0.5 mg/ml) (Sigma, USA) was added to the wells. The cells were incubated for 4 h, and then 150 μl DMSO (Sigma, USA) was added to dissolve the formazan product. Viable cells were evaluated by absorbance measurements at 490 nm using an auto microplate reader (Thermo1001027S, USA). Cell viability was expressed in optical density (OD) values. Cell proliferation was expressed as a fold of untreated cells, which served as control.

2.6. Flow cytometry

The expression of ICAM-1 in EPCs was identifid by a PerCP-labeled antibody. Cells were seeded at a density of 1 × 10^5 cells/well in 6-well plates. After incubation and intervention, EPCs were washed with ice-cold PBS three times, trypsinized to a single cell suspension in 100 μl PBS, stained with 0.625 μl PerCP-labeled anti-ICAM-1 (eBioscience, CA, USA) for 30 min at 4 °C, and then analyzed by a flow cytometer (BD, FACSCanto™II, USA).

2.7. Western blot analysis

Cells were harvested and lysed using a total protein extraction kit (APPLYGEN, Beijing, China). The lysates were homogenized, and centrifuged at 12,000 rpm for 10 min at 4 °C. The resulting supernatants were then collected. Protein concentrations were determined by using a BCA kit (APPLYGEN, Beijing, China). The protein samples were separated by 10% SDS–PAGE, and blotted onto polyvinylidene fluoride membranes (Millipore, USA). After being blocked in Tris buffered saline and Tween 20 (TBST) containing 5% nonfat dried milk for 2 h, the membranes were further incubated sequentially with specific antibodies in the following order: Anti-phosphorylated protein 65 (p-p65), anti-p65, anti-IκB-α(1:1000, CST, USA), anti-VEGF-A (1:500, Millipore, USA), anti-Flt-1 (1:800, Santa Cruz, USA), or anti-KDR(1:1000, Santa Cruz, USA). After 3 washes, the blots were subsequently incubated with horseradish-peroxidase conjugated secondary antibodies at room temperature for 2 h, and visualized with ECL Plus chemiluminescence reagent kit (Beyotime, Shanghai, China) by being exposed to an autoradiographic film (BIO-RAD, ChemiDoc XRS System, USA). Protein expression levels were quantified using Quantity One software.

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