Anti-adjuvant arthritis of recombinant human endostatin in rats via inhibition of angiogenesis and proinflammatory factors[1]

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ABSTRACT

AIM: To investigate the profile of endostatin on adjuvant arthritis (AA) and angiogenesis blockade in synovitis. **METHODS:** The model of rat AA was induced by injection of intradermal complete Freund's adjuvant (CFA). Hind paw volume of rat was measured by volume meter and the activities of interleukin-1 (IL-1) and IL-2 were measured by the assay of thymocytes proliferation. IL-1β and tumor necrosis factor-α (TNF-α) produced by synoviocytes was estimated with radioimmunoassay. The number of new blood vessels in knee joint synovium was counted under microscope by hematoxylin and eosin (HE) staining. **RESULTS:** The secondary inflammation of AA rats appeared on the 10th day after injection of CFA. The therapeutic administration of endostatin (0.1, 0.5, and 2.5 $mg \cdot kg^{-1} \cdot d^{-1}$, sc, ×7 d) was given from that time (d 10). It was found that endostatin significantly inhibited the secondary paw swelling and the number of new blood vessels in the synovium of AA rats. Endostatin significantly decreased the production of IL-1 derived from both peritoneal macrophages and synoviocytes and IL-2 from splenocytes, especially at the dose of 2.5 mg/kg. This effect of endostatin also was seen on TNF-α produced by synoviocytes. **CONCLUSION:** The recombinant human endostatin had an inhibitory effect on rat AA, which was related to its anti-angiogenesis and inhibition of proinflammatory cytokines.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease with swelling joints, inflammatory synovium, and degenerative cartilage. The chronic nature of this disease results in a progressive joint destruction, which leads to severe locomotive disability and deterioration quality of life. The prevalence of RA varies worldwide between 0.5 % and 2 %[1,2]. A major obstacle to develop rational treatment strategies is that the disease mechanisms, the causative environment, and genetic factors remain poorly understood. The higher secretion of synovial cells activated by proinflammatory factors such as IL-1 and TNF-α is thought to be a crucial process in the destruction of cartilaginous and bony tissues in RA joints. These cytokines, interacting with growth factors, stimulate the overgrowth of synovial cells to form a mass of synovial tissue with exuberant new blood vessels, called pannus, which invades the cartilage and bone via osteoclast activation and protease production. Recently, a large amount of investigations focus on the mechanism of RA and its relationship with the pathological angiogenesis. Angiogenesis or neovascularization from preexisting vasculature is not only necessary for a supply of nutrilites and oxygen,

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but also provides a route for metastasis[3]. The formation of new blood vessels is a complex multistage process involving close orchestration of endothelial cell, extracellular matrix, and soluble factors.

Rat adjuvant arthritis (AA), as an experimental model, resembles RA in histological pathology and pannus formation[4-6]. Moreover, a number of angiogenic mediators, including cytokines and growth factors, have been examined in RA joints. The similarities in joint pathology between AA and RA could be exerted for screening of new drugs for treatment of RA disease.

Endostatin, a 20 kDa C-terminal fragment of the NC1 domain of collagen XIII, is a proteoglycan found in vessel walls and basement membranes. It inhibits the growth of several primary tumor due to inhibition of angiogenesis. Recombinant human endostatin has been reported[7,8]. The ability of endostatin to inhibit angiogenesis in tumor suggested that it might be useful for treatment of other pathological neoangiogenic conditions such as RA[9,10]. Our data suggested that treatment with endostatin attenuated the progressive inflammatory-induced degeneration of synovium, cartilage, and bone in arthritic tissue. To further clarify the profile of endostatin on experimental arthritis, this study was designed to investigate the angiogenesis blockade of endostatin in synovitis.

MATERIALS AND METHODS

Reagents and drugs Recombinant human endostatin was obtained from Anhui Sunning Institute of Biotechnology. Bacille Calmette-Guerin (BCG) was bought from Shanghai Biochemical Factory (lot number: 200207001). Lipopolysaccharide (LPS), collagenase type II, trypsin, and MTT were purchased from Sigma Chemical Co (St Louis, MO, USA). RPMI-1640 medium was purchased from Gibco Co (CA, USA). The RPMI-1640 medium was supplemented with Hepes 10 μmol/L, *L*-glutamine 2 mmol/L, 2-mercaptoethanol 50 mmol/L, penicillin sodium 100 kU/L, streptomycin 100 mg/L, and 10 % new born bovine serum and pH was adjusted to 7.2. IL-1β and TNF-α commercial kits were purchased from Beijing North Institute Of Biological Technology. All other chemicals used in these experiments were of analytical grade from commercial sources.

Animals Sprague-Dawley (SD) rats (male), weighing 160-180 g were purchased from Shanghai BK Experimental Animal Center (Grade II,Certificate No 2002-0006). C_{57}BL/6J mice weighing 18-20 g were obtained from the Animal Department of Anhui Medical University. All rats were acclimatized under standard laboratory conditions. The lighting duration in the breeding room was 12 h (from 7:00 to 19:00). The room temperature was 24 ºC. The experiment was approved by the Committee of Laboratory Animals.

Preparation of rat AA model Adjuvant arthritis was induced on day 0 by a single intradermal injection of 100 μL of CFA. CFA was prepared by mixing 1 mg of heat-inactive (80 ºC, 1 h) BCG with 0.1 mL sterile mineral oil. The volume of rat hind paw was measured by means of Volume Meter (Mukomachi CD Kiai, Japan).

New blood vessels count The histological sections were stained by HE. The identical location of knee joint in the different samples was selected for observation. The new blood vessels localized in synovial lining were counted under microscope (20 multiplication). Five vision fields were counted for each sample and the average was calculated. The data were expressed as mean±SD of 4 samples from a group.

Measurement of IL-1 and IL-2 activity[11] AA Rats were killed on d 28 after immunization. Peritoneal macrophages and splenocytes were collected and seeded to 24-well plate. For IL-1 production, the non-adherent cells were removed by washing twice with RPMI-1640 medium after 2-h incubation at 37 ºC with 5 % CO_2. LPS 100 mL or ConA (4 mg/L) in 0.9 mL RPMI-1640 medium were added and incubated at 37 ºC with 5 % CO_2 for 48 h. The plate was centrifuged (1000×*g*, 10 min) and the supernatants containing IL-1 or IL-2 were collected and stored at -20 ºC until assay for activity. IL-1 and IL-2 activity was measured by ConA-induced thymocytes proliferation assay. Briefly, thymocytes (2×10^{10} cells/L) taken from C_{57}BL/6J mice were distributed to a flat-bottomed 96-well plate. The sample containing IL-1 and ConA (final concentration of 4 mg/L) were added. The cells were incubated at 37 ºC with 5 % CO_2 atmosphere for 48 h. MTT (5 g/L) was added 4 h before termination of culture. And then 120 mL isopropanol (containing HCl 0.04 mol/L) was added to each well and oscillated for 30 s. The absorbance was examined at 490 nm using an enzyme-linked immunosorbent assay plate reader.

Synoviocytes culture Synovium from rat knee joint was excised and digested with sequential incubation of 0.2 % (w/v) collagenase type II and 0.25 % (w/v) trypsin. Synoviocyte suspension was added to 24-well plate with LPS (10 mg/L). The supernatant containing

cytokines was collected and stored at -20 ºC after incubating the plate at 37 ºC with 5 % CO_2 for 48 h .

Assay for IL-1β and TNF-α by RIA They were assayed according to procedures described by the instruction manual using commercial kits.

Statistical analysis Data were expressed as mean±SD. The analysis of variance (ANOVA) were used to determine significant differences between groups. $P<0.05$ were considered to be significant.

RESULTS

Effects of endostatin on secondary inflammation in AA rats Inflammatory polyarthritis was induced in all immunized rats. The peak incidence occurred on d 10 after immunization. The therapeutic administration of endostatin (0.1, 0.5, and 2.5 mg·kg^{-1}·d^{-1}, sc, ×7 d) was given from that time (d 10). It was found that endostatin at the dose of 2.5 mg/kg significantly inhibited the secondary paw swelling on d 24, and so did at the doses of 0.1 and 0.5 mg/kg on d 28 (Fig 1).

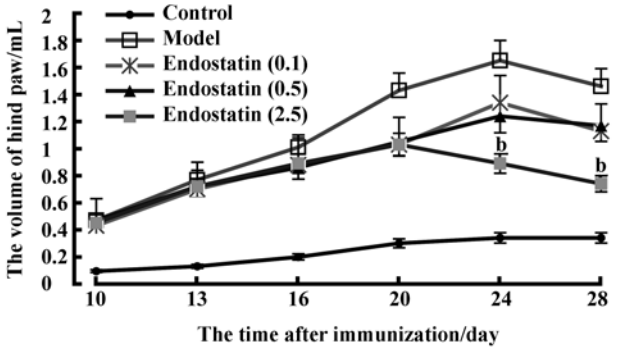

Fig 1. Inhibitory effect of endostatin on hindpaw swelling of rats with adjuvant arthritis. *n*=12. Mean±SD. $^{b}P<0.05$ *vs* model.

The number of new blood vessels of synovial lining Angiogenesis means the formation of new blood vessels from preexisting vessels. There were a few new blood vessels in the synovial lining of normal rats. The number of new blood vessels in hyperplasia synovium of AA rat increased significantly, which was observed under microscope after HE staining. However, the new blood vessels were less in the thin synovial tissue of rats treated with endostatin (0.5 and 2.5 mg/kg) than that in AA control on d 28 after immunization (Tab 1).

Effect of endostatin on cytokines The cytokines of AA rats were determined on d 28 after immunization. IL-1 produced by peritoneal macrophages and synoviocytes increased in AA rats compared with that in the normal group. So did TNF-α derived from synoviocytes. But IL-2 by splenocytes decreased. The administration of endostatin (0.5 and 2.5 mg·kg^{-1}·d^{-1}, sc) for 7 d significantly decreased the production of IL-1 by both peritoneal macrophages and synoviocytes and IL-2 (only at the dose of 2.5 mg·kg^{-1}) by splenocytes (Tab 2, 3). This effect of endostatin was also seen on TNF-α level at all the doses that were used in this experiment.

Tab 1. The antiangiogenesis of endostatin in adjuvant arthritis rats. *n*=4. Mean±SD. $^{c}P<0.01$ *vs* normal. $^{f}P<0.01$ *vs* model.

Group	Dose/mg·kg^{-1}	Number of new blood vessels
Normal	-	0.8±1.0
Model	-	29.0±5.5^{c}
Endostatin	0.1	22.2±5.3
	0.5	18.0±1.8^{f}
	2.5	9.8±1.7^{f}

Tab 2. Effects of endostatin on IL-1 and IL-2 production in adjuvant arthritis rats. *n*=12. Mean±SD. $^{c}P<0.01$ *vs* normal. $^{e}P<0.05$ *vs* model.

Group	Dose/mg·kg^{-1}	Peritoneal macrophages IL-1 ($A_{490\ nm}$)	Splenocytes IL-2 ($A_{490\ nm}$)
Normal	-	0.299±0.013	0.401±0.015
Model	-	0.395±0.015^{c}	0.304±0.046^{c}
Endostatin	0.1	0.370±0.015	0.273±0.054
	0.5	0.350±0.021^{e}	0.249±0.017
	2.5	0.335±0.014^{e}	0.228±0.070^{e}

Tab 3. Effects of endostatin on IL-1β and TNF-α by synoviocytes in AA rats. *n*=12. Mean±SD. $^{c}P<0.01$ *vs* normal. $^{e}P<0.05$, $^{f}P<0.01$ *vs* model.

Group	Dose/mg·kg^{-1}	TNF-α/kU·L^{-1}	IL-1β/kU·L^{-1}
Normal	-	0.64±0.14	0.07±0.04
Model	-	1.88±0.66^{c}	0.22±0.04^{c}
Endostatin	0.1	0.81±0.15^{e}	0.13±0.08
	0.5	0.75±0.33^{e}	0.12±0.04^{f}
	2.5	0.81±0.08^{e}	0.07±0.03^{f}

DISCUSSION

An attribute of RA that has been recognized but has only recently risen to prominence, because of an increased understanding of the underlying mechanisms, is the role of the vasculature in these invasive and destructive processes. The expansion of the synovial lining of joints in RA and the subsequent invasion by the pannus of underlying cartilage and bone necessitate an increase in the vascular supply to the synovium, to cope with the increased requirement for oxygen and nutrients. The formation of new blood vessels–termed "angiogenesis" – is now recognized as a key event in the formation and maintenance of the pannus in RA. This pannus is highly vascularized, suggesting that targeting blood vessels in RA may be an effective therapeutic strategy.

The main pathological changes of RA include synovitis and pannus formation, which lead to cartilage erosion and articular destruction. The therapeutic administration of endostatin significantly inhibited synovium hyperplasia and angiogenesis of lining, which contributed to inhibition of the secondary paw swelling in AA rats. Synoviocytes were ultimate effectual cells with pathologic change during this process. The activated synoviocytes proliferate and overexpress cytokines such as IL-1 and TNF-α, which are important in the pathogenesis of RA[12]. Endostatin inhibited both IL-1 and TNF-α produced by synoviocytes which derived from arthritic tissue in AA rats. Meanwhile, the production of IL-1 secreted from peritoneal macrophages and IL-2 from splenocytes decreased significantly after treatment of endostatin. Some evidence suggested that TNF-α might be more important in promoting mechanisms leading to inflammation, whereas IL-1 in processes led to cartilage and bone destruction. Inhibition of IL-1 reduced the extent of inflammation and bone destruction in various experimental models of arthritis[13-15]. IL-2 may be integral to many autoimmune process. Further investigation of the mechanisms of IL-2 in autoimmunity may lead to a better understanding in the pathogenesis of autoimmune arthritis and other autoimmune disorders.

In conclusion, our results indicated that recombinant human endostatin had an inhibitory effect on rat AA, which was related to its antiangiogenesis and inhibition of proinflammatory factors. These data suggested that endostatin was a potential agent for treatment of RA and other chronic inflammation mediated by pathological angiogenesis.

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