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Inhibition of IL-1β-mediated inflammatory responses by the IκBα super-repressor in human fibroblast-like synoviocytes

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ABSTRACT

The IL-1β-NF-κB axis is a key pathway in the pathogenesis of rheumatoid arthritis (RA) and is central in the production of proinflammatory mediators in the inflamed synovium. Therefore, we examined whether fibroblast-like synoviocytes (FLS) could be spared from IL-1β-induced toxicity by an over-expressing IκB super-repressor. Infection of FLS with Ad-IκBα (S32A, S36A), an adenovirus-containing mutant IκBα, inhibited IL-1β-induced nuclear translocation and DNA binding of NF-κB. In addition, Ad-IκBα (S32A, S36A) prevented IL-1β-induced inflammatory responses; namely, the production of chemokines, such as ENA-78 and RANTES, and activation of MMP-1 and MMP-3. Finally, increased cellular proliferation of FLS after IL-1β treatment was significantly reduced by Ad-IκBα (S32A, S36A). However, Ad-IκBβ (S19A, S23A), the IκBβ mutant, was not effective in preventing IL-1β toxicity. These results suggest that inhibition of IκBα degradation is a potential target for the prevention of joint destruction in patients with RA.

Rheumatoid arthritis (RA) is a chronic destructive disease of the joints that is characterized by proliferative synovitis, infiltration of inflammatory cells into synovial tissue of joints, and cartilage destruction. Fibroblast-like synoviocytes (FLS) and inflammatory cells, such as macrophages and T cells, produce proinflammatory cytokines, such as IL-1β and TNF-α, which play key roles in the pathogenesis of RA. Clinical studies have demonstrated that antagonists to the IL-1 receptor and monoclonal antibodies specific for TNF-α are effective in ameliorating RA [1,2]. In response to these cytokines, FLS produce chemokines, which further promote inflammation, hyperplasia, and cartilage destruction [3]. Chemokines can be divided into two major subfamilies, the CC and CXC subfamilies. CC chemokines, such as regulated on activation, normal T-cell expressed and secreted (RANTES), are implicated in chronic inflammatory disease, whereas CXC chemokines, such as epithelial neutrophil activating peptide-78 (ENA-78), are potent chemotactic factors for neutrophils and are implicated in acute inflammation [4,5].

In contrast to TNF-α, which is predominantly detected during the early stages of RA, IL-1β is detected long after the onset of the disease. IL-1β exerts its primary effects through the transcriptional nuclear factor κB (NF-κB) pathway. NF-κB activation in FLS contributes to the pathogenesis of RA by activating the transcription of a family of matrix metalloproteinases (MMPs) [6]. The MMP family includes over 20 members that differentially mediate the degradation of each component of the extracellular matrix, including MMP-1 and MMP-3, which are reportedly major enzymes involved in tissue destruction. MMP-1 and MMP-3 are found in higher levels in synovial fluids of RA patients [7]. These MMPs are major products of cytokine-stimulated FLS and efficiently degrade the collagenous components of cartilage and bone, which leads to joint deformity and a great deal of pain in RA patients.

In unstimulated cells, NF-κB is present in the cytoplasm in its inactive form complexed with IκB, an inhibitory factor of NF-κB. Various inducers cause dissociation of this complex, presumably by phosphorylation of IκB, which leads to NF-κB being released from the complex. NF-κB then translocates to the nucleus, where it interacts with specific DNA recognition sites to mediate gene transcription [8]. A number of different IκB proteins have been identified, including IκBα, IκBβ, IκBγ, and IκBε. Among these, IκBα and IκBβ are the most studied. Mutant forms of IκBα (S32A, S36A)

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or IκBβ (S19A, S23A), in which alanine residues are substituted for serine residues 32 and 36 or serine residues 19 and 23, respectively, have been shown to effectively prevent IκBα or IκBβ phosphorylation, degradation, and NF-κB activation in various systems [9–11]. In this study, we used an adenoviral construct of IκBα (S32A, S36A) and IκBβ (S19A, S23A) to block the NF-κB pathway in activated FLS. Ad-IκBα (S32A, S36A), but not Ad-IκBβ (S19A, S23A), was effective in preventing IL-1β-stimulated inflammatory responses in FLS.

Materials and methods

Isolation and culture of human FLS. FLS were isolated from primary synovial tissue obtained from 12 patients with RA who met the revised American Rheumatism Association criteria and had undergone total joint replacement surgery or synovectomy as we described previously [12]. Cells were used at passages 4–8, at which time they were comprised of a homogeneous population. Cells were grown at 37 °C under a humidified, 5% CO_2 atmosphere in high glucose-containing DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B. All treatments were performed in serum-free medium. Informed consent was obtained from all patients, and the study protocol was approved by the Chonbuk National University Hospital Ethical Committee.

Preparation of recombinant adenovirus. The adenoviruses containing the IκBα (S32A, S36A) or IκBβ (S19A, S23A) were kindly donated by Y.T. Kwak (UT Southwestern Medical Center, Dallas, TX). The control recombinant replication-defective adenoviruses containing β-galactosidase (Ad-β-gal) were obtained from R.H. Unger (UT Southwestern Medical Center). HEK293 cells were used for virus transfection and amplification. Viruses from 293 cell culture supernatants were purified by cesium chloride banding. Adenovirus infection of the FLS was performed by using a multiplicity of infection of 10 per cell for 24 h of exposure.

MTT assay. The proliferation potential of cultured cells was determined by measuring the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan as described previously [13].

Assay for chemokine production and MMP-1 and MMP-3. After treatment with IL-1β, the chemokine concentration and total MMP-3 in the cell culture supernatants were determined using Quantikine ELISA kits (R&D Systems). MMP-1 activity in the cell culture supernatants was quantified by fluorescent assay using the Fluorokine E Human Active MMP-1 Fluorescent Assay Kit (R&D Systems) according to the manufacturer's protocol.

Preparation of whole cell and nuclear protein extracts. FLS were seeded in 100 mm culture dishes at a density of 2×10^6 cells per dish and were then treated with IL-1β (5 ng/ml) for 3 h. Cells were immediately washed twice, scraped into 1.5 ml of ice-cold PBS (pH 7.9), and then pelleted at 12,000 *g* for 30 s. The cell pellets were then lysed in CytoBuster™ Protein Extraction Buffer (Novagen, Madison, WI). The lysate was centrifuged at 10,000g for 5 min at 4 °C, and the supernatant was used as the whole cell protein extract. Cytoplasmic and nuclear extracts were prepared from cells using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL).

Western blot analysis. FLS were treated with IL-1β (5 ng/ml) for 24 h and homogenized in 100 μl of ice-cold lysis buffer (20 mM Hepes, pH 7.2, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml aprotinin). The homogenates, which contained 20 μg of protein, were separated by SDS–PAGE, and then transferred to nitrocellulose membranes. The blot was probed with 1 μg/ml of primary antibodies for flag, p65, p50, β-actin, PCNA (Santa Cruz Biochemicals, Santa Cruz, CA), MMP-1, or MMP-3 (R&D Systems) and then detected with Horseradish peroxidase-conjugated IgG (Zymed, South San Francisco, CA).

Electrophoretic mobility shift analysis (EMSA). Activation of NF-κB was determined by a gel mobility shift assay using nuclear extracts from control and treated cells. An oligonucleotide containing the κ-chain binding site (κB, 5′-CCGGTTAACAGAGGGGGC TTTCCGAG-3′) was synthesized and used as a probe for the gel retardation assay. The two complementary strands were then annealed and labeled with [α-^{32}P]dCTP. Labeled oligonucleotides (10,000 cpm), 10 μg of nuclear extracts, and binding buffer (10 mM Tris–HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng poly(dI·dC), 1 mM dithiothreitol) were incubated for 30 min at room temperature in a final volume of 20 μl. The reaction mixtures were analyzed by electrophoresis on 4% polyacrylamide gels in 0.5× Tris–borate buffer, and the gels were dried and examined by autoradiography.

Statistical analysis. Statistical analyses of the data were performed using analysis of variance (ANOVA) and Duncan's multiple range test. *P*-values <0.05 were considered statistically significant.

Results

Effect of adenovirus-mediated IκBα super-repressor overexpression on IL-1β-induced NF-κB activity

To determine whether constitutive inactivation of NF-κB by regulation of IκBα or IκBβ could affect the inflammatory responses of IL-1β, we utilized recombinant adenoviruses containing cDNA of either flag-tagged mutant IκBα (S32A, S36A) or IκBβ (S19A, S23A). Western blot analysis with a flag monoclonal antibody confirmed the expression of the mutant IκBα and IκBβ proteins upon infection of FLS with the recombinant adenoviruses (Fig. 1A).

Each of these adenoviruses was then used to infect FLS, and nuclear extract prepared 3 h after IL-1β treatment was used to analyze NF-κB activity. The IL-1β-stimulated FLS showed increased p65 and p50 subunit levels in their nuclei (Fig. 1B) as well as an increase in binding activity to an NF-κB consensus sequence (Fig. 1C) when compared to unstimulated cells. However, nuclear extracts prepared from Ad-IκBα (S32A, S36A)-infected FLS revealed suppressed nuclear translocation and DNA binding of NF-κB. Neither Ad-IκBβ (S19A, S23A) nor Ad-β-gal altered the IL-1β-induced NF-κB activation. These results indicate that IκBα, but not IκBβ, is the major participant in IL-1β-induced NF-κB activation in FLS.

Effect of adenovirus-mediated IκBα super-repressor overexpression on IL-1β-induced chemokine production

To determine the effects of selective overexpression of either Ad-IκBα (S32A, S36A) or Ad-IκBβ (S19A, S23A) on chemokine production, infected FLS were incubated for 24 h with IL-1β, at which time the levels of chemokines present in the culture supernatants were determined using an ELISA kit. As shown in Fig. 2, IL-1β induced a 21.1-fold increase in the production of RANTES and a 45.2-fold increase in the production of ENA-78. However, prior infection with Ad-IκBα (S32A, S36A) significantly diminished IL-1β-mediated chemokine production. In contrast, overexpression of Ad-IκBβ (S19A, S23A) failed to reduce chemokine production.

Effect of adenovirus-mediated IκBα super-repressor overexpression on IL-1β-induced activation of MMP-1 and MMP-3

We used an ELISA kit to investigate the effect of Ad-IκBα (S32A, S36A) on IL-1β-induced MMP secretion. Incubation of FLS with IL-1β resulted in a 7.1-fold increase in production of MMP-1 and

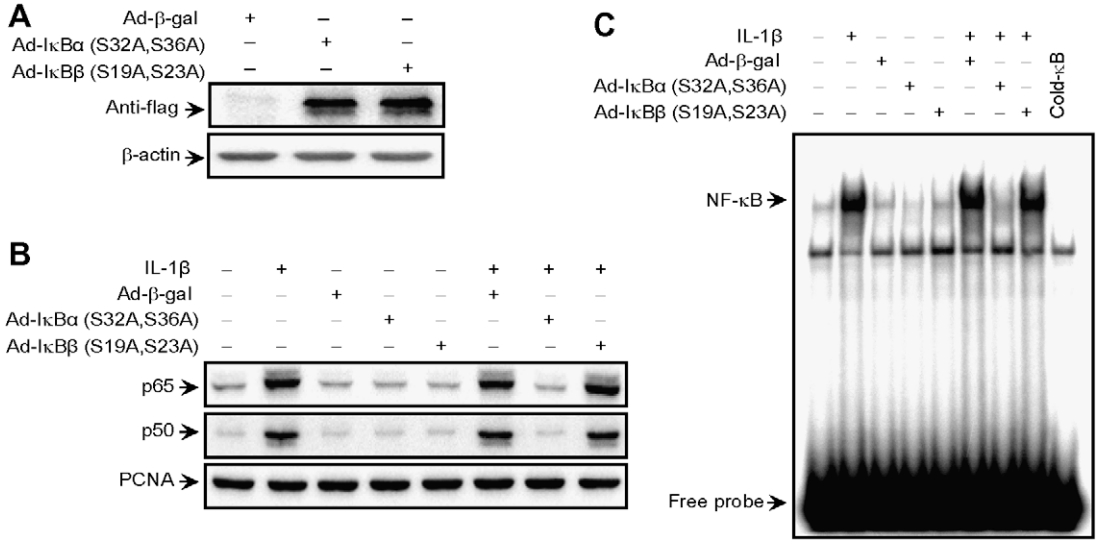

Fig. 1. Expression of IκB super-repressors and their effects on IL-1β-induced NF-κB activation. FLS (2×10^6) were infected with adenoviruses containing the IκBα (S32A, S36A) or IκBβ (S19A, S23A) super-repressor cDNA with FLAG tag. Ad-β-gal was used as a control. Cell extracts prepared 24 h after viral infection were subjected to immunoblot analysis with anti-flag antibody conjugated with horseradish peroxidase (A). The effect of the overexpressed IκB super-repressors on the IL-1β-induced NF-κB pathway was then analyzed. FLS infected with the IκB super-repressors or the control virus were treated with IL-1β (5 ng/ml). Following 3 h of incubation, the translocation of p65 and p50 to the nucleus was analyzed by Western blotting (B) and NF-κB DNA binding activity was analyzed by EMSA (C). PCNA was used as a nuclear loading control. Similar results were obtained in three independent experiments.

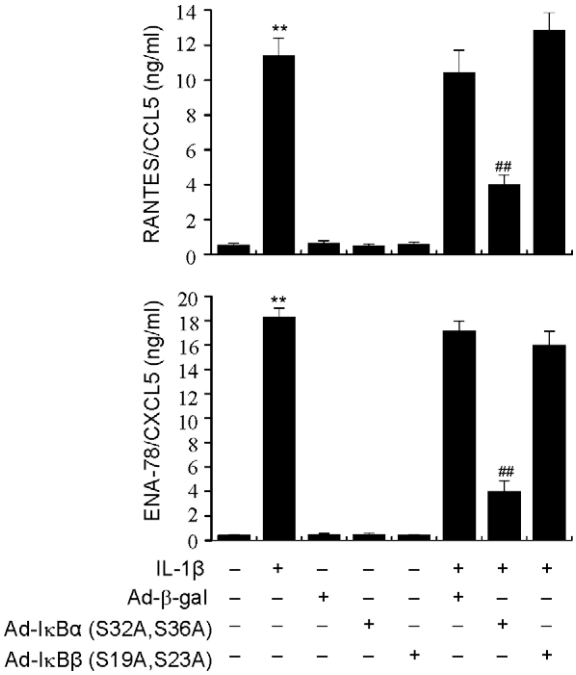

Fig. 2. Inhibition of IL-1β-induced chemokine production by the IκBα super-repressor. FLS (2×10^6) were infected with Ad-IκBα (S32A, S36A), Ad-IκBβ (S19A, S23A), or Ad-β-gal for 24 h and exposed to IL-1β (5 ng/ml) for 24 h. The levels of ENA-78 and RANTES were then determined in the cell-free culture supernatants by ELISA. Each value represents the mean ± the SEM of the three independent experiments. $^{**}p < 0.01$ vs. untreated control; $^{\#\#}p < 0.01$ vs. IL-1β.

a 10.5-fold increase in production of MMP-3 in the culture supernatants (Fig. 3A). However, prior infection with Ad-IκBα (S32A, S36A) significantly diminished IL-1β-mediated MMP-1 and MMP-3 production by 47.2% and 56.4%, respectively. The effect of Ad-IκBα (S32A, S36A) on IL-1β-induced MMP activation was further confirmed by Western blotting. As shown in Fig. 3B, IL-1β increased MMP-1 and MMP-3 protein levels in the total cell lysates, which was similar to the results observed when the ELISA was conducted. Prior infection with Ad-IκBα (S32A, S36A), but not with Ad-IκBβ (S19A, S23A), eliminated the action of IL-1β and decreased levels of MMP-1 and MMP-3.

Effect of adenovirus-mediated IκBα super-repressor overexpression on IL-1β-induced cell proliferation

Synovial hyperplasia is believed to play a central role in the development of pannus, a thickening of synovial tissue responsible for the cartilage and bone erosion seen in RA. Researchers have suggested that synovial hyperplasia is caused by an increased rate of proliferation of FLS. IL-1β stimulates the proliferation of FLS *in vitro* [12,14] and is known to act via signal transduction pathways including NF-κB [15] and mitogen-activated protein kinases [16]. To investigate whether Ad-IκBα (S32A, S36A) can affect the proliferation of IL-1β-stimulated FLS, cellular proliferation was measured using the MTT method (Fig. 4). Treatment with IL-1β for 72 h significantly increased the cell proliferative potential to 138.3 ± 3.6% of that of the control ($p < .05$). However, Ad-IκBα (S32A, S36A) reduced the proliferative potential of IL-1β-treated FLS by 105.3 ± 5.7%. Again, Ad-IκBβ (S19A, S23A) and Ad-β-gal were not effective in reducing IL-1β-induced proliferative potential.

Discussion

IL-1β is an important mediator of joint destruction in RA, and NF-κB is an IL-1β-inducible transcription factor that drives the expression of genes involved in inflammation. Unsurprisingly, NF-κB has been extensively studied to determine if it plays a role in the pathogenesis of RA, and NF-κB inhibition has been explored as a therapeutic approach for the disease. In the present study, our results indicated that the IκBα super-repressor inhibited the NF-κB pathway, which in turn inhibited inflammatory responses in IL-1β-stimulated FLS.

To investigate the critical role of NF-κB in IL-1β-induced inflammatory responses in FLS, we used the IκB mutant forms Ad-IκBα (S32A, S36A) and Ad-IκBβ (S19A, S23A). Prevention of NF-κB activation by overexpressing an IκB super-repressor has been reported previously in various systems, such as hepatic stellate cells [9],

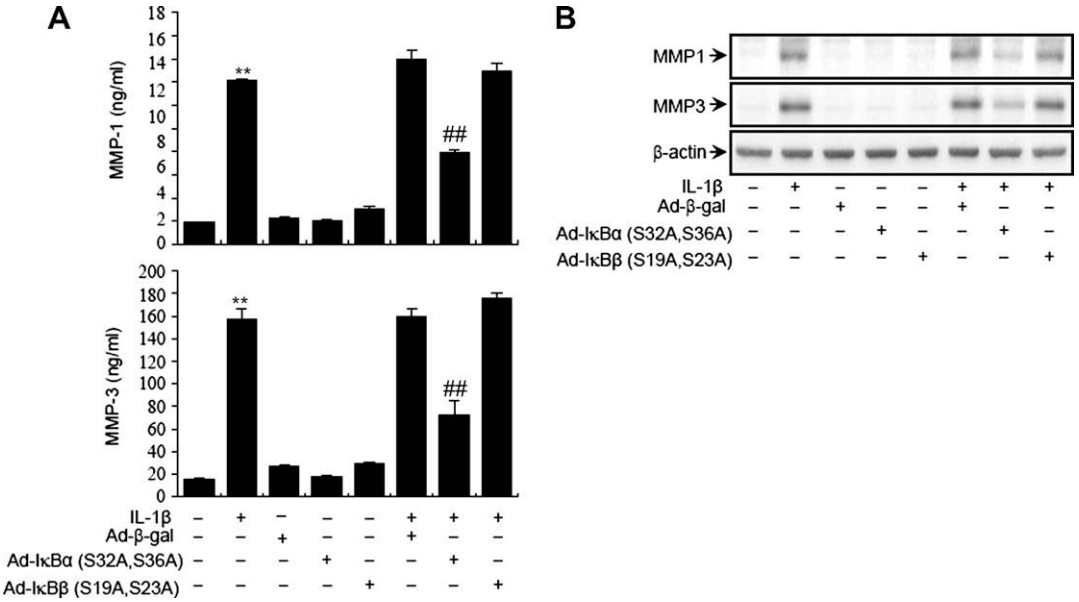

Fig. 3. Inhibition of IL-1β-induced MMP-1 and MMP-3 secretion from FLS by the IκBα super-repressor. FLS cultured in a 100 mm dish at a density of 2×10^6 cells were infected with Ad-IκBα (S32A, S36A), Ad-IκBβ (S19A, S23A), or Ad-β-gal for 24 h and exposed to IL-1β (5 ng/ml) for 24 h. The presence of MMP-1 and MMP-3 in the cell-free culture supernatants and total cell lysates were then evaluated by ELISA (A) and Western blotting (B), respectively. Each value represents the mean ± the SEM of the three independent experiments. $^{**}p < 0.01$ vs. untreated control; $^{\#\#}p < .01$ vs. IL-1β.

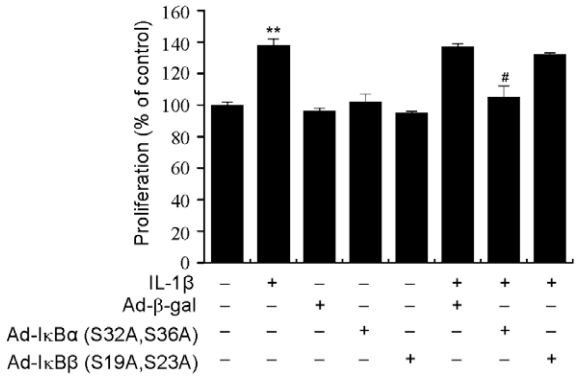

Fig. 4. Effect of the IκB super-repressors on IL-1β-induced cell proliferation. FLS (2×10^4) were infected with Ad-IκBα (S32A, S36A), Ad-IκBβ (S19A, S23A) or Ad-β-gal for 24 h and exposed to IL-1β (5 ng/ml) for 72 h. Cellular proliferation was measured using the MTT method. Each value represents the mean ± the SEM of the three independent experiments. $^{**}p < 0.01$ vs. untreated control; $^{\#}p < 0.05$ vs. IL-1β.

vascular smooth muscle cells [10], and prostate cancer cells [11]. We also previously demonstrated that adenoviral gene transfer of the IκBα super-repressor, but not the IκBβ super-repressor, to cultured pancreatic β cells prevented cytokine-mediated NF-κB activation [13]. In the present study, infection with Ad-IκBα (S32A, S36A) or Ad-IκBβ (S19A, S23A) induced a higher expression of non-degradable IκBα or IκBβ. Among these, Ad-IκBα (S32A, S36A) prevented IL-1β-induced NF-κB translocation to the nucleus, as indicated by EMSA and Western blotting analysis. This event further led to inhibition of chemokine production and MMP-1 and MMP-3 activation by IL-1β. In addition, Ad-IκBα (S32A, S36A) largely prevented cellular proliferation by IL-1β. However, Ad-IκBβ (S19A, S23A) was not effective at any step in preventing the aforementioned pathway, which suggests that IκBα, not IκBβ, is crucial for IL-1β-induced NF-κB activation and subsequent downstream inflammatory responses in FLS.

Treatment with IL-1β led to an increased production of ENA-78 and RANTES in FLS, which is in line with previous findings [4,12]. In addition, we showed that enhanced production of these chemokines by IL-1β was suppressed by the IκBα super-repressor. These chemokines are constitutively produced by FLS, and the expression of these chemokines has generally preceded the onset of clinical symptoms in animal models of RA [17]. Excessive production of chemokines by FLS has been shown to induce proliferation of these cells and to facilitate their invasion into the adjacent tissues [4]. Several mechanisms for regulation of chemokine gene expression have been suggested, including mRNA destabilization [18] and/or regulation of their transcription [19]. Promoters of ENA-78 [20] and RANTES [21] genes contain binding sites for NF-κB. Therefore suppression of nuclear translocation of NF-κB by the IκBα super-repressor might lead to decreased chemokine transcription.

Twenty-three members of the MMP family have been identified in humans to date. Of these, MMP-1 and MMP-3 are particularly important to RA because they are produced by FLS and macrophages in the synovium and are known to play a key role in tissue destruction. The levels of MMP-1 and MMP-3 are significantly higher in synovial fluid of RA patients [7,22]. FLS have been identified as one of the major sources of MMPs in RA, and the ability of FLS to destroy collagenous components of cartilage and bone appears to be associated with the level of MMP expression [23]. In this study, we showed that MMP-1 and MMP-3 were activated by IL-1β treatment, which was blocked by the IκBα super-repressor. This finding corroborates those of previous studies showing that NF-κB activation and nuclear translocation are necessary steps for IL-1β-induced MMP expression [24,25].

NF-κB has a dual role in RA pathology. As discussed above, activation of NF-κB in FLS is required for the inflammatory responses. On the other hand, suppression of NF-κB promotes cellular hyperplasia. The IκBα super-repressor inhibited the proliferative response to IL-1β in FLS. This result indicates that an activation of NF-κB is involved in the IL-1β-induced proliferation of FLS. It has been proposed that the activation of NF-κB protects the cells against apoptosis, thereby resulting in hyperplasia. We previously showed that pharmacological NF-κB inhibitor was effective in inhibiting the proliferation of FLS [12]. In addition, Miagkov et al. [26] demonstrated that suppression of NF-κB by intra-articular injection of the IκBα super-repressor profoundly enhanced apoptosis in the synovium of rats with streptococcal cell wall (SCW)-

and pristane-induced arthritis. It is therefore conceivable that the effect of the IκBα super-repressor on the proliferative response to IL-1β is due to inhibition of NF-κB activation.

In conclusion, we investigated the role of the NF-κB signal transduction pathway in RA by employing two IκB mutants; namely, super-repressors IκBα (S32A, S36A) and IκBβ (S19A, S23A). The IκBα super-repressor exerts a potent inhibitory effect on IL-1β-induced inflammatory responses in isolated FLS. These *in vitro* data provide a rationale for targeting NF-κB in attempts to prevent RA development. Additional *in vivo* animal experiments are required to determine whether using the IκBα super-repressor to block NF-κB activation will indeed prevent RA development.

Acknowledgments

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