Effect of Transcription Factor Decoy for NF-κB on the TNF-α Induced Cytokine and ICAM-1 Expression in Cultured HaCaT cells

Kyu Suk Lee, M.D., Jee Ook Kim, M.D., Byung Chun Kim, M.D., Young Wook Ryoo, M.D.

Department of Dermatology, Keimyung University, Colledge of Medicine, Taegu, Korea

**Background:** Psoriasis is the most prevalent T-cell-mediated inflammatory skin disease in humans. Numerous cytokines and adhesion molecules are expressed in the skin lesion of psoriasis such as TNF-α, IL-1, IL-6, VCAM-1 and ICAM-1. All of them contain at least one binding site for the transcription factor NF-κB. TNF-α activates NF-κB and many other transcription factors. Thus, transcription and expression of many genes involved in the inflammatory process may be influenced by TNF-α.

**Objective:** The purpose of this study was to study the effect of synthetic double-stranded DNA with high affinity for the NF-κB binding site on the TNF-α induced proinflammatory cytokines and ICAM-1 gene expression in the HaCaT cells.

**Material and Methods:** We examined whether inhibition of NF-κB activity by oligodeoxynucleotides (ODN) decoy for NF-κB blocks TNF-α induced cytokines such as IL-1α, IL-1β, IL-6 and ICAM-1 expression with electrophoretic mobility shift assay (EMSA) and reverse transcription-polymerase chain reaction (RT-PCR).

**Results:** In EMSA, TNF-α treatment (10 ng/ml) induced the activation of NF-κB. The NF-κB binding activity in the TNF-α treated HaCaT cells increased 5.0-fold compared to non-treated group. Next, we examined the effect of liposome mediated NF-κB decoy oligonucleotides (ODN) transfection. After transfection of the NF-κB decoy ODN, TNF-α increased NF-κB binding activity to 1.9-fold of non-treated group. Accordingly the transfection of NF-κB decoy ODN inhibited the TNF-α induced NF-κB binding activity up to 63%. RT-PCR analysis revealed that the transfection of NF-κB decoy ODN inhibited TNF-α induced cytokines and ICAM-1 mRNA expression.

**Conclusion:** Taken together, our results suggest the potential utility of NF-κB decoy technique for biologic therapy of psoriasis.


**Key Words:** NF-κB decoy, Cytokine, In vitro.

---

Received December 27, 2003
Accepted for publication July 15, 2003
Reprint request to: Kyu Suk Lee, M.D. Department of Dermatology, Keimyung University, Colledge of Medicine, 194 Dong san-dong, Jung gu, Taegu 700-310, Korea
Tel. 053-250-7625, Fax: 053-250-7626
E-mail. franzes@dsmc.or.kr

Psoriasis is a chronic inflammatory disease of the skin. It is a common and significant disease, with a prevalence of 1-2% world-wide. It has been shown that CD4 lymphocytes produce T-helper 1 cytokines, including interleukin (IL)-2, interferon-and that TNF-α plays a crucial part in early psoriatic skin lesions. TNF-α is increased in psoriatic lesions and the expression of several genes regulated by NF-κ B such as IL-1, IL-2, IL-6, ICAM-1, and
VCAM-1 is also increased in psoriatic lesions. The major sources of cellular TNF-α are activated monocytes and macrophages. But other cells capable of TNF-α synthesis include keratinocytes, Langerhans cells and activated T cells. TNF-α causes target cells to induce proinflammatory reaction or to die. Proinflammatory reaction by TNF-α starts as NF-κB is activated by signal transduction via tumor necrosis factor receptor (TNFR)1 and TNFR2. After NF-κB is activated, expression of adhesion molecules is upregulated and production of secondary cytokines as well as chemokines is stimulated.

The major alternative treatments for psoriasis, including retinoids, PUVA, phototherapy with UVB, methotrexate, and cyclosporin, all have well-known and characteristic effects1. Recently, several biologic agents that block the actions of IL-1 and TNF-α have been successfully used in experimental models. Moreover, anti-TNF-α antibody or soluble TNF-α receptor have been shown to be effective in human trials3, but there have been problems with rebound of symptoms, concomitant infection4, short half-life5, and cost. Recent progress in molecular biology has enabled us to apply gene transfer techniques for inhibiting expression of target genes, including decoy oligodeoxynucleotides (ODN)6,7. In this study, to investigate the capability of NF-κB transcription factor decoy for treatment of inflammatory skin diseases including psoriasis, we examined whether inhibition of NF-κB activity by ODN decoy for NF-κB blocks TNF-α induced cytokines such as IL-1α, IL-1β, IL-6 and ICAM-1 expression with electrophoretic mobility shift assay (EMSA) and reverse transcription-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Cell culture
HaCaT cells, the differentiated human keratinocyte cell line, were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (1 μg/ml). The cells were maintained in a humidified 5% CO₂ - 95% air incubator at 37 °C. Analyses of HaCaT cells cultures were carried out at 3-6 passages of subcultivation. The cells were seeded at a density of 1 × 10⁶ cells/100 mm dishes. After 48 h, the cells were washed with serum-free media, replaced with 10 ml of media without FBS and incubated in a humidified incubator for at least 24 h prior to experiments for TNF-α treatment.

Synthesis of ODN and selection of sequence targets
The following phosphorothioate double-strand ODN against NF-B binding sites and scrambled decoy ODN (as control) were used in this study.

NF-κB decoy ODN (consensus sequences are underlined):

5'-CTTGAAGGGATTTCCTCC-3'
3'-GGAACCTTCATAAAGGGAGG-5'
Scrambled decoy ODN
5'-TTGGCCTACCTGACTTAGCC-3'
3'-AACGGCATGGACTGATTCG-5'

NF-kB decoy ODNs have previously been shown to bind the NF-kB transcription factor2. Synthetic ODNs were washed with 70% ethanol, dried, and then dissolved in sterile Tris-EDTA buffer (10 mM Tris, 1 mM EDTA). The supernatant was purified over a nick column (Pharmacia, Piscataway, NJ) and quantified by spectrophotometry.

Decoy ODN transfection using liposome
This experiment was performed as described previously3. HaCaT cells were transfected with Lipofectamine Plus (Gibco-BRL, Gaithersburg, MD, USA). The cells were seeded at a density of 1-2 × 10⁵ cells/100 mm dishes with 2 ml of DMEM and incubated 24 h before transfection. At 40-60% confluency, a complex of decoy ODN (5 g) and DC-chol liposomes (50 nmol) (1:10, g DNA: nmole liposomes) was added to the washed cells and incubated in serum-free medium for 5 h. The transfected cells were further incubated in complete medium for 24 h and some transfected cells treated with TNF-α (10ng/ml) followed by EMSA assay or RT-PCR as described below.

Preparation of nuclear proteins
Cells were washed with cold PBS three times and scraped. After collection of cells by centrifugation at 2000 rpm for 5 min, the pellet was resuspended in buffer A (10 mM HEPES, 1.5 mM MgCl₂, 0.25% Nonidet P-40, pH 7.5) and incubated on ice for 5 min, followed by centrifugation at 4000 rpm for 2 min. The supernatant (the cytosol) was re-
Effect of Transcription Factor Decay for NF-κB on the TNF-α Induced Cytokine and ICAM-1 Expression in Cultured HaCaT Cells

Fig. 1. DNA binding activity of NF-κB is increased by TNF-α in HaCaT cells. Nuclear extracts from cells treated with TNF-α (10 ng/ml) at 1 h after treatment were incubated with the [32P] ATP-labeled NF-κB probe and analyzed by EMSA. The specificity of the bands was verified by adding 10-fold excess of competitor unlabelled NF-κB probe to TNF-α treated nuclear proteins. Lane 1 and 3: HaCaT cells, lane 2 and 4: Skin fibroblasts. * Significantly different from controls at p < 0.01.

RT-PCR
Total RNA was isolated from the cells treated with TNF-α (10 ng/ml) (Sigma, St. Louis, MO, USA) according to the single step guanidium thiocyanate-phenol-chloroform extraction procedure using RNAzol (Tel-Test, Houston, TX, USA) according to the manufacturers instructions. The cDNA was synthesized from 2 g of total RNA with 2.5 U/l of MMLV-RT (Promega Co., Madison, WI, USA) and 2.5 g of oligo-dT primer at 42 C for 1 h. For amplification of cDNAs, oligonucleotide primers for IL-1, IL-1, IL-6, ICAM-1 and GAPDH were designed as 5-GTCTCTGAAATGAAATCTTTCGATCT-3 (sense) and 5-CATGCACATTCTGCTTCATCC-3 (antisense) for the IL-1α (453bp), 5-AAGCAAGCTGGCAAGATCC-3′(sense) and 5-TCTTTAGGAAAGACAAAATTTC-3′ (antisense) for IL-1β (821bp), 5-CTCCGGTCACTTGTCTCTCCTC-3′ (sense) and 5-GCTCTGACTGCTCTGTTGGG-5′ (antisense) for IL-6 (398bp), 5-CTCATCGCTGCTGCTGGGT-3′ (sense) and 5-CCGGAGCTGGCTGACCTCAGG-3′ (antisense) for ICAM-1 (495bp), and 5-CCAGGGCTGGCTGACCTCAGG-3′ (sense) and 5-TCTTACAAGCTGGCTGACCTCAGG-5′ (antisense) for GAPDH (575bp). The reaction mixture was heated at 94°C for 1 min, annealed at 60°C, 2 min, and extended at 72°C for 1 min for 30 cycles. PCR products were electrophoresed in 8% polyacrylamide gels and visualized by ethidium bromide staining.

RESULTS

1. Effect of TNF-α on DNA binding activity of NF-κB
In EMSA, TNF-α treatment (10 ng/ml) induced an increase of NF-κB binding protein in the nuclear extract of HaCaT cells. NF-κB binding activity increased 5.0-fold compared to non-treated group in the TNF-α treated HaCaT cells (Fig. 1).

2. Effect of NF-κB decoy ODN on the TNF-α induced NF-κB binding activity
To examine the effect of transfection of NF-κB decoy ODN on the TNF-α induced NF-κB binding activity, cultured HaCaT cells were transfected with NF-κB decoy ODN and scrambled decoy ODN before treatment of TNF-α (10 ng/ml). In the NF-κ
Fig. 2. Reduction in the binding activity of NF-κB after decoy transfection in HaCaT cells. EMSA used the 
[32P] ATP-labeled NF-κB probe and nuclear extracts prepared from cultured HaCaT cells. Lane 1: non-treated control, lane 2: TNF-α (10ng/ml) treated group, lane 3: NF-κB decoy with TNF-α (10ng/ml) treated group. The mean results of densitometric analysis of three separate experiments are shown as a fold induction of untreated controls. Data are represented as the mean of three experiments SD. * Significantly different from controls and ** significantly different from only TNF-α treated groups at p < 0.01.

B decoy ODN transfected HaCaT cells, TNF-α treatment increased the NF-κB binding activity just 1.9-fold of non-treated group (Fig. 2). That means transfection of NF-κB decoy ODN inhibited TNF-α induced NF-κB binding activity up to 63%. In the scrambled decoy ODN transfected HaCaT cells, the TNF-α induced NF-κB binding activity was not affected (data not shown).

3. Effect of NF-κB decoy ODN on the TNF-α induced cytokine and ICAM-1 gene expression

To investigate the effect of transfection of NF-κB decoy ODN on the expression of TNF-α induced cytokine and ICAM-1, we examined IL-1α, IL-1β, IL-6 and ICAM-1 mRNA expression by RT-PCR in cultured HaCaT cells. Because cytokine gene expression can be induced in culture medium containing 10% FBS through activation of serum-responsive elements, throughout this study we transferred HaCaT cells to medium without FBS for 24 h before TNF-α treatment. RT-PCR analysis revealed that the transfection of NF-κB decoy ODN inhibited TNF-α induced cytokines and ICAM-1 mRNA expression, whereas GAPDH mRNA expression was not affected (Fig. 3).

Fig. 3. Changes in mRNA expression of cytokines and adhesion molecules by NF-κB decoy transfection. Total RNA was extracted from HaCaT cells treated with TNF-α (10 ng/ml) or treated with TNF-α 12 h after transfection, and subjected to RT-PCR for IL-1α, IL-1β, IL-6, ICAM-1, and GAPDH. Lane A: non-treated control, lane B: TNF-α (10 ng/ml) treated group, lane C: NF-κB decoy with TNF-α (10 ng/ml) treated group.

DISCUSSION

Over the past decade, the view of the pathogenesis of psoriasis had been changed. Previously it was assumed that keratinocyte hyperproliferation associated abnormal epidermal differetiation was the primary cause of psoriasis. However, it is now recognized that epidermal hyperplasia is a reaction to the activation of the immune system in focal skin regions. Plaques of psoriasis are characterized by the presence of multiple cytokines produced by T cells, keratinocytes and other cutaneous cells. These cytokines are predominantly of the Th1 subtype including IL-2, interferon-γ and IL-12 with relatively low in the Th2 cytokines, namely IL-4 and IL-10. IL-1 and TNF-α, which are proinflammatory cytokines, are increased in psoriatic plaques. These proinflammatory cytokines are important in initiating the inflammatory cascade. TNF-α plays a central part in the inflammatory process of psoriatic skin lesions. In addition, TNF-α strongly influences the function and maturation of antigen-presenting cells involved in the inflammatory process. Other inflammatory cytokines (eg, IL-1 and IL-6) have been shown to direct keratinocyte mitogens, so elaboration of cytokines from intraepidermal T cells could directly stimulate keratinocyte proliferation.
NF-κB can be activated within minutes by a variety of stimuli, including inflammatory cytokines, T-cell activation signals, growth factors, and stress inducers. The activation of NF-κB is normally associated with the induction of phosphorylation of IκB, followed by its degradation by the proteasome and nuclear translocation. NF-κB regulates the expression of several cytokines and adhesion molecules (IL-1α, IL-1β, IL-2, IL-6, IL-8, ICAM-1, and VCAM-1, to name a few) which contain in their promoter region of NF-κB-responsive element. Accordingly, we hypothesize that the final common pathway of NF-κB coordinated gene expression is an ideal target for the molecular therapy of many inflammatory diseases including psoriasis. Synthetic double-stranded ODN as transcription factor decoy can block the binding of specific nuclear factor to its corresponding cis element in the promoter regions of targeted genes, resulting in the inhibition of gene transactivation. In this study, we demonstrated that NF-κB decoy ODN inhibited NF-κB binding activity, which is increased by TNF-α. The specificity of the inhibitory effect of the decoy ODN against NF-κB is supported by evidence that NF-κB decoy ODN inhibited of NF-κB specific protein binding as documented by EMSA, whereas the scrambled ODN did not. TNF-α activates nuclear factor NF-κB in several cell types through different pathways. Kouba et al. demonstrated NF-κB activation of epidermal keratinocytes by TNFα utilized previously defined, NIK-dependent pathways while dermal fibroblasts utilized unique NF-κB-inducing kinase (NIK)/TNF-α receptor-associated factor 2-independent pathway. RT-PCR analysis revealed that transfection of NF-κB decoy ODN inhibited the expression of several cytokines and ICAM-1 mRNA which is induced by TNF-α, whereas GAPDH mRNA expression was not affected. The progression of many inflammatory diseases, such as arthritis and psoriasis depends upon the activation of the complicated network consisting of cytokines and adhesion molecules. Our data suggests that in vivo transfection of target cells of inflammatory diseases with sufficient quantities of the NF-κB decoy ODN prevent the transactivation of gene expression regulated by NF-κB. In this study, after transfection of NF-B decoy ODN, TNF-α-induced expression of IL-1α, IL-1β, and ICAM-1 mRNA was suppressed to near levels of controls, while that of IL-6 was suppressed by about 24%. Because IL-6 has another transcription factor, activator protein-1 (AP-1), only the blockage of NF-κB activation may be insufficient for the inhibition of IL-6 gene expression. For this reason, combination therapy of NF-κB decoy and AP-1 decoy could result in stronger inhibitory effect on IL-6. Given that effector cytokines are fairly downstream or distal in the inflammatory cascade, therapeutic blockade of cytokines might be expected to show clinical benefits rather rapidly. In addition, inhibition of NF-κB during treatment would provide the least likelihood of side effects targeting immune function. As such, this NF-κB decoy strategy may be particularly suited for short-term management of an inflammatory crisis. The therapeutic efficacy of NF-κB decoy ODN depends on the development of better delivery system and stable with safe ODN composition. Further studies are necessary to prove that the blockade of those cytokines and adhesion molecules by NF-κB decoy ODN is sufficient to block the inflammation and progression of diseases in vivo. Furthermore, the modification of ODN composition to enhance decoy stability and safety will be necessary. Despite these limitations, transcription factor decoy strategy may provide new a tool for treating inflammatory diseases.

In conclusion, transfection of NF-κB decoy ODN inhibited TNF-α induced NF-κB binding activity and also expression of cytokines and ICAM-1 mRNA which was induced by TNF-α. The results of present study suggest the potential utility of NF-κB decoy technique for biologic therapy of psoriasis.

REFERENCES