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Nuclear factor-κB regulates RANTES chemokine expression in response to tumor necrosis factor-α in fibroblast-like synoviocytes

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Abstract We investigated the role of nuclear factor (NF)-κB on tumor necrosis factor (TNF)-α-induced regulated upon activation, normal T-cell expressed and secreted (RANTES) expression in fibroblast-like synoviocytes from patients with rheumatoid arthritis (RA). Using cultured human fibroblast-like synoviocytes from patients with RA, semiquantitative reverse transcriptase-polymerase chain reaction, electrophoretic mobility shift assay, and Western blot were performed for RANTES expression, NF-κB activation, and degradation of IκB, respectively. In addition, the transcriptional effect of TNF-α on RANTES gene expression was analyzed by reporter gene assay. We found that TNF-α clearly induced RANTES protein production and expression of RANTES mRNA in a time-dependent manner. Furthermore, TNF-α persistently induced NF-κB activation caused by IκBα and IκBβ1 degradation. Supershift analysis revealed that TNF-α-induced DNA-binding complexes were composed principally of the p65 and p50 Rel family members. Moreover, transcriptional activation of the RANTES promoter by TNF-α was dependent on specific NF-κB response elements that were regulated by NF-κB. Results herein indicate that NF-κB activation caused by degradation of IκBα and IκBβ1 by TNF-α increased RANTES gene expression in fibroblast-like synoviocytes, suggesting that NF-κB plays an important role in the migration of inflammatory cells by RANTES to the synovium in patients with rheumatoid arthritis.

Key words Nuclear factor (NF)-κB · Regulated upon activation, normal T-cell expressed and secreted (RANTES) · Rheumatoid arthritis (RA) · Synoviocyte · Tumor necrosis factor (TNF)-α

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Introduction

Rheumatoid arthritis (RA) is characterized by excessive synovial membrane growth, causing inflammatory destruction of cartilage and bone.[1–3] In this disease, an autoimmune reaction leads to progressive recruitment of inflammatory cells from circulation to the synovium.[4–6] A variety of inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-8 (IL-8) are present at high levels in arthritic joints, and their blood concentration correlates with RA severity.[2,3] In these cytokine networks, TNF-α is considered to play a central role because it is known to induce IL-1, IL-6, and IL-8 in addition to itself.[7] Recently, TNF-α suppression with antibodies against TNF-α or soluble TNF receptor molecules has been shown to reduce RA symptoms and indicators.[8,9] Moreover, TNF-α transgenic mice develop chronic inflammatory arthritis.[10] Thus, it is thought that TNF-α is mainly related to RA pathogenesis.

Initiation and maintenance of these processes correlate with the coordinated chemokine production of two major families. The CXC chemokine family, typified by IL-8, is an important system for neutrophil chemoattraction, whereas the CC chemokines play a role in recruitment of monocytes, T cells, and eosinophils to areas of inflammation.[11] Regulated on activation, normal T-cell expressed and secreted (RANTES) is a CC chemokine that mainly migrates memory type CD4+ T cells[12] and is increased in the serum and synovial fluids in patients with active RA.[13] Ellingsen et al. reported that the synovial fluid levels of RANTES in patients with RA positively correlated to migration of mononuclear cells to synovial tissues.[14] Furthermore, high levels of serum RANTES during the active stage of RA were significantly lowered by treatment with methotrexate.[15] Additionally, polyclonal antibody to RANTES reportedly ameliorates symptoms in animals induced for adjuvant-induced arthritis.[16] Therefore, RANTES also plays an important role in RA pathogenesis. Also, TNF-α induces expression of RANTES mRNA in human RA synovial fibroblasts.[17] The upstream sequence of the RANTES

gene contains a number of putative *cis*-acting elements for transcription factors such as activator protein-1, nuclear factor-interleukin 6, and nuclear factor (NF)-κB.[18] However, little is known about the molecular mechanisms of TNF-α-induced RANTES expression in human RA fibroblast-like synoviocytes.

The present study examines the role of NF-κB in TNF-α-induced RANTES expression in fibroblast-like synoviocytes from patients with RA. Activation of NF-κB caused by degradation of IκBα and IκBβ1 in response to TNF-α was found to increase RANTES gene expression in fibroblast-like synoviocytes, which suggests that NF-κB played an important role in migration of inflammatory cells by RANTES to the synovium in patients with RA.

Materials and methods

Preparation of fibroblast-like synoviocytes

Synovial tissue samples were obtained during orthopedic surgery from knees of patients with RA, according to American College of Rheumatology criteria.[19] The tissue specimen was minced into small pieces, washed twice with phosphate-buffered saline (PBS), and treated with 0.1 mg/ml collagenase (Sigma, St. Louis, MO, USA) and 3.3 mg/ml dispase (Sanko Pharmacological, Tokyo, Japan) for 60 min at 37°C. Obtained cells were placed in tissue culture flasks and cultured in RPMI1640 medium (Gibco BRL, Grand Island, NY, USA) with 10% heat-inactivated fetal calf serum (FCS; Gibco BRL), 100 μg/ml penicillin, and 100 U/ml streptomycin in humidified air containing 5% CO_2 at 37°C. Cells passed from the third to fifth generation were used for the following experiments. Suspension HeLa cells were kept in Joklik modified minimum essential medium (Gibco BRL) with 1% nonessential amino acids, 100 μg/ml penicillin, 100 U/ml streptomycin, and 10% FCS.

Reagents and plasmid constructs

Recombinant human TNF-α was purchased from Boehringer Mannheim (Indianapolis, IN, USA) and dissolved in distilled water. RANTES promoter-luciferase reporter plasmid was a kind gift from Dr. Alan M. Krensky (Stanford University School of Medicine). This construct includes the 961 nucleotides of the region immediately upstream of the transcriptional start site and the complete RANTES 5′-untranslated region to the KpnI site. The κB1 site at positions −53 to −44 and the κB2 site at positions −39 to −30 of the RANTES promoter were respectively mutated in the 961-b 5′-flanking sequence of the RANTES gene by oligonucleotide-directed, site-specific mutagenesis according to instructions delivered with the site-directed mutagenesis kit (Promega, Madison, WI, USA). The mutant sequences utilized for the κB1 and the κB2 were GGAAACTtaC and GGtaATGCCC, respectively. Lowercase letters represent mutant nucleotides.

ELISA for RANTES

Cultured human fibroblast-like synoviocytes were grown to confluence in 60-mm collagen-coated culture dishes and treated with 10 ng/ml TNF-α for 0, 12, 24, 36, 48, 60, and 72 h. The supernatants were collected and analyzed for RANTES content. Levels of RANTES were measured using an RANTES monoclonal antibody sandwich enzyme-linked immunosorbent assay (ELISA) employing two anti-RANTES antibodies recognizing different, noncompeting determinants according to the instructions delivered with the Quantikine Human RANTES Immunoassay (R&D Systems, Minneapolis, MN, USA).

RNA extraction and semiquantitative RT-PCR analysis

Total RNA was extracted from cultured fibroblast-like synoviocytes according to the method of Chomczynski and Sacchi,[20] which includes a single step of acid guanidium thiocyanate (GTC) and phenol/chloroform extraction. RNA was quantified spectrophotometrically. Synthesis of the first strand of cDNA and polymerase chain reaction (PCR) analysis were performed according to instructions delivered with the RNA PCR Kit (AMV) Ver.2 (TaKaRa, Tokyo, Japan) as described previously.[21] In brief, 500 ng total RNA was subjected to first-strand cDNA synthesis in a 20-μl reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 μM of each deoxynucleoside triphosphate (dNTP), in presence of 2.5 μM random 9-mer nucleotides, 20 U RNase inhibitor, and 5 U avian myeloblastosis virus reverse transcriptase (RT). After completion of first-strand cDNA synthesis, the reaction was stopped by heat inactivation (5 min, 99°C). For semiquantitative RT-PCR analysis, cDNA amounts equivalent to 500 ng total RNA were subjected to PCR amplification in a 50-μl reaction containing 10 mM Tris-HCl (pH. 8.3), 50 mM KCl, 5 mM $MgCl_2$, 200 μM of each dNTP, 20 μM of each primer, and 2.5 U of TaKaRa Taq DNA polymerase. Samples from fibroblast-like synoviocytes were amplified at 94°C for 5 min, at 55°C for 30 s, and at 72°C for 90 s followed by 28 cycles at 94°C for 30 s, at 55°C for 30 s and at 72°C for 90 s. The following primers were used: for glyceraldehyde phosphate dehydrogenase (GAPDH), sense 5′-ACATCGCTC AGACACCATGG-3′, antisense 5′-GTAGTTGAGGTC AATGAAGGG-3′; for RANTES, sense 5′-GCTGTC ATCCTCATTGCTAC-3′, antisense 5′-TCCATCCTAG CTCATCTCCA-3′.

Then, 10 μl of the PCR products was electrophoresed through 1.5% agarose gels and visualized by ethidium bromide, and PCR was performed at different cycle numbers for each primer set to ensure that the assay was in the linear range for each molecule tested.

Preparation of whole-cell extracts and electrophoretic mobility shift assay

Whole-cell extracts were prepared as described previously.[22] Briefly, cells were washed twice with PBS and

incubated in 20 mM hydroxy ethylpiperazine ethane sulfonate (HEPES) (pH 7.9), 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM ethyleneglycoltetraacetic acid (EGTA), 1% Nonidet P-40, 0.5 mM dithiothreitol (DTT), and 0.4 mM 4-(2-amino-ethyl) benzenesulfonyl fluoride hydrochloride (Boehringer Mannheim) on ice at 15 min. After centrifugation at 10,000 *g* for 20 min, the supernatant was used as a whole-cell extract. For electrophoretic mobility shift assay (EMSA) experiments, equal amounts of whole-cell extracts (10 μg protein) were incubated with 30000 cpm of ^{32}P-labeled H_2k oligonucleotide probe for binding NF-κB. Reactions were performed in 20 μl binding buffer containing 20 mM HEPES (pH 8.4), 60 mM KCl, 4% Ficoll, 5 mM DTT, 1 μg bovine serum albumin, and 2 μg poly (dI-dC) for 20 min at 30°C. The reaction mixture was loaded on a 4% polyacrylamide gel and run in 1× Tris-borate + EDTA (TBE) buffer. The gel was dried and subjected to autoradiography.

Western blot analysis and antibodies for IκB

Whole-cell extracts (40 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinyldifluoride membrane as described previously.[22] Blots were blocked for 1 h in PBS-T (PBS and 0.1% Tween 20) containing 5% skim milk, washed with PBS-T, and incubated in primary antibody for 1 h at room temperature with agitation. Blots were washed with PBS-T and incubated in biotin-conjugated secondary antibody under similar conditions. After incubation in horseradish peroxidase streptavidin (Vector, Minneapolis, MN, USA) for 1 h at room temperature, Western blots were analyzed by ECL Western blotting detection reagents (Amersham, Arlington Heights, IL, USA). Anti-IκBα/MAD3 (sc-203; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-IκBβ1 (sc-945, Santa Cruz Biotechnology) were used for primary antibodies.

Transfection and luciferase assays

Transfection of suspension HeLa cells was performed by electroporation with a Bio-Rad (Hercules, CA, USA) Gene Pulser as described previously.[22] In detail, cells were grown at 5×10^5 cells/ml. Then, 0.4 ml of cells was mixed with 20 μg DNA at room temperature, electroporated at 250 V and 950 μF, and immediately transferred to small culture flasks containing 10 ml prewarmed medium. After 24 h, transfected cells were treated with TNF-α, and harvested after an additional 24 hours. For reporter gene assays, HeLa cells were transfected by electroporation with RANTES promoter-reporter plasmids (20 μg). Luciferase activities were measured with a Luminometer (Lumat LB9501; Berthold, Bad Wildbad, Germany).

Results

Production of antigenic RANTES by TNF-α

To examine the effect of TNF-α on RANTES production in fibroblast-like synoviocytes, conditioned media were collected from cells treated with TNF-α. TNF-α significantly increased RANTES protein production in a time-dependent manner (Fig. 1A). Moreover, RANTES mRNA expression was clearly upgraded by TNF-α (Fig. 1B). Cell viability was intact in the medium containing all TNF-α concentrations (data not shown).

Induction of DNA-binding activity of NF-κB by TNF-α

To determine the effect of TNF-α on NF-κB activation in fibroblast-like synoviocytes, EMSA was performed. Whole-cell extracts were prepared from fibroblast-like synoviocytes following 15-min to 24-h incubations with TNF-α (10 ng/ml). The κB-binding activity of the protein extracts was analyzed using an oligonucleotide encoding the NF-κB site of the major histocompatibility complex class I promoter. Here, DNA-binding activity of NF-κB was strongly induced by TNF-α and was maximal at 15 min (Fig. 2A, lane 3). Moreover, TNF-α continuously activated DNA-binding activity of NF-κB until 24 h (Fig. 2A, lanes 3–7). In addition, TNF-α-induced mobility shifts were abrogated by prior heat inactivation (95°C, 10 min) (data not shown). To identify Rel proteins associated with TNF-α-induced NF-κB-DNA complexes, competition and supershift analyses were performed. Whole-cell extracts from fibroblast-like synoviocytes activated by TNF-α (10 ng/ml) were used. Successful competition was observed using unlabeled NF-κB probe (Fig. 2B, lane 3), whereas an unrelated oligonucleotide was ineffective (Fig. 2B, lane 4). Moreover, TNF-α-induced DNA-binding activity of NF-κB was clearly supershifted by anti-p65 and p50 antibodies but not by anti-c-Rel antibody (Fig. 2B, lanes 5–7 compared with lane 2).

Degradation of IκBα and IκBβ1 by TNF-α

To evaluate persistent effects of TNF-α on NF-κB activation, Western blot analysis of the corresponding extracts with IκB isoform-specific antibodies was performed. Whole-cell extracts (40 μg) were separated by SDS-PAGE and blotted onto a polyvinyldifluoride membrane. Strong degradation was performed on IκBα and IκBβ1 with 10 ng/ml of TNF-α (Fig. 3). At 15 and 30 min after stimulation with TNF-α, IκBα was completely degraded (Fig. 3, lanes 2,3). Furthermore, IκBβ1 degradation was induced at 15 min after treatment with TNF-α and continued for 24 h (Fig. 3, lanes 8–12). Accordingly, these results imply that persistent NF-κB activation induced by TNF-α in fibroblast-like synoviocytes was a result of degradation of IκBβ1, possibly suggesting that persistent IκBβ1 degradation led to continuous RANTES production in fibroblast-like synoviocytes.

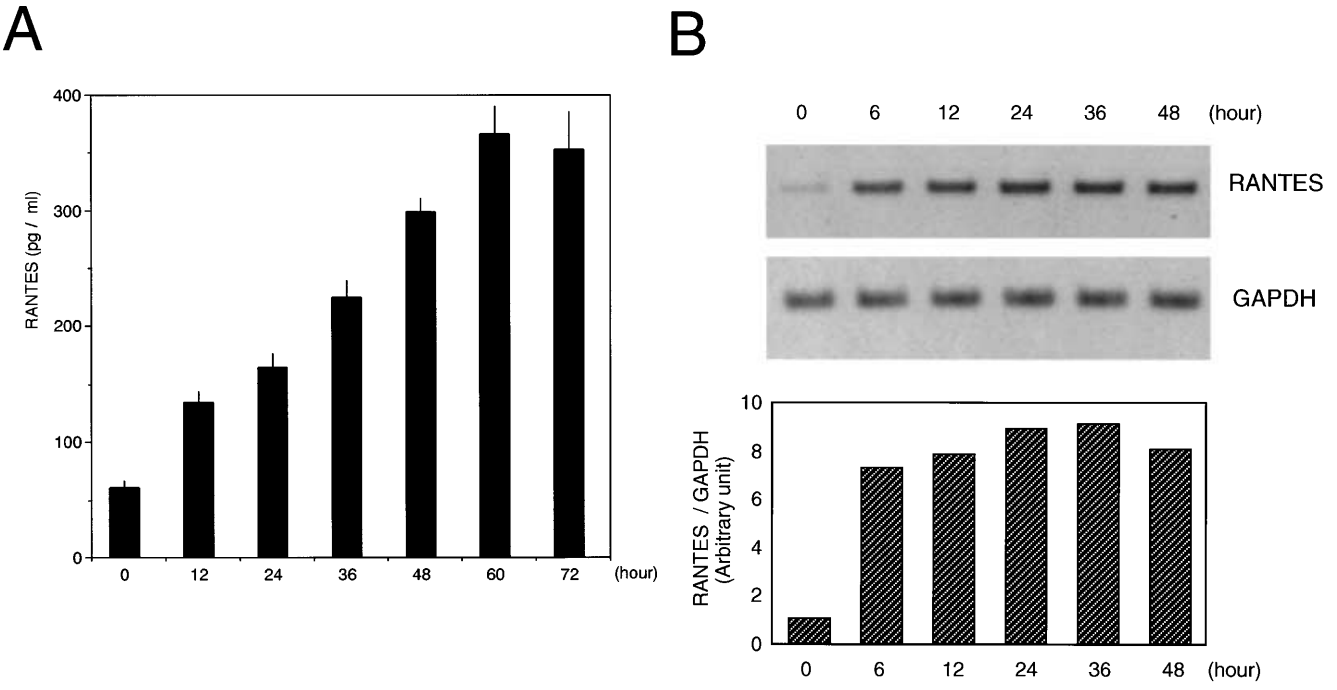

Fig. 1A,B. Effect of tumor necrosis factor-alpha (TNF-α) on regulated upon activation, normal T-cell expressed and secreted (RANTES) production in rheumatoid arthritis (RA) fibroblast-like synoviocytes. **A** RANTES protein in conditioned media. Enzyme-linked immuno sorbent assay (ELISA) was performed as described in Materials and methods. Conditioned media were collected after treatment with 10 ng/ml TNF-α for indicated times. Experiments were performed in quadruplicate. Results are presented as mean + SE of three independent experiments. **B** RANTES mRNA. Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed using total RNA in fibroblast-like synoviocytes from patients with RA as described in Materials and methods. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control. *Bottom*: densitometric quantification of RANTES mRNA/GAPDH mRNA ratio

Roles of NF-κB in TNF-α-induced RANTES promoter activity

To test effects of TNF-α on RANTES gene expression, a luciferase assay was performed using the reporter plasmid. According to dosage, TNF-α increased RANTES gene expression (Fig. 4B, lanes 1–4). Thus, TNF-α induced RANTES expression at the transcriptional level. Next, to define more precisely the roles of κB sites in TNF-α-controlling RANTES expression, each *cis*-acting element of NF-κB-binding sites was mutated individually. The RANTES gene upstream sequence contains the two putative NF-κB binding sites (κB1 and κB2; −44 and −30 relative to the transcription start site, respectively). Figure 4A presents mutations of individual *cis*-acting elements of NF-κB binding sites. The base vector for these constructions was the wild-type pN construct (Fig. 4A, upper). Because of the extremely poor transfection efficiency of fibroblast-like synoviocytes, the resulting wild-type, or mutant, constructions were transiently transfected into suspension HeLa cells that were highly transfectable. RANTES RNA blot hybridization showed that suspension HeLa cells responded to TNF-α with similar kinetics as the fibroblast-like synoviocytes (data not shown). Mutation of either κB1 or κB2 ablated TNF-α inducibility, suggesting that both sites were required for TNF-α induction of RANTES (Fig. 4B, lanes 5–12). These results might indicate TNF-α induced RANTES gene expression through NF-κB activation.

Discussion

Accumulation and activation of selected populations of inflammatory cells within an arthritic joint are salient RA characteristics. One putative signal for this process is the production, by resident cells, of a group of inflammatory mediators known as the chemokines, e.g., IL-8, monocyte chemotactic protein 1 (MCP-1), and RANTES.[4–6] Such chemokines are target cell specific chemoattractants produced by synovial fibroblasts in response to TNF-α stimulation.[7] Cellular composition of inflammatory synovial tissues reveals an extensive infiltrate of T cells and macrophages. In RA, T cells in inflamed joints are CC chemokine receptor 5 positive.[23] These findings suggest a role for RANTES in recruitment of proinflammatory T cells to the inflamed joint in RA.

Results herein demonstrate that TNF-α persistently induced RANTES expression in fibroblast-like synoviocytes from patients with RA through NF-κB activation. For genes involved in the immune and inflammatory response, NF-κB is an essential regulator.[24–27] In most cell types, NF-κB is

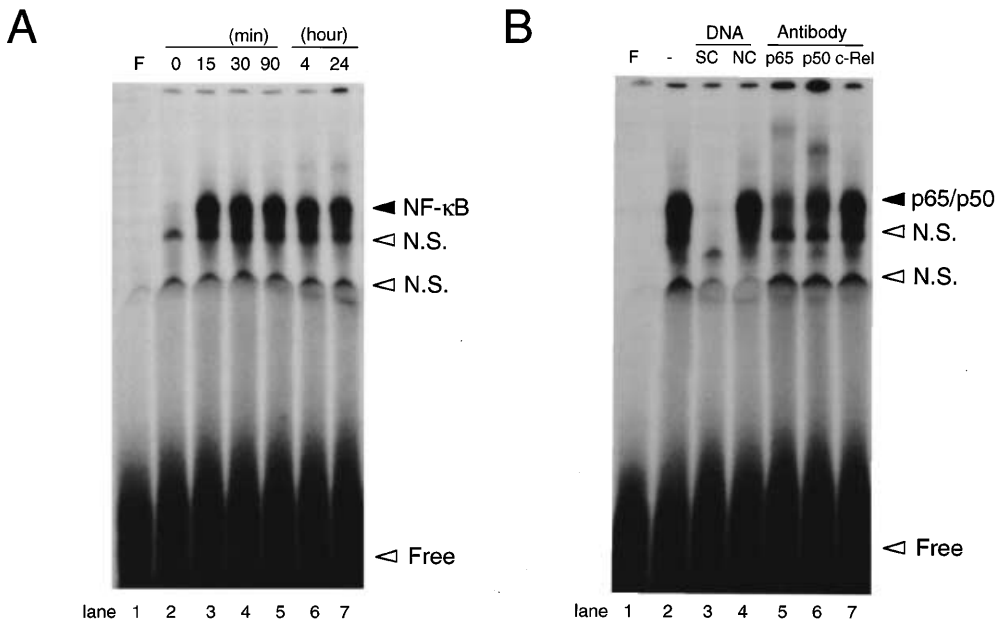

Fig. 2A,B. DNA-binding activity of nuclear factor (NF)-κB was induced by TNF-α in fibroblast-like synoviocytes. Electrophoretic mobility shift assay (EMSA) using H_2k oligonucleotide for a probe was performed as described in Materials and methods. **A** Time course. Fibroblast-like synoviocytes were treated with 10 ng/ml TNF-α for indicated times. Specific NF-κB bands, *closed triangle*; nonspecific bands and unbound labeled oligonucleotides, *open triangles* (*N.S.* and *Free*), respectively. Data are representative of three similar experiments. **B** Competition and supershift analysis. For competition, EMSA was performed using specific (*SC*) or nonspecific (*NC*) oligonucleotides on extracts obtained following 30 min of 10 ng/ml TNF-α. For supershift analysis, EMSA was performed using anti-p65, p50, or c-Rel antibody on extracts obtained following 30 min of 10 ng/ml TNF-α. Specific NF-κB bands (p65 and p50), *closed triangle*; nonspecific bands and unbound labeled oligonucleotides, *open triangles* (*N.S.* and *Free*), respectively. Data represent three similar experiments

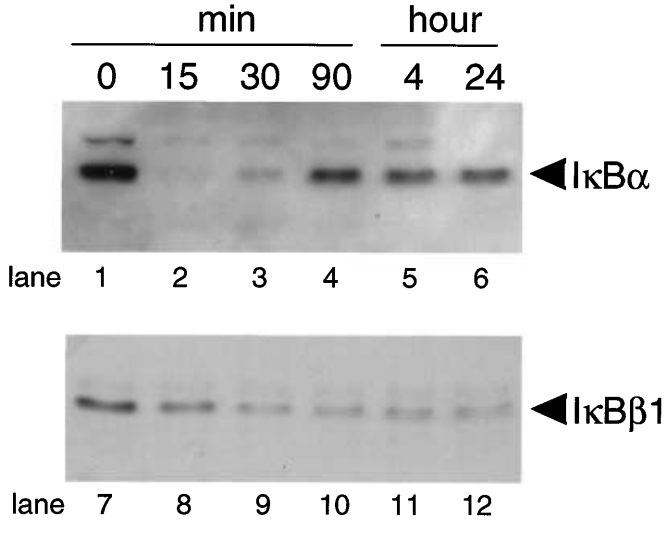

Fig. 3. Degradation of IκBα and IκBβ1 induced by TNF-α in fibroblast-like synoviocytes. Western blot analysis was performed as described in Materials and methods. Cells were treated with TNF-α for indicated times. Positions of IκBα (*lanes 1–6*) and IκBβ1 (*lanes 7–12*) in the Western blot are indicated. Data represent three similar experiments

present as a heterodimer comprising a 50-kDa (p50) and a 65-kDa subunit (p65) that is sequestered in the cytoplasm by a member of the IκB family of inhibitory proteins, which includes IκBα (also called MAD-3), IκBβ1, IκBβ2, IκBγ, IκBε, and Bcl-3.[22,28–32] Such IκB proteins mask the NF-κB nuclear localization signal, thereby preventing NF-κB nuclear translocation. Fujisawa et al. reported that NF-κB played a pivotal role in synovial cell proliferation by treatment with TNF-α, as *N*-acetyl-L-cysteine inhibited both TNF-α-induced NF-κB activation and synovial cell proliferation.[33] In that report, TNF-α was shown to upregulate mRNA expression of TNF-α itself and to persistently induce DNA-binding activity of NF-κB for more than 24 h in synovial cells from patients with RA.[33] However, they did not show the effect of TNF-α on IκBs degradation. In this report, we present for the first time that continuous NF-κB activation stimulated by TNF-α was, at least in part, also due to IκBβ1 degradation in RA fibroblast-like synoviocytes. Degradation of IκBβ1 is not associated with its rapid resynthesis, probably because the gene encoding IκBβ1 is not upregulated by NF-κB.[34–37] Treatment with TNF-α was well correlated with late-phase induction of NF-κB containing p65 and p50. Consequently, these findings suggest that degradation and downregulated expression of IκBβ1 might be an important mechanism for sustained induction of NF-κB nuclear expression by TNF-α. However, further study should examine degradation of other inhibitory molecules such as IκBβ2 and IκBε.

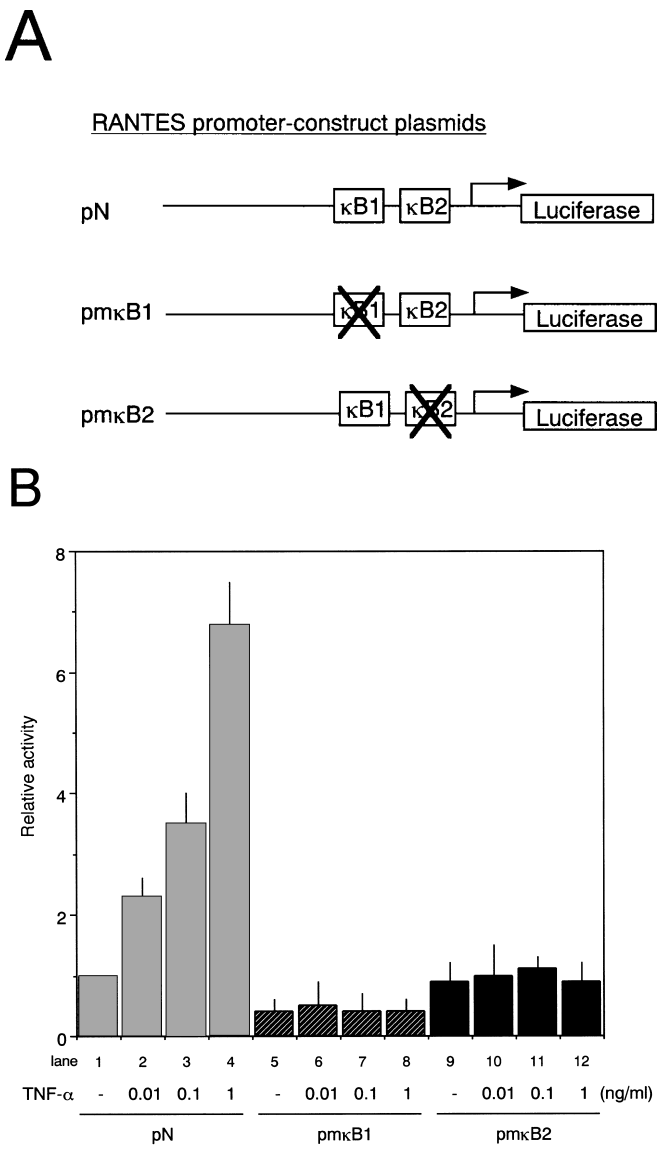

Fig. 4A,B. TNF-α-induced RANTES gene expression through two *cis*-acting elements of κB sites on the RANTES promoter. **A** Schematic maps of the RANTES reporter constructions. Two NF-κB binding sites are shown as κ*B1* and κ*B2*. Here, pN was the wild-type RANTES promoter construct plasmid. Mutated sites, *X*. Mutation of κB1 or κB2 site is shown as pmκB1 or pmκB2, respectively. **B** Transcriptional effect of TNF-α on RANTES gene expression. Cells were transfected with 20 μg of pN, pmκB1, or pmκB2. After transfection, cells were incubated with indicated concentrations of TNF-α. After 24 h, cellular extracts were prepared for luciferase enzyme assay. Experiments were performed in quadruplicate. Levels of luciferase activity of pN in unstimulated cells were taken as 1.0. Results are mean + SE of three independent experiments

Functional studies indicate that multiple *cis*-acting elements interspersed within the RANTES promoter sequence contribute to promoter activity on cell activation.[18] This study asserts that mutation on NF-κB-binding sites markedly reduced TNF-α inducibility, possibly indicating that NF-κB is a potent inducer of RANTES expression in response to TNF-α. Aupperle et al. reported that only inhibition of IκB kinase-2 (IKK2) function could prevent TNF-α-induced expression of intercellular adhesion molecule 1 in RA fibroblast-like synoviocytes, identifying IKK2 as the key convergence site for the TNF-α-induced NF-κB-mediated pathway.[38] Moreover, IKK2 induces IκBα and IκBβ1 degradation in human monocytic cells.[39] Thus, in synoviocytes, TNF-α might activate IKK2 and induce NF-κB activation, which transcriptionally upregulates RANTES expression.

In summary, these results might indicate that NF-κB activation is required for TNF-α-induced RANTES expression through two NF-κB-binding sites of the RANTES promoter in fibroblast-like synoviocytes.

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