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Naringin enhances endothelial progenitor cell (EPC) proliferation and tube formation capacity through the CXCL12/CXCR4/PI3K/Akt signaling pathway

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ABSTRACT

Endothelial progenitor cells (EPCs) have been shown to be involved in the process of physiological neovascularization in vivo. Because increasing evidence has indicated that naringin, a major active ingredient in the Chinese herb *Drynaria fortunei*, can promote angiogenesis and inhibit endothelial cell apoptosis, our study was designed to determine the role of naringin in EPC proliferation and tube formation capacity and examine the potential mechanism for these effects. EPCs were isolated from bone marrow and treated with naringin. An MTT assay was used to investigate EPC proliferation and the tube formation capacity of these EPCs, which were seeded on Matrigel. The protein levels of CXCL12, its receptor (chemokine receptor 4 (CXCR4)) and a downstream signaling molecule (Akt and phosphorylated Akt (pAkt)) were examined using Western blotting. A CXCR4 antagonist (AMD3100) and a phosphatidylinositol 3-kinase (PI3K) antagonist (LY294002) were used to characterize the underlying mechanisms. The results showed that naringin-induced EPC proliferation reached a maximum at day 3 and that the optimal dose of naringin was 500 ng/ml. Treatment with naringin facilitated the EPC tube formation capacity and increased the levels of CXCL12, CXCR4 and pAkt ($P < 0.05$) relative to those in the control group. Moreover, the naringin-induced EPC tube formation capacity was significantly attenuated by AMD3100 or LY294002. In conclusion, we showed here that the naringin-enhanced EPC proliferation and tube formation were mediated by the activation of the PI3K/Akt signaling pathway via the CXCL12/CXCR4 axis, which suggests that naringin could serve as a new therapeutic medicine and has the potential to be applied for the treatment of ischemic disease.

1. Introduction

Naringin is the major active ingredient in the Chinese herb *Drynaria fortunei* and can be obtained from grape, tomato and citrus fruit species [1]. Studies have demonstrated that naringin possesses numerous potential therapeutic properties: antioxidant, anti-inflammatory, anti-apoptotic, anti-ulcer, anti-osteoporotic, etc. [1,2]. Kandhare et al. [3] found that naringin can promote angiogenesis and inhibit endothelial apoptosis by modulating the expression of inflammatory and growth factors in diabetic foot ulcers in rats. Kandhare et al. [4] concluded that naringin can up-regulate the expression of the growth factors vessel endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β), thus promoting angiogenesis and leading to wound healing. In our previous studies, naringin promoted angiogenesis by up-regulating the expression of VEGF at the fracture sites in rats with osteoporosis [5]. Rong et al. [6] found that naringin can be used as a promising therapeutic agent in the treatment of spinal cord injury by up-regulating VEGF.

Endothelial progenitor cells (EPCs) were first isolated from adult peripheral blood in 1997 [7]. Accumulating evidence has indicated that EPCs are involved in blood vessel regeneration, a process defined as post-natal vasculogenesis [8,9]. Under stimulation with various factors, EPCs can migrate to damaged sites to replenish the injured endothelium and enhance neovascularization. CXCL12 (also called stromal cell-derived factor-1 (SDF-1)), a member of the CXC family of chemokines, is thought to have an important role in the proliferation and migration of EPCs. CXC chemokine receptor 4 (CXCR4) is a specific receptor for SDF-1 and has been shown to play a vital role in vascular development. Kawakami et al. [10] used Tie2-Cre (ER) CXCR4 conditional knockout mice to demonstrate that the SDF-1/CXCR4 axis in EPCs plays an important role in bone fracture healing. CXCL12/CXCR4 could also stimulate phosphatidylinositol 3-kinase (PI3K)/Akt signaling activity, thereby promoting EPC proliferation [11].

In summary, we hypothesize that naringin may promote the

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proliferation and tube formation capacity of EPCs and then promote angiogenesis and that the potential mechanism may be mediated by the CXCL12/CXCR4/PI3K/Akt signaling pathway.

2. Material and methods

2.1. Reagents and antibodies

The PI3K inhibitor LY294002 was purchased from Selleckchem (Houston, TX, USA), and the CXCR4 antagonist (AMD 3100) and naringin were purchased from Sigma (St. Louis, MO, USA). The Akt, pAkt and β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). The SDF-1α, CXCR4 and vessel endothelial growth factor receptor-2 (VEGFR-2) antibodies were from Abcam (Cambridge, MA, USA). EGM-2 SingleQuots was purchased from Lonza (Lonza, Basel, Switzerland). The CD31 and vWF antibodies were purchased from Boster Company (Boster, Wuhan, China). The CD34 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the CD133 antibody was purchased from Biorbyt (Biorbyt, UK). DiI-acLDL was obtained from Molecular Probes (Molecular Probes, Eugene, OR, USA), and fluorescein isothiocyanate (FITC)-labeled Ulex europaeus agglutinin (UEA) was acquired from Vector Laboratories (Vector Laboratories, Burlingame, CA, USA).

2.2. EPC isolation and culture

EPCs were obtained from bone marrow mononuclear cells (BMNCs) as previously reported [12]. Briefly, bone marrow was flushed out of the tibias and femurs of Sprague Dawley rats (180–200 g, from Tianjin Hospital, Tianjin, China), and the BMNCs were isolated using density gradient centrifugation in lymphocyte separation medium (Hao Yang Biological Manufacturing Company, Tianjin, China). Then, the BMNCs were plated at a density of 1×10^6/ml in a culture flask pre-coated with fibronectin (50 μg/ml, Solarbio Biological Company, Beijing, China) and cultured in endothelial cell basal medium-2 (EBM-2) supplemented with 7% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and EBM-2 SingleQuots. After 3 days of culture, the non-adherent cells were removed, and new medium was applied. Thereafter, the culture medium was changed every 3 days. All animals used in the experiments received humane care, and the experimental protocol was approved by the Ethics Committee of Tianjin Hospital (2018YLS003).

2.3. Identification of EPCs

As described in a previous study, EPCs were characterized with immunofluorescence analysis of the specific EPC surface markers VEGFR-2, vWF and VEGF [13]. On day 7, the cells were double-labeled for CD34 and CD133 to identify the EPCs. The cellular morphology at various phases was also used to identify the EPCs. To confirm their endothelial phenotype, the uptake of DiI-acLDL and UEA was also evaluated. The EPCs were washed in phosphate-buffered saline (PBS) and stained with DiI-acLDL (2.5 mg/ml) for 30 min at 37 °C and then stained with UEA.

2.4. Cell proliferation assay

The proliferation activity of EPCs in the presence of naringin was examined using a tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assay according to the manufacturer's instructions. Briefly, EPCs were seeded into 96-well culture plates at a density of 5×10^3 cells per well, cultured in EBM-2 with 10% FBS containing naringin (0 ng/ml, 5 ng/ml, 50 ng/ml, 500 ng/ml, 5 μg/ml, or 50 μg/ml) and followed for 1 d, 3 d and 5 d at 37 °C in 5% CO_2. The cells were incubated with the MTT solution for 4 h before it was replaced with 100 μl dimethyl sulfoxide (DMSO). The plate was then read using an enzyme-linked immunosorbent assay (ELISA) reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA, USA) at 560 nm.

2.5. Tube formation assay

EPC tube formation was assessed in Matrigel as described previously [14]. After the EPCs were cultured in EGM-2 with 7% FBS containing naringin (0 ng/ml or 500 ng/ml) for three days, equal numbers of EPCs were seeded into plates that had been coated with Matrigel. The plates were cultured at 37 °C and 5% CO_2 for 12 h; then, images of the tube-like structures were acquired in a blinded manner using a light microscope (Leica, Germany). The length and area of the tube-like structures in the image were measured with ImageJ software (Rawak Software, Inc., Germany). At least eight fields were examined per well, and the experiment was repeated with three independent EPC cultures.

2.6. Western blotting

To explore the mechanisms underlying naringin-mediated EPC proliferation, the CXCR4 inhibitor AMD3100 and the PI3K inhibitor LY294002 were used. EPCs were pretreated with AMD3100 (20 μM) or LY294002 (10 μM) for 2 h, followed by stimulation with 500 ng/ml naringin for 24 h. The inhibitor concentrations were chosen based on previous studies [15–17]. A Western blot assay was performed to evaluate the levels of CXCL12, CXCR4, total Akt and pAkt. The total cell protein was isolated with RIPA buffer containing a phosphatase inhibitor and a protease inhibitor (PMSF). The supernatants were obtained, and the protein concentrations were measured using a BCA protein kit (Boster, Wuhan, China). SDS-polyacrylamide gel electrophoresis was used to separate the proteins, which were then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated overnight at 4 °C with primary antibodies against pAkt (1:1000), CXCL12 (1:2000), CXCR4 (1:2000), Akt (1:1000) or β-actin (1:1000). The PVDF membranes were washed and then incubated with horseradish peroxidase-conjugated (HRP) secondary antibodies (1:5000) for 30 min. The membranes were washed again and then treated with a chemiluminescence (ECL) solution (Boster, Wuhan, China) and visualized by exposure to film. Finally, the expression of each protein relative to β-actin was determined using ImageJ (Rawak Software, Inc. Germany) software.

2.7. Statistical analysis

All data are shown as the means ± standard deviation (SD), and at least three independent experiments were performed for all analyses. SPSS for Windows 2007 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The difference between the naringin group and control group was evaluated with an independent sample *t*-test. One-way ANOVA followed by the LSD post hoc test was used to assess the effects of treatments and time on the changes in EPC proliferation activity and the relative expression of protein. All data were considered statistically significant at $P < 0.05$.

3. Results

3.1. Identification of EPCs

After being cultured in vitro for 7 days, bone marrow-derived EPCs showed a spindle-like morphology and tended to form cluster-like colonies (Fig. 1 A). The immunofluorescence staining for vWF and CD31 was positive and is shown in Fig. 1 B and Fig. 1 C, respectively. The EPCs also co-expressed CD34 (Fig. 1 D) and CD133 (Fig. 1 E), shown in yellow in the merged image (Fig. 1 F), which demonstrates the EPC markers. The immunofluorescence assays demonstrated that EPCs were positive for VEGFR-2 (Fig. 1 G, H, I). Meanwhile, the EPCs can bind to UEA-I-FITC (Fig. 1 J) and take up DiI-acLDL (Fig. 1 K).