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# Human β-defensin 2 is induced by interleukin-1β in the corneal epithelial cells

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Abbreviations: EMSA, electrophoretic mobility shift assay; HCE, human corneal epithelial cells; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-κB

## Abstract

**Mammalian epithelia produce the various antimicrobial peptides against the bacterial or viral infection, thereby acting as the active immune modulators in the innate immunity. In this study, we examined the effects of the various proinflammatory cytokines or LPS on cell viability and antimicrobial β-defensin gene expressions in human corneal epithelial cells. Results showed that the cytokines or LPS did not exert severe cytotoxic effects on the cells, and that β-defensin 1 was constitutively expressed, while β-defensin 2 was specifically induced by IL-1β, supporting the idea that these cytokines or LPS involve the defense mechanism in the cornea. Furthermore, the reporter and gel shift assay to define the induction mechanism of β-defensin 2 by IL-1β demonstrated that the most proximal NF-κB site on the promoter region of β-defensin 2 was not critical for the process. Data obtained from the normal or patients with the varying ocular diseases showed that our *in vitro* results were relevant in the clinical settings. Our results clearly demonstrated that β-defensin 1 and 2 are important antimicrobial peptides in the corneal tissues, and that the mechanistic induction process of β-defensin 2 by IL-1β is not solely dependent on proximal NF-κB site activation, thus suggesting that the long distal portion of the promoter is needed for the full responsiveness toward IL-1β.**

**Keywords:** antimicrobial cationic peptides; β-defensins; cornea; corneal epithelium; gene expression regulation; interleukin-1β

## Introduction

Mammalian epithelial cells represent a major barrier to the environment and provide the first line of defense against invading microorganisms. In addition, mammalian epithelia produce a wide spectrum of antimicrobial peptides, thereby acting as an active immune modulator against infection by a variety of microorganisms (Zasloff, 2002).

Defensins are small cationic peptides that can be grouped into the α-and β-defensins; the α-defensins human neutrophil peptides 1-4 are expressed in human leukocytes (Tang *et al*., 1999) and human defensin 5 and 6 are expressed in Paneth cells in the small intestine (Jones and Bevines, 1993). Human β-defensins produced by epithelial or epithelial-derived tissues show antimicrobial activity against predominantly Gram-negative bacteria, and are also effective against Gram-positive bacteria such as *Staphylococcus aureus* (Harder *et al*., 1998; Harder *et al*., 2001).

In the human eye, the β-defensin 1 was found to be constitutively expressed in the cornea, conjunctiva, and lens, whereas the β-defensin 2 was only observed in some of the tested samples or in some pathological samples (Mcnamara *et al*., 1999; Lehmann *et al*., 2000). These observations strongly suggest that the β-defensin 2 gene might be induced at the transcriptional level in some conditions, unless otherwise silenced. In this study, therefore, we examined that the effects of the proinflammatory cytokines on β-defensin genes using corneal epithelial cells and further demonstrated that β-defensin 2 was specifically induced by IL-1β.

## Materials and Methods

### Materials

All materials for cell culture were obtained from Invitrogen (Carlsbad, CA). Recombinant human EGF, cholera toxin, LPS and Griess' reagent were from Sigma Chemical Co. (St. Louis, MO). Human IL-1β, TNF-α, and IFN-γ were purchased from R&D Systems, Inc. (Minneapolis, MN). All other reagents were analytical grade and obtained from Sigma Chemical.

Co. unless otherwise specified.

### Culture of human corneal epithelial cell (HCE) line

HCE line was purchased from the American Type Culture Collection (ATCC), and routinely grown in DMEM/F-12 media (1 : 1 mixture) supplemented with 5 μg/ml of insulin, 10 ng/ml of EGF, 0.1 μg/ml cholera toxin, and 15% FBS at 37°C under a 95% air, 5% $CO_2$ atmosphere.

### MTT assay and nitrite measurements

HCEs were detached by trypsin treatment, and seeded at $2\times10^4$ cells/well on 24-well plates (Nalge Nunc International, IL). After the cultivation for 1-2 days, cells were treated with various concentrations of IL-1β, TNF-α, IFN-γ, or LPS for 24 h. Then, culture supernatant was collected, and the amount of nitrite was measured by Griess' reagent using sodium nitrite as a standard. Following cytokine treatment, cells were washed with cold PBS, and were incubated for 3 h in media containing MTT (0.5 mg/ml, C, *N*-diphenyl-*N*'-4,5-dimethylthiazol-2-yl tetrazolium bromide). At the end of incubation, media were carefully withdrawn, and formazan crystal was solubilized with acidified isopropanol containing 10% (v/v) Triton X-100. The optical density was recorded with an automatic microplate reader at 570 nm.

### RT-PCR

RT-PCR was performed as previously described (Shin *et al.*, 2004). Briefly, HCEs were treated with cytokines as above, and total RNA was extracted with Trizol reagent (Invitrogen). The first strand cDNA was synthesized out of 2 μg of the total RNA in 20 μl reaction mixture, containing Superscript II RNase $H^-$ reverse transcriptase and oligo$(dT)_{18}$ primer. Target regions (Table 1) were then amplified in a 20 μl of volume for 30 cycles using the following schedule: 1 min at 94°C followed by 30 cycles of 1 min at 94°C, 50 s at 59°C, 1 min at 72°C, and a final extension step of 10 min at 72°C. PCR products were run on a 1.5 or 2% agarose gel, and the bands were photographed.

### Plasmid construction and transient transfection

Sequences from the upstream region of the human β-defensin 2 gene (GenBank accession no. AF071216) were isolated by PCR amplification and subcloned into the multiple cloning site of the promoterless reporter vector, pGL-2 Basic (Promega Corp., Madison, WI). Transcription start site of the β-defensin 2 gene is designated +1. Plasmid pGL-1522, -1152, -752, and -350 contain bases -1522 to +1, -1152 to +1, -752 to +1, and -350 to +1, respectively. HCEs ($2\times10^5$ cells per well) were dispensed into 6-well plate, and were transfected with 1.2 μg of plasmid DNA by using Lipofectamine (Invitrogen), followed by cultivation for 48 h before reporter gene analysis. After 16 h of incubation with 2.5 ng/ml of IL-1β, cells were lysed and assayed for enzymatic activity according to the manufacturer's instruction. Luciferase activity was measured with luciferase assay system (Promega Corp.) in a luminometer, and data were normalized to total protein concentration.

### Electrophoretic mobility shift assay (EMSA)

HCEs were treated with various cytokines as above, and nuclear extracts were prepared by the method of Moon *et al.* (2002). EMSA was carried out in the presence of poly (dI/dC) by a standard protocol with 6% nondenaturing polyacrylamide gel electrophoresis in 0.25× Tris-borate-EDTA, except for the labeling of oligonucleotides by non-radioactive dioxygenin (DIG)

**Table 1.** Oligonucleotide sequences used in this study.

| Name | Sequence | Accession | Product size (bp) |
|---|---|---|---|
| β-defensin 1 | F: 5'-GTGGTAACTTTCTCACAGGC-3'<br>R: 5'-AGTTCATTTCACTTCTGCGT-3' | NM_005218 | 189 |
| β-defensin 2 | F: 5'-ATCTCCTCTTCTCGTTCCTC-3'<br>R: 5'-ACCTTCTAGGGCAAAAGACT-3' | Z71389 | 126 |
| β-defensin 3 | F: 5'-AGCCTAGCAGCTATGAGGATC-3'<br>R: 5'-CTTCGGCAGCATTTTGCGCCA-3' | AJ237673 | 206 |
| Dermcidin | F: 5'-AGCATGAGGTTCATGACTCTC-3'<br>R: 5'-CACGCTTTCTAGATCTTCGAC-3' | NM_053283 | 290 |
| GAPDH | F: 5'-GAAATAAAGCCACCACCGCC-3'<br>R: 5'-ATCAACCCTTCCACGATCCC-3' | BC026907 | 593 |

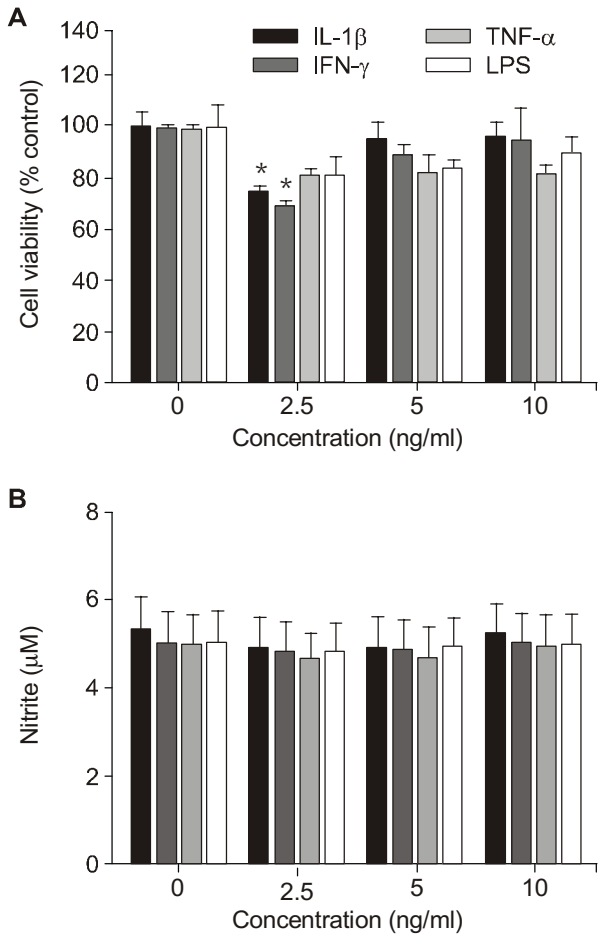


**Figure 1.** The effects of various cytokines on cell viability and the accumulated nitrite level. HCEs were treated with various concentrations of cytokines for 24 h, and the cell viability and the accumulated nitrite were measured by MTT assay and Griess' reaction, respectively. Asterisk (*) denotes the statistical significance ($P < 0.05$ by ANOVA).

method according to the manufacturer' instruction (Roche Applied Biosciences, Mannheim, Germany). The sequence of the sense NF-κB consensus oligonucleotide is 5'-AGTTGAGGGGACTTTCCCAGGC-3', and that of proximal NF-κB binding site in the native β-defensin 2 gene is 5'-TTTTCTGGGGTTTCCTGA-3'. The nonspecific oligonucleotides used for the competition experiments are mutant forms with the sequence 5'-AGTTGAGGTAACTTTCCCAGGC-3' (for consensus NF-κB) and 5'-TTTTCATAGGTTTAATGA-3' (for proximal NF-κB).

### Clinical samples

Clinical diagnosis was made by one of the authors (Dr. Jae-Chan Kim), and human biopsy was obtained after the patient's consent. Corneal tissues were immediately stored in RNA Later solution (Ambion, Austin, TX), and RNA isolation and RT-PCR were performed as described above.

### Statistics

Values are mean ± SEM, and statistical significance was evaluated by unpaired Student's *t* test or by one-way ANOVA followed by a test of Newman-Keuls when more than two groups were compared.

## Results

### Cell viability and the accumulated nitrite level

To test the expression patterns of the antimicrobial peptides in the corneal tissues, we used the corneal epithelial cells as a model system, and selected several proinflammatory cytokines and LPS as gene inducers, since these cytokines or bacterial component has been widely used in the surrogate experiments mimic to the bacterial infection *in vivo* (Mcnamara *et al.*, 1999; Harder *et al.*, 2000; Hao *et al.*, 2001). When HCEs were treated with these cytokines as indicated in Figure 1, their viability and the accumulated nitrite level were not significantly different between the samples, except in the lower concentration of IFN-γ and IL-1β, cell viabilities were decreased by 30% and 25%, respectively compared to untreated control ($P < 0.05$ by ANOVA). These results clearly demonstrated that the cytokines used in this study did not exert severe cytotoxic effects on the HCEs, thereby supporting the idea that they involve the defense mechanism against the corneal infection. Results also preclude the possibility that the nitric oxide acts as a secondary effector molecule upon treatment of the cytokines or LPS.

### The effects of various cytokines and LPS on β-defensin genes in HCEs

As shown in Figure 2A, β-defensin 1 was expressed in all conditions tested, while β-defensin 2 was induced only in IL-1β-treated cells. β-defensin 2 gene was specifically induced by IL-1β as little as 2.5 ng/ml concentration, and its expression persisted over 48 hrs with the maximal level at 16 h (Figure 2B). β-defensin 3 and dermcidin recently identified antimicrobial peptide in the skin (Schittek *et al.*, 2001) were not detected in our condition (data not shown). These results were compatible with the previous reports (Mcnamara *et al.*, 1999; Lehmann *et al.*, 2000), and suggested that IL-1β could induce β-defensin 2 at the transcriptional level. To test this possibility, we have constructed several reporter plasmids spanning the promoter region of the β-defensin 2 gene, and examined that the reporter activity could be induced by IL-1β using transient transfection method. By the

computer analyses, several consensus DNA-binding sites in the promoter region of β-defensin 2 gene including the NF-IL6 and NF-κB were identified (Figure 3A). Upon the transfection of each reporter construct followed by IL-1β treatment, the reporter activity containing only the full length construct (-1522 to +1 at the transcription start site) was increased up to 2-fold compared to untreated control, while other deleted reporter genes were not activated by IL-1β treatment (Figure 3B). These results strongly suggested that β-defensin 2 induction by IL-1β might need the long distance of the upstream portion of the promoter, and is not solely dependent on proximal NF-κB site unlike other studies (O'Neil *et al.*, 1999; Diamond *et al.*, 2000; Wada *et al.*, 2001).

### The effects of various cytokines and LPS on NF-κB complex binding on specific oligonucleotides

Because our reporter assay could not show the specific role of proximal NF-κB site in the promoter region of β-defensin 2 gene, we further tested whether the NF-κB complex could bind to NF-κB site *in vitro*. To this end, the oligonucleotides containing the consensus NF-κB or proximal NF-κB sequence on β-defensin 2 were used in EMSA. As shown in Figure 4A, none of nuclear proteins bind to proximal NF-κB site on β-defensin 2 irrespectively of the presence of various cytokines or LPS. By contrast, specific NF-κB complex was observed in untreated, IFN-γ, and LPS-treated cells, thereby precluding the possibility that the limited amount of NF-κB complex was present in our nuclear extracts (Figure 4B). Taken together, our results demonstrated that IL-1β could specifically induce β-defensin 2 gene transcription, but the induction pathway was not solely dependent on proximal NF-κB site activation in HCEs.

### Defensin expressions in clinical samples

To extend our results into the clinical settings, several specimen representing the various ocular diseases were obtained, and β-defensin genes were analyzed by semi-quantitative RT-PCR. β-defensin 1 was observed in all samples, while the β-defensin 2 gene was only detected in samples from the patients with ocular diseases (Table 2). None of β-defensin 3 was detected in all samples (data not shown). It should be noted that the various ocular diseases accompany the increased proinflammatory cytokines including the IL-1β, IL-6 in the cornea (Xue *et al.*, 2002), and the clinical data were completely compatible with our results obtained *in vitro*.

## Discussion

In the present study, we have demonstrated that among the proinflmmatory cytokines or LPS, only

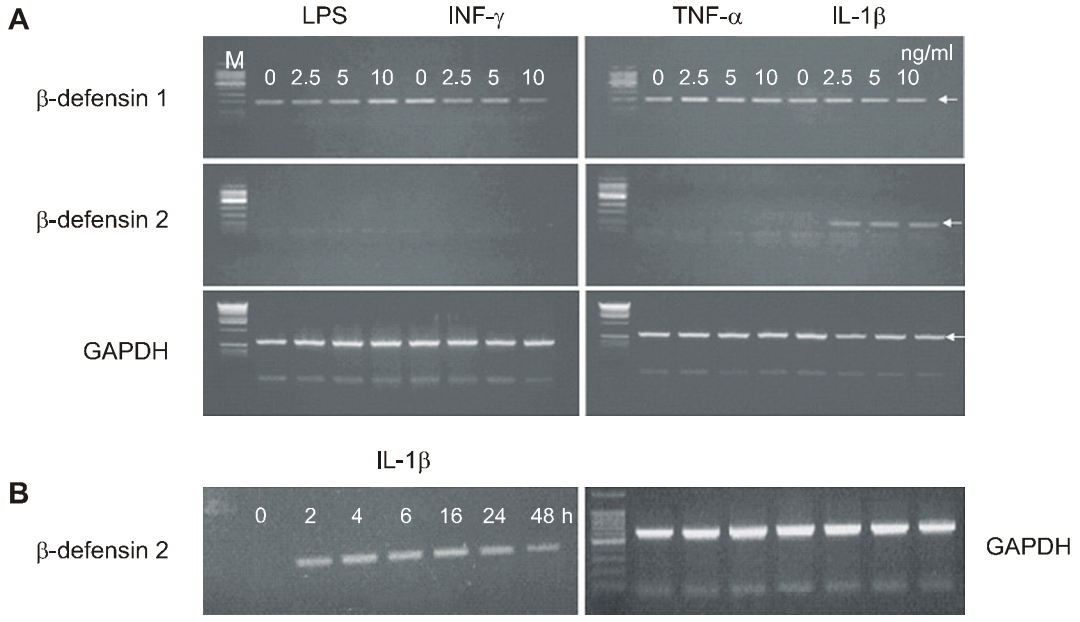


**Figure 2.** The effects of various cytokines on β-defensin gene expressions in HCEs. HCEs were treated with varying amount of indicated cytokines for 24 h, and then RT-PCR was performed as described in the Materials and Methods (A). Time course of β-defensin 2 gene induction by IL-1β (2.5 ng/ml) was shown in (B). All experiments were performed at least 3 times, and the representative photographs are shown here.

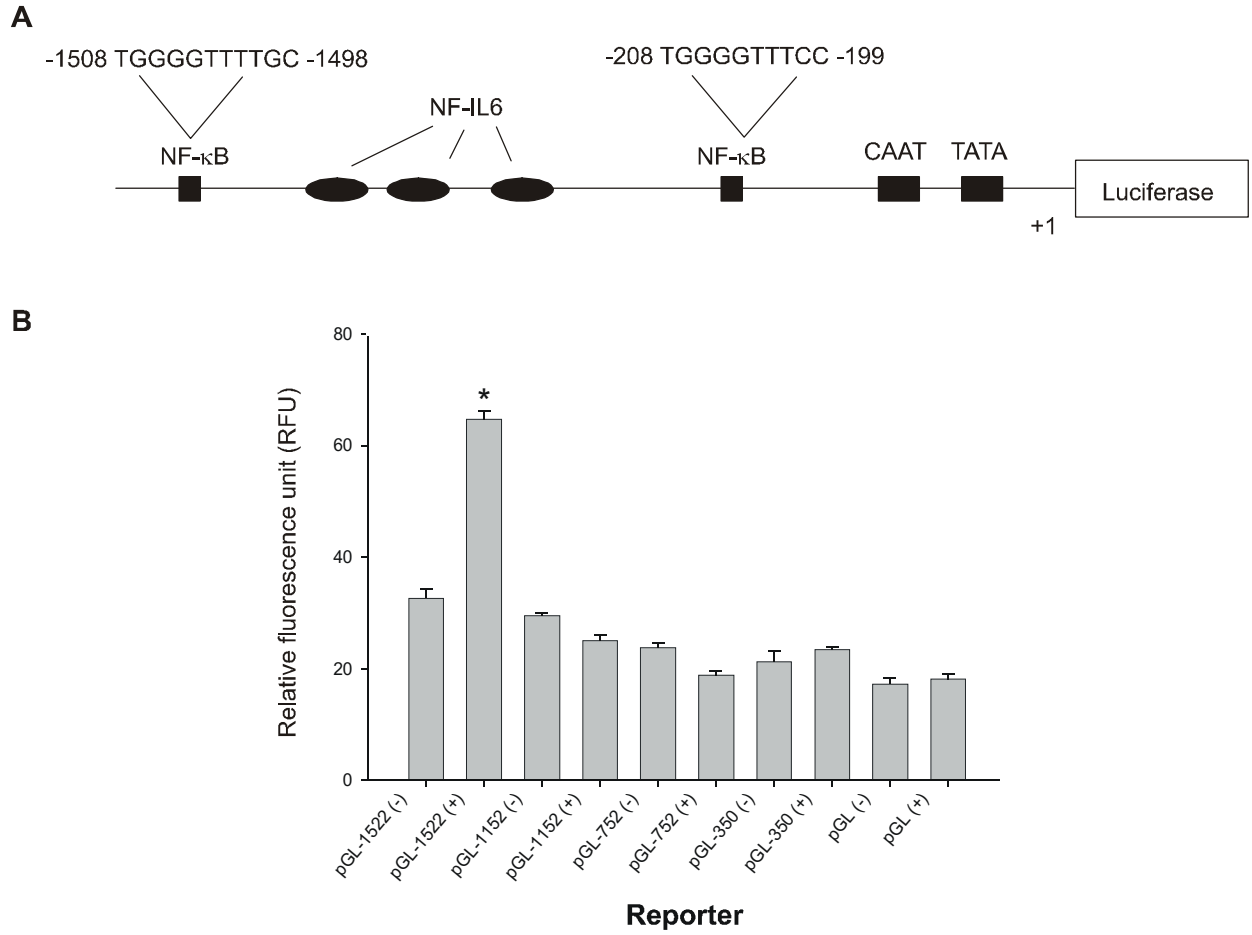


**Figure 3.** Promoter activity of reporter constructs by transient transfection. Promoter region of β-defensin 2 gene was analyzed with TransFac computer program (A). HCEs were transfected with indicated reporter plasmids, and relative luciferase activity was measured in the absence (-) or presence (+) of IL-1β. Values are means±SEM of three independent experiments, and the statistical significance was evaluated by Student's *t* test (*$P < 0.05$)

**Table 2.** Clinical diagnosis and the expression levels of β-defensins. Band intensity of each gene product was digitized using flatbed scanner and Scion Image analysis software (Scion Corp., Frederick, MD, USA), and the expression levels were denoted in relative arbitrary unit normalized to GAPDH expression level.

| Patient (sex/age) | Diagnosis | β-defensin 1 | β-defensin 2 |
|---|---|---|---|
| A, M/34 | Normal | 4.0 | 0.0 |
| B, M/49 | Herpetic corneal ulcer | 9.6 | 3.4 |
| C, M/47 | Herpetic ulcer | 9.6 | 0.4 |
| D, F/41 | Staphylococcal corneal ulcer, Allergic conjunctivitis | 4.2 | 0.3 |
| E, M/61 (right eye) | Normal | 4.9 | 0.9 |
| E, M/61 (left eye) | Corneal ulcer | 5.4 | 0.8 |

IL-1β specifically induced β-defensin 2 gene transcription in HCEs, but induction pathway was not solely dependent on proximal NF-κB site activation. In the clinical settings, furthermore, we have shown that β-defensin 2 was detected only in specimen from patients with ocular diseases, thereby strengthening our results obtained *in vitro.*

While this manuscript is being prepared, McDermott group has reported that β-defensin 2 could be induced by IL-1β in corneal epithelial cells and

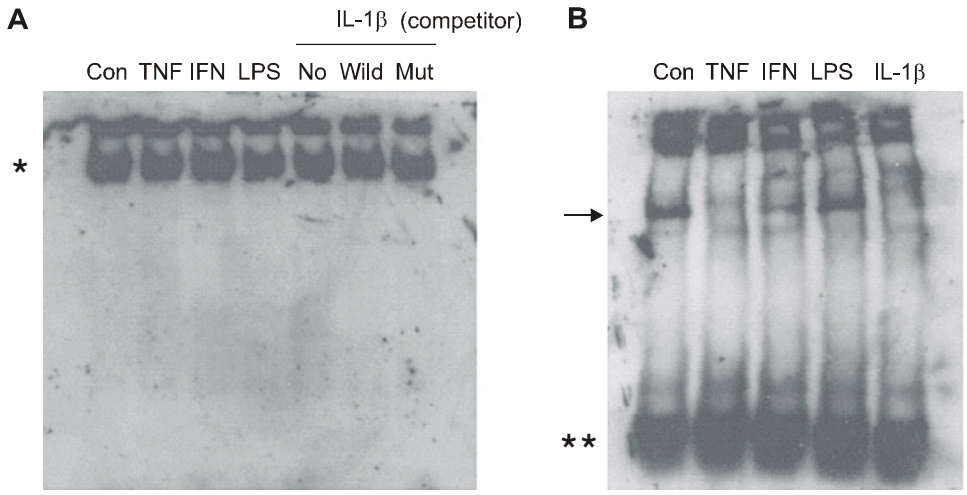


**Figure 4.** The effects of various cytokines on the binding of NF-κB complex to the oligonucleotides. Nuclear extracts were prepared after the treatment as indicated, and the binding reaction was carried out with the oligonucleotides containing the putative NF-κB site in the β-defensin 2 gene (A) or consensus sequence (B). Asterisks * and ** denote non-specific complex and the free probes, respectively, and the photograph is a representative of three independent experiments.

conjunctival epithelial cells, and its induction was mediated *via* multiple pathways including p38 mitogen activated protein kinase, c-Jun $NH_2$-terminal kinase, and NF-κB pathway (Mcdermott *et al*., 2003; Narayanan *et al*., 2003). Among the signal pathways, the authors argued that NF-κB pathway was predominantly involved in IL-1β-induced β-defensin 2 gene transcription, by showing that the β-defensin 2 expression was completely suppressed by the specific inhibitor of NF-κB pathway such as pyrrolidinedithiocarbamate, MG 132, and caffeic acid phenethyl ester. These results were fully compatible with our data, however, our results further suggested that the most proximal NF-κB site on the promoter of β-defensin 2 is not involved in β-defensin 2 induction by IL-1β, and the full responsiveness toward IL-1β need the long distal portion of the promoter as like to human iNOS gene regulation (De Vera *et al*., 1996; Chu *et al*., 1998; Taylor *et al*., 1998). It is also worthwhile to stress that β-defensin 2 was only detected in samples obtained from the patients with ocular diseases in our study, since the amount of proinflammatory cytokines, in particular, IL-1β and IL-6 was shown to be increased in the disease state (Xue *et al*., 2002).

Finally, the caveats of the data from this study and other *in vitro* studies must be interpreted with the caution, since the corneal epithelial cells *in vivo* are stratified to 4-5 cell thickness with each layer having different differentiation state (West-Mays *et al*., 2003). Therefore, it is possible that the Toll-like receptors essential for transmitting the external stimuli such as LPS or other bacterial components into intracellular signaling are not properly expressed in monolayer HCEs. Further studies will be required to address this important possibility.

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