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Pristimerin inhibits angiogenesis in adjuvant-induced arthritic rats by suppressing VEGFR2 signaling pathways

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ABSTRACT

Rheumatoid arthritis (RA) is a progressive, inflammatory autoimmune disease. As RA progresses, the hyperplastic synovial pannus creates a hypoxic, inflammatory environment that induces angiogenesis. Further vascularization of the synovial tissue promotes pannus growth and continued infiltration of inflammatory leukocytes, thus perpetuating the disease. Pristimerin inhibits inflammation and tumor angiogenesis. The present study focused on the inhibition of angiogenesis by Pristimerin in adjuvant-induced arthritic rats and the underlying molecular mechanisms. Our results clearly demonstrate for the first time that Pristimerin significantly reduces vessel density in synovial membrane tissues of inflamed joints and reduces the expression of pro-angiogenic factors in sera, including TNF-α, Ang-1, and MMP-9. Pristimerin also decreased the expression of VEGF and p-VEGFR2 in the synovial membrane, whereas the total amount of VEGFR2 remained unchanged. Pristimerin suppressed the sprouting vessels of the aortic ring and inhibited VEGF-induced HFLS-RA migration in vitro. Pristimerin also inhibited VEGF-induced proliferation, migration and tube formation by HUVECs, blocked the autophosphorylation of VEGF-induced VEGFR2 and consequently downregulated the signaling pathways of activated PI3K, AKT, mTOR, ERK1/2, JNK, and p38 in VEGF-induced HUVECs. Our results indicate that Pristimerin suppressed synovial angiogenesis in our rat model and in vitro by interrupting the targeting of VEGFR2 activation. Therefore, Pristimerin has potential as an angiogenesis inhibitor in the treatment of rheumatoid arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disease characterized by proliferation of synovial cells, resulting in the formation of a pannus that invades cartilage and bone and damages joints [1,2]. Although the etiology of RA remains unclear, angiogenesis plays a pivotal role in the development of this disease [3–5]. Angiogenesis is a highly regulated process of growth of new blood vessels from existing blood vessels [6]. These new blood vessels maintain the inflammatory state by transporting inflammatory cells to the synovium and supplying nutrients for the pannus [7]. Angiogenesis is essential for the development of RA [8], and thus a pharmaceutical agent that can effectively inhibit or reduce angiogenesis may be clinically relevant for RA treatment.

Angiogenesis is a complex process that is regulated by many growth factors and cytokines. Among them, vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis and has been implicated in various biological activities, such as stimulating endothelial cell proliferation, migration, and tube formation [9–11]. VEGF binds to vascular endothelial growth factor receptor 2 (VEGFR2), one of the primary receptors in angiogenesis [12–14]. VEGFR2 is upregulated at sites where VEGF is produced or increased in the synovium in response to pro-inflammatory cytokines [10,13,15,16]. The binding of VEGF to VEGFR2 leads to the activation of diverse intracellular signaling molecules, including extracellular signal-regulated kinases (ERKs), phosphoinositide 3-kinase/AKT kinase, and p38 mitogen-activated protein kinase (p38-MAPK) [17,18]. Thus, characterizing the effects of Pristimerin on the interactions of VEGFR2 with these intracellular signaling molecules may facilitate the elucidation of the molecular mechanisms underlying the anti-angiogenic effects of Pristimerin in RA pathogenesis.

Celastrus aculeatus Merr. (*Celastrus*), a traditional Chinese herbal medicine belonging to Celastraceae, has been used in China for centuries to treat various rheumatoid diseases. We previously reported that *Celastrus* exhibits anti-inflammatory activity and reduces the severity of clinical arthritis and bone destruction in an adjuvant-induced arthritis (AA) rat model of human RA [19,20]. Celastraceae plants contain the bioactive natural triterpenoid quinine methide Pristimerin

Abbreviation: RA, Rheumatoid arthritis; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; AA, adjuvant-induced arthritis; SD, Sprague–Dawley; DMSO, dimethyl sulfoxide; DMEM-high glucose, Dulbecco's modified Eagle's medium with high glucose; FBS, heat-inactivated fetal bovine serum; HUVECs, Human umbilical vein endothelial cells; HFLS-RA, Human fibroblast-like synoviocytes of rheumatoid arthritis; ELISA, Enzyme-linked immunosorbent assay; TNF-α, tumor necrosis factor-α; Ang-1, Angiopoietin-1; MMP-9, matrix metalloproteinases-9.

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(Fig. 4A). Pristimerin exhibits antimicrobial, antiperoxidative, and anti-inflammatory effects and inhibits tumor angiogenesis during cancer treatment [21–23]. Cytokines, hypoxia, and new blood vessel formation (angiogenesis) are involved in both tumorigenesis and RA pathogenesis [7,8]. Therefore, we studied the anti-angiogenic potential of Pristimerin for the treatment of angiogenesis in RA and the underlying mechanisms.

2. Methods and materials

2.1. Medicines and reagents

Purified (≥98%) Pristimerin ($C_{30}H_{40}O_4$) was purchased from Pi and Pi Technology (Guangzhou, China). A stock solution of Pristimerin was prepared in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and diluted in culture medium to the required concentration at a final DMSO concentration <1‰. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (St Louis, MO). Matrigel was purchased from BD Bioscience Company (NJ, USA).

Tumor necrosis factor-alpha (TNF-α) ELISA kits were purchased from Dakewe Biotech Co., Ltd. (Beijing, China). Angiopoietin-1 (Ang-1) and matrix metalloproteinases-9 (MMP-9) ELISA kits were obtained from Panera Biotech Co., Ltd. (Guangzhou, China). Recombinant human $VEGF_{165}$, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were acquired from PeproTech Company (Rockyhill, NJ). Rabbit polyclonal antibody against CD34 was purchased from Abnova Biotech (Taipei, Taiwan). Rabbit polyclonal antibody against VEGF was purchased from Santa Cruz Biotechnology (CA, USA). Rabbit polyclonal antibody against β-actin was obtained from Sangon Biotech (Shanghai, China), whereas rabbit polyclonal antibodies against VEGFR2, phospho-VEGFR2, PI3K, and phospho-PI3K were procured from Biowarld Technology (Nanjing, China). Rabbit polyclonal antibodies against AKT, phospho-AKT, mTOR, phospho-mTOR, ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, p38 and phospho-p38 were acquired from Cell Signaling Technology (Danvers, MA).

2.2. Animals

Male Sprague–Dawley (SD) rats (five to six weeks of age; Permit Number: SCXK (Yue) 2011–0015) were purchased from the Animal Experiment Center of Southern Medical University (Guangzhou, China). All animals were housed in a temperature and humidity controlled animal facility (temperature: 25 ± 1 °C; relative humidity: 50 ± 5) under a 12 h light–dark cycle with access to standard diet and water ad libitum. The protocol was approved by the Animal Care Welfare Committee of Southern Medical University.

2.3. Induction and evaluation of rats with adjuvant arthritis (AA)

AA was induced by the same procedures described in previous studies [24]. Arthritis was induced in SD rats by subcutaneous injection at the base of the tail with 200 μl of heat-killed *Mycobacterium tuberculosis* $H_{37}R$ (Mtb) (Difco, Detroit, MC) at a dose of 1.0 mg/rat in mineral oil. After immunization was complete, the rats were examined once daily and graded in terms of signs observed since the onset of arthritis (on day 9). The four limbs of the rats were evaluated at an interval of 3 days by visually assessing inflammation or swelling and scored on a scale from 0 to 4, as described previously [25]. The arthritis score corresponded to the total score for each of the four limbs (the maximum possible arthritis score is 16). Paws were marked with a red line 0.5 cm above the ankle joint, and the volume of the inflamed hind paws was determined at an interval of 3 days using a water-replacement plethysmometer (YLS-7A; Jinan Yiyan Technology and Science Development Co., LTD, China).

2.4. Treatment of arthritic rats with Pristimerin

Pristimerin was dissolved in DMSO (0.4%) and intraperitoneally injected daily into rats in the experimental group (low-dose group, 0.40 mg/kg of body weight; high-dose group, 0.80 mg/kg of body weight) from day 11 to day 24 of immunization. The model group received vehicle (DMSO, 0.4%), and the normal control group received normal saline (NS). Methotrexate (positive control) was suspended in NS and orally administered in the autoimmune phase at an interval of 5 days.

2.5. Histological examination, quantification and evaluation of the angiogenesis in rat synovium

Twenty-four days after immunization, the rats were sacrificed by collecting blood from the abdominal aorta. The joints were isolated, and knee joints were fixed in formalin, decalcified, and embedded in paraffin. Paraffin-embedded sections were then sliced to a thickness of 5 μm.

The sections were stained with hematoxylin and eosin to evaluate arthropathologic and histological characteristics.

A polyclonal antibody recognizing CD34 was used as a microvessel marker to determine vessel density in synovial membrane tissues of inflamed joints [26–28]. Five images of each section were obtained and evaluated microscopically at high magnification (200×), as described in a previous study [29]. To calculate the density, the number of vessel-like structures in the synovial membrane tissue was divided by the area of the synovial membrane tissue to calculate density.

2.6. Rat aortic ring assay

A rat aortic ring assay was performed as described previously [30,31]. Thoracic aortas were isolated from SD rats and immediately transferred to a culture dish filled with PBS. After gentle removal of the periadventitial fat and fibroadipose tissues, the aortas were cut into rings with a width ranging from 1.0 mm to 1.5 mm. The rings were rinsed three times with PBS and placed in 96-well plates pre-coated with Matrigel (45 μl/well). Additional Matrigel (60 μl/well) was added to the aortic rings for solidification. After incubation at 37 °C and 5% CO_2 for 30 min, the aortic rings were placed in Dulbecco's modified Eagle's medium with high glucose (DMEM-high glucose) and 5% heat-inactivated fetal bovine serum (FBS) containing various concentrations of Pristimerin (0, 0.5, 1.0, and 2.0 μM) with or without VEGF (100 ng/ml). The aortic rings were cultured at 37 °C in a humidified atmosphere containing 5% CO_2; the culture medium and the drug were replaced every 3 days. After 9 days, microvessel growth was quantified using a microscopic camera system at 100× magnification by manually counting the number of microvessels sprouting from the rat aortic rings. Three independent assays were performed for each group in three parallel wells.

2.7. Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords using type II collagenase as a previously described [32], and the cells were characterized using CD31. HUVECs were cultured in DMEM/F12 supplemented with 20% FBS, bFGF (10 ng/ml), EGF (20 ng/ml), 100 U/ml penicillin, and 80 U/ml streptomycin at 37 °C in an atmosphere of 5% CO_2. HUVECs were used at passages three to six in this study.

Human fibroblast-like synoviocytes of rheumatoid arthritis (HFLS-RA) were isolated from synovial tissues obtained from RA patients undergoing synovectomy at the Integrated Chinese and Western Medicine Hospital of Southern Medical University (Guanzhou, China). All RA patients fulfilled the 1987 revised criteria of the American College of Rheumatology. All patients provided informed consent, and study approval

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