Curcumin (Diferuloylmethane) Downregulates the Constitutive Activation of Nuclear Factor κB and IκBα Kinase in Human Multiple Myeloma Cells Leading to Suppression of Proliferation and Induction of Apoptosis

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Running title: Curcumin inhibits NF-κB and induces apoptosis in multiple myeloma

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Abbreviations used: DEVD, N-Acetyl-Asp-Glu-Val-Asp-CHO, EMSA, electrophoretic mobility shift assay; IKK, I-κBα kinase; IL-6, interleukin-6; FBS, fetal bovine serum; IκB, inhibitory subunit of NF-κB; MM, multiple myeloma; NF-κB, nuclear transcription factor-κB; NEMO, NF-κB essential modifier; NBD, NEMO-binding domain peptide; PI, propidium iodide; PIS, preimmune serum; HRP, horse reddish peroxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, YVAD, N-Acetyl-Tyr-Val-Ala-Asp-CHO.

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Abstract

Because of the central role of the nuclear transcription factor NF-κB in cell survival and proliferation in human multiple myeloma (MM), we explored the possibility of using it as a target for MM treatment by using curcumin (diferuloylmethane) an agent known to have very little or no toxicity in humans. We found that NF-κB was constitutively active in all human MM cell lines examined and that curcumin, a chemopreventive agent, downregulated NF-κB in all cell lines as indicated by electrophoretic mobility gel shift assay and prevented the nuclear retention of p65 as shown by immunocytochemistry. All MM cell lines showed constitutively active IκB kinase (IKK) and IκBα phosphorylation. Curcumin suppressed the constitutive IκBα phosphorylation through the inhibition of IKK activity. Curcumin also downregulated the expression of NF-κB-regulated gene products including IκBα, Bcl-2, Bcl-xL, cyclin D1 and interleukin-6. This led to the suppression of proliferation and arrest of cells at the G1/S phase of the cell cycle. Suppression of NF-κB complex by IKKγ/NF-κB essential modulator-binding domain peptide also suppressed the proliferation of MM cells. Curcumin also activated caspase-7 and caspase-9 and induced PARP cleavage. Curcumin-induced downregulation of NF-κB, a factor that has been implicated in chemoresistance, also induced chemosensitivity to vincristine and melphalan. Overall, our results indicate that curcumin downregulates NF-κB in human MM cells, leading to the suppression of proliferation and induction of apoptosis; thus providing the molecular basis for the treatment of MM patients with this pharmacologically safe agent.
Introduction

Multiple myeloma (MM) is a B cell malignancy characterized by the latent accumulation in bone marrow of secretory plasma cells with a low proliferative index and an extended life span\(^1\). MM accounts for 1% of all cancers and >10% of all hematologic cancers. Various agents used for the treatment of myeloma include combinations of vincristine, BCNU, melphalan, cyclophosphamide, adriamycin, and prednisone or dexamethasone\(^2\). Usually, patients younger than 65 years are treated with high-dose melphalan with autologous stem-cell support, and older patients who can not tolerate such intensive treatment receive standard-dose oral melphalan and prednisone. Despite these treatments, this malignancy remains incurable, with a complete remission rate of 5% and a median survival of 30-36 months\(^3,4\).

The dysregulation of the apoptotic mechanism in plasma cells is considered a major underlying factor in the pathogenesis and subsequent chemoresistance in MM. It is established that IL-6, produced in either an autocrine or paracrine manner, has an essential role in the malignant progression of MM by regulating the growth and survival of tumor cells\(^5,6\). The presence of IL-6 leads to constitutive activation of Stat 3 which in turn results in expression of high levels of the anti-apoptotic protein Bcl-x\(_L\)\(^7\). Bcl-2 overexpression, another important characteristic of the majority of MM cell lines\(^8\), rescues these tumor cells from glucocorticoid-induced apoptosis\(^4\). Furthermore, treatment of MM cells with TNF activates NF-\(\kappa\)B, induces secretion of IL-6, induces expression of various adhesion molecules and promotes proliferation\(^9\). Besides, MM
cells have been shown to express the ligand for the receptor that activates NF-κB (RANKL), a member of the TNF superfamily, which could mediate MM-induced osteolytic bone disease\textsuperscript{10-12}.

One of the potential mechanisms by which MM cells could develop resistance to apoptosis is through the activation of nuclear transcription factor NF-κB\textsuperscript{13,14}. Under normal conditions, NF-κB is present in the cytoplasm as an inactive heterotrimer consisting of p50, p65, and IκBα subunits. On activation, IκBα undergoes phosphorylation and ubiquitination-dependent degradation by the 26S proteosome, thus exposing nuclear localization signals on the p50-p65 heterodimer, leading to nuclear translocation and binding to a specific consensus sequence in the DNA (5’-GGGACTTTC-3’). The binding activates gene expression, which in turn results in gene transcription\textsuperscript{15}. The phosphorylation of IκBα occurs through the activation of IκB kinase (IKK)\textsuperscript{16}. The IKK complex consists of three proteins IKKα, IKKβ and IKKγ/NF-κB essential modulator (NEMO)\textsuperscript{16}. IKKα and IKKβ are the kinases that are capable of phosphorylating IκBα, whereas IKKγ/NEMO is a scaffold protein that is critical for the IKKα and IKKβ activity. Extensive research during the past few years has indicated that NF-κB regulates the expression of various genes that play critical roles in apoptosis, tumorigenesis, and inflammation\textsuperscript{17}. Some of the NF-κB-regulated genes include IκBα, cyclin D1, Bcl-2, bcl-x\textsubscript{L}, COX-2, IL-6, and the adhesion molecules ICAM-1, VCAM-1, and ELAM-1. Recently it was reported that NF-κB is constitutively active in MM cells, leading to bcl-2 expression, which rescues these cells from glucocorticoid-induced apoptosis\textsuperscript{4,18}. Since MM cells express IL-6, various adhesion molecules, Bcl-x\textsubscript{L}, and Bcl-2\textsuperscript{4+}}
all regulated by NF-κB, and since their suppression can lead to apoptosis, we propose that NF-κB is an important target for MM treatment.

To identify a pharmacologically safe and effective agent with which to block constitutive NF-κB in MM, we selected curcumin (diferuloylmethane). We can cite the following reasons. First, curcumin has been shown by us and others to suppress NF-κB activation induced by various inflammatory stimuli. Second, curcumin inhibits the activation of IKK activity needed for NF-κB activation. Third, curcumin has been shown to downregulate the expression of various NF-κB-regulated genes including bcl-2, COX2, MMP-9, TNF, cyclin D1, and the adhesion molecules. Fourth, curcumin has been reported to induce apoptosis in a wide variety of cells through sequential activation of caspase-8, BID cleavage, cytochrome C release, caspase-9, and caspase-3. Fifth, numerous studies in animals have demonstrated that curcumin has potent chemopreventive activity against a wide variety of different tumors. Sixth, administration of curcumin in humans even at 8 g per day in phase I clinical trials has been shown to be quite safe.

Our results demonstrate that all MM cell lines expressed constitutively active NF-κB, which was suppressed by curcumin through inhibition of IKK activity. This led to downregulation of expression of gene products regulated by NF-κB, thus suppressing proliferation and inducing apoptosis in MM cells.
Materials and Methods

Materials: Human MM cell lines U266, RPMI 8226, and MM.1 were obtained from the American Type Culture Collection (Rockville, MD). Cell lines U266 (ATCC#TIB-196) and RPMI 8226 (ATCC#CCL-155) are plasmacytomas of B cell origin. U266 is known to produce monoclonal antibodies and IL-6\(^5,34\). RPMI 8226 produces only immunoglobulin light chains and there is no evidence for heavy chain or IL-6 production. The MM.1 (also called MM.1S) cell line, established from the peripheral blood cells of a patient with IgA myeloma, secretes lambda light chain, is negative for the presence of EBV genome, and expresses leukocyte antigen DR, PCA-1, T9 and T10 antigens\(^35\). MM.1R is a dexamethasone-resistant variant of MM.1 cells\(^36\) and was kindly provided by Dr. Steve T. Rosen of Northwestern University Medical School (Chicago, Il).

The rabbit polyclonal antibodies to IκBα, p50, p65, cyclin D1, Bcl 2, Bcl-x\(_L\), PARP and annexin V kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against cleaved-PARP, phospho-IκBα, procaspase –7 and procaspase-9 and the polynucleotide kinase kit were purchased from New England Biolabs, Inc. (Beverly, MA). Anti-IKKα and anti-IKKβ antibody were kindly provided by Imgenex (San Diego, CA). Goat anti-rabbit-HRP conjugate purchased from Bio-Rad Laboratories (Hercules, CA), goat anti-mouse-HRP was purchased from Transduction Laboratories (Lexington, KY) and goat anti-rabbit-Alexa 594 was purchased from Molecular Probes (Eugene, OR). Cell-permeable NEMO (NF-κB essential modifier; also called IKKγ)-binding domain peptide (NBD), NH\(_2\)-DRQIKIWFQNRRMKWKKTALDWSWLQTE-CONH\(_2\),
and control peptide NEMO-C, NH₂-DRQIKIWFQNRRMKWKK-CONH₂ were kind gifts from Imgenex (San Diego, CA). Curcumin, vincristine, melphalan, caspase inhibitors (Ac-DEVD-CHO or Ac-YVAD-CHO), Hoechst 33342 and MTT were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Curcumin was prepared as a 20 mM solution in dimethyl sulfoxide and then further diluted in cell culture medium. RPMI-1640, fetal bovine serum (FBS), 0.4% trypan blue vital stain and antibiotic-antimycotic mixture were obtained from Life Technologies Inc. (Grand Island, NY). Protein A/G-Sepharose beads were obtained from Pierce (Rockford, IL), γ-p³²-ATP was from ICN Pharmaceuticals (Costa Mesa, CA). A human IL-6 kit was purchased from BioSource International (Camarillo, CA). Apo Logix carboxyfluorescein caspase detection kit was a gift from Cell Technology (Minneapolis, MN).

**Cell culture:** All the human multiple myeloma cell lines were cultured in RPMI 1640 medium containing 1 X antibiotic-antimycotic. U26634, MM.135, and MM.1R36 were cultured in 10% FBS, whereas cell line RPMI 822637 was grown in 20% FBS. Occasionally cells were tested by Hoechst staining and by custom PCR for mycoplasma contamination.

**Preparation of nuclear extracts for NF-κB:** The nuclear extracts were prepared according to Schreiber et al.38. Briefly, 2x 10^6 cells were washed with cold PBS and suspended in 0.4 ml of hypotonic lysis buffer containing protease inhibitors for 30 min. The cells were then lysed with 12.5 μl of 10% Nonidet P-40. The homogenate was centrifuged, and supernatant containing the cytoplasmic extracts was stored frozen at -80°C. The nuclear pellet was resuspended in 25-μl ice-cold nuclear extraction buffer.
After 30 min of intermittent mixing, the extract was centrifuged, and supernatants containing nuclear extracts were secured. The protein content was measured by the Bradford method. If they were not used immediately, they were stored at –80 °C.

**Electrophoretic mobility shift assay for NF-κB:** NF-κB activation was analyzed by electrophoretic mobility gel shift assay (EMSA) as described previously. In brief, 8-µg nuclear extracts prepared from curcumin-treated or untreated cells were incubated with ³²P end-labeled 45-mer double-stranded NF-κB oligonucleotide from human immunodeficiency virus-1 long terminal repeat (5′-TTGTTACAAAGGACTTTCCGCTGGGGACTTTCCAGGGGCGTGG-3′) for 15 min at 37 °C, and the DNA-protein complex resolved in a 6.6 % native polyacrylamide gel. The radioactive bands from the dried gels were visualized and quantitated by the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

**Immunocytochemistry for NF-κB p65 localization:** Curcumin treated MM cells were plated on a glass slide by centrifugation using a Cytospin 4 (Thermoshendon, Pittsburg, PA), air-dried for 1 h at room temperature, and fixed with cold acetone. After a brief washing in PBS, slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-human p65 antibody (dilution, 1:100). After overnight incubation, the slides were washed and then incubated with goat anti-rabbit IgG-Alexa 594 (1:100) for 1 h and counter stained for nuclei with Hoechst (50 ng/ml) for 5 min. Stained slides were mounted with “Mounting medium” (Sigma Co.) and analyzed under an epifluorescence microscope (Labophot-2, Nikon, Tokyo, Japan). Pictures were captured using Photometrics Coolsnap CF color camera (Nikon,
Western blot: Thirty to fifty micrograms of cytoplasmic protein extracts, prepared as described, were resolved on 10% SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with antibodies against either I\(\kappa\)B\(\alpha\), phospho-I\(\kappa\)B\(\alpha\), Bcl-2, Bcl-x\(_L\), or cyclin D1 (1:3000) for 1 h. Thereafter, the blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally detected by chemiluminescence (ECL, Amersham Pharmacia Biotech. Arlington Heights, IL).

For detection of cleavage products of PARP, whole cell extracts were prepared by lysing the curcumin-treated cells in lysis buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, pH 8.0, 0.1% Triton -X100, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.4 mM PMSF, and 4 mM NaVO\(_4\)). Lysates were then spun at 14000 rpm for 10 min to remove insoluble material). Lysates were resolved on 7.5% gel and probed with PARP antibodies. PARP was cleaved from the 116-kDa intact protein into 85-kDa and 40-kDa peptide products. To detect cleavage products of procaspase 7 and procaspase 9, whole cell extracts were resolved on 10% gel and probed with appropriate antibodies.

I\(\kappa\)B kinase assay: The I\(\kappa\)B kinase assay was performed by a modified method as described earlier. Briefly, 200 \(\mu\)g cytoplasmic extracts were immunoprecipitated with 1 \(\mu\)g of anti-IKK\(\alpha\) and IKK\(\beta\) antibodies each, and the immune complexes so formed were precipitated with 0.01 ml of protein A/G-Sepharose beads for 2 h. The beads were washed first with lysis buffer and then with the kinase assay buffer (50 mM HEPES pH
7.4, 20 mM MgCl₂, and 2 mM DTT). The immune complex was then assayed for the kinase activity using kinase assay buffer containing 20 µCi [γ-³²P]ATP, 10 µM unlabeled ATP, and 2 µg/sample glutathione S-transferase- IκBα (1-54). After incubation at 30°C for 30 min, the reaction was stopped by boiling the solution in 6x SDS sample buffer. Then the reaction mixture was resolved on 12% SDS-PAGE. The radioactive bands of the dried gel were visualized and quantitated by PhosphorImager. To determine the total amount of IKK complex in each sample, 60 µg of the cytoplasmic protein was resolved on a 7.5% acrylamide gel and then electrotransferred to a nitrocellulose membrane; the membrane was blocked with 5% nonfat milk protein for 1 h and then incubated with either anti-IKKα or anti-IKKβ antibodies for 1 h. The membrane was then washed and treated with HRP-conjugated secondary anti-mouse IgG antibody and finally detected by chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL).

**MTT assay:** The antiproliferative effects of curcumin against different MM cell lines were determined by the MTT dye uptake method as described earlier. Briefly, the cells (5000/well) were incubated in triplicate in a 96-well plate in the presence or absence of indicated test samples in a final volume of 0.1 ml for 24 h at 37°C. Thereafter, 0.025 ml of MTT solution (5 mg/ml in PBS) was added to each well. After a 2 h incubation at 37 °C, 0.1 ml of the extraction buffer (20% SDS, 50% dimethylformamide) was added, incubation was continued for overnight at 37 °C, and then the OD at 590 nm was measured using a 96-well multiscanner autoreader (Dynatech MR 5000), with the extraction buffer as blank.
Percent cell viability = (OD of the experiment samples/ OD of the control) X 100.

**Thymidine incorporation assay:** The antiproliferative effects of curcumin were also monitored by the thymidine incorporation method. For this, 5000 cells in 100 µl medium were cultured in triplicate in 96-well plates in the presence or absence of curcumin for 24 h. Six hours before the completion of experiment, cells were pulsed with 0.5 µCi ³H-thymidine, and the uptake of ³H-thymidine was monitored using a Matrix-9600 β-counter (Packard Instruments, Downers Grove, IL).

**Flow cytometric analysis:** To determine the effect of curcumin on the cell cycle, MM cells were treated for different times, washed, and fixed with 70% ethanol. After incubation for overnight at –20°C, cells were washed with PBS prior to staining with propidium iodide (PI), and then suspended in staining buffer (PI, 10 µg/ml; Tween-20, 0.5%; RNase, 0.1%  in PBS). The cells were analyzed using a FACS Vantage flowcytometer that uses CellQuest acquisition and analysis programs (Becton Dickinson, San Jose, CA). Cells were gated to exclude cell debris, cell doublets and cell clumps.

To determine the apoptosis, curcumin-treated cells were washed in phosphate-buffered saline, resuspended in 100 µl binding buffer containing FITC conjugated annexin V and analyzed by flow cytometry. As curcumin also emits the fluorescence in the same range as FITC, unstained treated cells were also analyzed in parallel.

**Determination of IL-6 protein:** Cell free supernatants were collected from untreated or curcumin-treated cultures of multiple myeloma cells. One hundred micro liter aliquots were removed, and IL-6 contents were determined by ELISA kit (Biosource
International).
Results

Human MM U266, RPMI 8226, MM.1 and MM.1R cell lines used in our study are well-characterized. We used them to investigate the effect of curcumin on constitutively active NF-κB, NF-κB-regulated gene expression, cell proliferation, and apoptosis. The time and dose of curcumin used to downregulate NF-κB had no effect on the cell viability.

Curcumin suppresses constitutive NF-κB expressed by multiple myeloma cells: We first investigated the NF-κB status in four different MM cell lines by EMSA. The results shown in Fig. 1 indicate that all the four cell lines expressed constitutively active NF-κB, resolved as an upper and a lower bands. We then investigated the effect of curcumin on constitutively active NF-κB. We first examined the dose of curcumin required for complete suppression of NF-κB. For this all the MM cell lines were treated with different concentrations of curcumin for 4 h and then examined for NF-κB by EMSA. Densitometric analysis of the retarded radiolabeled probe showed a decrease in NF-κB DNA binding activity. These results showed that 50 μM curcumin was sufficient to fully suppress the constitutive NF-κB activation in U266 (Fig. 1A), MM.1 (Fig. 1B) and MM.1R (Fig. 1C) and RPMI 8226 (Fig. 1D). We then examined the minimum duration of exposure to curcumin required for suppression of NF-κB. For this cells were incubated with 50 μM curcumin for different times, the nuclear extracts were prepared and examined for NF-κB by EMSA. The results showed that curcumin downregulated constitutive NF-κB in all four cell lines but with different kinetics. Complete
downregulation of NF-κB occurred at 4 h in U266 (Fig. 1E), MM.1 (Fig. 1F) and MM.1R (Fig. 1G) cells, whereas it took 8 h to downregulate NF-κB in RPMI 8226 cells (Fig. 1H). Curcumin downregulated only the upper band and not lower band of NF-κB in most cases. In the case of RPMI 8226 cells, both bands were downregulated.
Since NF-κB is a family of proteins, various combinations of Rel/NF-κB protein can constitute an active NF-κB heterodimer that binds to a specific sequence in DNA. To show that the retarded band visualized by EMSA in MM cells was indeed NF-κB, we incubated nuclear extracts from MM cells with antibody to either the p50 (NF-κB1) or the p65 (RelA) subunit of NF-κB. Both shifted the band to a higher molecular mass (Fig. 1-I), thus suggesting that the major NF-κB band in MM cells consisted of p50 and p65 subunits. A nonspecific minor band was observed in some MM cell lines which was not supershifted by the antibody. Neither preimmune serum nor the irrelevant antibody as anti-cyclin D1 had any effect. Excess unlabeled NF-κB (100-fold), but not the mutated oligonucleotides, caused complete disappearance of the band.

When NF-κB is activated, the p65 subunit of the NF-κB containing transactivation domain is translocated to the nucleus. In the inactive state, the p65 subunit of NF-κB is retained in the cytoplasm. To confirm that curcumin suppresses nuclear retention of p65, we used immunocytochemistry. Curcumin-treated and untreated cells were cytospun on a glass slide, immunostained with antibody p65, and then visualized by the Alexa-594 conjugated second antibody as described in Methods. The results in Fig. 2 clearly demonstrate that curcumin prevented the translocation of the p65 subunit of NF-κB to the nucleus in all four MM cell lines. These cytological findings were consistent with the NF-κB inhibition observed by EMSA.
Curcumin inhibits IκBα phosphorylation and IκB kinase activity: The degradation of IκBα and subsequent release of NF-κB (p65:p50) requires prior phosphorylation at ser 32 and ser 36 residues. Therefore, in order to investigate whether the inhibitory effect of curcumin is mediated through the alteration of phosphorylation of IκBα, U266 cells were treated with curcumin and their protein extracts were checked for phospho-IκBα expression. Results in figure 3A show that untreated U266 cells constitutively expressed ser 32-phosphorylated IκBα. Upon curcumin treatment, the phosphorylated IκBα content decreased rapidly.
A. 

![Graph](Curcumin Time Course)

B. 

![Graph](Curcumin Concentration Course)

C. 

![Graph](Curcumin Concentration Effects)
Phosphorylation of IκBα is mediated through IKK\textsuperscript{45}. In vitro kinase assay using immunoprecipitated IKK from untreated U266 cells and the GST-IκBα as substrate showed constitutive IKK activity, whereas under similar conditions immunoprecipitated IKK from curcumin-treated cells showed a decreased kinase activity that corresponded to the duration of curcumin treatment (figure 3B; upper panel). However, immunoblotting analysis of the cell extracts of untreated and curcumin-treated cells showed no significant change in the protein levels of the IKK subunits IKKα and IKKβ, in treated cells (figure 3B; middle and lower panel).

IKK has been shown to be regulated by several upstream kinases\textsuperscript{44,45}. Whether curcumin inhibited IKK activity directly or suppressed the activation of IKK was investigated. To determine if curcumin acted as a direct inhibitor of IKK activity, the IKK was immunoprecipitated from untreated U266 cells and then treated with different concentrations of curcumin for 30 min. After the treatment, the samples were examined for IKK activity using GST-IκBα as a substrate. Results in figure 3C (upper panel) showed that curcumin inhibited the IKK activity directly in a dose-dependent manner. These results suggest that curcumin is a direct inhibitor of IKK. Since we did not use purified IKK, we can not completely rule out the possibility that curcumin suppressed an upstream kinase required for IKK activation.

**Curcumin downregulates the expression of NF-κB-regulated gene products:** Because IκBα, Bcl-2, Bcl-x\textsubscript{L}, and cyclin D1, all have been shown to be regulated by NF-κB\textsuperscript{17}, we examined the effect of curcumin on the expression of these gene products by
immunoblotting. As depicted in Fig. 4, all four gene products were expressed in U266 cells. The treatment of cells with curcumin downregulated the pools of IκBα (Fig. 4A), Bcl-2 (Fig. 4B), Bcl-xL (Fig. 4C) and cyclinD1 (Fig. 4E) proteins in a time-dependent manner, although the kinetics of suppression followed by each protein were different. Cyclin D1 showed the most abrupt and complete depletion within 4 h of curcumin. Bcl-2 also showed a complete decline but it achieved the lowest level by 8 h. On the other hand IκBα and Bcl-xL showed only a partial decline.
Interleukin-6 is another NF-κB-regulated gene\textsuperscript{17} and has been shown to serve as a growth factor for MM cells\textsuperscript{5-7}. As shown in Fig. 4E, U-266 cells produced a significant amount of IL-6 protein in a time-dependent manner whereas neither MM.1 nor RPMI 8226 produced any detectable amount of IL-6 as measured by the ELISA method.

Curcumin treatment inhibited the production of IL-6 by U266 cells.

**Curcumin suppresses the proliferation of MM cells:** Because NF-κB has been implicated in cell survival and proliferation\textsuperscript{13,14}, we examined the effect of curcumin on proliferation of MM cell lines. U266, RPMI 8226, MM.1, and MM.1R cells were cultured in the presence of different concentrations of curcumin, and the number of viable cells examined by trypan blue dye-exclusion method. Results in figure 5 show that curcumin at a concentration as low as 1 μM inhibited the growth by 27%, 23%, 45% and 51% in U266 (panel A), RPMI 8226 (panel B), MM.1 (panel C) and MM.1R (panel D), respectively. At 10 μM curcumin completely suppressed the growth in all cell lines. These results indicate that curcumin suppresses the proliferation of all MM cell lines tested, including MM.1R (the line resistant to dexamethasone-induced apoptosis).
We also examined the antiproliferative effects of curcumin by thymidine incorporation in U266 cells. Curcumin suppressed thymidine incorporation within 24 h in a dose-dependent manner (Fig. 6A). The MTT method (which indicates the mitochondrial activity of the cells) showed that curcumin suppressed the mitochondrial activity of U266 cells within 24 h and the suppression occurred in a dose-dependent manner (Fig. 6B).
Curcumin induces apoptosis in MM cells: Whether suppression of NF-κB in MM cells also leads to apoptosis, was investigated. The curcumin-induced apoptosis was examined by the annexin V method. The latter binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic feature of cells entering apoptosis. This allows for live cells (unstained with either fluorochrome) to be discriminated from apoptotic cells (stained only with annexin V)\textsuperscript{43}. To check this, U266 cells were treated for 24 h with different concentrations of curcumin and then stained with annexin V-FITC. Results in figure 6C show a dose-dependent increase in cells positive for annexin V indicating the onset of apoptosis in curcumin-treated cells.

Another hallmark of apoptosis is activation of caspases. To determine this, U266 cells were treated with curcumin for different times, and the whole cell extracts were prepared and analyzed for activation of caspase-9 (an upstream caspase), caspase-7 (a downstream caspase) and cleavage of PARP, a well-known substrate for caspase-3, -6 and -7\textsuperscript{46}. Immunoblot analysis of the extracts from cells treated with curcumin for different times clearly showed a time-dependent activation of caspase-9 (Fig. 7A), as indicated by the disappearance of 47-kDa band and an appearance of 37-kDa band. Similarly, the western blot analysis also showed an activation of caspase-7 (Fig. 7B), as indicated by the disappearance of the 35-kDa band and an appearance of 20-kDa band. Activation of downstream caspases led to the cleavage of a 118-kDa PARP protein into an 89-kDa fragment, another hallmark of cells undergoing apoptosis (figure 7C), whereas untreated cells did not show any PARP cleavage. Antibodies that recognize only the cleaved 89-kDa PARP species, increased with increase in duration of curcumin
treatment (Fig. 7C, lower panel). These results clearly suggest that curcumin induced apoptosis in MM cells.
To further demonstrate the activation of caspases by curcumin in situ, we also labeled the cells with a cell permeable carboxyfluorescein analog of zVAD-FMK (a general caspase inhibitor) that irreversibly binds to the activated caspases and gives a green fluorescence in the same range as FITC. Untreated cells did not show any fluorescence but curcumin-treated cells showed an intense fluorescence indicating the presence of active caspases in these cells (Fig. 7D).

To determine whether caspase activation is needed for curcumin-induced PARP cleavage, U266 cells were treated with curcumin in the presence of caspase inhibitors, Ac-DEVD-CHO (caspase 3 inhibitor) or Ac-YVAD-CHO (caspase 1 inhibitor) and analyzed for PARP cleavage. As shown in Fig. 7E, caspase-3 inhibitor suppressed the curcumin-induced PARP cleavage whereas caspase-1 inhibitor did not.

To further determine whether caspase activation is needed for the suppression of cell growth induced by curcumin, U266 cells were treated with caspase inhibitors, Ac-DEVD-CHO or Ac-YVAD-CHO and then examined for curcumin-induced cytotoxicity by the MTT method. Results shown in Fig. 7F demonstrate a dose-dependent protection of cells from curcumin-induced cytotoxicity by caspase 3 inhibitor but not by caspase 1 inhibitor. These results, thus suggest that caspase-3 activation is essential for curcumin-induced cytotoxicity.

**Curcumin arrests the cells at the G1/S phase of the cell cycle:** D-type cyclins are required for the progression of cells from the G1 phase of the cell cycle to S phase (DNA synthesis)\(^4\). Since we observed a rapid decline of cyclin D1 in curcumin-treated MM cells, we wished to determine the effect of curcumin on U266 cell cycle. Flow
cytometric analysis of the DNA from curcumin-treated cells showed a significant increase in the percentage of cells in the G1 phase, from 61% to 70%, and a decrease in the percentage of cells in the S phase, from 20% to 9%, within 24 h of curcumin (10 μM) treatment (Fig. 8). These results clearly show that curcumin induces G1/S arrest of the cells.
NEMO-binding domain (NBD) peptide suppresses constitutive NF-κB and proliferation of MM cells: IKK is composed of IKKα, IKKβ and IKKγ (also called NEMO). The amino-terminal α-helical region of NEMO has been shown to interact with the C-terminal segment of IKKα and IKKβ. A small peptide from the C-terminus of IKKα and IKKβ NEMO has been shown to block this interaction. To make it cell permeable, the NBD peptide was conjugated to a small sequence from the antennapedia homeodomain. This peptide has been shown to specifically suppress NF-κB activation. The peptide without the antennapedia homeodomain protein sequence, was used as a control.

Our results to this point have shown that curcumin suppressed constitutive NF-κB which in turn led to suppression of cell proliferation and induction of apoptosis. To establish that NF-κB suppression is linked to proliferation and apoptosis, we used the NBD and control peptide. As shown in Fig. 9A, treatment of U266 cells with NEMO-control peptide had no effect, whereas NBD peptide suppressed the constitutive NF-κB in a time-dependent manner with complete suppression occurring at 12 h. The suppression of NF-kB activation in MM cells was also confirmed independently by the immunocytochemistry. The results indicated a decrease in the nuclear pool of the p65 subunit of NF-κB (Fig. 9B). Suppression of NF-κB by NBD peptide also led to inhibition of cell proliferation of U266 cells. Approximately 32% suppression of cell growth was observed after NBD treatment for 24 h (Fig. 9C). These results thus suggest that NF-κB suppression is indeed linked to the antiproliferative effects in MM cells.
**Curcumin potentiates the cytotoxic effects of chemotherapeutic agents:** Because NF-κB has been implicated in chemoresistance of cells, we investigated the effects of curcumin on chemosensitivity. We investigated the effect of curcumin on the cytotoxic effects of vincristine and melphalan against various multiple myeloma cell lines. These two chemotherapeutic agents showed variable cytotoxic effects in different multiple myeloma cell lines when treated alone (Fig. 10). MM.1 cells were clearly most sensitive to both the drugs (Fig. 10C). The presence of curcumin enhanced the cytotoxic effects of both vincristine and melphalan against all the multiple myeloma cell lines. For instance, U266 cells were least sensitive to vincristine but presence of curcumin enhanced the cytotoxicity from less than 10% to greater than 70% (Fig. 10A). When compared with MM.1 cells, dexamthasone-resistant MM.1R cells were also found to be relatively resistant to both vincristine as well as melphalan. The treatment of these chemoresistant cells with curcumin enhanced the cytotoxic effects of both the chemotherapeutic agents (Fig. 10D). As a control, curcumin had minimal cytotoxic effect on normal human peripheral blood mononuclear cells under these conditions (data not shown). These results indicate that curcumin may sensitize the multiple myeloma cells to the cytotoxic effects of vincristine and melphalan.
Discussion

Because of the central role of nuclear factor NF-κB in cell survival and proliferation, we explored this transcription factor as a target for the treatment of MM by using curcumin (diferuloylmethane). Our results indicate that NF-κB is constitutively active in all human MM cell lines examined and that curcumin downregulated the nuclear pool, or active form of NF-κB and suppressed constitutive IκBα phosphorylation, IKK kinase activity, and expression of the NF-κB-regulated gene products IκBα, Bcl-2, Bcl-xL, cyclin D1, and interleukin-6. This led to the suppression of proliferation, arrest of cells at the G1/S phase boundary of the cell cycle, and induction of apoptosis as indicated by the activation of caspase-7 and caspase-9 and PARP cleavage. Curcumin also induced chemosensitivity to vincristine and melphalan.

Our results indicate that all four MM cell lines (U266, RPMI 8226, MM.1 and MM.1R) expressed constitutively active NF-κB. These results are in agreement with two recent reports by Feinman et al.⁴ and Ni et al.¹⁸ who showed constitutive NF-κB in U266 and RPMI 8226 cells by EMSA. We now show that MM.1 and MM.1R, a dexamethasone-resistant cell line, also express constitutive NF-κB. Our results differ from those of Hideshima et al., who showed lack of constitutively active NF-κB in MM.1S (same as MM.1)⁴⁸. Because the constitutive activation of NF-κB leads to nuclear translocation of p65, we confirmed the presence of nuclear p65 in all the cell lines by immunocytochemistry. Why do MM cells constitutively express NF-κB? Our results indicate that MM cells exhibit constitutively active IKK, the kinase required for NF-κB
activation. This is the first report to show an elevated IKK activity in MM cells. Why these cells express an elevated IKK is, however, not clear at present.

We found that curcumin suppressed constitutive NF-κB activation in all four MM cell lines. These results are in agreement with previous reports from our laboratory and others that curcumin is a potent inhibitor of NF-κB activation²⁰⁻²⁷. Curcumin inhibits NF-κB activation by blocking the constitutively active IKK present in MM cells. Because curcumin inhibited IKK activity both inside the cells and in vitro, we suggest that curcumin may be a direct inhibitor of IKK. Because we did not employ recombinant enzyme, we can not completely rule out the possibility of indirect inhibition of IKK by curcumin. In any case, curcumin appears to suppress IKK activation, which leads to inhibition of IκBα phosphorylation as reported here and thus abrogation of IκBα degradation. Our results are in agreement with previous reports which showed inhibition of IKK by curcumin in colon cancer cells and macrophages²²,²⁴. A recent report showed that PS-1145, a rationally designed IKK inhibitor, blocked TNF-induced NF-κB activation in MM.1 cells⁴⁸. Because MM.1 cells in our study constitutively expressed NF-κB, no TNF induction was required. The concentration of curcumin required to block IKK activity in the cells was comparable to that reported for PS-1145⁴⁸.

We found that suppression of NF-κB by curcumin downregulated the expression of several gene products regulated by NF-κB. The expression of IκBα, Bcl-2, Bcl-xL, IL-6, and cyclin D1¹⁴,¹⁷,⁴⁹, whose synthesis is known to be regulated by NF-κB, was suppressed by curcumin. We found that only U266 cells produced IL-6. Neither RPMI
8226 nor MM.1 produced any detectable IL-6. Previous reports on the production of IL-6 by these MM cell lines has been controversial (49-52).

The suppression of cell proliferation by curcumin in MM cells is in agreement with our previous reports that curcumin-induced suppression of NF-κB leads to inhibition of cellular proliferation of cutaneous T cell lymphoma and acute myelogenous leukemia. Our results on the antiproliferative effects of curcumin are in agreement with those of Hideshima et al., who showed that PS-1145, an IKK blocker, inhibits cell proliferation. These workers reported that 50 µM PS-1145 inhibits the proliferation of the MM cell lines MM.1S, RPMI-8226 and U266 by less than 50%. In contrast, we found almost complete inhibition of proliferation of all these cell lines with as little as 10 µM curcumin.

Several potential mechanisms could explain why NF-κB downregulation by curcumin leads to suppression of proliferation of MM cells. One of the potential mechanisms involves suppression of IL-6 production as shown in our studies. Numerous studies indicate that IL-6 is a potent growth factor for MM. Whether it is a paracrine or an autocrine growth factor for MM cells is highly controversial. In our studies it is unlikely, however, that curcumin suppressed the growth of MM cells through suppression of IL-6 production because three out of the four cell lines examined produced no detectable IL-6. It is also unlikely that curcumin inhibits cell growth through downregulation of the constitutively active Stat3 signaling because the proliferation of cells which do not express constitutively active Stat3 (e.g; RPMI 8226), are also inhibited by curcumin. In our study curcumin downregulated bcl-2 and bcl-xL.
expression, the proteins that have been implicated in the cell survival of MM cells. Thus it is possible that downregulation of bcl-2 and bcl-xL by curcumin could lead to suppression of cell proliferation.

We also found that MM cells overexpress cyclin D1, another NF-κB-regulated gene, and that this expression is downregulated by curcumin. The overexpression of cyclin D1 has been noted in a wide variety of tumors, but its role in MM cells has not been reported. Given that cyclin D1 is needed for cells to advance from the G1 to S phase of the cell cycle, it is not surprising we found that curcumin induced G1/S arrest and thus caused suppression of cell proliferation.

Suppression of NF-κB by curcumin also led to apoptosis of MM cells, as indicated by activation of caspases and cleavage of PARP. These results are in agreement with reports indicating that NF-κB mediates antiapoptotic effects. Downregulation of NF-κB also sensitized MM cells to vincristine and melphalan. Even the MM.1R cells, which have been shown to be resistant to dexamethasone, were sensitive to curcumin.

MM is an incurable aggressive B cell malignancy, and more than 90% of MM patients become chemoresistant. Several agents have been tested in the search for more effective treatment of MM. Besides curcumin, these include PS341 (a proteosome inhibitor) and thalidomide (an inhibitor of TNF production). The nonspecific drug-toxicity is one of the major problem in drug development. Numerous studies have shown that curcumin is pharmacologically safe. It was recently demonstrated in phase 1 clinical trials that humans can tolerate up to 8 grams of curcumin per day when taken
orally\textsuperscript{32}. Additionally, curcumin has been shown to downregulate the expression of ICAM-1, VCAM-1 and ELAM-1, all NF-κB-regulated gene products\textsuperscript{21} that have been implicated in activation of stromal cells by MM cells. TNF, another cytokine known to play a pathological role in MM\textsuperscript{9}, has also been shown to be downregulated by curcumin\textsuperscript{27}. The results presented here clearly demonstrate that curcumin can suppress NF-κB, IKK, bcl-2, bcl-x\textsubscript{L}, cyclin D1 and cell proliferation in MM cells. Our studies provide enough rationale for considering curcumin worthy of clinical trial in patients with multiple myeloma.
Legend to Figures

Fig. 1. Curcumin inhibits constitutive nuclear NF-κB in multiple myeloma cells. Dose responses of NF-κB to curcumin treatment in U266 (panel A), MM.1 (panel B), MM1R (panel C), RPMI 8226 (panel D) cells. Two million cells/ml were treated with the indicated concentration of curcumin for 4 h and tested for nuclear NF-κB by EMSA as described. Effect of exposure duration on curcumin-induced NF-κB suppression in U266 (panel E), MM.1 (panel F), MM.1R (panel F), RPMI 8226 (panel G) cells. Cells were treated with curcumin (50 µM) for the indicated times and tested for nuclear NF-κB by EMSA as described. The binding of NF-κB to the DNA is specific and consists of p50 and p65 subunits (panel I). Nuclear extracts were prepared from U266 cells (2x10^6/ml), incubated for 30 min with different antibodies or unlabeled NF-κB oligonucleotide probe, and then assayed for NF-κB by EMSA.

Fig. 2. Curcumin induces redistribution of p65. U266 and RPMI 8226 cells were incubated alone or with curcumin (50 µM) for 4 h and then analyzed for the distribution of p65 by immunocytochemistry. Red stain indicates the localization of p65 and blue stain indicates nucleus (magnification 200X).

Fig. 3. Curcumin inhibits IκBα phosphorylation and IκB kinase. For panel A and B, five million U266 cells/2.5 ml were treated with curcumin (50 µM) for indicated times.
and cytoplasmic extracts were prepared to check; A, the level of phosphorylated IκBα by Western blotting; or B, Immunoprecipitated IKK and performed the kinase assay to check the IKK activity (upper panel) or performed Western blotting for the analysis if total IKKα and IKKβ proteins in cytoplasmic extracts. C, Five millions were taken, Cytoplasmic extracts were prepared from 5 x 10⁶ U266 cells, IKK was immunoprecipitated and kinase assay was performed in the absence or presence of the indicated concentration of curcumin (upper panel). Lower panel indicates the amount of GST-κBα protein stained with Coomassie Blue in each well in the same dried gel.

**Fig. 4. Effect of curcumin on NF-κB regulated gene products.** Two million U266 cells were treated with curcumin (50 μM) for indicated times, and cytoplasmic extracts were prepared. Sixty micrograms of cytoplasmic extracts were resolved on 10% SDS-PAGE gel, electrotransferred on a nitrocellulose membrane, and probed for the following: A, IκBα; B, Bcl-2; C, Bcl-xL and D, cyclin D1. The same blots were stripped and reprobed with anti-β-actin antibody to show equal protein loading (lower panel in each figure). E. Curcumin downregulates IL-6 production. U266, MM.1 or RPMI 8226 cells (2 x 10⁶ cells/ml) were treated with curcumin (10 μM), supernatants were harvested after 24 h and levels of IL-6 assayed by IL-6 ELISA-kit as described in Methods. Values are mean IL-6 levels (± s. d.) obtained from three independent treatment of cell with curcumin.

**Fig. 5. Curcumin inhibits the growth of human multiple myeloma cells.** A, U266; B, RPMI 8226 cells; C, MM.1; or D. MM.1R (5000 cells/0.1 ml) were incubated at 37°C
with curcumin (1 µM and 10 µM) for indicated time duration and the viable cells were counted using standard trypan blue dye exclusion test. The results are shown as the mean (± s. d.) cell count from triplicate cultures.

**Fig. 6. Curcumin inhibits the growth of human multiple myeloma cells and induces apoptosis.** A, U266 cells (5000 cells/0.1 ml) were incubated with different concentrations of curcumin for 24 h, and cell proliferation assay was performed as described in Materials and Methods. Results are shown as mean (± s.d.) of percent [³H]-thymidine incorporation of triplicate cultures compared to the untreated control. B, U266 cells (5000 cells/0.1 ml) were incubated with different concentrations of curcumin for 24 h, and cell viability was determined by MTT method, as described in Methods. The results are shown as the mean (± s. d.) percent viability from triplicate cultures. C, Flow cytometric analysis of annexin V-FITC stained cells after treatment with different concentrations of curcumin. U266 cells were incubated alone or with indicated concentrations of curcumin for 24 h, thereafter either cells were left unstained (left panel) or stained with annexin V-FITC (right panel). Unstained cells exhibited autofluorescence due to curcumin.

**Fig. 7. Curcumin-induced apoptosis of human multiple myeloma cells is mediated through caspase activation.** U266 cells (2 x 10⁶ cells/ml) were incubated in the absence or in presence of curcumin (50 µM) for indicated times. The cells were washed and total proteins were extracted by lysing the cells. Sixty microgram of extracts were resolved on 10% SDS-PAGE gel, electrotransferred to a nitrocellulose membrane, and probed
with A, anti-caspase-9, B, anti-caspase-7; and, C, anti-PARP (upper panel) and anti-cleaved PARP (lower panel) antibodies as described in Methods. D, Detection of caspase activation by fluorescence microscopy. Untreated or curcumin-treated U266 cells (12 h) were examined for caspase activation by Apo Logix carboxyfluorescein caspase detection kit. Cells were analyzed under light microscopy (LM) and by fluorescence microscopy (FM). Green fluorescence indicates activated caspases. E, Suppression of curcumin-induced PARP cleavage by caspase-3 inhibitor. U266 cells (2 x 10^6 cells/ml) were preincubated with caspase inhibitors, Ac-DEVD-CHO (10 µM) or Ac-YVAD-CHO (10 µM) for 2 h and then treated with curcumin (50 µM) for 24 h. Thereafter, cell extracts were prepared and analyzed for PARP cleavage by using either anti-PARP antibody (upper panel) or antibodies which recognize only cleaved PARP (lower panel) as described in Materials and Methods. F, Caspase-3 inhibitor protects cells from curcumin-induced cytotoxicity. U266 cells (5000 cells/0.1 ml) were incubated with different concentrations of caspase inhibitors, Ac-DEVD-CHO or Ac-YVAD-CHO, for 2 h and then treated with curcumin. After 24 h, cell viability was determined by MTT method, as described in Methods. The results are shown as the mean (± s.d.) percent viability from triplicate cultures.

Fig. 8. Curcumin arrests the cells at G1/S phase of the cell cycle. U266 cells (2 x 10^6 cells/ml) were incubated in the absence or in presence of curcumin (10 µM) for the indicated times. Thereafter, the cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flowcytometry as described in Methods.

Fig. 9. NEMO-binding domain (NBD) peptide inhibits constitutive NF-κB and
induces cytotoxicity in human multiple myeloma cells. A. U266 cells (2 x 10^6 c/ml) were treated with indicated concentrations of NEM-O-control or NBD-peptide (100 µM) for indicated times. A. Nuclear extracts were checked for the presence of NF-κB DNA-binding activity by EMSA. B. Untreated or NBD-peptide-treated (100 µM; 12 h) U266 cells were cytospun, and p65 immunocytochemistry was performed as described in Methods. Red stain indicates the localization of p65 and blue stain indicates nucleus (magnification 200X). C. U266 cells (2 x 10^6 cells/ml) were treated with indicated concentrations of NEMO-control or NBD-peptide (100 µM) for indicated time periods, and cell viability was monitored by the trypan blue dye exclusion method. Percent cell killing was determined as follows: Percent killing = (number of trypan blue stained cells/total cells) x 100.

Fig. 10. Curcumin potentiates the cytotoxic effect of vincristine and melphalan in human multiple myeloma cells. Multiple myeloma cells (10000 cells/0.1 ml), U266 (panel A), RPMI 8226 (panel B), MM.1 (panel C), and MM.1R (panel D) were incubated with either medium, vincristine (50 µM) or melphalan (10 µM) in the absence (black bars) or presence of curcumin (10 µM; hatched bars) for 24 h, and then the cell viability was determined by the MTT method as described. Values are mean (± s. d.) of triplicate cultures.
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Curcumin (Diferuloylmethane) downregulates the constitutive activation of nuclear factor κB and IκBα kinase in human multiple myeloma cells leading to suppression of proliferation and induction of apoptosis

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