European Journal of Pharmacology 723 (2014) 7–14

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Immunopharmacology and inflammation

Anti-proliferative effect of recombinant human endostatin on synovial fibroblasts in rats with adjuvant arthritis

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ARTICLE INFO

Article history:
Received 12 May 2013
Received in revised form
2 September 2013
Accepted 28 October 2013
Available online 13 November 2013

Keywords:
Recombinant human endostatin
Rheumatoid arthritis
Synovial fibroblast
Proliferation

ABSTRACT

Rheumatoid arthritis (RA) is characterized by pronounced synovial hyperplasia resulting in pannus formation, cartilage erosion and ultimately joint destruction. Activated RA synovial fibroblasts (SFs) mediate the invasion and destruction of cartilage and bone. We previously demonstrated that recombinant human endostatin (rhEndostatin) is sufficient to induce SF apoptosis in adjuvant arthritis (AA) rats. However, the effect of this protein on SF proliferation is unknown. This study was designed to assess the inhibitory effect and mechanisms of rhEndostatin on the proliferation of cultured AA SFs. MTT assay and flow cytometric detection were performed to investigate SF proliferation and cell cycle progression, respectively. Also, the expression levels of p53, p21, cyclin D1, CDK4 and PCNA in AA SFs were detected by real-time PCR and western blotting assays. Our data revealed that AA SF proliferation was significantly inhibited by rhEndostatin in a concentration-dependent manner. In addition, rhEndostatin (50 μg/ml) caused the G0/G1 cell cycle arrest of AA SFs. There were significant decreases in the expression levels of p53, p21, cyclin D1 and PCNA in AA SFs treated with rhEndostatin, and a significant increase in CDK4 expression. Collectively, our data suggest that rhEndostatin inhibits AA SF proliferation, which is preceded by cell cycle arrest at the G0/G1 phase. This is partly due to the inhibitory effect of rhEndostatin on cyclin D1 and PCNA by a p53–p21–CDK4-independent mechanism. Taken together, these findings highlight the potential use of rhEndostatin for RA treatment.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by hyperplasia of the synovial lining cells, angiogenesis and infiltration of mononuclear cells resulting in pannus formation, cartilage erosion and ultimately joint destruction (Michael and Alisa, 2000). Although the inflammatory reaction contains numerous cell types, RA synovial fibroblasts (SFs) show an activated destructive phenotype independent of the surrounding inflammatory environment in the synovium, Therefore, SFs contribute to inflammation, angiogenesis and matrix degradation by producing inflammatory cytokines, proangiogenic factors and matrix-degrading enzymes. Thus, activated RA SFs mediate the invasion and destruction of cartilage and bone (Bartok and Firestein, 2010; Muller-Ladner et al., 2007; Neumann et al., 2010; Schett, 2012). It is generally accepted that RA synovium hyperplasia is reminiscent of tumor-like expansion, which may result from the imbalance between RA SF proliferation and apoptosis (Firestein, 1999). Therefore, inhibition of proliferation in RA SFs has been proposed as a promising strategy for the treatment of RA by way of reducing synovial hyperplasia in situ.

Rat models are often used to gain further insight into the pathological mechanisms of joint inflammation as well as for preclinical evaluation of therapeutic agents. In this context, adjuvant arthritis (AA) induced by injection of complete Freund's adjuvant is the most widely studied animal model for RA. It models pathology in the joint and the cellular and humoral immunities similar to that observed in human disease (Bendele et al., 1999).

Endostatin, a 22-kDa fragment of collagen XVIII, was originally isolated and characterized as a specific inhibitor of endothelial cell proliferation from conditioned murine hemangioendothelioma (EOMA) cell media (O'Reilly et al., 1997). It is one of the most

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http://dx.doi.org/10.1016/j.ejphar.2013.10.068

active natural inhibitors of angiogenesis and is activated by proteolytic processing with little toxicity to normal cells (O'Reilly et al., 1997). The antiangiogenic activity of endostatin is specifically mediated by inhibiting endothelial cell adhesion, migration and proliferation, and by inducing cellular apoptosis (Dhanabal et al., 1999a; O'Reilly et al., 1997). Recently, direct antitumor effects of endostatin on cancer cells have been reported. Functional endostatin receptors, such as integrins, exist not only on endothelial cells, but also on cancer cells, and endostatin signaling through these receptors directly inhibits *in vitro* cancer cell migration and proliferation, and induces apoptosis through its accumulation at the G1 phase (Cui et al., 2007; Dkhissi et al., 2003; Jin et al., 2012; Wilson et al., 2003). Synovial fibroblasts are the ultimate target cells of the pathologic changes in arthritis and have similar properties to transformed cells (Hui et al., 1997; Michael and Alisa, 2000). Previous studies have shown that systemic administration of recombinant human endostatin (rhEndostatin) attenuates arthritis severity and blocks synovial thickening in AA rats *via* inhibition of angiogenesis and proinflammatory factors (Yue et al., 2007, 2004). We have also demonstrated that rhEndostatin can induce SF apoptosis in AA rats, which may be strongly associated with the elevated expression of c-Jun, Fas and activation of caspase-3 (p20) in AA SFs (Huang et al., 2008). These findings suggest that endostatin may be a potential therapeutic agent for RA. However, it remains unclear whether endostatin directly inhibits SF proliferation. The aim of the current study was to determine whether endostatin could inhibit SF proliferation in AA rats and what mechanism is involved.

2. Materials and methods

2.1. Rats

Male Sprague Dawley rats weighing 160–180 g were obtained from the Animal Center of Anhui Medical University (Hefei, China). The animals were tagged and housed in plastic cages (four rats per cage) under standard laboratory conditions with a 12-h light/12-h dark cycle, a constant temperature of 22 °C, and humidity of 48%. Food and water were provided *ad libitum* with a standard rodent diet. The animals were allowed 1 week to adjust to the housing conditions prior to the initiation of studies. The study was approved by the Ethics Committee of Anhui Medical University. All animal care and experimental procedures were carried out in accordance with the Ethical Regulations for the Care and Use of Laboratory Animals of Anhui Medical University, which conform to the Guidelines for Laboratory Animals of the National Research Council of the USA (1996).

2.2. Adjuvant-induced arthritis

On day 0, rats were injected intradermally into the left hind paw with 1 mg of heat-killed Bacillus Calmette-Guerin (Shanghai Institute of Biological Products, Shanghai, China) in 0.1 ml of paraffin oil as previously described (Huang et al., 2008). Arthritis, as determined by the first signs of redness or swelling of the ankle joints, was observed approximately 10 days after immunization. On day 26, the animals were euthanized under anesthesia with sodium pentobarbital (45 mg/kg intraperitoneally) and the knee joints were promptly removed for histopathological analysis and subsequent experiments.

2.3. Primary cells and cultures

Primary cultures of SFs were isolated, characterized and established from fresh synovial tissues as previously described (Huang et al., 2008). The specimens were freshly minced aseptically, seeded in cell culture flasks with RPMI 1640 (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10 mM HEPES (pH 7.2), 20% fetal calf serum (FCS) (Invitrogen Corporation), 2 mM glutamine, 100 U/ml penicillin sodium, and 100 μg/ml streptomycin. The macerated tissues were cultured for 7 days at 37 °C in a humidified 5% CO_2-containing atmosphere. After removal of the synovial pieces, the adherent cells (passage 0) were cultured in the same medium for an additional period. At 70–80% confluence, nonadherent cells were removed and adherent cells were subcultured (passage 1) after dissociation with trypsin (Sigma-Aldrich, St. Louis, MO, USA). The synoviocytes were used in experiments from passage 2 onwards. After two passages, most of the cultured synoviocytes comprised a homogeneous population of SFs. The cells were identified by their morphology and expression of vascular cell adhesion molecule-1 (VCAM-1). Phycoerythrin (PE) mouse anti-rat VCAM-1 Kit containing PE-conjugated mouse IgG (negative control) was purchased from Becton Dickinson Biosciences (Pharmingen, San Diego, CA, USA).

2.4. Cell proliferation assay

To determine anti-proliferative effect of rhEndostatin, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich) assay was performed. Briefly, cells were incubated in 96-well plates (5×10^3 cells/well) with RPMI-1640 medium (100 μl) supplemented with 10% FCS at 37 °C in a humidified 5% CO_2-containing atmosphere. After 24 h, cells were treated with rhEndostatin (Anhui Sunning Institute of Biotechnology, Hefei, China) in serum-supplemented RPMI-1640 (200 μl/well) at various final concentrations of 0, 3.125, 6.25, 12.5, 25 and 50 μg/ml and were incubated for 48 h. At the end of the treatment, cells were further incubated for 4 h followed by addition of 20 μl of MTT solution (5 mg/ml in PBS). The medium was removed. The cells were lysed with dimethyl sulfoxide (150 μl; Sigma-Aldrich), and plates were gently shaken for 10 min at room temperature. The optical density of each condition was measured by microplate reader (Dynatech Laboratories, Chantilly, VA) at a wavelength of 570 nm with a reference wavelength of 630 nm. Each experiment was repeated in triplicate.

2.5. Cell cycle analysis

Synovial fibroblasts obtained from normal and AA rats were seeded in six-well plates (2×10^5 cells/well) and incubated in serum-free medium for 24 h at 37 °C. After harvesting, AA SFs were randomly divided into AA control and rhEndostatin (50 μg/ml)-treated groups. For the treated group, cells were washed with PBS, replaced with fresh serum-supplemented RPMI-1640 and treated with rhEndostatin (50 μg/ml). The cells were then incubated for 48 h, while the normal and AA control groups were incubated with drug-free medium. Next, cells were trypsinized, washed with ice-cold PBS and centrifuged for 5 min at 1500 rpm at 4 °C. The cells were then fixed with ice-cold 70% ethanol and kept overnight at 4 °C. Fixed cells were washed with cold PBS and incubated with 5 μg/ml RNase (Sigma-Aldrich) at 37 °C for 30 min, followed by staining with propidium iodide (PI) (50 μg/ml; Sigma-Aldrich) for 30 min at 37 °C in the dark. The cells were analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA, USA) and expressed as a percentage of cells in each cell cycle phase.

2.6. RNA purification and reverse transcription (RT)

Total RNA was extracted from SFs in normal, AA control and rhEndostatin (50 μg/ml)-treated groups using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The obtained RNA samples were rinsed with ethanol and dissolved