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Involvement of DDAH/ADMA pathway in the pathogenesis of rheumatoid arthritis in rats

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ABSTRACT

Endothelial dysfunction is the early stage of atherosclerosis, which is typically associated with rheumatoid arthritis (RA), a chronic inflammatory autoimmune disorder. Asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor, is not only an independent predictor for endothelial dysfunction but also a proinflammatory mediator. It has been shown that the level of ADMA was elevated in patients with RA. In the present study, we investigated the potential effect of ADMA on inflammation process in collagen-induced arthritis (CIA) animal model and primary cultured fibroblast-like synoviocytes (FLS) exposed to tumor necrosis factor-α (TNF-α). In CIA rats, the plasma levels of inflammatory cytokines TNF-α, interleukin-1β (IL-1β) and IL-6 were markedly increased, while the plasma levels of ADMA did not increase. The expression of dimethylarginine dimethylohydrolase2 (DDAH2), the key enzyme for ADMA degradation, was markedly reduced in inflamed joint synovium of CIA rats. Moreover, the expression of anti-inflammatory factor cortistatin (CST) was markedly decreased in joint synovium of CIA rats. Treatment of cultured FLS with TNF-α significantly increased the levels of ADMA, and decreased the expression of DDAH2 mRNA and protein accompany with an increase in the levels of IL-1β and IL-6 and a reduction in the expression of CST mRNA and protein, and the effects of TNF-α were abolished by DDAH2 overexpression. Treatment of FLS with ADMA also significantly increased the levels of IL-1β and IL-6, and reduced the expression of CST. These findings suggest that DDAH/ADMA participates in the pathogenesis of RA, and that the effect of DDAH/ADMA may be mediated by CST.

1. Introduction

Over the last years, rheumatoid arthritis (RA), is closely associated with high morbidity and mortality of cardiovascular events [1]. There is abundant evidence that in the pathogenesis of RA, systemic inflammation induces persistent endothelial cell activation and dysfunction, resulting in cardiovascular complications [2]. However, the mechanism of cardiovascular complications of RA remains unclear. It is known that inflammation has been considered to be a critical step not only in initiation and progression of RA but also in endothelial activation and dysfunction of atherosclerosis [3,4]. Asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor, has been shown to be related with endothelial dysfunction in multiple cardiovascular diseases including atherosclerosis [5]. Interestingly, elevated ADMA could also be detected in RA patients with atherosclerosis or subclinical carotid atherosclerosis [6–9]. A positive correlation between plasma ADMA levels and anticitrullinated protein antibodies, highly specific for RA, has been observed in RA patients without cardiovascular diseases [10]. Moreover, ADMA has been implicated in inflammation response through activation of leukocyte adhesion and production of inflammatory cytokine tumor necrosis factor α (TNF-α) in cultured endothelial cells or monocytes [11–13]. These results suggest that ADMA may be involved in the pathogenesis of RA.

Dimethylarginine dimethylaminohydrolase (DDAH) is the key enzyme for ADMA metabolism. It has been demonstrated that the elevated ADMA is associated with reduction of DDAH expression in some cardiovascular diseases such as atherosclerosis and hypertension. Based on the fact that the expression of DDAH reduced in endothelial cells pretreated with TNF-α [14], a crucial inflammatory mediator within the inflamed synovium in RA, it is possible that elevated ADMA in RA is related to alteration of DDAH expression. DDAH exists in two isoforms of DDAH-1 and DDAH-2 with distinct tissue-relevant distribution. DDAH-1 is predominantly expressed in tissues expressing neuronal NOS, whereas DDAH-2 is located mainly in vasculature tissues containing the endothelial NOS [15–17]. However, which isoforms of DDAH play critical roles in RA also remain unclear.

Cortistatin (CST), a member of somatostatin (SST) neuropeptide family, is widely distributed in central nervous system, peripheral organs and immune organs. CST exerts various biological functions,

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such as protecting neurons against ischemic injury and cytotoxic damage, suppressing the proliferation of tumor cells, inducing immune tolerance, and regulating endocrine secretions and metabolic functions [18]. Recently, CST has been documented to be a very potent anti-inflammatory peptide [19]. Evidences showed that CST inhibited the proliferation of Th1 cells and production of a wide range of inflammatory mediators [20–23]. Others reported that CST strongly reduced the inflammatory response in CIA mice [24]. These findings suggest that CST may be involved in the pathogenesis of RA. The aim of this study is to investigate the potential role of ADMA on inflammation response of RA, and its correlation with CST.

2. Materials

2.1. Animals

Male Sprague Dawley rats (body weight 200–250 g) were purchased from the Experimental Animal Center of Central South University. The animals were housed in a controlled environment of SPF level throughout the experiment under a 12:12 h light-dark cycle, and provided with standard rodent diet and water ad libitum. All animals received human care in the strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. The experimental protocols were reviewed and approved by the Animal Care and Use Committee at Medical School of Central South University.

2.2. Collagen-induced arthritic model

Bovine collagen-II (Chondrex) was dissolved in acetic acid 0.1 mol/l and emulsified with an equal volume of incomplete Freund's adjuvant (IFA, Sigma). 0.5 ml of emulsion (1 mg/ml collagen-II) was injected intradermally into the base of the tail. At day 7 after primary immunization, rats were given a booster injection intradermally on the other side of the tail with 0.25 ml emulsion of collagen-II and IFA. Control group received the injection of an equal volume of 0.1 mol/l acetic acid at the same location.

Animals were randomly assigned two groups: control group and CIA model group. All animals were tested daily and recorded the arthritis index (AI) before first immunization and before being killed (60 days after the first immunization) by two independent judges. These scores indicate the range and degree of joint swelling and deformation, and were recorded according to the following criteria: score 0, no swelling; score 1, single swelling site was affected in paw or in paw pad; score 2, two or more sites were affected in paw, paw pad or ankle joint; score 3, the whole paw was affected; score 4, severe swelling causing anchylosis, deformity and functional disturbance. Maximum score of a single paw was 4 and a single rat was 16 [25].

The animals were sacrificed 60 days after the first immunization and plasma were obtained for determination of ADMA and inflammatory cytokines. Right hindpaw was obtained for histological observation and immunohistochemistry. Knee joint synovium was obtained for real-time PCR.

2.3. Histological observation

The right hindpaw was fixed in 4% phosphate-buffered formaldehyde, decalcified with 10% EDTA at 4 °C for 12 weeks, dehydrated in ethanol, cleared with dimethyl benzene, embedded in paraffin blocks, and cut serially into slices of 4 μm thickness.

Routine hematoxylin and eosin staining (HE staining) was performed. Histological score was determined on the basis of infiltration of inflammatory cells, synovial hyperplasia, cartilage and bone destruction. Inflammatory cell infiltration: score 0, no inflammatory cell infiltration; score 1, mild infiltration; score 2, moderate infiltration; score 3, severe infiltration of large number of inflammatory cells was observed. Synovial hyperplasia: score 0, no hyperplasia was observed; score 1–9, 1–9 layers of synovial lining cells; score 10, 10 or more layers of synovial lining cells. Destruction of cartilage: score 0, no destruction; score 1, mild destruction; score 2, severe destruction plus vasculogenesis. Destruction of bone: score 0, no destruction; score 1, mild pannus destruction; score 2, severe pannus destruction [25].

2.4. Immunohistochemistry

To test the localization and expression of DDAH2 and CST in joint synovia membranes, immunohistochemical analysis was performed. The sections were deparaffinized in xylene 3× for 10 min followed by dehydration in a graded alcohol series. Antigen retrieval was performed in 1 mM citrate buffer for 2 h at 100 °C. Primary antibodies (anti-DDAH2, 1:100, Abcam, or anti-CST antibody, 1:100, Santa Cruz) were applied to sections and incubated at 4 °C overnight. Sections were washed 3× for 5 min in 1× PBS and incubated with secondary biotinylated antibodies at room temperature for 1 h followed by streptavidin-conjugated enzyme and chromogenic reagent, separately for 10 min. Slides were developed for 10 min with diaminobenzidine (DAB) and counterstained with hematoxylin. Finally, sections were observed using Olympus BX53 microscope.

2.5. Fibroblast-like synoviocytes culture

Fibroblast-like synoviocytes (FLS) were isolated from knee of rats. Synovia membranes were minced and digested with a mixture of 1 mg/ml type II collagen (Sigma) in serum-free DMEM (Hyclone) at 37 °C in 5% CO_2 for 1 h. After centrifugation and washing, the cells were resuspended in DMEM supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 U/ml streptomycin (Beyotime). Non-adherent cells were removed after 24 h. And adherent cells were cultured in DMEM medium containing 10% fetal bovine serum, trypsinized with 0.25% trypsin (Hyclone) at confluence, and plated in cultured flask. To obtain a homogeneous population of synoviocytes, confluent cultures from passages 3 to 7 were used. FLS were identified by immunocytochemistry (BOSTER) as a homogeneous population (phenotype: >99% vimentin and <1% CD68, data not shown).

2.6. Determination of ADMA

The plasma and cellular medium were collected after treatment and stored at −80 °C until analysis. The levels of ADMA were measured by high-performance liquid chromatography (HPLC). HPLC was carried out using a Shimadzu LC-6A liquid chromatograph with Shimadzu SCL-6A system controller and Shimadzu SIC-6A autosampler. o-Phthaldialdehyde adducts of methylated amino acids and internal standard ADMA produced by precolumn mixing were monitored using a model RF 530 fluorescence detector set at k ex = 338 and k em = 425 nm on a Resolve C18 column. Samples were eluted from the column using a linear gradient containing mobile phase A composed of 0.05 M (pH 6.8) sodium acetate–methanol–tetrahydrofuran (81:18:1 v/v/v) and mobile phase B composed of 0.05 mM sodium acetate–methanol–tetrahydrofuran (22:77:1 v/v/v) at a flow-rate of 1 ml/min [26].

2.7. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits (BOSTER) were used to measure the levels of TNF-α, IL-1β and IL-6 in plasma and medium according to the manufacturer's protocol.