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Inhibition of interleukin-12 production in lipopolysaccharide-activated macrophages by curcumin

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Abstract

Pharmacological control of interleukin-12 production may be a key therapeutic strategy for modulating immunological diseases dominated by type-1 cytokine responses. In this study we investigated the effects of curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione) on the production of interleukin-12 from mouse macrophages stimulated with lipopolysaccharide. Curcumin potently inhibited the production of interleukin-12 in a dose-dependent manner. The effect of curcumin on interleukin-12 p40 promoter activation was analyzed by transfecting RAW264.7 monocytic cells with p40 promoter/reporter constructs. The repressive effect mapped to a region in the p40 promoter containing a binding site for nuclear factor κB (p40-κB). Furthermore, activation of macrophages by lipopolysaccharide resulted in markedly enhanced binding activity to the κB site, which significantly decreased upon addition of curcumin. These results suggest that curcumin-induced inhibition of interleukin-12 production in macrophages may explain some of the biological effects of curcumin including its anti-inflammatory activity.

Keywords: Curcumin; Interleukin-12; Macrophage; Nuclear factor κB

1. Introduction

Interleukin-12, a heterodimeric cytokine composed of two disulfide-linked subunits of 35 (p35) and 40 (p40) kDa encoded by two separate genes, is produced by phagocytic cells and other antigen-presenting cells in response to stimulation by a variety of microorganisms as well as their products (Trinchieri, 1998). Interleukin-12 exerts multiple biological activities mainly through T and natural killer cells by inducing their production of interferon-γ, which augments their cytotoxicity, and by enhancing their proliferation potential. Interleukin-12 production is critical for the development of T helper type 1 cells and the initiation of cell-mediated immune responses. Recent evidence points to a critical role for interleukin-12 in the pathogenesis of rodent models of Th1-mediated autoimmune diseases such as type-1 diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and acute graft-versus-host disease (Romagnani, 1996). Thus, pharmacological control of interleukin-12 production may be a key strategy in modulating specific immune-mediated diseases dominated by type-1 cytokine responses.

Curcumin, widely used as a spice and responsible for the yellow color of curry, is a natural product of plants obtained from *Curcuma longa* Linn (tumeric). Curcumin is known to exhibit a variety of pharmacological effects including anti-tumor, anti-inflammatory, anti-HIV and anti-infectious activities (Allen et al., 1998; Chan et al., 1998; Vlietinck et al., 1998), and is under preclinical evaluation as a cancer preventive and anti-inflammatory drug (Gescher et al., 1998).

In this study we investigated the effect of curcumin on interleukin-12 production in mouse macrophages. Here we demonstrate that curcumin inhibited interleukin-12 production in lipopolysaccharide-activated macrophages. This inhibition was dependent, at least in part, on the downregulation of nuclear factor kappaB (NFκB) binding to the p40-κB sequence.

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2. Materials and methods

2.1. Mice, cell culture, and transient transfection

Female DBA/2 mice were obtained from Japan SLC (Tokyo, Japan) and used at 6–10 weeks of age. RAW264.7 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY) at 37°C in a 5% CO_2 humidified air atmosphere. Spleen cell populations and macrophages from mice were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics. For transfections, cells were grown in 24-well plates with medium supplemented with 10% fetal bovine serum for 24 h and transfected with the indicated plasmid in the presence of Superfect according to the manufacturer's protocol (Qiagen, Valencia, CA). After 12 h, cells were washed and refed with DMEM containing 10% fetal bovine serum. Cells were harvested 24 h later, luciferase activity was assayed as described (Ausubel et al., 1995), and the results were normalized to *Lac*Z expression.

2.2. Interleukin-12 p40 promoter constructs

The $-689/+98$ fragment of the murine interleukin-12 p40 promoter from pXP2 (Ma et al., 1996) was subcloned into the *Kpn*I/*Xho*I sites of the pGL3-basic luciferase vector (Promega, Madison, WI). All the deletion mutants were generated by polymerase chain reaction using an upstream primer containing *Bam*HI site. A linker-scanning mutant was generated by a two-step polymerase chain reaction procedure with overlapping internal primers that contain mutated sequences for the NFκB site.

2.3. Preparation of splenic macrophages stimulated with lipopolysaccharide

Spleen cells were cultured at 10^6 cells/ml for approximately 3 h at 37°C. The non-adherent cells were removed by washing with warm DMEM until visual inspection revealed a lack of lymphocytes (> 98% of the cell population). The cell population was mostly macrophages as confirmed by flow cytometry (Epic V, Coulter Electronics, Hialeah, FL). The adherent cells were removed from plates by incubating them for 15 min with ice-cold phosphate-buffered saline solution and rinsing them repeatedly. The isolated adherent cell population was stimulated with 5 μg/ml lipopolysaccharide in the absence or presence of curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione; Sigma, St. Louis, MO) at 0.5, 1.0, 2.5, 5.0 μg/ml at 1×10^5 cells per well in 96-well culture plates for 48 h.

2.4. Cytokine assays

The quantities of interleukin-12 p40, interleukin-12 p70 and interleukin-10 in culture supernatants were determined by a sandwich enzyme-linked immunosorbent assay using monoclonal antibodies specific for each cytokine as previously described (Kim et al., 1997). The monoclonal antibodies for coating the plates and the biotinylated second monoclonal antibodies (from PharMingen, San Diego, CA) were as follows: for interleukin-12 p40, C17.8 and C15.6; for interleukin-12 p70, Red-T/G297-289 and C17.8; for interleukin-10, JES-2A5 and SXC-1. Standard curves were generated using recombinant cytokines. The lower limit of detection was 30 pg/ml for interleukin-12 p40, 50 pg/ml for interleukin-12 p70, and 0.2 ng/ml for interleukin-10.

2.5. Electrophoretic gel shift assay

The nuclear extracts were prepared from the cells as previously described (Dignam et al., 1983). An oligonucleotide containing an NFκB-binding site within the immunoglobulin κ-chain (5′ CCG GTT AAC AGA GGG GGC TTT CCG AG 3′) was used as a probe. Specific binding was confirmed by competition experiments with a 50-fold excess of unlabeled, identical oligonucleotides or cAMP response element-containing oligonucleotides.

2.6. Statistical analysis

Student's *t*-test was used to determine the statistical differences between various experimental and control groups. A P value of < 0.01 was considered significant.

3. Results

3.1. Curcumin inhibited interleukin-12 production from lipopolysaccharide-activated macrophages

We examined the effect of curcumin on the production of interleukin-12 by primary macrophages stimulated with lipopolysaccharide. Lipopolysaccharide readily induced the production of interleukin-12 heterodimer as well as the p40 subunit, as expected (Fig. 1). However, curcumin significantly inhibited this lipopolysaccharide-induced interleukin-12 production in a dose-dependent manner ($P <$ 0.01 at 2.5 and 5.0 μg/ml curcumin, relative to a group with no treatment). In contrast, treatment with curcumin had little influence on interleukin-10 production from lipopolysaccharide-stimulated macrophages, suggesting that the inhibition of interleukin-12 production by curcumin was not the result of a general dampening of cellular activation.

3.2. Curcumin inhibited NFκB-mediated activation of interleukin-12 p40 promoter by lipopolysaccharide

An interleukin-12 p40 subunit is known to be a highly inducible and tightly regulated component of interleukin-12 (Kang et al., 1996). To identify the region involved in

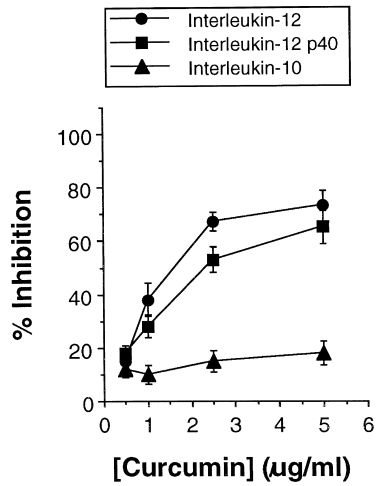

Fig. 1. Inhibition of interleukin-12 production in macrophages by curcumin. Primary macrophages were stimulated with lipopolysaccharide (5 μg/ml) for 48 h in the absence or presence of various concentrations of curcumin. Culture supernatants were harvested and cytokine levels were evaluated by enzyme-linked immunosorbent assay specific for each cytokine, and results are presented as means ± S.D. of the percentage response of cytokine production of curcumin-treated macrophages compared with untreated control macrophages stimulated with lipopolysaccharide. The data are representative of three similar experiments. Mean cytokine levels in the absence of curcumin were 1300 pg/ml (interleukin-12 p70), 3800 pg/ml (interleukin-12 p40), 1250 ng/ml (interleukin-10).

these curcumin actions, we generated a series of luciferase reporter constructs containing the p40 promoter sequences from positions −689, −231, and −185 to +98 relative to the transcription initiation site (Fig. 2A). Mouse RAW264.7 monocytic cells were transfected with each of these constructs and stimulated with lipopolysaccharide either in the absence or in the presence of curcumin, and the luciferase activity was determined. All of these constructs showed strong stimulation with lipopolysaccharide in the absence of curcumin but impaired stimulation with curcumin (Fig. 2B). In particular, deleting sequences to −185 (p40/185) did not diminish the lipopolysaccharide-dependent promoter activity and the inhibitory effect of curcumin was still observed, suggesting that the target site for curcumin resides within this region. To directly test the role of a κB site found between −121 and −131 of the p40 promoter in the curcumin-mediated inhibitory actions, we introduced a linker scanning mutation into the κB site within the context of the −689/+98 construct (p40/LS). Lipopolysaccharide-dependent promoter activation was still observed with p40/LS, although it was significantly reduced (Fig. 2B), consistent with the previous findings in which the κB site was shown to be important for the lipopolysaccharide induction of p40 promoter (Murphy et al., 1995). However, addition of curcumin to lipopolysaccharide-stimulated cells had only a slight repressive effect with p40/LS, clearly indicating that the inhibitory effect of curcumin on interleukin-12 production was mediated through the κB site.

3.3. Curcumin inhibited NFκB binding to the κB site by forming a complex with NFκB and curcumin

To gain more insight into the mechanisms of curcumin-mediated inhibition of p40-κB function, we analyzed the κB binding activity present in nuclear extracts of unstimulated or lipopolysaccharide-stimulated primary macrophages, either in the absence or presence of curcumin. As expected, nuclear extracts from lipopolysaccharide-stimulated macrophages exhibited strong κB-binding activity in the electrophoretic mobility shift assays using a labeled oligonucleotide containing a consensus immunoglobulin-κB site (Fig. 3A). The binding was specific since it was inhibited with an unlabeled, identical oligonucleotide, but not with an unrelated, nonspecific oligonucleotide, and was absent with nuclear extracts from nonstimulated cells. Nuclear extracts from macrophages stimulated by lipopolysaccharide in the presence of curcumin showed diminished κB-binding activity in a dose-dependent manner (Fig. 3A). To determine whether curcumin forms a complex with NFκB, curcumin was directly added to the binding reactions, along with nuclear extracts from lipopolysaccharide-stimulated macrophages. In these experiments, the κB binding activity decreased in a curcumin-dose dependent manner, suggesting that the curcumin may directly modulate the NFκB–DNA interaction by forming

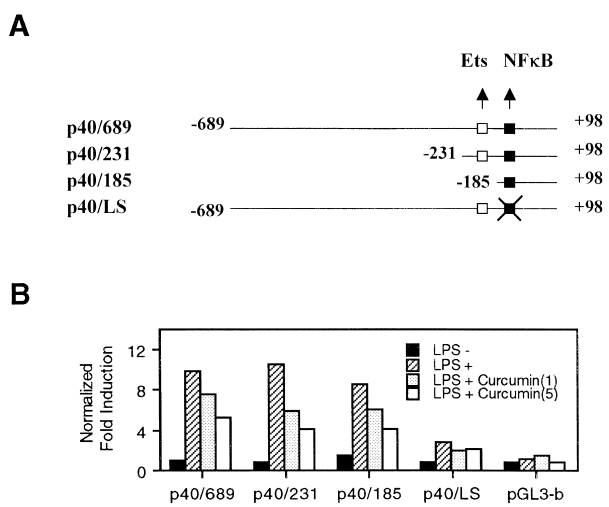

Fig. 2. Analysis of curcumin-mediated transcriptional repression of interleukin-12 p40 promoter constructs activated by lipopolysaccharide. (A) Schematic representation of the mouse interleukin-12 p40 promoter constructs and of a linker-scanning mutant of the NFκB site are as shown, along with Ets and NFκB binding sites. The nucleotide sequence numbers for each construct are shown. (B) Transient transfection of RAW264.7 cells with the p40 promoter constructs, followed by stimulation with lipopolysaccharide either in the absence or in the presence of curcumin (1 or 5 μg/ml). The results are expressed as induction over the value obtained with the unstimulated RAW264.7 cells transfected with the −689/+98 construct, which was given an arbitray value of 1. The data are representative of three similar experiments.

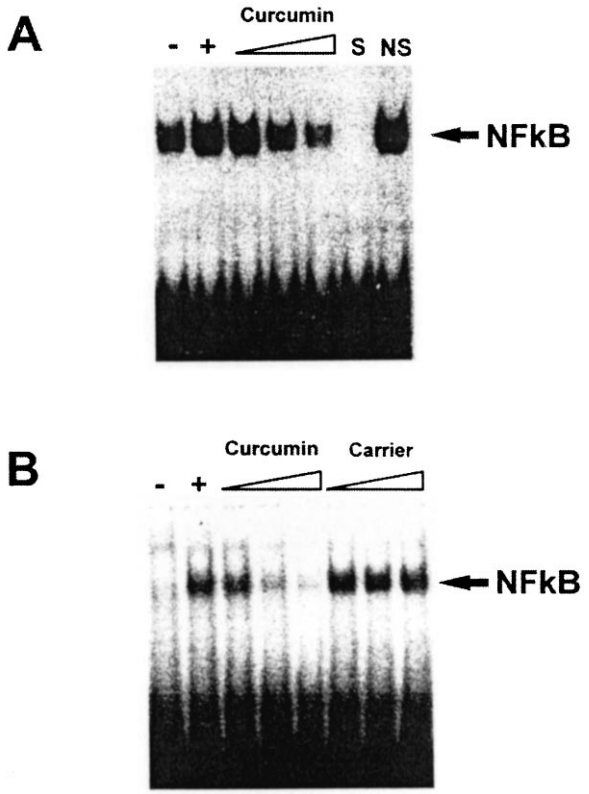

Fig. 3. Curcumin-mediated inhibition of κB binding by NFκB. (A) Nuclear extracts prepared from monocytic cells stimulated with lipolysaccharide either in the absence or presence of curcumin (1.0, 5.0 and 25 μg/ml each) were examined for κB binding activity in the electrophoretic mobility shift assay using a labeled oligonucleotide containing a consensus immunoglobulin-κB site, as indicated. S and NS indicate the presence of an unlabeled, identical oligonucleotide and nonspecific oligonucleotide, respectively. The specific NFκB complexes are as indicated. (B) Curcumin was directly added to nuclear extracts prepared from macrophages stimulated by lipopolysaccharide in the absence of curcumin, and κB binding activity was examined in the electrophoretic mobility shift assays. Increasing amounts of curcumin (1.0, 5.0 and 25 μg/ml) or carrier (ethanol) were used as indicated.

a complex with NFκB that is unable to bind κB sites (Fig. 3B).

4. Discussion

Inhibition of the action of interleukin-12 has been shown to prevent the development and to block the progression of disease in experimental models of autoimmunity. These findings have raised great interest in the identification of inhibitors of interleukin-12 production for the treatment of T helper type 1 cell-mediated diseases such as type-1 diabetes, inflammatory bowel disease and acute graft-versus-host disease. The key role of interleukin-12 in inflammation as well as in cell-mediated immune responses has raised considerable interest in the mechanisms of interleukin-12 gene transcription. In lipopolysaccaride- and interferon-γ-treated monocytes, the expression of interleukin-12 p40 has been shown to be primarily regulated at the transcriptional level, involving at least two transcription factors that belong to the NFκB and Ets families (Ma et al., 1996). Expression of interleukin-12 p35 is also known to be subject to similar transcriptional regulation, although this has been less well characterized than that for p40 (Yoshimoto et al., 1996). In this study we demonstrated that curcumin inhibited NFκB-mediated interleukin-12 production in macrophages in a dose-dependent manner. The lack of inhibition of interleukin-10 secretion by curcumin indicated that the effect of interleukin-12 was not the result of a general dampening of cellular events.

The mechanism by which curcumin inhibits interleukin-12 production in lipopolysaccharide-stimulated macrophages seems to be through the downregulation of NFκB-mediated activation and binding to the p40-κB site. This point was supported by several lines of evidence. First, an interleukin-12 p40 subunit is known to be a highly inducible and tightly regulated component of interleukin-12 (Kang et al., 1996). The inhibitory effect of curcumin on a series of 5′ deletions of the p40 promoter was retained within −185 bp upstream of the transcription initiation site (Fig. 2B), suggesting that curcumin may interfere with the inducible binding of NFκB at position −121/−131 bp in the p40 promoter. Linker scan mutation of the p40-κB site abolished the inhibitory effect of curcumin on the p40 promoter, indicating that this site plays a role in transcriptional repression of the p40 gene (Fig. 2B). In addition, curcumin significantly decreased binding to the κB site in lipopolysaccharide-activated macrophages, as demonstrated by the electrophoretic mobility assays (Fig. 3A). Direct addition of curcumin to nuclear extracts from lipopolysaccharide-stimulated macrophages decreased the κB-binding activity in a curcumin-dose dependent manner (Fig. 3B). This result suggests that curcumin may inhibit interleukin-12 production, at least in part by directly modulating the NFκB–DNA interactions via forming a complex with NFκB that is unable to bind κB sites. The NFκB-mediated inhibition of interleukin-12 production is in accord with previous observations that 1,25-dihydroxyvitamin D_3 and retinoids inhibit interleukin-12 production by downregulating NFκB activation and binding to the κB sequence of the p40 gene (D'Ambrosio et al., 1998; Na et al., 1999).

Further work will be required to elucidate the mechanism(s) by which curcumin inhibits NFκB in lipopolysaccharide-activated macrophages. One mechanism regulating NFκB activation is its binding in the cytoplasm to members of a family of ankyrin-containing molecules, the IκBs (Ghosh et al., 1998). In most cells, IκBα is the predominant inhibitory molecule, and activation and translocation of NFκB are therefore contingent upon its release from IκBα. In vivo, IκBα is rapidly degraded in response to numerous stimuli including phorbol, bacterial

lipopolysaccharide, and tumor necrosis factor α (Baldwin, 1996).

In conclusion, we have shown that curcumin inhibits interleukin-12 production in lipopolysaccharide-activated macrophages. Curcumin-induced inhibition of interleukin-12 production in macrophages may explain some of the biological effects of curcumin including its anti-inflammatory activity.

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