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Attenuation of Interleukin-8 Production by Inhibiting Nuclear Factor-κB Translocation Using Decoy Oligonucleotides*

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ABSTRACT. Interleukin-8 (IL-8), a monocyte-derived neutrophil chemoattractant factor, is a polymorphonuclear neutrophil chemotaxin that is involved in a number of inflammatory disorders. Transcription of the IL-8 gene is controlled by regulatory proteins, including nuclear factor-κB (NF-κB), a family of proteins that is important in the transcriptional control of a number of genes. When cells are activated, NF-κB translocates from the cytoplasm to the nucleus, where it activates transcription by binding to a specific sequence within the 5′ untranslated region of the gene. During translocation, NF-κB is potentially susceptible to diversion by oligonucleotides that contain the binding sequence for this protein. In the current study, we produced phosphorothioate-modified oligonucleotides containing the specific DNA sequence that NF-κB binds within the IL-8 gene. We then investigated the effects of transfection of monocytes with these oligonucleotides on interleukin-1β (IL-1β)-stimulated IL-8 production, IL-8 mRNA expression, and NF-κB binding activity. We found that transfection with these oligonucleotides significantly inhibited monocyte IL-8 production. A single-stranded oligonucleotide with two copies of the NF-κB-binding sequence was the most potent of those tested. This single-stranded oligonucleotide also inhibited IL-1β-induced translocation of NF-κB to the nucleus and reduced IL-8 mRNA expression. These studies demonstrated that monocyte production of IL-8 can be attenuated using a single-stranded oligonucleotide that binds a transcriptional activating protein before it translocates to the cell nucleus. This approach ultimately may be useful in the control of inflammation involved in a number of diseases. BIOCHEM PHARMACOL **59**;6:605–613, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. inflammation; interleukin-8; NF-κB; monocytes; oligonucleotides; northern blotting

IL-8‡, a monocyte-derived neutrophil chemoattractant factor, is a CXC chemokine that attracts and activates polymorphonuclear neutrophils. IL-8 is involved in a number of disease processes characterized by acute inflammation [1]. Increased IL-8 protein or mRNA levels have been noted in psoriatic skin [2], nasal secretions of individuals with allergic rhinitis [3], lung tissue from patients with idiopathic pulmonary fibrosis [4], coronary circulation of post-ischemic hearts [5], joint fluid from patients with rheumatoid arthritis [6], areas of gingivitis [7], and other inflammatory sites.

The gene coding for human IL-8 has been characterized [8]. Within the 5′ untranslated region of this gene exist regulatory regions containing consensus binding sequences for transcriptional activating factors, including the NF-κB family. The NF-κB family is a family of proteins related to the Rel family of transcriptional regulatory proteins [9]. NF-κB was characterized initially as a B lymphocyte and plasma cell nuclear protein that binds to a specific 10-bp sequence in the kappa intronic enhancer region [10]. Subsequently, numerous cell types have been shown to possess NF-κB binding activity [9]. NF-κB is important for the transcription of a large number of cytokine genes including IL-1β, IL-2, IL-2 receptor antagonist, tumor necrosis factor-α, β-interferon, granulocyte/macrophage colony stimulating factor, and serum amyloid protein [9]. There are a number of inducers of NF-κB activity [9] including reactive oxygen species, viral or bacterial pathogens, and cytokines, including IL-1β and tumor necrosis factor-α.

Proteins with NF-κB binding activity that have been best characterized include 50-kDa (p50 subunit), 52-kDa (p52 subunit), and 65-kDa (RelA p65 subunit) molecules [9]. The originally described NF-κB protein is a heterodimer composed of the p50 and RelA p65 subunits. More recently, NF-κB binding activity has been character-

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‡ *Abbreviations*: IL, interleukin; NF-κB, nuclear factor-κB; PMSF, phenylmethylsulfonyl fluoride; LDH, lactate dehydrogenase; SSC, 0.15 M sodium chloride + 0.015 M sodium citrate; and DTT, dithiothreitol.

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ized in homo- or heterodimers of all these subunits and other proteins that contain a conserved 300-amino-acid segment termed the Rel homology domain [9]. The 10-bp consensus DNA sequence recognized by this Rel homology domain is GGGGYNNCCY. In the 5′ untranslated region of the IL-8 gene, the sequence GGAATTTCCT is a putative NF-κB binding domain [11]. After cellular activation, NF-κB proteins are freed from an inhibitor protein, and the active complex translocates from the cytoplasm to the nucleus where it binds to the appropriate DNA sequence and initiates transcription.

Use of oligonucleotides to act as "decoys" for transcriptional activating proteins has emerged as an active field of investigation [12]. Introduction of oligonucleotides into cell cytoplasm to bind NF-κB-related proteins has been investigated previously. In one study [13], transfection with a double-stranded oligonucleotide containing the consensus binding sequence for NF-κB attenuated phorbol myristate acetate-induced adhesion in HL-60 cells. To date, no studies have examined whether transfection with oligonucleotides containing the NF-κB consensus binding sequence can alter IL-8 or other chemokine production. In the present study, we assessed whether transfection with single- or double-stranded oligonucleotides containing variable repeats of the IL-8 gene NF-κB consensus sequence can alter NF-κB translocation and IL-8 gene transcription. Our studies suggest that IL-8 production can be reduced by using oligonucleotides that decoy NF-κB proteins.

MATERIALS AND METHODS

Oligonucleotides Employed

Single-stranded oligonucleotides were produced commercially (Genosys Biotechnologies). Certain oligonucleotides used for the study were phosphorothioate-modified to reduce intracellular nuclease digestion. Single-stranded phosphorothioate-modified oligonucleotides employed as NF-κB decoys were (i) an oligonucleotide containing a single copy of the binding sequence for NF-κB contained in the IL-8 gene, 5′-GGAATTTCCT-3′ (termed NF-κB1), and (ii) an oligonucleotide containing two copies of the consensus binding sequence, 5′-GGAATTTCCTGGAATTTCCT-3′ (termed NF-κB2). Complementary phosphorothioate-modified oligonucleotides were annealed to NF-κB1 and NF-κB2 to produce double-stranded molecules (termed DSNF-κB1 and DSNF-κB2). Control oligonucleotides included an unrelated single-stranded oligonucleotide (5′-ACCACACTATTG-3′) that was not phosphorothioate-modified and a phosphorothioate-modified oligonucleotide with two copies of the IL-8 gene NF-κB locus containing mutations (underlined) in the NFκB binding site (CTAATCTCCTCTAATCTCCT), termed MNF-κB2.

Monocyte Purification

Whole blood was obtained from normal volunteers, and mononuclear cells were isolated using Ficoll–Hypaque gradient centrifugation as previously described [14]. After isolation by gradient centrifugation, mononuclear cells were washed three times in modified Hanks' balanced salt solution without calcium, resuspended in Hanks' balanced salt solution with calcium at 40×10^6 cells/mL (total of 16×10^6 cells), and placed in a glass-based tissue culture chamber (LabTek dishes, Miles) for adherence. Monocytes were allowed to adhere for 2 hr at 37° in the presence of 5% CO_2, after which nonadherent cells, mostly lymphocytes, were removed and fresh medium (RPMI 1640 without serum) was added.

Transfection of Monocytes with Oligonucleotides Using Lipofectin as Control

Monocytes were transfected with oligonucleotides using Lipofectin as vehicle or without any vehicle. When Lipofectin was employed, cells were transfected with 10 or 1 μM NF-κB1, NF-κB2, or DSNF-κB1 oligonucleotides in 200 μL of RPMI with 10.6 μL of Lipofectin. Control exposure involved RPMI with Lipofectin alone. Cells were incubated with Lipofectin/oligonucleotide or Lipofectin alone for 5 hr prior to removal of supernatant and replacement with medium containing serum and recombinant IL-1β for further culture.

Determination of Optimal Lipofectin Concentration

The volume of Lipofectin that most effectively facilitated oligonucleotide transfection was determined using NF-κB1 labeled with digoxigenin. The oligonucleotide was labeled with digoxigenin according to the protocol of a commercially available kit (Genius system, Boehringer). Labeled oligonucleotide (0.3 μg) was added to adherent preparations of monocytes for 3 hr at 37°. Then cells were washed and fixed, and intracellular oligonucleotide was detected using an alkaline phosphatase-labeled antibody directed at digoxigenin and an alkaline phosphatase substrate. The color reaction was quantitated using an image analysis system (Olympus Instruments) blinded to the specific treatment, and the volume of Lipofectin that resulted in the greatest apparent intracellular oligonucleotide was subsequently used for future transfection.

Determination of Endotoxin Content

The concentration of endotoxin in Lipofectin was determined by the *Limulus* lysate test (Associates of Cape Cod) as previously described [15]. Sensitivity of the test was 1.0 pg/mL.

Transfection with Naked Oligonucleotides

The effect of naked NF-κB2, transfected without Lipofectin, on monocyte cytokine production also was examined. Monocytes were exposed to 10 or 1 μM NF-κB2 or the unrelated, control oligonucleotide in RPMI without serum for 3 hr prior to the removal of supernatant and replace-

ment of medium with serum and IL-1β for further incubation. The effect of transfection with naked NF-κB2 also was compared with the effect of transfection with MNF-κB2 in a similar manner to establish that effects on IL-8 release were specific for the NF-κB binding sequence of the oligonucleotide.

Cellular Stimulation After Oligonucleotide Transfection

After transfection, medium was changed, and RPMI with 10% fetal bovine serum and recombinant IL-1β (50 ng/mL) (Promega) was added. Cell cultures were continued for an additional 16 hr; then supernatant was removed and nonadherent cells were separated by centrifugation. Adherent cells were harvested by scraping, and then lysed for total protein determination, RNA recovery, or nuclear protein purification for quantitation of NF-κB binding activity.

Quantitation of IL-8 Release

An enzyme-linked immunoassay was used to measure levels of IL-8 in cell culture supernatants [16]. Microtiter plates were coated overnight with a 1:1000 dilution of a monoclonal antibody directed at IL-8 (Upstate Biotechnologies). Wells were washed, and then cell culture supernatants or known concentrations of recombinant IL-8 diluted in PBS with 0.05% Tween and 0.1% BSA were added. Plates were incubated at 37° for 1.5 hr, then wells were washed, and a 1:2000 dilution of a secondary polyclonal antibody generated in rabbits (R&D Systems) and directed at IL-8 was added. Plates were incubated for 1.5 hr, then washed, and a 1:10,000 dilution of a horseradish peroxidase-labeled antibody directed at rabbit IgG was added (Calbiochem). Plates were incubated at 37° for 1.5 hr and washed, and a solution containing a peroxidase substrate was added. The IL-8 concentration was directly related to the absorbance at 490 nm, and the concentration of IL-8 in cell culture supernatants was determined by comparing to the standard curve generated with recombinant IL-8. Protein concentrations of monocytes lysed in a buffer containing 1% Triton X-100 (20 mM Tris buffer, pH 8.0, 137 mM NaCl, 10% glycerol, 1 mM PMSF, 0.15 units/mL of aprotinin, and 1 mM sodium *o*-vanadate) also were measured by the Lowry method (see below). Cellular total protein was used as a measure of the number of cells present, and IL-8 concentrations were divided by this value to adjust for any variability in the number of cells present during the incubation.

Total Protein Assay

Cellular and nuclear total protein were quantitated using the Lowry method by adapting [17] a commercially available assay for use in a microtiter plate (Sigma). All incubation times and relative amounts of reagents remained the same. Sample and reagent volumes were reduced proportionately to allow performance of the assay in a microtiter plate.

Assessment of Cellular Viability

Polymorphonuclear leukocyte viability after transfection with oligonucleotides was assessed by determining LDH release using a commercially available kit (Sigma) that was adapted for performance in microtiter plates [18].

Preparation of Cellular Protein Extracts

Proteins were extracted from monocytes using methods described by Moynagh and colleagues [19]. After transfection with unlabeled or digoxigenin-labeled oligonucleotide, followed by IL-1 stimulation overnight, adherent monocytes were exposed to hypotonic buffer (10 mM HEPES–NaOH, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, pH 7.9) and scraped off the culture chamber. Cellular debris was harvested by centrifugation (14,000 rpm for 10 min in a microcentrifuge) and resuspended in 0.1% Nonidet P-40 in 10 mM HEPES–NaOH (pH 7.9). The material was placed on ice for 10 min. The supernatant, containing cytoplasmic solubilized material, was saved for further analysis. The insoluble fraction, including nuclei, was resuspended in 15 μL of 20 mM HEPES–NaOH (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF and maintained on ice for 15 min, then centrifuged at 14,000 rpm for 10 min. The supernatant was removed and added to 75 μL of 10 mM HEPES–NaOH (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM PMSF, 0.5 mM DTT. This material contained solubilized nuclear proteins. The amount of cytoplasmic and nuclear protein was determined by the Lowry assay as described above.

Subcellular Location of Transfected Naked Oligonucleotide

Location of transfected NF-κB2 oligonucleotide was determined using the single-stranded oligonucleotide labeled with digoxigenin. The oligonucleotide was labeled according to the directions of the commercially available kit (DIG oligonucleotide 3′-end labeling kit, Boehringer Mannheim). Adherent monocytes were exposed to 10 μM labeled NF-κB2 in RPMI 1640 for 4 hr at 37°. Untransfected cells served as a negative control. Supernatant was removed and replaced with RPMI 1640 containing 10% fetal bovine serum and 50 ng/mL of recombinant human IL-1β (Promega). Cells were incubated overnight, supernatant was removed, and cells were processed to obtain cytoplasmic and nuclear extracts as described above. These extracts were separated on a 12% nondenaturing polyacrylamide gel, and then were transferred to a nitrocellulose membrane, which was probed with an antibody directed at digoxigenin according to the instructions of a commercially available kit (DIG nucleic acid detection kit, Boehringer Mannheim). To identify specific proteins that bound the labeled oligonucleotide, extracts from transfected IL-1β stimulated monocytes were incubated for 2 hr with a 1:10

dilution of commercially available purified polyclonal antibodies generated to p50 (Santa Cruz Biotechnology) and p65 (UBI) subunits of NF-κB. The mixture was electrophoresed over a 12% nondenaturing gel, then transferred to a nitrocellulose membrane, and probed with antibody to digoxigenin. A reduction in binding of the digoxigenin-labeled oligonucleotide to a protein in the extract or a noted shift of the binding complex by a specific antibody was taken as evidence that the specific NF-κB subunit recognized by that antibody bound NF-κB2.

Gel Mobility Shift Assay

NF-κB binding activity in nuclear protein extracts was determined using a commercially available double-stranded consensus oligonucleotide for NF-κB (sense strand 5′-AGTTGAGGGGACTTTCCCAGGC-3′, Promega) [20]. Both strands of the oligonucleotide were labeled with ^{32}P using [γ-^{32}P]ATP and T4 polynucleotide kinase. Five micrograms of protein extracted from nuclei of exposed monocytes or a recombinant NF-κB p50 subunit (Promega), used as a positive control, was incubated with a 10-fold excess of unlabeled consensus oligonucleotide in gel shift buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM TrisñHCl, pH 7.5, and 80 ng/mL of calf thymus DNA) for 10 min at room temperature. Labeled oligonucleotide (175 nmol) was added, and the mixtures were incubated for an additional 20 min at room temperature. The reaction then was stopped by adding nondenaturing sample loading buffer, and the reaction mixture was electrophoresed on an 8% nondenaturing polyacrylamide gel. The gel was dried and exposed to film to establish the amount of labeled oligonucleotide bound by specific nuclear proteins. Binding was quantitated by densitometry.

In vitro competition of single-stranded oligonucleotides with the commercially available double-stranded consensus oligonucleotide also was assessed by gel shift assay. Five micrograms of nuclear protein from monocytes exposed overnight to IL-1β was incubated for 20 min with ^{32}P-labeled double-stranded NF-κB consensus oligonucleotide in the absence or presence of a 10-fold excess of unlabeled consensus oligonucleotide or unlabeled single-stranded NF-κB2. The mixture was run over a nondenaturing polyacrylamide gel, which was dried and exposed to film.

Northern Blot Analysis

After transfection with oligonucleotides and subsequent stimulation with IL-1β, monocytes were lysed with guanidine isothiocyanate, and residual adherent cells were scraped off the culture chamber. The cellular lysate was layered over CsCl (5.7 M CsCl, 0.1 M EDTA) and centrifuged in a Beckman SW-41 rotor at 151,000 *g* overnight. The RNA pellet was resuspended in TES [10 mM tris(hydroxymethyl)aminomethane, 5 mM EDTA, 1% SDS] and extracted with phenol/chloroform followed by chloroform alone. RNA was precipitated by adding 3 M sodium acetate and absolute ethanol, pelleted in a microcentrifuge, and dried prior to resuspension in RNase-free water. Thirty micrograms of total RNA, suspended in a solution containing dimethyl sulfoxide, glyoxal, and 0.5 M sodium phosphate (pH 7.0), was loaded onto a 1% agarose gel (in 0.01 M sodium phosphate) and electrophoresed for 4–6 hr at 60 V. RNA markers were run in parallel to establish the size of detected transcripts in the sample. Material on the gel was transferred to nylon membranes by capillary action and probed with cDNA specific for IL-8 (provided by Dr. Teizo Yoshimura at the National Cancer Institute) that had been labeled with ^{32}P using random primers and purified using a G50 push column. Membranes also were probed with similarly labeled γ-actin cDNA (Clone No. HFBCE81, American Type Culture Collection) to verify equal loading of RNA. Labeled probes were added to achieve a concentration of 1.5×10^6 cpm/mL. Membranes first were prehybridized at 65° in a solution containing 50 mM $NaPO_4$ (pH 6.5), 100 mM NaCl, 50 mM PIPES (pH 6.8), 1 mM EDTA, and 5% SDS, then the probe was added, and membranes were hybridized in the same solution overnight at 65°. To determine whether the NF-κB2 oligonucleotide bound directly to the IL-8 transcript, potentially reducing subsequent binding of the IL-8 cDNA, 10 μM NF-κB2 was added to the prehybridization solution of a membrane containing 10 μg of total RNA extracted from monocytes exposed to IL-1β. Then the membrane was probed with the IL-8 cDNA, and the signal was compared with that obtained using prehybridization solution without NF-κB2. The next day, membranes were washed four times at 65° in 5% SDS, 0.667x SSC, and then were exposed overnight to film [16].

Statistics

Data were stored and analyzed using a software package (SPSS) and a Gateway P5–133 computer. Differences in means of normally distributed data were assessed by Student's *t*-test. When multiple groups were involved, differences were assessed by ANOVA, and individual group differences were assessed by the NeumanñKeuls test. When data were not normally distributed, differences were determined by the Friedman test, if multiple groups were involved, and by the Wilcoxon signed ranks test for differences between two measurements. All data are means ± SEM. P values ≤ 0.05 were considered significant [21].

RESULTS

Effects on IL-8 Production of Transfection with Oligonucleotides Using Lipofectin as a Vehicle

Initial experiments used Lipofectin as a vehicle for transfection of monocytes with single-stranded NF-κB1 and NF-κB2 oligonucleotides or NF-κB1 annealed to its complement (DSNF-κB1). Transfection with 10 μM NF-κB1, NF-κB2, or DSNF-κB1 attenuated IL-1β-stimulated IL-8

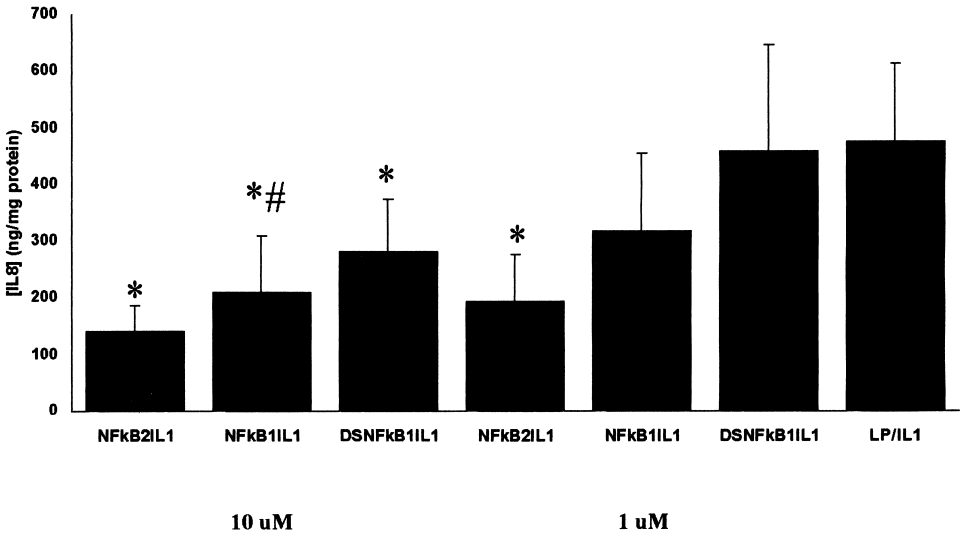

FIG. 1. Effects of transfection of monocytes, using Lipofectin as vehicle, with single-stranded oligonucleotides containing one (NFκB1IL1) or two (NFκB2IL1) copies of the IL-8 gene NF-κB binding sequence, or an annealed double-stranded oligonucleotide containing one copy of the IL-8 NF-κB binding sequence (DSNFκB1IL1) on IL-1-stimulated IL-8 production (ordinate). Monocytes were exposed to a 10 or 1 μM concentration of oligonucleotide in the presence of Lipofectin, and then were stimulated with IL-1β. LP/IL1 = supernatant from monocytes exposed to Lipofectin alone, and then stimulated with IL-1 (N = 7, means ± SEM). Key: (*) $P < 0.05$ vs LP/IL1; and (#) $P < 0.05$ vs 10 μM DSNFκB1IL1.

production significantly when compared with Lipofectin alone (Fig. 1). Only NF-κB2 inhibited significantly at a 1 μM concentration. Ten micromolar single-stranded NF-κB1 inhibited IL-8 production more significantly than 10 μM DSNF-κB1.

Effect of Lipofectin on IL-8 Production

Monocytes exposed to Lipofectin alone produced significantly more IL-8 than those exposed to buffer (Table 1). The degree of stimulation for IL-8 production that Lipofectin induced was similar to that induced by recombinant IL-1β. However, the two stimuli were additive. The effect of Lipofectin on monocyte IL-8 production was not due to endotoxin contamination, as there was no detectable (<1 pg/mL) endotoxin in the Lipofectin preparation employed.

In Vitro *Competition of NF-κB2 Oligonucleotide with NF-κB Consensus Oligonucleotide*

When 10-fold excess single-stranded NF-κB2 oligonucleotide was added to ^{32}P-labeled double-stranded NF-κB consensus oligonucleotide, the binding of monocyte nuclear protein to the labeled consensus oligonucleotide was prevented completely (Fig. 2). A 10-fold excess of unlabeled consensus oligonucleotide also completely prevented binding of nuclear protein to labeled consensus oligonucleotide.

TABLE 1. Effect of Lipofectin on monocyte IL-8 production

Treatment	[IL-8] (ng/mg protein)
Buffer	26.5 ± 23
Lipofectin	280 ± 155*
IL-1β	254 ± 110*
Lipofectin + IL-1β	475 ± 141*†

Values are means ± SEM, N = 7.
*$P < 0.05$ vs buffer.
†$P < 0.05$ vs IL-1β, by Wilcoxon signed ranks test.

Effects of Transfection with Naked NF-κB2 on IL-8 Production

Because Lipofectin induced an increase in monocyte IL-8 production, effects of transfection with single-stranded NF-κB2 in the absence of Lipofectin also were examined.

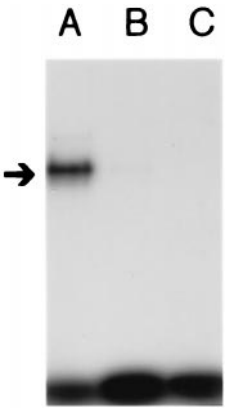

FIG. 2. *In vitro* competition of single-stranded NF-κB2 (B) or double-stranded consensus NF-κB oligonucleotide (C) with ^{32}P-labeled double-stranded consensus NF-κB oligonucleotide. Nuclear protein from monocytes stimulated with IL-1 was exposed to a 10-fold excess of the noted unlabeled oligonucleotides in the presence of labeled consensus oligonucleotide, and specific binding was determined by gel shift. Lane A shows nuclear protein incubated with ^{32}P-labeled consensus oligonucleotide alone. Similar findings were noted in two other independent experiments.

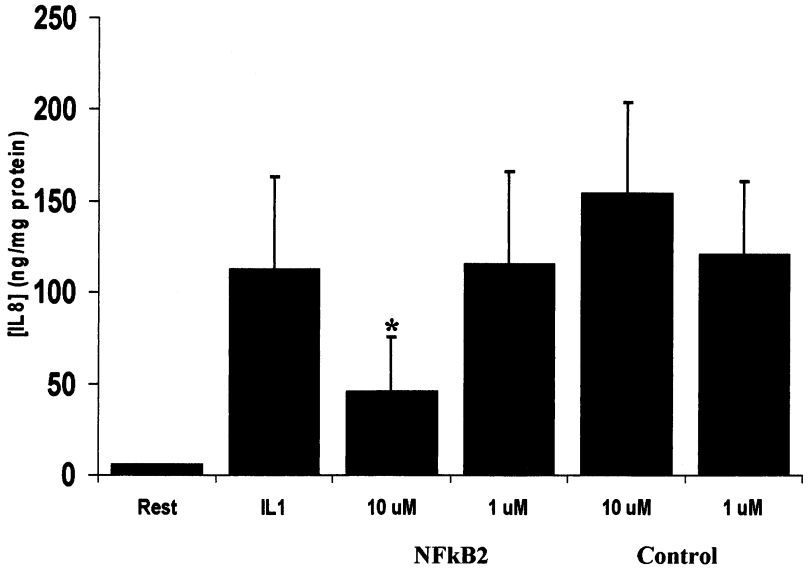

FIG. 3. Effects of transfection with naked single-stranded NF-κB2 oligonucleotide or control single-stranded oligonucleotide on IL-1-stimulated IL-8 production (ordinate). Rest = monocytes exposed to buffer alone; IL-1 = monocytes exposed to buffer, and then to IL-1 (N = 7, means ± SEM). Key: (*) $P < 0.05$ vs 10 μM control oligonucleotide exposure.

Monocytes exposed to a 10 μM concentration of naked oligonucleotide produced significantly less IL-8 when compared with a 10 μM concentration of control oligonucleotide. However, 1 μM NF-κB2 did not alter IL-8 production significantly (Fig. 3). The IC_{50} value of transfected naked NF-κB2 oligonucleotide was approximately 10 μM, compared with an IC_{50} value of less than 1 μM for NF-κB2 transfected using Lipofectin as vehicle. To determine whether the NF-κB binding sequence in the oligonucleotide was specific for the effect, we also compared IL-8 production in monocytes transfected with a 10 μM concentration of naked single-stranded NF-κB2 to cells transfected with a 10 μM concentration of single-stranded MNF-κB2, an oligonucleotide containing the specific mutation in the NF-κB binding site (Table 2). There was a significant difference between these two treatments, suggesting that the effect was specific for the NF-κB binding site of the NF-κB2 oligonucleotide.

Subcellular Location of Transfected Naked NF-κB2

After monocyte transfection, digoxigenin-labeled NF-κB2 could be detected in cytoplasmic and nuclear cellular fractions. The labeled oligonucleotide was bound to a higher molecular weight molecule, presumably a protein, in each of these fractions (Fig. 4A). Treatment of the nuclear fraction with polyclonal antibody to the p65 NF-κB subunit decreased detection of the oligonucleotide/protein complex noted in control and p50 antibody-treated extracts and also caused a faint shift upwards of the bound oligonucleotide on the blot (Fig. 4B).

TABLE 2. Comparison of effects of NF-κB2 to MNF-κB2 on IL-1-stimulated monocyte IL-8 production

Treatment	[IL-8] (ng/mg protein)
NF-κB2	20 ± 4.7*
MNF-κB2	174 ± 94

Values are means ± SEM, N = 7.
*$P < 0.05$ vs MNF-κB2, by Wilcoxon signed ranks test.

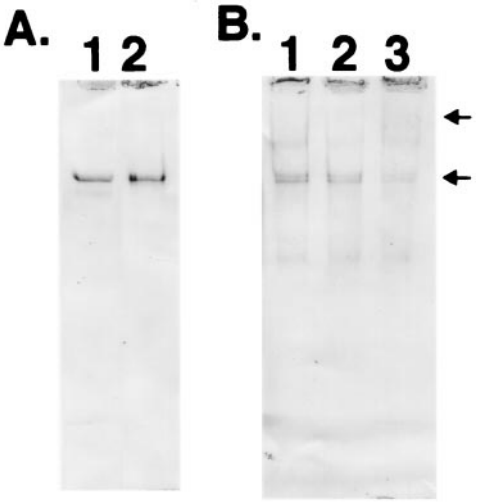

FIG. 4. Subcellular location of transfected NF-κB2 (A) and identification of the protein binding transfected labeled NF-κB2 (B). In panel A, cells transfected with a 10 μM concentration of naked digoxigenin-labeled NF-κB2 were processed to obtain cytoplasmic (lane 1) and nuclear (lane 2) extracts. Western blotting using an anti-digoxigenin antibody detected the oligonucleotide, bound to a protein, in both fractions. In panel B, when the nuclear extract was incubated with an antibody to the p65 subunit of the NF-κB protein (lane 3), a reduction in the noted band (lower arrow) was noted due to a faint shift of the binding complex to a higher level (upper arrow). Incubation with antibody to the NF-κB p50 subunit had no effect (lane 2) when compared with exposure to buffer alone (lane 1).

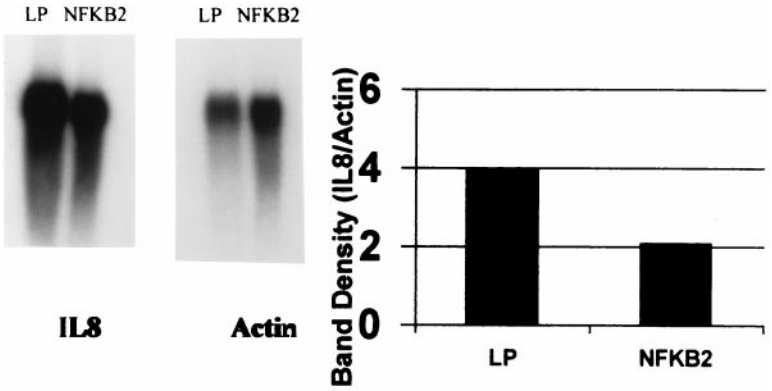

FIG. 5. Northern blot analysis showing effects of transfection with a 10 μM concentration of single-stranded NF-κB2 oligonucleotide, using Lipofectin as vehicle, on IL-1-stimulated IL-8 mRNA levels. Cells were transfected with the oligonucleotide, using Lipofectin (NF-κB2) as vehicle, or were exposed to Lipofectin alone (LP), and IL-8 or actin transcript levels were assessed. The autoradiogram is shown on the left; densitometry values for the blot, expressed as density of IL-8 band divided by the actin band, are shown on the right. Similar findings were noted on two other distinct northern blots.

Effect of Transfection with Oligonucleotides on Cellular Viability

No detectable LDH was noted in supernatants of monocytes transfected with any concentration of oligonucleotides employed, suggesting that cells remained viable. In addition, cellular protein was no different among the groups.

Effect of Transfection with NF-κB2 on Monocyte IL-8 mRNA Levels

When monocytes were exposed to 10 μM NF-κB2, with Lipofectin as vehicle, and then stimulated with IL-1β, there was a reduction in IL-8 mRNA compared with cells exposed to the control unrelated oligonucleotide in the presence of Lipofectin alone (Fig. 5). Actin mRNA levels were not altered by cellular transfection with NF-κB2. The addition of 10 μM NF-κB2 to prehybridization solution did not affect binding of the IL-8 cDNA to RNA from IL-1β-stimulated monocytes (data not shown).

Attenuation of NF-κB Binding Activity by Transfection with NF-κB2

Extracts from nuclei of monocytes exposed to IL-1β contained two protein complexes that bound the double-stranded, commercially available NF-κB consensus oligonucleotide. Transfection of monocytes with 10 μM NF-κB2 in the presence of Lipofectin inhibited NF-κB binding activity in nuclear extracts after IL-1β stimulation (Fig. 6), suggesting that this oligonucleotide inhibited IL-8 production in part through competition for the NF-κB protein.

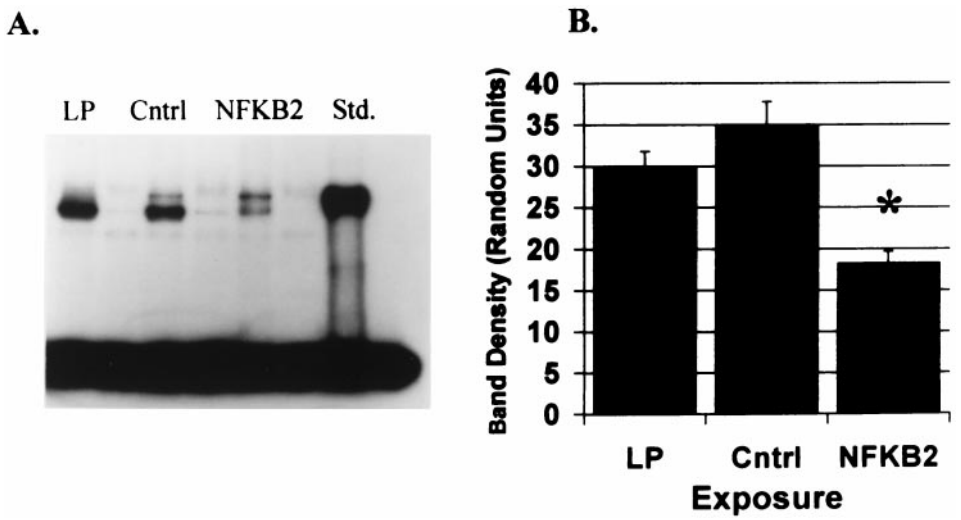

FIG. 6. Effects of transfection with a single-stranded NF-κB2 oligonucleotide (NF-κB2), single-stranded control oligonucleotide (Cntrl), or Lipofectin exposure (LP) on NF-κB activity in monocytes stimulated with IL-1. NF-κB activity was measured by conventional methods, using the gel shift assay and a commercially available double-stranded consensus oligonucleotide. A representative gel shift assay is shown on the left, and densitometry values for four different experiments (means ± SEM) are shown on the right. Key: (*) $P < 0.05$ vs Cntrl or Lipofectin alone; Std = NF-κB p50 subunit protein standard.

DISCUSSION

We have demonstrated that transfection of monocytes with oligonucleotides containing binding sequences for NF-κB attenuated IL-1β-stimulated IL-8 production. The alteration in IL-8 production appeared to be due to binding of these oligonucleotides to the NF-κB complex, specifically the p65 subunit, with subsequent reduced binding of NF-κB to the promoter region of the IL-8 gene. Because IL-8 is important in a number of disease processes [1] and NF-κB is important in the transcription of a number of different genes [9], these findings have significant ramifications for the control of inflammatory diseases.

Our study also suggests that single-stranded oligonucleotides are at least as effective in reducing IL-8 production as annealed double-stranded forms. Other investigators have used double-stranded, phosphorothioate-modified oligonucleotides to inhibit certain cellular functions [12]. However, previous studies [22] that have examined binding characteristics of the p50 subunit of NF-κB suggest that this protein preferentially binds one strand of double-stranded genomic DNA. Our studies suggest that the single-stranded NF-κB2 oligonucleotide employed in the current study competes with a double-stranded oligonucleotide containing the consensus binding sequence for NF-κB. These findings suggest that the NF-κB protein studied in our project efficiently binds to single-stranded DNA, and a single-stranded oligonucleotide can compete with a double-stranded form for binding to these proteins.

There has been evidence that phosphorothioate-modified oligonucleotides can bind to transcriptional regulatory factors in a nonspecific manner [23]. Although our studies used Lipofectin alone or a non-thiol modified oligonucleotide as initial controls, we did determine specific effects by comparing the phosphorothioate-modified oligonucleotide containing the NF-κB binding sequence with a thiol-modified oligonucleotide containing a mutated NF-κB-binding sequence. We also showed that an intracellular molecule, presumably the p65 subunit of NF-κB, bound the single-stranded oligonucleotide when it was introduced into the cell. These studies suggest that inhibition of IL-8 secretion by the oligonucleotides containing the NF-κB binding sequence is specific for that sequence.

Numerous diseases that are associated with IL-8 overproduction are accessible to local control using oligonucleotides as decoys for transcriptional regulatory proteins. Increased concentrations of IL-8 have been noted in allergic rhinitis [3], pulmonary fibrosis [4], post-ichemic myocardial injury [5], psoriasis [2], gingivitis [7], and other inflammatory diseases. Oligonucleotides are relatively easy to introduce into tissues. In fact, a previous study [13] has demonstrated that a double-stranded oligonucleotide, used as a decoy for the NF-κB p50 subunit, can be transfected into endothelial cells without the use of any vehicle. We have confirmed that naked oligonucleotide can be transfected into cells with resultant inhibition of a specific cellular function. Although Lipofectin enhances the effect, Lipofectin also appears to stimulate IL-8 release. Therefore, the use of naked oligonucleotides as decoy molecules to turn off gene transcription *in vivo* seems more feasible.

These studies suggest that single-stranded oligonucleotides with the appropriate DNA sequence for binding to a transcriptional activating protein can be used as decoys to attenuate IL-8 gene transcription and subsequent production of IL-8 protein. These studies lay the groundwork for developing strategies to suppress cytokine production and dampen subsequent inflammation through the decoy approach.

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