Transcription factor decoy for NFkB inhibits cytokine and adhesion molecule expressions in synovial cells derived from rheumatoid arthritis


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Abstract

Objective. Numerous cytokines are expressed in lesions of synovial hyperplasia of patients with rheumatoid arthritis (RA), and their pathophysiological contributions have been the subject of speculation. These genes are regulated by the transcription factor NFkB which in turn is activated by tumour necrosis factor-α (TNF-α) and cytokines. In this study we examined the inhibition of the production of pro-inflammatory cytokines, adhesion molecule and matrix metalloproteinase (MMP) from synovial tissue of patients with RA by the introduction of synthetic double-stranded DNA with high affinity for the NFkB binding site.

Method. NFkB decoy oligonucleotides (ODN) were introduced with the aid of the haemagglutinating virus of Japan (HVJ)-liposome method into synovial tissue or synovial cells derived from patients with RA. The levels of interleukin-1β (IL-1β), IL-6, TNF-α, intercellular adhesion molecule-1 (ICAM-1) and MMP-1 were determined by means of enzyme-linked immunosorbent assay (ELISA) and Northern blotting analysis. A cell counting kit was used to study the effect of NFkB decoy ODN on synovial cell proliferation.

Results. The production of these mediators was significantly inhibited by the introduction of NFkB decoy ODN compared with the effect of scrambled decoy ODN. Transfection of NFkB decoy ODN resulted in a significant inhibition of synovial cell proliferation as compared with that of scrambled decoy ODN.

Conclusion. The results demonstrated in this study suggest the potential usefulness of NFkB decoy ODN for gene therapy of inflammatory synovitis of RA.

Key words: Transcription factor decoy, Gene therapy, Rheumatoid arthritis, HVJ-liposome, Synovial cells.

One of the characteristic features of rheumatoid arthritis (RA) is synovial hyperplasia with infiltration of various inflammatory cells resulting in degradation of articular cartilage and bone [1, 2]. Overexpression of inflammatory cytokines, especially interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α), is thought to play an important role in the pathogenesis of joint destruction in the arthritic joints [3, 4]. Synovial cells are the major source of these pro-inflammatory cytokines [5], and IL-1 and TNF-α expression is particularly clearly recognizable at the sites of cartilage-pannus junction [6]. Monoclonal anti-TNF-α, soluble TNF-α receptor fusion protein, soluble IL-1 receptor, and IL-1 receptor antagonist have been successfully used to block the activity of TNF-α and IL-1 both in experimental models and human trials [7–13]. These results suggest the importance of such inflammatory cytokines in the pathogenesis of arthritis, and that the blockade of these cytokines or augmentation of anti-inflammatory cytokines may provide an alternative therapy for arthritis.

NFkB is a heterodimetric DNA-binding protein, and a critical element in the regulation of many genes involved in the complex formed by NFkB and 1xB. It has been reported that dissociation of the transcription factor NFkB from this complex plays a pivotal role in the regulation of inflammatory cytokine production by inducing a co-ordinated transactivation of genes including TNF-α, IL-1, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and intercellular adhesion molecule-1 (ICAM-1) [14–18]. In RA, activa-
tion of NFkB in synovium has been reported [19–21]. Recently NFkB has been reported to regulate matrix metalloproteinase-1 (MMP-1) transcription [22]. In this study we examined the role of NFkB in synovial tissue derived from RA. We hypothesized that the transfection of a sufficient quantity of decoy oligodeoxynucleotides (ODN) containing the NFkB cis element into inflammatory cells in the synovial tissues of RA would effectively bind NFkB, thus preventing those cells from transactivating the gene expression of inflammatory cytokines, adhesion molecules and MMP-1.

To deliver the transcription factor decoy into synovial cells, we used the haemagglutinating virus of Japan (HVJ)–liposome-mediated method of gene transfer which has been shown to enhance the efficiency of transcription of oligodeoxynucleotides [23–26]. In this study we examined the inhibition of the production of pro-inflammatory cytokines, adhesion molecule and MMP from synovial tissue of patients with RA by the introduction of synthetic double-stranded DNA with high affinity for the NFkB binding site.

Materials and methods

Patients

Synovial tissues were obtained, with informed consent, from five patients with RA who were undergoing joint reconstructive surgery at Osaka University Hospital. All of the patients with RA satisfied the 1987 revised diagnostic criteria of the American College of Rheumatology (formerly the American Rheumatism Association) [27]. Normal synovial tissues were obtained from five patients who were seen at the hospital for trauma and had no evidence of arthritis.

Organ culture

The tissue specimen was cut into small pieces, washed three times in phosphate-buffered saline (PBS), and its wet weight determined. Synovial tissues were cultured on 24-well plates at 150 mg/well in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Grand Island, New York, USA) supplemented with 10% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT, USA).

Synthesis of ODN and selection of sequence targets

The sequences of phosphorothioate double-stranded ODN against the NFkB binding site and of scrambled ODN used in this study were reported previously [26]. The phosphorothioate ODN utilized in this study had the following sequences:

NFkB ODN (consensus sequences are shown in bold)

5’-CTTGAAGGAGTTCCCTCC-3’

5’-GGACTTCCTAAGGGAGG-5’

Scrambled ODN

5’-CTTGAAGGAGTTCCCTCC-3’

5’-GGACTTCCTAAGGGAGG-5’

The NFkB ODN have been shown to bind the NFkB transcription factor [26]. Synthetic ODN were washed in 70% ethanol, dried and dissolved in sterile Tris-ethylene diamine tetra acetic acid (EDTA) buffer (10 mM Tris, 1 mM EDTA). The supernatant was purified over a nucleic acid purification-10 (NAP-10) column (Pharmacia LKB Biotechnology, Piscataway, NJ, USA), and the ODN concentration was quantitated by spectrophotometry. The single-stranded ODN were annealed for 2 h while the temperature descended from 80 to 25°C.

Gel mobility shift assay

The nuclear extract was prepared from cultured synovial tissues using methods described previously [25]. In brief, synovial tissues were homogenized with Potte-Elvehjem homogenizer in 4 volumes of ice-cold homogenization buffer [10 mM HEPES pH 7.5, 0.5 mM sucrose, 0.5 mM spermidine, 0.15 mM spermin, 5 mM EDTA, 0.25 mM ethylene glycol tetra acetic acid (EGTA), 7 mM β-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride]. After centrifugation at 12 000 g for 30 min at 4°C, the pellets were lysed and homogenized in a Dounce homogenizer in 1 volume of ice-cold homogenization buffer containing 0.1% NP-40. They were then centrifuged at 12 000 g for 30 min at 4°C and the pellets were washed twice with ice-cold buffer containing 0.35 mM sucrose. The nuclei were pre-extracted with 1 volume of ice-cold homogenization buffer containing 0.05 mM NaCl and 10% glycerol for 15 min at 4°C. The nuclei were then extracted with homogenization buffer containing 0.3 mM NaCl and 10% glycerol for 1 h at 4°C and the concentration of DNA was adjusted to 1 mg/ml. After the nuclear extract was pelleted at 12 000 g for 30 min at 4°C, the supernatant was brought to 45% (NH₄)₂SO₄ and stirred for 30 min at 4°C. The precipitated protein was collected at 17 000 g for 30 min, resuspended in homogenization buffer containing 0.35 mM sucrose, and stored in aliquots at −70°C. NFkB ODN were labelled as probes at the 3’ end by means of a 3’ end-labelling kit (Clontech Inc., Palo Alto, CA, USA). After end-labelling, 32P-labelled ODN were purified over a nick column (Pharmacia LKB Biotechnology). Binding reactions (10 μl) including the 32P-labelled probe (0.5–1 ng, 10 000–15 000 c.p.m.), and 1 μg of polydeoxyinosinic-deoxycytidic acid (Sigma Chemical Co., St Louis, MO, USA) were incubated with nuclear extract for 30 min at room temperature and then loaded on to a 5% polyacrylamide gel. The gels were subjected to electrophoresis, dried, and pre-incubated with parallel samples 10 min before the addition of the labelled probe.

Preparation of HVJ-liposomes containing ODN and transfection

Phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in a weight ratio of 1:4:8:2 [23–26]. The lipid mixture (10 mg) was deposited on the sides of a flask by removal of tetrahydrofuran in a rotary evaporator. Dried lipid was hydrated in 200 μl of balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6) containing synthetic double-stranded ODN. Liposomes were prepared by shaking and sonication. Purified HVJ (Z strain) was inactivated

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by ultraviolet irradiation (110 erg/mm²/s) for 3 min just before use. The liposome suspension (0.5 ml, containing 10 mg of lipids) was mixed with HVJ (10 000 haemagglutinating units) in a total volume of 4 ml of BSS. The mixture was incubated at 4°C for 10 min and then for 60 min with gentle shaking at 37°C. Free HVJ was removed from the HVJ-liposomes by sucrose density gradient centrifugation. The top layer of gradient containing purified HVJ-liposomes was collected for use.

Synovial tissues were placed in a serum-free medium 6 h prior to the transfection, then washed three times with BSS containing 2 mM CaCl₂. We transfected decoy ODN into synovial tissues by utilizing the HVJ-liposome method. The HVJ-liposome complex (15 μM of encapsulated ODN) was added to the synovial tissues and the cells were incubated at 37°C for 30 min. Finally, fresh medium was added to the cells, which were incubated in a CO₂ incubator.

Estimation of the transfection efficiency
To check the localization of transfected FITC-labelled ODN, cryostat sections of synovium transfected with decoy ODN were prepared for fluorescence microscopy. To identify the localization of FITC-labelled ODN, the sections were also stained with propidium iodide (Sigma Chemical Co.), and observed under an ultraviolet laser scanning confocal microscope (PCM 2000: Nikon, Tokyo, Japan).

RNA extraction and Northern blot analysis
Twenty-four hours after transfection of the HVJ-liposome solution, RNA was extracted from synovial tissues by means of RNAzol (Tel-Test Inc., Friendswood, TX, USA) for Northern blot analysis, which was performed as described previously [25]. Briefly, samples (30 μg) were electrophoresed on a 1.5% agarose formamide denaturing gel and transferred to a nitrocellulose membrane (Amersham International, Amersham, UK). The filter was baked, pre-hybridized and hybridized with 32P-labelled IL-1β, TNF-α, ICAM-1 and GAPDH ODN probes (Clontech Laboratories Inc.). The filters were washed rigorously and then exposed to X-ray film.

Densitometric analysis
The relative intensities of bands of interest were analysed with the use of a NSF-300G scanner (Microtek, Anaheim, CA, USA) and scan analysis software (Biosoft, Palo Alto, CA, USA). The results were expressed as the mean ratio of relative intensities to that of the control (synovial tissues with RA transfected with HVJ-liposome solution alone) (determined by the gel mobility shift assay).

Measurement of generated cytokines, MMP-1 and adhesion molecule by enzyme-linked immunosorbent assay (ELISA)
Three days after transfection, the conditioned medium was collected, centrifuged at 600g for 10 min and stored at −20°C. The synovial tissues were homogenized in 1 ml of PBS buffer and centrifuged and the supernatant was stored at −20°C. Two hundred microlitres of supernatant was used for measurement. The assays for human IL-1β, IL-6, TNF-α, and MMP-1 in the supernatant and human ICAM-1 in synovial tissues were performed by ELISA kit (IL-1β, IL-6, TNF-α, ICAM-1: R&D Systems Inc, Minneapolis, MN, USA; MMP-1: Amersham International) by following the manufacturer’s protocol.

Synovial cell preparation
The synovial specimens were finely minced into small pieces, soaked in a solution of enzyme cocktails containing 0.1% type IV collagenase, 0.1% hyaluronidase, and 0.01% DNase (all from Sigma Chemical Co.), and incubated for 2 h at 37°C in a shaking water bath. After the removal of debris by filtration, the cells thus obtained were suspended in DMEM, washed twice, resuspended with DMEM with 10% FCS, and seeded in culture dishes. After overnight culture, non-adherent cells were removed, while adherent cells were re-cultured. The synovial cells used in the experiments were the third passage.

Cell proliferation assay
Synovial cells were seeded on to uncoated 24-well tissue culture plates (Corning Inc., Corning, NY, USA) at 4000 cells/well. The cells were then incubated in DMEM with 10% FCS for 48 h. After transfection of decoy ODN, the medium was changed to fresh DMEM with 10% FCS. Four days after transfection, an index of cell proliferation was determined by using sulphonated tetrazolium salt, and a 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) cell counting kit, which is similar to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [28]. This compound produces a highly water-soluble formazan dye, which makes the assay procedure easier to perform.

Statistical analysis
All values are expressed as mean ± standard error of the mean (s.e.m.). Analysis of variance with subsequent Dunnett’s test was used to determine significant differences in multiple comparisons. P < 0.05 was considered significant. All experiments were carried out at least three times.

Results
Transfection of FITC-labelled ODN into synovial tissue
We first verified that double-stranded ODN tagged with FITC at either the 3’ or the 5’ end could be introduced efficiently into synovial cell nuclei using the HVJ-liposome method. Synovial tissues were fixed 1 and 5 days after transfection and observed by fluorescence microscopy. One day after transfection with the HVJ-liposome method, fluorescences were detected in both the nuclei and the cytoplasm. We detected FITC-labelled ODN in the nuclei of approximately 75% of the cells (Fig. 1A–C). One day after direct transfection (without
 HVJ-liposome), fluorescences were also detected in both the nuclei and the cytoplasm, but we detected FITC-labelled ODN in the nuclei of only 30% of the cells (Fig. 1D–F). Even 5 days after transfection with HVJ-liposomes, FITC-labelled ODN were detected in both nuclei and cytoplasm (Fig. 1G–I). In tissues not treated with the HVJ-liposome method, there was no uptake of FITC-ODN by synovial cells 5 days after transfection (Fig. 1J–L). At least 7 days after transfection with HVJ-liposomes, FITC-labelled ODN were detected in both nuclei and cytoplasm. No fluorescent signal was seen in the non-transfected cells.

**NFκB activation in synovium derived from RA**

We ascertained that increases in NFκB binding activity in synovial cells derived from patients with RA. We could also demonstrate by means of the gel mobility shift assay that the NFκB binding activity was enhanced in synovial cells from patients with RA but not in synovial cells from trauma patients (Fig. 2). This NFκB binding was eliminated by pre-incubation of nuclear extracts with excess amounts of unlabelled NFκB ODN, but not with excess unlabelled scrambled ODN (Fig. 2).

**Effect of NFκB decoy on mRNA expression of IL-1β, TNF-α, and ICAM-1**

We also examined the effect of NFκB decoy ODN on the RNA expression level of several cytokines and adhesion molecule genes whose expressions are known to be up-regulated in RA. Total RNA was extracted from synovial tissues 24 h after transfection of ODN and Northern blot analysis was performed using the extracted RNA. As shown in Fig. 3, RNA expressions of IL-1β, TNF-α and ICAM-1 in synovial tissues were inhibited by *in vitro* transfection of NFκB decoy ODN, but not of scrambled decoy ODN.

**Reduction in the binding of NFκB activity after decoy transfection**

We then examined changes in NFκB binding activity after transfection of decoy ODN. Twelve hours after transfection, NFκB decoy ODN reduced NFκB binding activity in nuclear extracts of synovial cells derived from RA patients (Fig. 4A). This effect was not seen in the case of transfection with scrambled ODN. This reduction was dose dependent of transfected NFκB decoy ODN. A higher concentration (15 µM of decoy ODN) showed a stronger effect than a lower concentration (5 µM). The results of densitometric analysis showed significant differences (Fig. 4B).

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**Fig. 1.** FITC-labelled ODN uptake into synovial tissues. FITC-labelled double-stranded ODN (1 µM) were transfected into synovial tissues with or without the HVJ-liposome method. Synovial tissues were fixed with methanol at 1 and 5 days after transfection, and examined by fluorescence microscopy. (A) Transfection using HVJ-liposome method (1 µM; day 1, ×400). (D) Direct transfection (10 µM; day 1, ×400). (G) Transfection using HVJ-liposome method (1 µM; day 5, ×400). (J) Direct transfection (10 µM; day 5, ×400). (B), (E), (H), (K) The section was counterstained with propidium iodide (B, E; day 1, H, K; day 5, ×400). (C), (F), (I), (L) The section was observed under confocal microscopy to identify the localization of FITC-labelled ODN in the nuclei of synovial tissue (day 1, ×400). (C) Transfection using HVJ-liposome method (1 µM; day 1, ×400). (F) Direct transfection (10 µM; day 1, ×400). (I) Transfection using HVJ-liposome method (1 µM; day 5, ×400). (L) Direct transfection (10 µM; day 5, ×400).
Changes in RNA expression of cytokines and adhesion molecules by decoy transfection. Total RNA was extracted from synovial tissues 24 h after transfection with NFκB decoy ODN or scrambled decoy ODN, and subjected to Northern blot analysis for IL-1β, TNF-α, ICAM-1 and GAPDH. Lane 1, RNA from synovial tissues from trauma patient; lane 2, mRNA from synovial tissues with RA after transfection with NFκB decoy ODN; lane 3, mRNA from synovial tissues with RA after transfection with scrambled decoy ODN. Thirty micrograms of total RNA was used for each blot.

**Effect of NFκB decoy on synovial cell proliferation**

Finally, we assessed the effect of NFκB decoy ODN on synovial cell proliferation. The level of synovial cell proliferation was determined by using the WST-1 cell counting kit at 4 days after transfection. The index of cell proliferation determined by absorbance at 450 nm using the WST-1 cell counting kit was 0.286 ± 0.28 with synovial cells transfected with NFκB decoy ODN and 0.356 ± 0.41 with synovial cells transfected with scrambled decoy ODN. Transfection of NFκB decoy ODN resulted in a significant inhibition of synovial cell proliferation as compared with scrambled decoy ODN transfected synovial cells and non-transfected synovial cells (P < 0.01) (Fig. 6).

**Discussion**

One of the important clinical problems in RA is progressive joint destruction. Although the precise mechanism of joint destruction remains partly unknown, we do know that one of the important processes is an inflammatory response that is mediated by the activation of cytokines and other inflammatory mediators [29], and the migration of leucocytes into the vascular endothelium also plays an important role in inflammation and immune reactions [30]. It has been demonstrated that pro-inflammatory cytokines produced locally in the joints stimulate the production of MMPs and may strongly mediate joint destruction through the degradation of bone and cartilage [31–34]. The blocking of
inflammatory mediator genes at more than one point to achieve the maximum inhibitory effect is usually due to the redundancy and complexity of the interactions of these genes. In view of this, our NFκB decoy strategy can be expected to be simple and more effective in targeting the activation of multiple inflammatory mediators to treat arthritis. The transcription factor NFκB has been implicated in the up-regulation of the expression of numerous cytokines and adhesion molecules [19–21]. The results of our study led us to hypothesize that the inhibition of stimulated NFκB activity in synovial cells by NFκB decoy ODN results in the inhibition of the expression of key cytokines and adhesion molecules which may play a pivotal role in the pathogenesis of arthritis. Our study demonstrates the validity of the concept of NFκB decoy strategy in vitro by transfection of decoy ODN containing the NFκB cis element into cultured synovial tissues and synovial cells. The specificity of the inhibitory effect of the decoy ODN against NFκB on synovial cells is supported by several lines of evidence: (1) NFκB decoy ODN exhibited competitive and specific binding for NFκB as shown by the gel shift assay, (2) NFκB decoy ODN reduced the stimulation of NFκB activity in synovial cells, (3) our experiments proved that the NFκB decoy ODN selectively inhibits the expression and production of IL-1β, TNF-α, and ICAM-1, but not of β-actin which does not contain the NFκB binding sequence in its promoter regions as assessed by Northern blot analysis, and (4) no effect could be detected as a result of the transfection of scrambled decoy ODN.

We previously reported that the HVJ-liposome method is an effective ODN delivery system, which enhances the uptake and intracellular stability of ODN in cultured cells [25]. As the decoy effect is apparently located in the nucleus, bypassing the endocytotic pathway and translocation of decoy ODN from the cytoplasm are extremely important. The fusion with the cell membrane mediated by the envelope proteins of HVJ (HN and F proteins) results in intracellular ODN delivery, which bypasses endocytosis. Consequently, this method enhances and prolongs the effect of ODN in vitro. In this study, we confirmed that the HVJ-liposome method is also effective for ODN transfer into synovial cells. We speculate that the enhanced efficiency of this system may result in fewer non-specific toxic effects observed at high concentrations of ODN. Indeed, this method has been used recently for the transfection of plasmid DNA encoding SV 40 large T antigen and ODN into rat knee joints in vivo [35, 36]. As previously shown, the expression of the transgene was recognized in the synovial cells. HVJ-liposome may thus be an effective method for decoy ODN transfer for the purpose of gene therapy for RA.

The progression of arthritis depends upon the activation of the complicated network consisting of cytokines and adhesion molecules. In this study, the production of IL-1β and TNF-α was suppressed to almost below detectable levels, while the production of IL-6 was suppressed by about 55%. Because IL-6 has another
NFκB decoy transfer to synovial cells of RA

Fig. 5. Effect of transfection on cytokine production into medium. Medium was harvested 72 h after transfection. (A) IL-1β protein concentration, (B) TNF-α protein concentration, (C) IL-6 protein concentration, (D) MMP-1 protein concentration, and (E) ICAM-1 protein concentration as assessed by ELISA. *, **, and *** $P < 0.01$ vs scrambled decoy ODN-treated group, and #, ## $P < 0.05$ vs scrambled decoy ODN-treated group.

transcriptional factor, activator protein-1 (AP-1) [37, 38], only the blockage of NFκB activation may be insufficient for the production of IL-6. MMP-1 directly and efficiently degrades type I, II, and III collagen, and contributes to the destruction of articular cartilage and bone in RA. The inhibitory effect of NFκB decoy on the production of MMP-1 as shown in this study was about 40% of that achieved with the treatment with the scrambled decoy. This is because in the promoter region of MMP-1, AP-1 also plays an important role in transcriptional induction by extracellular stimulation [39–41]. For this reason, combination therapy of NFκB decoy and AP-1 decoy could result in a stronger inhibitory effect. Although an inhibitory effect on synovial cell proliferation in vitro was shown in this study, the biological significance of NFκB decoy on synovial proliferation in vivo still remains unknown. The previous in vivo study using an experimental arthritis model showed marked reduction of the weight of synovial tissue in the joints transfected with NFκB decoy compared with the joints transfected with scrambled decoy or untreated joints [36]. These results suggest that NFκB decoy may provide a biologically significant effect on synovial proliferation in vivo.

Here we reported a novel therapeutic strategy to suppress the inflammatory process in synovial cells by
transfecting decoy ODN to block the binding of the critical transcription factor NF-κB to its promoter sequence of the target genes, thereby inhibiting the co-ordinated transactivation of genes of key cytokines and adhesion molecules necessary for the progressive joint destruction in RA. Further studies are necessary to prove that the blockade of those cytokines and adhesion molecules by NF-κB decoy ODN is sufficient to block joint inflammation and destruction in vivo. Furthermore, modification of ODN composition in order to prolong decoy stability will be essential for the enhancement of therapeutic efficacy. Despite these limitations, transcription factor decoy technology provides a new tool for identifying biological processes and for treating pathological conditions in the joints. Even though the data derived from in vitro experiments, the results of this study clearly demonstrate that the transcription factor NF-κB is one of the key regulators which promote joint destruction in arthritis.

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