Thrombin receptor mediated signals induce expressions of interleukin 6 and granulocyte colony stimulating factor via NF- κ B activation in synovial fibroblasts

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Abstract

Objective—To clarify the mechanism of thrombin receptor mediated signal transduction and the induction of cytokines by thrombin stimulation in rheumatoid synovial fibroblasts.

Methods—Cytokines were measured by enzyme linked immunosorbent assay (ELISA) in the supernatants of cultured rheumatoid synovial fibroblasts stimulated by thrombin. To assess the mechanism of thrombin receptor mediated signal transduction in the rheumatoid synovial fibroblasts, electrophoretic mobility gel shift assay (EMSA), immunoglobulin κ -chloramphenicol acetyltransferase (CAT) assay, and immunostaining for NF- κ B subunit molecule was performed.

Results—Thrombin stimulation activated the inducible transcription factor NF- κ B, and then induced subsequent expressions of interleukin 6 (IL6) and granulocyte colony stimulating factor (G-CSF) in the cells.

Conclusion—Thrombin receptor mediated signal transduction could induce the expressions of IL6 and G-CSF, and increase inflammatory events in the cavum articulare via NF- κ B activation.

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We recently reported that the level of thrombin-antithrombin III complex was considerably increased in the synovial fluid of rheumatoid arthritis (RA) patients compared with those of osteoarthritis patients, and that thrombin acts on synovial cells as a mitogen through the thrombin receptor.¹ Thrombin is known to act as a mitogen and motogen on several cell types including endothelial cells,² vascular smooth muscle cells (VSMC),³ and fibroblasts.⁴ The receptor responsible for the signal transduction was cloned from distinct species, and structural analysis revealed it to be a putative G-coupled receptor with seven transmembrane domains. Activation of the thrombin receptor entails a novel mechanism in which thrombin cleaves the extracellular domains of the receptor and exposes a new N-terminal, and in which thrombin receptor agonist peptide (TRAP) acts as a ligand for the receptor.⁵ In addition, Morris *et al* recently reported thrombin receptor expression in rheumatoid and osteoarthritic synovial tissues.⁶

Previous studies have implicated the involvement of nuclear factor- κ B (NF- κ B) in the expressions of a variety of genes including inflammatory cytokines,⁷ oncogenes,⁸ and cell adhesion molecules.⁹ Recently, we demonstrated that NF- κ B is involved in thrombin signal transduction through the receptor in human VSMC.^{10–11} However, the mechanism of thrombin receptor mediated signal transduction in synovial cells remains to be clarified.

In this study we investigated the intracellular events in thrombin/thrombin receptor signalling and subsequent cellular events in synovial cells. Based on these results, the pathophysiological role of the marked increase of thrombin in the synovial fluid of RA patients is discussed.

Methods

CELLS AND CELL CULTURE

The fresh synovium was obtained from three RA patients during synovectomy. Diagnosis of RA was based on the criteria of the American Rheumatism Association.¹² The patients were a 23 year old men with stage III and class III, and four years of RA duration; a 45 year old woman with stage III and class III, and eight years of RA duration; and a 58 year old woman with stage IV and class IV, and 12 years of RA duration. Synovial fibroblasts were prepared as described elsewhere.^{13–14} Briefly, the synoviums were washed three times in phosphate buffered saline (PBS) to remove blood components and then incubated with Dulbecco's modified essential medium (DMEM) (Gibco Laboratories, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Gibco), 100 units/ml of penicillin (Gibco), 100 mg/ml of streptomycin (Gibco), and 1% of fungizone (Gibco), at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. We used synovial cell culture passaged 5 to 8 times in this study. Almost all our synovial cells were fibroblastic-like cells and strongly reactive to the antihuman fibroblast antibody (DAKO, Denmark) but not reactive to the antihuman muscle actin antibody (Biomedica Corporation, CA) or to the antihuman factor VIII monoclonal antibody (DAKO, Denmark). In the cell culture we used, <1% of the total population was reactive to the antihuman macrophage antibody—that is, the anti-CD68 antibody (DAKO, Denmark). As a control, we used MT-2 cells—that is, a human T cell leukaemia virus type I (HTLV-I) infected T cell line established by

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cocultivation of human cord blood lymphocytes of a normal subject with peripheral blood mononuclear cells of an adult T cell leukaemia patient.¹⁵ MT-2 cells were cultured in RPMI 1640 supplemented with 10% FBS.

ASSAY OF CYTOKINES IN CONDITIONED MEDIUM
G-CSF, GM-CSF, TNF α , IL1 α , IL1 β , IL2, and IL6 were measured using a specific ELISA in culture supernatants.¹⁶ In brief, synovial cells were prepared from the synovium collected from the three RA patients, and both five and eight passaged cells were examined. The cultured cells were starved for 48 hours in medium containing 0.5% FBS, then stimulated with 1.5 ml of medium containing thrombin (10 units/ml). The thrombin used in this study was purchased from Sigma Chemical Corporation (St Louis, MO). At 30 minutes and 1, 2, 4, 8, 12, and 24 hours after the addition of thrombin, the supernatant was collected and the amount of each cytokine was determined. At each measurement time after thrombin stimulation, mean (SD) values were obtained for six samples each. Statistical analysis was done using Student's *t* test and analysis of variance. A level of *p* < 0.05 compared with the value at hour 0 was considered statistically significant.

INCREASE IN IL6 AND G-CSF mRNA EXPRESSIONS BY THROMBIN

Synovial fibroblasts (2×10^5 cells) were starved for 48 hours in the medium containing 0.5% FBS, then stimulated with thrombin (10 units/ml). At 30 minutes, and 1, 2, 4, 8, 12, and 24 hours after the addition of thrombin, total RNA (2 μ g) was isolated using the acid guanidinium thiocyanate-phenol-chloroform method.¹⁷ After treatment with RNase free DNase, the purified total RNA was converted to cDNA by reverse transcriptase, and then polymerase chain reaction¹⁸ was used for the detection of IL6 or G-CSF cDNA. The following oligonucleotide primer sets were used: 5'-¹³⁹⁶GCG CCT TCG GTC CAG TTG CCT TCT C¹⁴²⁰-3' (IL6-1) and 5'-²⁶⁷³CCT CTT TGC TGC TTT CAC ACA TG²⁶⁵¹-3' (IL6-2),¹³ which are specific to human IL6 mRNA (GeneBank Accession No M62424); and 5'-⁶⁰¹ACA GTG CAC TCT GGA CAG T⁶¹⁹-3' (GCSF-1) and 5'-¹⁴⁶⁸TCC AGC TGC AGT GTG TCC A¹⁴⁸⁶-3' (GCSF-2),¹⁹ which are specific to human G-CSF mRNA (GeneBank Accession No X03656). Primers

specific to β actin mRNA—that is, 5'-¹⁴⁷¹AAG AGA GGC ATC CTC ACC CT¹⁴⁹⁰-3' (BAC1) and 5'-²¹²⁹TAC ATG GCT GGG GTG TTG AA²¹¹⁰-3' (BAC2)¹⁴ (GeneBank Accession Nos X00351, J00074, and M10278)—were also used. The reaction mixture (50 μ l) consisted of 100 ng of DNA, 1 μ M of each primer, 200 μ mol of each deoxynucleotide triphosphate, and 2.0 units of Taq polymerase (Promega Corporation, Madison, WI). The reaction mixture was subjected to 30 cycles of denaturation for one minute at 95°C, primer annealing for one minute at 57°C, and chain elongation for two minutes at 72°C using a DNA thermal cycler (TSR-300, IWAKI, Chiba, Japan). The RT-PCR with β actin primers was carried out with 25 cycles in the same way as described above. One fifth of the amplified DNA was then electrophoresed on a 1.3% agarose gel and visualised by ethidium bromide fluorostaining. The amount of visualised activity within the same area in each band was measured using an image analyser system (Adobe Photoshop System, 2.01J, Adobe Systems, Inc, San Jose, CA).

NUCLEAR EXTRACT PREPARATION AND ELECTROPHORETIC MOBILITY GEL SHIFT ASSAY (EMSA)

The nuclear extract preparation and EMSA were conducted as previously described.^{10, 11} Briefly, nuclear proteins (5 μ g) were incubated with a 22 base pair (bp) double stranded ³²P end labelled probe (2×10^4 cpm) encoding the common sequence of κ B—that is, 5'-AGT TGA GGG GAC TTT CCC AGG C-3',²⁰ in 15 μ l of a binding solution (Promega) containing 10 mM TRIS-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 4% glycerol, 1 mM MgCl₂, 0.5 mM dithiothreitol, and 50 μ g poly(dI-dC) (Sigma Chemical Co, St Louis, MO). The mixture was incubated at 37°C for 30 minutes, and then analysed on native 4% polyacrylamide gels. The gels were dried and subjected to autoradiography. For competition assays, a 25-fold or 50-fold molar excess of unlabelled probe was added as a competitor at 30 minutes before the addition of the labelled probe. For characterisation of κ B binding proteins, the reactions were supplemented with affinity purified rabbit polyclonal antibodies against human p50 (ap50), p65 (ap65) or c-Rel (uc-Rel) (Santa Cruz Biotechnology, Santa Cruz, CA), 30 minutes before electrophoresis.

Table 1 Cytokine induction by thrombin in synovial fibroblasts. The synovial fibroblasts were collected from three RA patients, and both five and eight passage cells (2×10^5 cells, three samples each) were examined for each patient. The synovial fibroblasts were cultured for 48 hours in DMEM containing 0.5% FBS, then stimulated with thrombin (10 units/ml). At 4, 8, 12, and 24 hours after the stimulation, the supernatants of cultured cells were collected and measured using ELISA. Student's *t* test and analysis of variance were used for statistical analyses, and *p* < 0.05 compared with the value at hour 0 (*) was evaluated as being statistically significant. Figures represent mean (SD) for the six samples per patient

Time (h)	0	4	8	12	24
IL6 (pg/ml)	14 (5.4)	16 (6.2)	108 (28.5)*	178 (34.7)*	288 (48.5)*
G-CSF (pg/ml)	10 (4.3)	14 (5.5)	20 (7.5)	39 (5.5)*	58 (8.8)*
IL1 α (pg/ml)	6 (3.1)	8 (3.8)	5 (3.3)	7 (4.5)	8 (4.3)
IL1 β (pg/ml)	12 (6.2)	15 (6.8)	13 (7.1)	16 (8.8)	15 (5.9)
IL2 (pg/ml)	32 (8.7)	26 (6.7)	40 (8.3)	37 (7.5)	42 (9.5)
TNF α (pg/ml)	13 (5.6)	12 (7.7)	17 (9.6)	13 (8.8)	15 (7.5)
GM-CSF (pg/ml)	18 (8.8)	17 (6.5)	16 (7.9)	20 (6.6)	25 (8.5)

Table 2 IL 6 and G-CSF productions by thrombin dose dependence. The synovial fibroblasts were cultured for 48 hours in DMEM containing 0.5% FBS, then stimulated with thrombin (from 0 to 200 units/ml). At 12 hours after the thrombin stimulation, the supernatants of cultured cells were collected and measured using ELISA. Student's *t* test and analysis of variance were used for statistical analysis, and $p < 0.05$ compared with the value at no thrombin stimulation (*) was evaluated as being statistically significant. Figure represent mean (SD) for six samples per patient

Thrombin (units/ml)	0	1	10	50	100	200
IL6 (pg/ml)	14 (5.4)	32 (13.3)	178 (34.7)*	201 (68.5)*	220 (75.5)*	268 (99.8)*
G-CSF (pg/ml)	10 (4.3)	14 (5.5)	39 (5.5)*	48 (8.2)*	54 (11.3)*	60 (15.5)*
IL1 α (pg/ml)	6 (3.1)	5 (2.7)	7 (4.5)	8 (4.3)	10 (6.5)	9 (5.2)
TNF α (pg/ml)	13 (5.6)	15 (4.2)	13 (8.8)	16 (8.5)	17 (8.6)	18 (8.1)

CAT ANALYSIS FOR NF- κ B EXPRESSION

Synovial fibroblasts were transfected by using the DEAE-Dextran transfection method. DEAE-Dextran (Promega) plus 2 μ g of immunoglobulin κ (Igk)-chloramphenicol acetyl transferase (CAT) reporter plasmid, which contains seven copies of κ B binding motif of the Igk light chain enhancer²¹ (kindly provided by Dr Shoji Yamaoka, Kyoto University, Japan), and 1 μ g of SV- β gal control plasmid (Promega) as an internal reference for transfection efficacy, were co-transfected into cells in PBS for three hours. The cells were washed and cultured in 5% FBS and the medium containing the indicated reagents for another 48 hours. Cells were then harvested by a scraper into 0.25 M TRIS-HCl (pH 7.5) and then subjected to three cycles of freezing and thawing. The transfection efficacy was analysed by a 4-methylumbelliferyl β -D galactoside assay, and protein concentrations were determined by the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). CAT activity was determined by thin layer chromatography with [¹⁴C]chloramphenicol (Amersham, Buckinghamshire, UK) and quantified by Fuji Computed Radiogram^{10 11} (Fuji Photo Film).

IMMUNOFLUORESCENT STAINING FOR NF- κ B

Synovial fibroblasts were seeded onto glass coverslips and incubated overnight at room temperature. Cells were then permeabilised with acetone/methanol (1:1) for 20 minutes at -20°C . Incubation with rabbit polyclonal antibody against the p50 subunit of NF- κ B (1:50 in PBS, 1% bovine serum albumin) for one hour at room temperature was followed by one hour treatment with fluorescein isothiocyanate

conjugated goat antirabbit IgG (Organon Tekunika Corp, Durham, NC, 1:100 in PBS, 1% bovine serum albumin). Coverslips with stained cells were mounted in 80% glycerol in PBS.

Results

CYTOKINE INDUCTION BY THROMBIN IN SYNOVIAL FIBROBLASTS

Table 1 shows the concentrations of IL1 α , IL1 β , IL2, IL6, TNF α , GM-CSF, and G-CSF in the supernatants of thrombin stimulated synovial cell cultures. IL6 and G-CSF were significantly ($p < 0.05$) increased at 12 hours after the stimulation, and their induction was found to be time dependent. GM-CSF was weakly detected at 24 hours after the stimulation. Supernatant concentrations of IL1 α , IL1 β , IL2 and TNF α , however, did not change up to 24 hours after thrombin stimulation.

As the next step of examination, the synovial cells were stimulated with various concentrations (1–200 units/ml) of thrombin, and then IL6 and G-CSF concentrations in culture supernatants were examined with ELISA at 12 hours after the stimulation. IL6 concentrations increased to a maximum 17-fold higher level and G-CSF increased to a sixfold higher level, in a dose dependent manner (table 2).

Figure 1 Induction of IL6 or G-CSF mRNA expressions by thrombin. Synovial fibroblasts (2×10^5 cells) were starved for 48 hours in DMEM containing 0.5% FBS, then stimulated with thrombin (10 units/ml). At 30 minutes, and 1, 2, 4, 8, 12, and 24 hours after the stimulation, total RNA (2 μ g) was isolated and converted to cDNA. RT-PCR analysis was performed as described in Methods. The RT-PCR products for β actin were used as an internal control. Results shown are obtained in a representative examination of two sets of measurements.

Figure 2 Activation of nuclear κ B binding proteins by thrombin. Synovial cells were either untreated or stimulated with thrombin (10 units/ml) for the indicated times. Five micrograms of nuclear protein were incubated for 30 minutes at room temperature with ³²P-labelled probe. The mixtures were then run on a native 4% polyacrylamide gel. Nuclear protein from MT-2 cells was used as a positive control. Results shown are from two sets of experiments.

Figure 3 Identification of NF- κ B protein in RA. Supershift assay was performed using antibodies against the NF- κ B subunits, p50 (ap50), p65 (ap65), or c-Rel (ac-Rel). DNA binding mixture containing nuclear extracts from thrombin treated synovial cells and 32 P-labelled κ B probe was treated with each antibody. The mixture was applied to a native 4% polyacrylamide gel. Competition assay was performed using a 25- ($\times$ 25) or 50- ($\times$ 50) fold molar excess of unlabelled probe. Results shown are obtained in a representative examination of three sets of measurements.

INDUCTION OF IL6 AND G-CSF MRNA EXPRESSIONS BY THROMBIN IN SYNOVIAL CELLS

We examined the expressions of IL6 and G-CSF mRNA in synovial fibroblasts after thrombin stimulation. The expressions of these cytokine mRNAs were confirmed by RT-PCR analysis, and activity levels of the signals were quantified by the image analyser. Synovial fibroblasts were stimulated for the indicated times with thrombin (10 units/ml), and the levels of IL6 and of G-CSF mRNAs showed a time dependent increase during two to eight hours after the stimulation (fig 1). The PCR products for β actin were amplified as an internal control, and its mRNA level was not changed by thrombin stimulation. In the mixture without the addition cDNA, no DNA product was detected by PCR (data not shown).

ACTIVATION OF NF- κ B BINDING PROTEINS BY THROMBIN

We performed EMSA to determine whether thrombin receptor signalling activates nuclear κ B binding proteins in fibroblasts. Nuclear extracts were isolated from synovial fibroblasts at 0, 0.5, 1, 4, or 8 hours after 10 units/ml of thrombin stimulation. As shown in fig 2,

nuclear proteins were incubated with a 32 P-labelled 22 bp probe containing the NF- κ B binding site. The proteins (5 μ g) with the probe were then applied to a native 4% polyacrylamide gel. NF- κ B activation was observed within 30 minutes of thrombin stimulation and reached a peak at four hours. As a positive control, nuclear proteins (1 μ g) isolated from MT-2 cells were applied to a native gel, and no activation was observed (data not shown).

IDENTIFICATION OF NF- κ B BINDING PROTEINS IN SYNOVIAL CELLS

We used the supershift assay with antibodies against NF- κ B subunits and a competition assay with an unlabelled probe to characterise the nuclear κ B-binding proteins (fig 3). Supershift analyses were performed with antibodies to NF- κ B subunits NF- κ B p50, p65, and c-Rel (Santa Cruz Biotechnology, Inc). Anti-p50 and p65 antibodies caused a supershift of nuclear κ B proteins, while anti-c-Rel did not affect the nuclear κ B binding proteins. Nuclear proteins were isolated from the synovial cells that had been stimulated by thrombin (10 units/ml) for four hours, then preincubated with a 25-fold or 50-fold molar excess of unlabelled probe before the exposure to the 32 P-labelled probe. Addition of the unlabelled probe caused a decrease in the intensity of the κ B binding protein band. Normal rabbit serum did not affect the nuclear κ B binding proteins (data not shown).

NUCLEAR TRANSLOCATION OF NF- κ B MOLECULES ON THROMBIN STIMULATION

We used immunofluorescence staining with ap50 to determine whether NF- κ B molecules were translocated in the nucleus on thrombin stimulation (fig 4). NF- κ B molecules were detected in the cytosol but not in the nucleus of unstimulated synovial cells. However, thrombin stimulation induced a marked translocation of NF- κ B molecules into the nucleus, with positive fluorescence observed at 30 minutes after the stimulation. This nuclear translocation of NF- κ B by thrombin stimulation was observed in all synovial fibroblasts. As activation of inducible NF- κ B has been reported to be involved in the removal of the inhibitory subunit, I κ B, from a latent cytoplasmic complex, we examined the immunofluorescence staining of I κ B using affinity purified rabbit polyclonal antibodies against human I κ B. I κ B was positively stained in the cytoplasm without translocation to the nucleus after thrombin stimulation (data not shown). We did not observe fluorescence positive cells on staining with control rabbit IgG (data not shown).

EFFECT OF THROMBIN ON TRANSACTIVATION IN SYNOVIAL FIBROBLASTS

To investigate the effect of thrombin on transcriptional responses mediated by NF- κ B activation, we used the Ig κ -CAT assay, because the CAT expressing plasmid contains seven copies of the κ B binding motif of Ig κ light chain enhancer.²¹ In synovial cells, a threefold increase in CAT transactivation was seen after the stimulation using 10 units/ml of thrombin

Figure 4 Nuclear translocation of NF- κ B molecules on thrombin stimulation. Synovial cells were stimulated by thrombin (10 units/ml) and were fixed using acetone/methanol (1:1). The fixed cells were incubated with ap50, polyclonal anti-p50 antibody. The bound antibody was visualised with fluorescein isothiocyanate conjugated goat antirabbit IgG.

Figure 5 Effect of thrombin on immunoglobulin κ -CAT transactivation in synovial fibroblasts. Synovial fibroblasts were transfected with immunoglobulin κ (Ig κ)-CAT plasmid by the DEAE dextran method. The cells were cotransfected with 2 μ g of Ig κ -CAT and 1 μ g of SV- β gal control plasmid. The transfected cells were stimulated with vehicle (thrombin 0 units/ml), thrombin (5, 10, 50 units/ml) or TNF α (500 units/ml) for 48 hours. CAT activity was assayed by thin layer chromatography, and the relative CAT activities were quantified using Fuji computed radiographs. Results shown are obtained in a representative examination of three sets of measurements.

(fig 5). We also confirmed in the IL6 5'-promoter CAT assay that thrombin induced IL6 5'-promoter CAT transactivation. A 1.88-fold increase in the CAT transactivation was seen after thrombin (10 units/ml) stimulation, and it increased with the dose increase—that is, a 1.4-fold increase with 1 unit/ml thrombin and a 1.83-fold increase with 5 units/ml thrombin (unpublished observations). TNF α was used as the positive control of NF- κ B transactivation, and a 3.2-fold increase was observed.¹⁰

Discussion

Recent studies have indicated a close association of homeostatic mechanism with the inflammation of RA.^{1, 22–25} Cirino *et al* reported thrombin functions as an important mediator of inflammatory responses by using a rat model of inflammation.²⁶ Thrombin is known to cause degranulation with the release of histamine in mast cells,²⁷ increase vascular permeability²⁸ and the number of cell adhesion molecules,⁹ and induce such oncogenes as *c-myc*.⁸ In this study, we demonstrated that thrombin induced the expression of the inflammatory cytokines, IL6 and G-CSF, through activation of the transcription factor NF- κ B in synovial fibroblasts. We believe that thrombin receptor mediated signal transduction is crucial in extending inflammation in the articular region of RA patients.

We recently reported that the concentration of thrombin-antithrombin III complex in the synovial fluid of RA patients was considerably increased compared with those of osteoarthritis patients, and that thrombin acts on synovial cells as a mitogen through the thrombin receptor.¹ We also reported involvement of NF- κ B activation in thrombin induced VSMC proliferation.^{10, 11} However, the mechanism of thrombin induced human synovial cell proliferation and the involvement of thrombin in the inflammation in RA have yet to be investigated.

In this study we investigated the signal transduction of thrombin from the thrombin receptor and the cellular responses in synovial fibroblasts. The pathophysiological significance of the increased thrombin expression is thought to be attributable to the marked increase in the concentration of thrombin-antithrombin III complex in the synovial fluid in RA.

Fujisawa *et al*²⁹ recently reported TNF α induced NF- κ B activation in synovial cells, and the involvement of NF- κ B in the proliferation of synovial cells and pathogenesis of RA. Marok *et al* reported activation of NF- κ B in inflamed synovial tissue.³⁰ The activation of NF- κ B may have a central role in the inflammation and synovial proliferation in RA.

To determine whether a link between NF- κ B and cytokine production is in response to thrombin, we previously examined the effect of NF- κ B p65 antisense ODNs.³¹ NF- κ B protein synthesis was monitored with EMSA, and antisense p65 ODNs treatment induced approximately an 80% decrease of NF- κ B activity compared with the level by sense p65 ODNs treatment. Treatment of synovial cells with antisense p65 ODNs showed a 78% decrease of thrombin stimulated IL6 production compared with sense p65 ODNs treatment, and G-CSF also decreased 32% by antisense p65 ODNs treatment (unpublished observations).

It is known that the IL6⁶ and G-CSF³² promoter regions contain NF- κ B binding sites. In this study, we confirmed that the levels of IL6 and G-CSF proteins in culture medium increased at eight hours or 12 hours after thrombin stimulation (table 1), and that IL6 and G-CSF mRNA expressions were induced at four hours after stimulation (fig 1). However, we did not detect any expression of IL1 α , IL1 β , IL2, and TNF α up to 24 hours after giving the stimulation. It is known that IL1 α and IL1 β mRNA expressions are induced by the transcription factor, Spi-1/PU.1, but not induced by NF- κ B activation.³³ It is also known that IL2 is secreted by T cells but not by synovial fibroblasts. Duckett *et al*³⁴ reported a relation between NF- κ B activation and cell cycle. In this study, we confirmed that thrombin induced synovial cell proliferation may depend on the NF- κ B activation. Ohba *et al*³⁵ recently reported thrombin mediated proliferation of synovial fibroblast-like cells by induction of platelet derived growth factor (PDGF). However, it is unknown whether PDGF induces NF- κ B activation. It is known the promoter regions of TNF α and GM-CSF contain NF- κ B binding sites and both are NF- κ B

dependent cytokines.³⁶ We guess that thrombin may selectively regulate the expression of various inflammatory cytokine responses to NF- κ B activation. Transcriptional regulation for each cytokine in synovial fibroblasts stimulated by thrombin is an important subject for further investigation.

In summary, we were able to demonstrate that thrombin receptor mediated stimulation induced the expressions of IL6 and G-CSF after activation of the transcription factor NF- κ B in synovial fibroblasts. Thrombin induced NF- κ B activation may also cause synovial fibroblast proliferation. These findings suggest that thrombin acts as an inducer of inflammatory cytokines such as IL6 and G-CSF and as an enhancer of the inflammatory response in RA. Antithrombin reagents, such as antithrombin III or thrombomodulin, and inhibitors of NF- κ B activation may have a therapeutic potential in the treatment of RA.

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