Involvement of lymphocytes with a Th1 cytokine profile in bone cell damage associated with MMP-9 production in collagen-induced arthritis

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Abstract. Objective: To investigate whether lymphocytes could directly cause the destruction of bone cells in collagen-induced arthritis (CIA) mice and the mechanism of this destruction.

Methods: Arthritis was induced with chick type II collagen in Kunming mice. The activities of TNF-α and IFN-γ were measured by biological methods. The mRNA of TNF-α, IFN-γ, T-bet, a Th1 specific transcription factor and Fas was detected by reverse transcript-polymerase chain reaction (RT-PCR). Matrix metalloproteinase (MMP)-9 was measured by gelatin zymography assay. The cytotoxicity of spleen cells to bone cell populations and the proliferation of spleen cells against chick type II collagen (CII) were examined by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) method.

Results: Arthritis was successfully induced by chick CII in Kunming mice, which had remarkable paw swelling and typical features of arthritis. Compared with naive mice, the arthritic mice showed an increased IFN-γ and TNF-α production in serum, and the spleen cells from the arthritic mice displayed a Th1 cytokine profile and directly damaged bone cells in vitro. Interestingly, naive spleen cells treated by phorbol 12-myristate 13-acetate (PMA) also damaged bone cells as did spleen cells from CIA mice. Cyclosporine A blocked the cytotoxicity of spleen cells from CIA mice to bone cells. Only anti-CD4 and complement system together inhibited the effect of spleen cells from CIA mice on bone cells. Only anti-MMP-9 but not anti-MMP-2 antibody inhibited the cytotoxicity of spleen cells from CIA mice to bone cells.

Conclusions: The elevated proinflammatory factors such as TNF-α and IFN-γ may promote the MMP-9 activity in local inflammatory cells that are actually involved in the bone cell damage in CII-induced arthritis.

Key words: Collagen-induced arthritis – Spleen cells – Cytokines – MMP – Bone cell damage

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory synovitis, which affects about 1% of the population, in a female/male ratio of 2.5/1. This inflammation has many concurrent diseases including rheumatoid vasculitis, rheumatoid cachexia and cardiovascular events such as atherogenesis [1–5]. Though the etiology of RA remains unclear, the pathogenesis of RA has been extensively and deeply investigated during the past decades. The pathogenetic cells of RA include type A (macrophage-like) and type B (fibroblast-like) synoviocytes in the hyperplastic synovial lining, and mononuclear cells such as T cells, B cells, macrophages and plasma cells in the synovial sublining [1, 6]. These cells could directly interact with each other through costimulatory and adhesive molecules, or indirectly interact through soluble factors such as cytokines, chemokines and proteinases [7–9]. These interactions usually lead to infiltration of inflammatory cells, proliferation of synovial fibroblasts, activation of osteoclasts, production of proteinases like matrix metalloproteinases (MMPs) and induction of angiogenesis [10–13]. Among the factors involved in the interactions, cytokines, especially TNF-α, play an essential role in the inflammatory events occurred in RA. For example, etanercept, a recombinant version of soluble human tumor necro-
sis factor receptor (sTNFR), and infliximab, a chimeric human/mouse monoclonal antibody for TNF-α, have been evidenced as excellent therapeutic agents for RA patients [14, 15]. Moreover, intravenous injection of recombinant adenoviruses containing sTNFR gene ameliorated collagen-induced arthritis (CIA) in rats [16]. The transgenic mice that overexpressed human TNF-α developed an erosive polyarthritis with many characteristics observed in RA patients [17]. Furthermore, TNF-α, as well as other cytokines such as IL-1, IL-17, could affect the activities and expressions of MMPs in synovial fibroblasts and chondrocytes [18–20]. These cytokines could also regulate the activation and function of osteoclasts that play a central role in irreversible destruction of bone and cartilage [19–21].

On the other hand, animal models of arthritis have been used for the elucidation of arthritic inflammations as well as for the evaluation of remedies for RA. One of them, CIA, was found to resemble human RA in many ways, including induction of synovitis, destruction of bone and cartilage, susceptibility linking to major histocompatibility complex II (MHC II), production of cytokines and activation of proteinases like MMPs [22–24]. Many reports have indicated the essential role of T lymphocytes in the pathogenesis of CIA [1, 7, 25]. However, there remains lack of direct evidence for lymphocytes to cause the destruction of bone and cartilage, as a result of the arthritic inflammation. In the present study, therefore, CIA was successfully induced with chick type II collagen (CII) in mice. Lymphocytes isolated from these CIA mice were found to show a Th1 cytokine profile with high MMP-9 activity. The direct damage of bone cell populations by the lymphocytes was then confirmed.

### Materials and methods

#### Animals and cell lines

Male Kunming mice, 6–8 weeks old, were purchased from Experimental Animal Center of Jiangsu Province, China, maintained with free access to pellet food and water in plastic cages at 21 ± 2 °C and kept on a 12-h light/dark cycle. This study complied with current ethical regulation on animal research of our university, and all mice used in the experiment were treated humanely. Murine fibroblast L929 and macrophage J774A.1 cells were purchased from Shanghai Institute of Cell Biology (China) and growing in RPMI 1640 medium (GIBCOBRL, NY, USA) supplemented with 10% fetal calf serum (FCS, GIBCOBRL, NY, USA), 100 IU/ml penicillin (Shandong Lukang Pharmaceutical Group Co., Ltd, China), and 100 µg/ml streptomycin (Shandong Lukang Pharmaceutical Group Co., Ltd, China).

#### Reagents

Phorbol 12-myristate 13-acetate (PMA), 3,4, 5-dimethyl-2-thiazoly1-2, 5-diphenyl-2H-tetrazolium bromide (MTT), Type IV collagenase, gelatin, guinea pig complement system and cyclosporine A were purchased from Sigma (MO, USA). Recombinant mouse TNF-α and IFN-γ were obtained from eBioscience (CA, USA). Anti-CD4 antibody was purchased from R&D (MN, USA). Chick type II collagen (Chick CII, Shanghai Institute of Bencao Biomedical Engineering, China), complete Freund’s adjuvant (GIBCOBRL, NY, USA), anti-MMP-2 and anti-MMP-9 antibodies (Wuhan Boster Biological Technology Co., Ltd, China) were obtained commercially.

#### Induction of CIA [26]

Chick CII was dissolved at 2 mg/ml in 0.2% (w/v) acetic acid, and then emulsified with an equal volume of complete Freund’s adjuvant. The mice were injected intradermally with 0.1 ml of emulsion near the base of the tails. The day of the first immunization was defined as day 0. Twenty days after the primary immunization, the mice were challenged with the same dose of the immunogen by the same route. Observations were made on daily basis for presence of distal joint swelling and erythema. Animals were identified to be arthritic if there was evidence of redness or erythema on any digits or elsewhere on the paws. The edema of the joint was measured with a digit micrometer (0.001 mm, Mitutoyo, Tokyo, Japan) every three days. Paw swelling was calculated as the difference in the paw thickness before and after the first immunization.

#### Preparation of tissue homogenates

The joint tissues (about 100 mg) were scissored scrappilly and homogenized by a homogenizer. After centrifuged at 12,000 g for 10 min at 4°C, the supernatants were collected and total proteins in which were quantified by Bradford method, a rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding [27].

#### Isolation and culture of spleen cells and bone cell populations [28, 29]

Spleens aseptically taken from CIA or naive mice were crushed gently and separated into single cells by squeezing in Hank’s solution containing 5 U/ml heparin. The cells obtained were passed though a gauze of eight-layers and centrifuged at 1000 rpm for 10 min at 4°C. The pellet of spleen cells was added into 10 ml sterile Tris-NH₄Cl, pH 7.5 followed by centrifugation to remove erythrocytes. After washed twice with RPMI 1640 medium containing 10% FCS, they were resuspended in the medium and used for culture. Bone cell populations were obtained by sequential enzymatic digestion of 14-day fetal mouse calvaria as previously described with minor modification [29]. Briefly, calvariae were dissected from fetuses of pregnant mice, and bisected along the sagittal suture. They were then sequentially digested by a five-step procedure in a collagenase-containing enzyme mixture at 37°C. The first digest for 5 min was discarded. The cells obtained from the next four digests were pooled in flasks in a Dulbecco’s modified Eagle medium (DMEM, GIBCO, BRL) supplemented with 10% FCS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. After 24 h, cultures were washed with DMEM to remove nonadhering cells and debris. The cells were then used for further culture. Before they were used in our experiments, the bone cells were identified by modified Gomori’s alkaline phosphatase stain [30, 31]. The purity of bone cells was more than 80%.

#### MTT assay

MTT reduction assay determined the number of viable cells according to formazan production formed by cleavage of MTT by dehydrogenase enzymes of metabolically active cells [32]. This method was used to evaluate the cytotoxicity of cells to other cells or the proliferation of cells against some stimuli [33, 34]. Briefly, MTT was dissolved in phosphate-buffered saline (PBS) at 2 mg/ml, and 40 µl of the solutions were added to 96-well plates containing 200 µl medium per well. After incubation at 37°C for 4 h, MTT formazan products were solubilized by replacement of the culture medium with dimethyl sulfoxide (DMSO). The optical density at 540 nm (OD₅₄₀) was measured with a microplate reader.