THROMBIN AFFECTS ACTIVATION OF CATHEPSIN B VIA IL-8 IN RHEUMATOID ARTHRITIS

+Mishiro, T; *Shinohara, K; **Shimizu, H; *Imagawa, M; **Nakano, S; **Yasui, N
+*Clinical Research Center and Department of Orthopaedic Surgery, Kochi National Hospital
**Department of Orthopaedic Surgery, School of Medicine, University of Tokushima, Japan

INTRODUCTION: Rheumatoid arthritis (RA) is a complicated inflammatory disease characterized by chronic joint inflammation that leads to the destruction of articular tissues. We previously reported that both thrombin and cathepsin B-like protease activities were higher in the SF of RA patients than in those of OA patients. Both of these proteases appear to be related to the progression of synovitis and the destruction of the extracellular matrix. Active thrombin stimulates the proliferation of fibroblasts, numerous cellular responses, synthesis of the extracellular matrix, cytokine release from fibroblasts and degradation of the extracellular matrix. And cathepsin B is also known as an important enzyme to degrade not only collagen but also proteoglycans. Few studies have examined the relationship between these two proteases. In the present study, we investigated the relationship between thrombin and cathepsin B in RA joint.

METHODS: Synovial fluid and synovial tissue specimens SF was collected from the knee joints of RA patients by puncture. It was then centrifuged at 1, 000 x g for 10 min at 4 °C. The resultant supernatants were immediately frozen at -20 °C until use. The precipitates were used as materials of synovial fluid cell extracts.

Assay of protease activity and IL-8 of SF Thrombin-like activity was measured by spectrophotometry using the synthetic fluorogenic peptide Boc-Val-Pro-Arg-MCA as the substrate, by the method of Yasuoka et al. and Cathepsin B-like activity was measured with Z-Phe-Arg-MCA by the method of Barrett and Kirschke with some minor modifications. The amount of AMC released was calculated from a standard curve. One unit of enzymatic activity was defined as that releasing one μmole of AMC per min. The concentration of IL-8 in the SF samples was measured using specific ELISA kit (ENDOGEN). Before assay, each SF was treated with hyaluronidase (Sigma, St Louis, MO) at concentration of 10 U/ml for one hour at 37 °C and centrifuged at 15,000 rpm to remove particles.

Cells culture Synovial fibroblast-like cells (SFCs) were obtained from fresh synovial tissues of RA patients at joint surgery. The synovial tissues were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin G sodium, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The cells from the first to third passages were used for the following experiments.

Effect of thrombin on the release of IL-8 and cathepsin B from SFCs After the cells were starved for 24 h in DMEM / 0.5% FBS, 500 μl of DMEM/ 0.5% FBS containing various concentrations of human thrombin (0.1-10 unit/ml) were added to each well. After two days, the concentration of IL-8 and the activity of cathepsin B in the culture medium was also measured as described above.

Effect of thrombin on the expression of IL-8 mRNA in SFCs The expression of IL-8 mRNA was estimated by real-time quantitative PCR as follows. SFCs were stimulated with 500 μl of DMEM/ 0.5% FBS containing various concentrations of human thrombin (0.1-10 unit/ml). After 3-24 hours, the culture medium was removed, and then the cells were lysed in ISOGEN, a mixture of guanidium isocyanate and phenol. From the lysed cells, total RNA was extracted with chloroform and precipitated with isopropanol. cDNA from total RNA were synthesized with TaqMan Reverse Transcription Reagent (Applied Biosystems, Tokyo) using random hexamers according to the protocol provided by the manufacturer. Pre-Developed TaqMan Assay reagents were used for specific primer and probe of IL-8 and GAPDH. cDNA standard curves were generated using serial dilutions of cDNA obtained from the RNA sample. Then, cDNA sample, no template control and cDNA standard were amplified using TaqMan Universal PCR Master Mix and ABI Prism 7000 Sequence Detection System (Applied Biosystems).

Statistical analysis Statistical analysis was performed by Student’s t-test for the expression of IL-8 mRNA and the significance of correlation between the concentrations of IL-8 and the activity of cathepsin B-like activities in SF were calculated by Spearman’s rank correlation coefficient.

RESULTS: Cathepsin B-like activity The cathepsin B-like activity (mU/ml) in the supernatants of SF was significantly higher (about 3.5-fold) in the RA group (2.68 ± 1.75 mU/ml, n = 39) than in the OA group (0.77 ± 0.51 mU/ml, n = 33, p < 0.0001). The thrombin-like activity in the supernatants of SF was significantly higher (about 6.3-fold) in the RA group (10.9 ± 11.3 mU/ml, n = 39) than in the OA group (1.74 ± 0.75 mU/ml, n = 33, p < 0.001). In the case of SF cell extracts, cathepsin B-like activity expressed as mU/mg protein was also significantly higher (about 5.6-fold) in the RA group (6.33 ± 7.26 mU/mg protein, n = 14) than in the OA group (1.13 ± 1.40 mU/mg protein, n = 10, p = 0.0017). In the case of synovial tissue extracts, it was also significantly higher (about 2.7-fold) in the RA group (77.8 ± 59.4 mU/mg protein, n = 21) than in the OA group (29.2 ± 17.5 mU/mg protein, n = 10, p = 0.018).

Effect of thrombin on the release of IL-8 and cathepsin B from SFCs Thrombin markedly increased the release of IL-8 from RA SFCs in a dose-dependent fashion. Cathepsin B-like activity was not detectable in the culture medium. The time-dependent change in IL-8 mRNA level after treatment with thrombin was quantified by densitometric analysis. Peak level of IL-8 mRNA was observed eight hours after treatment with thrombin. And thrombin increased IL-8 mRNA level in a dose-dependent manner in the range of 0.1 to 10 units / ml. There was a significant correlation between cathepsin B-like activity and IL-8 concentration in the SF of RA patients (n = 35, r = 0.688, p < 0.0001).

DISCUSSION: Our previous studies showed that in the SF, thrombin-like and cathepsin B-like activity was very high and the purified cathepsins isolated from SF of RA patients were mainly composed of cathepsin B. And the cathepsin B purified from SF of RA patients was capable of activating urokinase-type plasminogen activator in vitro. The increase in thrombin and cathepsin B level in SF of RA patients has been shown in several previous studies, but there are no previous reports that examined the relationship between thrombin and cathepsin B in SF from RA patients.

Cathepsins, including cathepsin B, are mainly localized in intracellular spaces. However, the pathologies of inflammatory and neoplastic diseases suggest roles for cathepsin B outside the cells, although the origin of extracellular active cathepsin B has not been well defined. The cells in the SF of our RA patients were composed mainly of neutrophils, and the activity of cathepsin B was very high compared with OA. These results support the idea that the cathepsin B present in the RA SF derives not only from synovial cells but also from inflammatory cells (neutrophils) recruited into the RA lesion.

On the other hand, IL-8 is a potent chemoattractant and activator of neutrophils-mediated cartilage degradation. Inflammatory cells, such as neutrophils, may contribute to cartilage destruction through the release of lysosomal enzymes, including cathepsin B. Since our results revealed thrombin enhanced IL-8 release from SFCs obtained from RA patients, it is plausible that thrombin is related to the enhancement of neutrophils migration in RA. And the significant correlation between cathepsin B activities and concentrations of IL-8 in RA SF supports the hypothesis that thrombin could enhance proteolytic arthritis indirectly in RA. It is therefore possible that thrombin plays important roles in the pathophysiology of RA by increasing the release of IL-8 and cathepsin B from SFCs and neutrophils in inflammatory lesions.

50th Annual Meeting of the Orthopaedic Research Society
Poster No: 0953