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Curcumin (diferuloylmethane) inhibits constitutive NF-κB activation, induces G1/S arrest, suppresses proliferation, and induces apoptosis in mantle cell lymphoma☆

Shishir Shishodia [a], Hesham M. Amin [b], Raymond Lai [b,1], Bharat B. Aggarwal [a,*]

[a] *Cytokine Research Section, Department of Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA*
[b] *Department of Hematopathology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA*

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Abstract

Human mantle cell lymphoma (MCL), an aggressive B cell non-Hodgkin's lymphoma, is characterized by the overexpression of cyclin D1 which plays an essential role in the survival and proliferation of MCL. Because of MCL's resistance to current chemotherapy, novel approaches are needed. Since MCL cells are known to overexpress NF-κB regulated gene products (including cyclin D1), we used curcumin, a pharmacologically safe agent, to target NF-κB in a variety of MCL cell lines. All four MCL cell lines examined had overexpression of cyclin D1, constitutive active NF-κB and IκB kinase and phosphorylated forms of IκBα and p65. This correlated with expression of TNF, IκBα, Bcl-2, Bcl-xl, COX2 and IL-6, all regulated by NF-κB. On treatment of cells with curcumin, however, downregulated constitutive active NF-κB and inhibited the consitutively active IκBα kinase (IKK), and phosphorylation of IκBα and p65. Curcumin also inhibited constitutive activation of Akt, needed for IKK activation. Consequently, the expression of all NF-κB-regulated gene products, were downregulated by the polyphenol leading to the suppression of proliferation, cell cycle arrest at the G1/S phase of the cell cycle and induction of apoptosis as indicated by caspase activation, PARP cleavage, and annexin V staining. That NF-κB activation is directly linked to the proliferation of cells, is also indicated by the observation that peptide derived from the IKK/NEMO-binding domain and p65 suppressed the constitutive active NF-κB complex and inhibited the proliferation of MCL cells. Constitutive NF-κB activation was found to be due to TNF, as anti-TNF antibodies inhibited both NF-κB activation and proliferation of cells. Overall, our results indicate that curcumin inhibits the constitutive NF-κB and IKK leading to suppression of expression of NF-κB-regulated gene products that results in the suppression of proliferation, cell cycle arrest, and induction of apoptosis in MCL.

Keywords: MCL; NF-κB; IκBα; IKK; Curcumin

1. Introduction

Mantle cell lymphoma (MCL) is a type of B cell non-Hodgkin lymphoma (NHL) that accounts for 3–10% of all NHL in Western countries [1] and leads to higher fraction of deaths, given that it is an incurable malignancy [2]. MCL patients are most often elderly men who present with advance stage of disease, and most often with extranodal involvement [3]. The length of survival of MCL patients following diagnosis is quite variable with median survival of around 3 years. Depending upon the severity, it may vary between 1 and 10 years.

There is no clear standard approach for treating mantle cell lymphoma. Chemotherapy with chlorambucil [4], CVP

Abbreviations: EMSA, electrophoretic mobility shift assay; IKK, IκB kinase; FBS, fetal bovine serum; IκBα, inhibitory subunit of NF-κB; MCL, mantle cell lymphoma; NF-κB, nuclear transcription factor-κB; NEMO, NF-κB essential modifier; NBD, NEMO-binding domain peptide; PI, propidium iodide; PIS, pre-immune serum; HRP, horse radish peroxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PMSF, phenylmethylsulfonyl fluoride; EBV, Epstein-Barr virus; PTD, protein transduction domain

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* Corresponding author. Tel.: +1 713 792 3503; fax: +1 713 794 1613.
E-mail address: aggarwal@mdanderson.org (B.B. Aggarwal).
[1] Present address: Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alta., Canada.

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(cyclophosphamide, vincristine, and prednisone), or CHOP (cyclophosphamide, hydroxydoxorubicin, oncovin, and prednisone) [5] can be used in patients who are not candidates for aggressive therapy. Allogeneic transplant is promising for young patients with matched donors [6]. High-dose therapy with autologous stem cell transplantation may extend the time to progression and the duration of overall survival. Other agents, including rituximab [7], fludarabine [8], and cladribine [9] have demonstrated activity, but these agents do not appear to offer survival advantages over combination chemotherapy. Despite these treatment options, this malignancy remains incurable.

MCL is characterized cytogenetically by the presence of a non-random chromosomal abnormality, the t(11;14)(q13;q32) chromosomal translocation, as a result of which the cyclin D1 gene is brought under the control of the immunoglobulin heavy chain gene enhancer, leading to overexpression of cyclin D1 [10–12]. The latter is a hallmark of this disease and is believed to contribute to deregulated cellular proliferation in MCL [2]. Additionally, the anti-apoptotic protein Bcl2 is also overexpressed in MCL [13]. Recent gene profiling studies have shown that the genes involved in TNF and NF-κB signaling pathways are overexpressed in MCL [14]. Both cyclin D1 and Bcl-2 are regulated by NF-κB [15,16].

NF-κB is a transcription factor present in the cytoplasm as an inactive heterotrimer consisting of p50, p65, and IκBα subunits. On activation, IκBα undergoes phosphorylation and ubiquitination-dependent degradation leading to nuclear translocation and binding to a specific consensus sequence in the DNA which results in gene transcription [17]. The kinase which phosphorylates IκBα is termed IκB kinase (IKK) composed of IKKα, IKKβ and IKKγ (also called NEMO [18]). NF-κB regulates the expression of genes involved in antiapoptosis (e.g. *bcl-2* and *bcl-xl*); proliferation (COX2 and cyclin D1) and metastasis (e.g., MMP-9).

Curcumin, a diferuloylmethane derived from turmeric (*Curcuma longa*) is a pharmacologically safe agent that has been shown to suppress NF-κB activation and NF-κB gene products [19,20]. In the current report, we targeted NF-κB pathway in MCL cells by using curcumin. We found that all four MCL cell lines expressed constitutively active NF-κB and NF-κB-regulated gene products (Bcl-2, Bcl-XL, cyclin D1, COX2, TNF, IL-6, RANK, and RANKL); and treatment with curcumin suppressed NF-κB activation and downregulated the expression of these gene products leading to cell cycle arrest, suppression of proliferation and induction of apoptosis.

2. Materials and methods

2.1. Materials

The four MCL cell lines included in this study were JeKo-1, Mino, SP-53, and Granta 519. JeKo-1 [21] was kindly provided by T. Akagi (Chosun University Medical School, Kwangju, Korea). The cell line Mino was established and characterized at The University of Texas M. D. Anderson Cancer Center by Dr. Raymond Lai [22]. SP-53 [23] was a generous gift from M. Daibata (Kochi Medical School, Kochi, Japan). The cell line Granta 519 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). Granta 519 was established using Epstein-Barr virus (EBV), whereas other three cell lines were EBV-negative.

The rabbit polyclonal antibodies to IκBα, p50, p65, cyclin D1, Bcl-2, Bcl-xL, and PARP and the 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 Bio Labs, Inc. (Beverly, MA). Phospho specific Akt antibody was purchased from Cell Signaling (Beverly, MA). TNF-A5 purified mouse anti-human TNFα monoclonal antibody was purchased from BD Pharmingen. Anti-IKKα and anti-IKKβ antibody were kindly provided by Imgenex (San Diego, CA). Goat anti-rabbit-horseradish peroxidase (HRP) conjugate was 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 (NBD) peptide, NH2-DRQIKIWFQNRRMKWKKTALDWSW-LQTE-CONH2, PTD-p65-P1 peptide (amino acid residues 271-282 of p65 linked with a peptide transduction domain (PTD) derived from the third helix sequence of antennapedia), and the control peptide NEMO-C, NH2-DRQIKIWFQNRRMKWKK-CONH2 were kind gifts from Imgenex (San Diego, CA). Hoechst 33342 and MTT were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Curcumin with a purity of greater than 98% was purchased from LKT laboratories (Minneapolis, MN) and prepared as a 20 mM solution in dimethyl sulfoxide and then further diluted in cell culture medium. RPMI-1640, DMEM, 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-P^{32}-ATP was purchased from ICN Pharmaceuticals (Costa Mesa, CA).

2.2. Cell culture

All the human MCL cell lines except Granta 519 were cultured in RPMI 1640 medium containing 10% FBS and 1× antibiotic–antimycotic. Granta 519 was cultured in DMEM supplemented with 10% FBS.

2.3. Electrophoretic mobility shift assay for NF-κB

To determine NF-κB (which has a well-established role in inflammation, tumor proliferation, promotion, invasion and metastasis), we carried out EMSA essentially as previously described [24].

2.4. Immunocytochemistry for NF-κB p65 localization

Curcumin-treated MCL 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. The p65 was examined by an immunocytochemical method using an epifluorescence microscope (Labophot-2; Nikon, Tokyo, Japan) and a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX) as described previously [25].

2.5. Western blot analysis

Thirty to fifty micrograms of cytoplasmic protein extracts, prepared as described [24], were resolved on 10% SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% non-fat milk, and probed with antibodies against either IκBα, phospho-IκBα, Bcl-2, Bcl-xL, p65, phosphorylated p65, COX2, MMP-9, or cyclin D1 according to manufacturer's protocol. Thereafter, the blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally detected by ECL chemiluminescence reagents (Amersham Pharmacia Biotech, Arlington Heights, IL). For detection of caspases and cleavage products of PARP, whole-cell extracts were prepared by lysing the curcumin-treated cells and Western blot was performed as described previously [26].

2.6. IκB kinase assay

The IκB kinase assay was performed by a modified method as described earlier [27]. Briefly, IKK complex was precipitated from whole-cell extracts with antibody to IKKα and IKKβ followed by treatment with 20 μl of protein A/G-sepharose (Pierce, Rockford, IL). After 2 h, the beads were washed with lysis buffer and then assayed in kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 2 mM DTT, 20 μCi [γ-^{32}P] ATP, 10 μM unlabeled ATP, and 2 μg of substrate GST-IκBα (1–54). After incubation at 30 °C for 30 min, the reaction was terminated by boiling with 5 μl of 5× SDS sample buffer for 5 min. Finally, the protein was resolved on 10% polyacrylamide gel under reducing conditions, the gel was dried, and the radioactive bands were visualized using a PhosphorImager. To determine the total amounts of IKKα and IKKβ in each sample, 30 μg of the whole-cell extract protein was resolved on a 7.5% acrylamide gel and then electrotransferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk protein for 1 h and then incubated with either anti-IKKα or anti-IKKβ (1:1000 dilution) for 1 h. The membrane was then washed and treated with horseradish peroxidase-conjugated secondary anti-mouse IgG antibody and proteins were detected by chemiluminescence (Amersham).

2.7. MTT assay

The antiproliferative effects of curcumin against different MCL cell lines were determined by the MTT dye uptake method as described earlier [28].

2.8. Thymidine incorporation assay

To determine the cell proliferation, 5000 cells in 0.1 ml medium were cultured in triplicate in 96-well plates in the presence or absence of anti-TNF antibody (50 ng/ml) for indicated time points. Cells were pulsed with 0.5 μCi (0.0185 MBq) ^{3}H-thymidine 6 h before harvesting, and the uptake of ^{3}H-thymidine was monitored by means of a Matrix-9600-counter (Packard Instruments, Downers Grove, IL).

2.9. Flow cytometric analysis

To determine the effect of curcumin on the cell cycle, MCL cells were treated for different times, washed, and fixed with 70% ethanol. After incubation overnight at −20 °C, cells were washed with PBS, and then suspended in staining buffer (Propidium iodide, 10 μg/ml; Tween-20, 0.5%; RNase, 0.1% in PBS). The cells were analyzed using a FACS Vantage flow cytometer that uses CellQuest acquisition and analysis programs (Becton Dickinson, San Jose, CA). Gating was set to exclude cell debris, cell doublets, and cell clumps. To determine 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.

2.10. RNA analysis and RT-PCR

MCL cells were left untreated or treated with 50 μM curcumin for various times, washed, and suspended in Trizol reagent. Total RNA was extracted according to the manufacturer's instructions (Invitrogen, Life Technologies, Grand Island, NY). Two micrograms of total RNA was converted to cDNA by Superscript reverse transcriptase and then amplified by Platinum Taq polymerase using Superscript One Step RT-PCR kit (Invitrogen). The relative expression of TNF, IL-6, RANK and RANKL was analyzed using quantitative RT-PCR with β-actin as an internal control.

The RT-PCR reaction mixture contained 25 μl of 2× reaction buffer, 2 μg of total RNA and 0.2 μM each of sense and anti-sense primers and 2 units of RT-Platinum Taq in a final volume of 50 μl. The primer sequences for TNF were sense: 5′ACAAGCCTGTAGCCCATGTT 3′; anti-sense: 5′AAAGATGACCTGCCCAGACT 3′; IL-6, sense: 5′GTCTCCTCATTGAATCCAGATTGG3′; anti-sense: 5′AGCTCAGCTATGAACTCCTTCTC3′; RANK, sense: 5′ GGGAAAGCACTCACAGCTAATTTG 3′; anti-sense: 5′ CAGCTTTCTGAACCCACTGTG 3′; and RANKL, sense: 5′ CGTTGGATCACAGCACATCAG 3′; anti-sense: 5′ AGTATGTTGCATCCTGATCCG 3′. For β-actin the primer sequences were as follows: sense 5′GGGTCAGAAGGATTCCTATG3′ and anti-sense 5′ GGTCTCAAACAT GATCTGGG 3′. The reaction was performed at 50 °C for 30 min, 94 °C for 2 min, 94 °C for 35 cycles of 15 s each, 60 °C for 30 s, and 72 °C for 1 min with extension at 72 °C for 10 min. PCR products were run on 2% agarose gel and then stained with ethidium bromide. Stained bands were visualized under UV light and photographed.

2.11. Live and dead assay

To measure apoptosis, we used the *Live and Dead assay* (Molecular Probes), which determines intracellular esterase activity and plasma membrane integrity. This assay employs calcein, a polyanionic dye, which is retained within the live cells and provides green fluorescence. It also employs the ethidium monomer dye (red fluorescence), which can enter the cells only through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membrane of live cells. Briefly, 1×10^5 cells are incubated with 150 μM each of p65 free peptide, only antennapedia domain or p65 inhibitory peptide for 12 h at 37 °C. Cells were stained with the *Live and Dead reagent* (5 μM ethidium homodimer, 5 μM calcein-AM) and then incubated at 37 °C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2).

3. Results

The aim of this study was to investigate the effect of curcumin on the proliferation and survival of MCL. Curcumin was selected because it is a pharmacologically safe agent that has been shown to downregulate cyclin D1 expression (for references see [20]). Four MCL cell lines that have been previously characterized by us were used in the present study [29]. As the MCL cells are characterized by overexpression of cyclin D1, so we also examined the expression of cyclin D1 in MCL cells. All four MCL cell lines showed constitutive expression of cyclin D1 protein (Fig. 1A). The time and dose of curcumin used to downregulate NF-κB had no effect on the viability of these cells.

3.1. MCL cell lines express constitutive active NF-κB

We first examined the level of NF-κB in all the four MCL cell lines. EMSA indicated that all the four cell lines examined expressed constitutively active NF-κB. In comparison, chronic myeloid leukemia (KBM-5) cells showed NF-κB only upon TNF treatment and multiple myeloma (U266) cells, like MCL, had constitutively active NF-κB (Fig. 1B). The level of NF-κB expression was lowest in Granta 519 cells. Since various combinations of Rel/NF-κB protein can constitute an active NF-κB heterodimer that binds to a specific sequence in DNA [30], we incubated nuclear extracts from MCL 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. 1C), thus suggesting that the major NF-κB band in MCL cells consisted of p50 and p65 subunits. Neither pre-immune serum nor the irrelevant antibody as anti-cyclin D1 had any effect. Excess unlabeled NF-κB (100-fold), but not the mutated oligonucleotides, caused the band to disappear completely.

3.2. MCL cell lines express constitutive active IκB kinase

We next examined whether IKK was constitutive active in the MCL cell lines. IKK has been implicated in the phosphorylation of IκBα and of p65, and is required for the activation of NF-κB. The results demonstrate that IKK was constitutive active in all the four MCL cell lines examined (Fig. 1D). Whether IκBα and p65 were constitutively phosphorylated was also examined. It was found that all the four MCL cell lines examined had constitutively phosphorylated IκBα (Fig. 1E) and p65 (Fig. 1F).

3.3. MCL cell lines express NF-κB regulated gene products

As NF-κB is known to regulate the expression of a number of genes involved in cell survival, we examined the expression of IκBα, Bcl-2, Bcl-xL, COX2 and cyclin D1 in all the four MCL cell lines. As shown in Fig. 1G, all of these gene products were expressed constitutively in MCL cells. The expression of IL-6, RANK, and RANKL, all regulated by NF-κB, was also examined. All 4 MCL cell lines expressed the mRNA for IL-6; however, IL-6 was very low in SP-53 and Mino cell lines. RANK mRNA was expressed in SP 53 and Granta 519 cells, whereas RANKL was expressed only in Granta 519 cell lines (Fig. 1H).

3.4. Curcumin inhibits constitutive NF-κB activity

We next examined the effect of curcumin on constitutive NF-κB activation in MCL cell lines. To determine the dose of curcumin required for complete suppression of NF-κB,

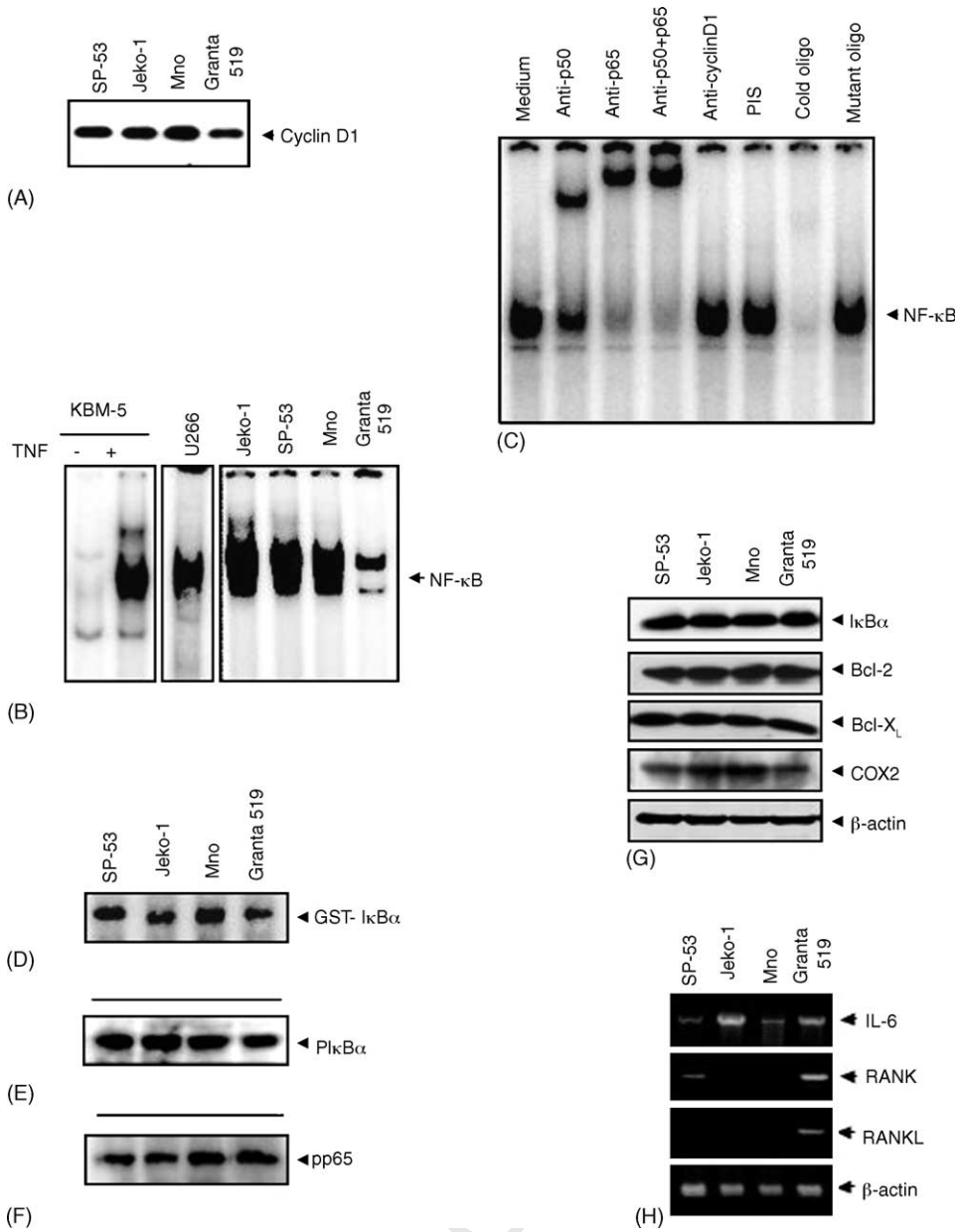

Fig. 1. NF-κB and cyclin D1 is constitutively active in MCL. Two million cells/ml were tested (A) for cyclin D1 by Western blot and (B) for nuclear NF-κB by EMSA. Untreated and TNF-treated KBM-5 cells and multiple myeloma (U266) cells were used as a negative and positive control. (C) The binding of NF-κB to the DNA is specific and consists of p50 and p65 subunits. Nuclear extracts were prepared from SP-53 cells (2×10^6 ml^{-1}), incubated for 30 min with different antibodies or unlabeled NF-κB oligonucleotide probe, and then assayed for NF-κB by EMSA. NF-κB-regulated genes are overexpressed in MCL. Two million cells/ml were tested (D) for IKK by immunecomplex kinase assay, (E) for phosphorylated IκBα by Western blot, (F) for phosphorylated p65 by Western blot, (G) for IκBα, Bcl-2, Bcl-XL and COX2 by Western blot, and (H) for IL-6, RANK and RANKL by RT-PCR. β-actin was used as a loading control.

all the MCL cell lines were treated with various concentrations of curcumin for 3 h and then examined for NF-κB by EMSA. A dose of 50 μM curcumin was sufficient to fully suppress the constitutive NF-κB activation in SP-53, Jeko-1 and Mino MCLs, and 100 μM was sufficient in the Granta 519 cell line (Fig. 2A). An EMSA examination of the kinetics of curcumin-induced NF-κB downregulation showed that downregulation was complete at less than 4 h in SP-53 and in less than 2 h in Jeko-1 and Mino cells, whereas it took 8 h to downregulate NF-κB in Granta 519 cells (Fig. 2B).

3.5. Curcumin inhibits the phosphorylation of IκBα and IκB kinase activity

As all the four MCL cell lines demonstrated constitutively phosphorylated IκBα, so we next determined whether curcumin affected phosphorylation of IκBα [31]. It was found that curcumin inhibited the phosphorylation of IκBα in SP-53 cells (Fig. 3A). Because IKK is implicated for the phosphorylation of IκBα [31], we performed an in vitro kinase assay of immunoprecipitated IKK from treated and untreated SP-53 cells. Curcumin

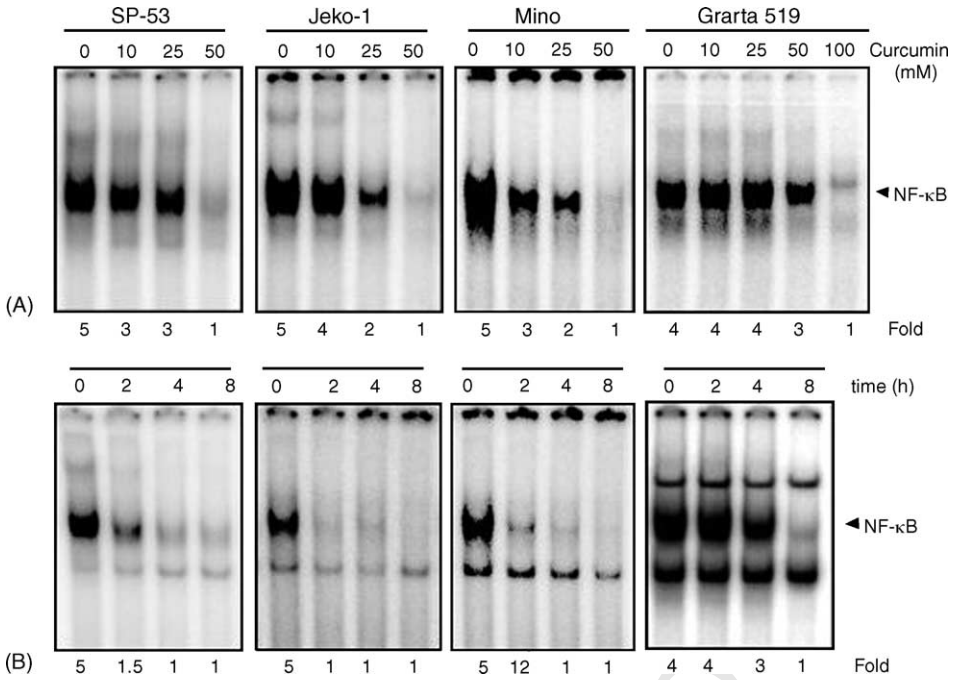

Fig. 2. Curcumin inhibits constitutive nuclear NF-κB in MCL. (A) Dose responses of NF-κB to curcumin treatment in MCL cells. Two million cells/ml were treated with the indicated concentration of curcumin for 3 h and tested for nuclear NF-κB by EMSA as described in Section 2. (B) Time course of curcumin-induced NF-κB suppression in MCL cells. Cells were treated with curcumin (50 μM) for the indicated times and tested for nuclear NF-κB by EMSA as described in Section 2.

inhibited the constitutive IKK activity in a time-dependent manner with complete inhibition occurring at 2 h (Fig. 3B; upper panel). Immunoblot analysis of the cell extracts of untreated and curcumin-treated cells showed no significant difference in the protein levels of the IKK subunits, IKKα and IKKβ (Fig. 3B; middle and lower panel).

Akt has been linked to the activation of IKK and NF-κB activation [32]. Whether curcumin inhibits IKK activation through inhibition of Akt activation was examined. We found that Akt was constitutively active in MCL cells and curcumin inhibited the Akt activation in a time-dependent manner (Fig. 3C).

3.6. Curcumin inhibits phosphorylation and nuclear translocation of p65

Because p65 was constitutively phosphorylated in all the four MCL cell lines, we also examined the effect of curcumin treatment on the phosphorylation of p65. Results in Fig. 3D showed that curcumin suppressed p65 phosphorylation in a time-dependent manner.

The effect of curcumin on the nuclear-retention of p65 was also examined independently by immunocytochemistry in curcumin-treated and untreated SP-53 MCL cells. The results demonstrate that curcumin prevented the nuclear-retention of p65 in SP-53 cell lines (Fig. 3E). These results were consistent with the curcumin-induced NF-κB inhibition observed by EMSA.

3.7. Curcumin downregulates the expression of NF-κB-regulated gene products

Because IκBα, Bcl-2, Bcl-xL, COX2 and cyclin D1 have all been shown to be overexpressed in MCL, we examined the effect of curcumin on the expression of these gene products by Western blotting. The treatment of SP-53 MCL cells with curcumin downregulated the expression of all these proteins in a time-dependent manner. IκBα, Cyclin D1 and Bcl-XL required 8 h of curcumin treatment for their suppression, whereas Bcl-2 was suppressed at 4 h. The suppression of COX2 by curcumin started as early as 8 h and was completely suppressed at 24 h (Fig. 3F). NF-κB upregulates the expression of a number of genes implicated in facilitating tumor cell survival, including cIAP1, xIAP, TRAF1, survivin. We found that all these proteins were constitutively expressed in MCL cells and curcumin downregulated the expression of all these proteins (Fig. 3G).

Because interleukin-6 (IL-6), RANK, and RANKL are also overexpressed in MCL, we also examined the effect of curcumin on their mRNA expression by RT-PCR. As shown in Fig. 1H, all four MCL cell lines expressed the mRNA for IL-6; however, IL-6 was very low in SP-53 and Mino cell lines. RANK mRNA was expressed in SP 53 and Granta 519 cells, whereas RANKL was expressed only in Granta 519 cell lines. Because Granta 519 cells expressed IL-6, RANK and RANKL mRNA, we examined the effect of curcumin on their expression in Granta 519 cells. Curcumin treatment inhibited the expression of all cytokines (Fig. 3H).

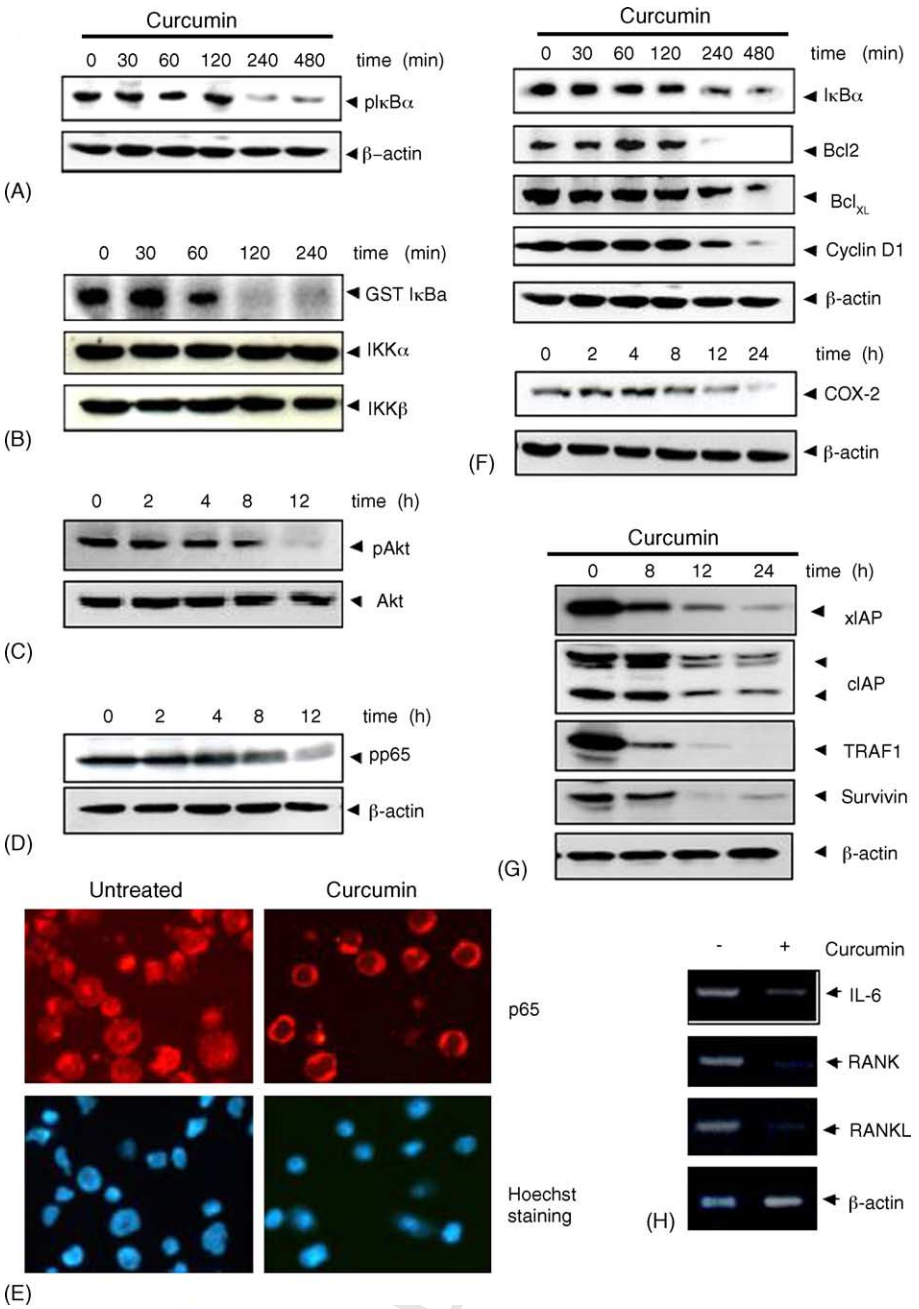

Fig. 3. Curcumin inhibits IκBα phosphorylation, IκB kinase, p65-phosphorylation, and induces redistribution of p65. (A) Two million SP-53 MCL cells were treated with curcumin (50 μM) for indicated times, and cytoplasmic extracts were prepared and examined for the level of phosphorylated IκBα by Western blotting. (B) Five million SP-53 MCL cells were treated with curcumin (50 μM) for indicated times, and whole-cell extracts were prepared and immunoprecipitated with IKK antibodies and examined by the immunecomplex kinase assay for IKK activity (upper panel) or by Western blotting for total IKKα and IKKβ proteins (middle and lower panel). (C) Two million SP-53 MCL cells were treated with curcumin (50 μM) for indicated times, and whole-cell extracts were prepared and examined for the level of phosphorylated Akt by Western blotting. (D) Two million SP-53 MCL cells were treated with curcumin (50 μM) for indicated times, and cytoplasmic extracts were prepared and examined for the level of phosphorylated p65 by Western blotting. (E) SP-53 cells were incubated with or without curcumin (50 μM) for 3 h and then analyzed for the distribution of p65 by immunocytochemistry. Red stain indicates the localization of p65, and blue stain indicates the nucleus (magnification, 200×). Curcumin inhibits NF-κB-regulated gene products. (F) Two million SP-53 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 onto a nitrocellulose membrane, and probed for IκBα; Bcl-2, Bcl-xL, cyclin D1, and COX2. The same blots were stripped and reprobed with anti-β-actin antibody to show equal protein loading (lower panel in each figure). (G) Two million SP-53 cells were treated with curcumin (50 μM) for indicated times, and whole-cell extracts were prepared. Fifty micrograms of cytoplasmic extracts were resolved on 10% SDS-PAGE gel, electrotransferred onto a nitrocellulose membrane, and probed for xIAP, cIAP, TRAF1 and survivin. Same blots were stripped and reprobed with anti-β-actin antibody to show equal protein loading (lower panel in each figure). (H) Five million Granta 519 cells were treated with curcumin (50 μM) for 3 h, and total mRNA was extracted and examined for expression of IL-6, RANK, and RANKL mRNA by RT-PCR. β-actin mRNA was used as an internal control to show equal RNA loading.

3.8. Curcumin suppresses the proliferation of MCL cells and arrested the cells at the G1/S phase of the cell cycle

Because NF-κB has been implicated in cell survival and proliferation [15,16], we examined the effect of curcumin on proliferation of MCL cell lines by the MTT method. Curcumin at a concentration as low as 1 μM inhibited growth of SP-53, Jeko-1, Mino and Granta 519 (Fig. 4A–D). At 10 μM, curcumin completely suppressed the growth in all cell lines.

How soon after NF-κB suppression follows antiproliferative effects of curcumin, was further investigated. We found that 50 μM curcumin-induced approximately 30% cytotoxicity within 8 h and about 60% cytotoxicity at 24 h (Fig. 4E and F). Because D-type cyclins are required for the progression of cells from the G1 phase of the cell cycle to S phase (DNA synthesis) [33] and we observed a rapid

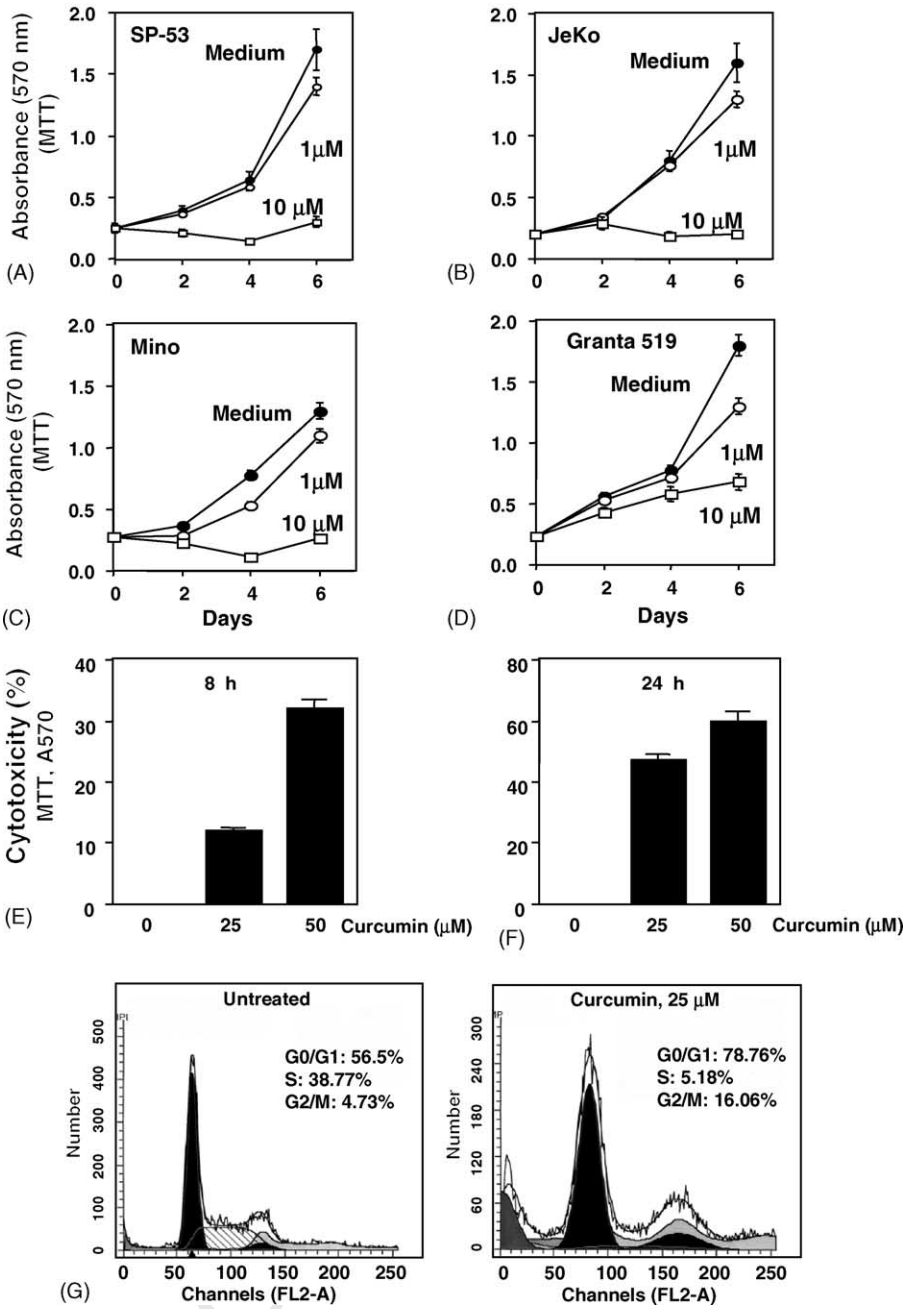

Fig. 4. Curcumin inhibits proliferation in MCL cells. (A) SP-53; (B) JeKo-1; (C) Mino; or (D) Granta 519 (5000 cells/0.1 ml) were incubated at 37 °C with curcumin (1 and 10 μM) for indicated time duration, and the viable cells were assayed using MTT reagent. The results are shown as the mean ± S.D. from triplicate cultures. (E, F) SP-53 cells (5000/0.1 ml) were incubated at 37 °C with curcumin (25 and 50 μM) for 8 and 24 h, and then cell viability determined using MTT method. The results are shown as the mean ± S.D. of triplicates. (G) Curcumin arrests the cells at G1/S phase of the cell cycle. Serum-starved SP-53 cells (2×10^6 cells/ml) were incubated in the absence or in presence of different doses of curcumin for 36 h. Thereafter, the cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry as described in Section 2.

decline of cyclin D1 in curcumin-treated MCL cells, we wished to determine the effect of curcumin on SP-53 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 57 to 79%, and a decrease in the percentage of cells in the S phase, from 39 to 5%, within 36 h of curcumin (25 μM) treatment (Fig. 4G). These results clearly show that curcumin induces G1/S arrest of the cells.

3.9. Curcumin-induced apoptosis in MCL cells

Whether suppression of NF-κB in MCL cells also leads to apoptosis was investigated by determining the activation of caspases. SP-53 cells were treated with curcumin for different times, and the whole-cell extracts were prepared and analyzed by Western blotting for activation of caspase-9, caspase-7, and cleavage of PARP [34]. The results showed a time-dependent activation of caspase-9 (Fig. 5A), as indicated by the disappearance of the 47 kDa band and the appearance of a 37 kDa band. Similarly, the Western blot analysis also showed an activation of caspase-7 (Fig. 5B), as indicated by the disappearance of the 35 kDa band and the appearance of a 20 kDa band. Furthermore, curcumin also induced the activation of caspase-3 (Fig. 5C). Activation of downstream caspases led to the cleavage of a 118 kDa PARP protein into an 89 kDa fragment (Fig. 5D), whereas untreated cells did not show any PARP cleavage. These results clearly suggest that curcumin-induced apoptosis in MCL cells.

Curcumin-induced apoptosis in MCL cells was also confirmed independently by the annexin V method. Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic of cells entering apoptosis. This allows live cells (unstained with either fluorochrome) to be discriminated from apoptotic cells (stained only with annexin V) [31]. SP-53 cells were treated for 24 h with different concentrations of curcumin and then stained with annexin V-FITC. Results in Fig. 5E show a dose-dependent increase in cells positive for annexin V, indicating the onset of apoptosis in curcumin-treated cells.

3.10. Suppression of constitutive NF-κB activation is linked to the inhibition of proliferation of MCL cells

Curcumin suppresses NF-κB and suppresses proliferation of MCL cells. To determine whether suppression of

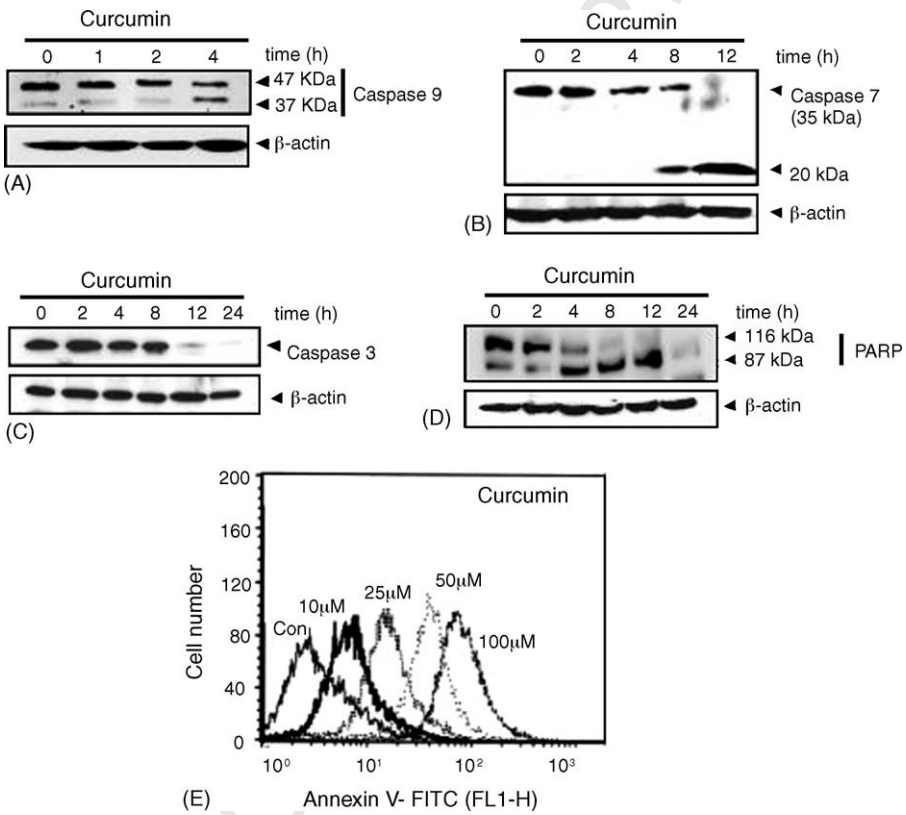

Fig. 5. Curcumin-induced apoptosis of MCL cells is mediated through caspase activation. SP-53 cells (2×10^6 cells/ml) were incubated in the absence or in presence of curcumin (50 μM) for indicated times. The cells were washed and total proteins were extracted by lysing the cells. Sixty micrograms of extracts was resolved on 10% SDS-PAGE gel, electrotransferred to a nitrocellulose membrane, and probed with (A) anti-caspase-9, (B) anti-caspase-7, (C) anti-caspase-3, and (D) anti-PARP antibodies as described in Section 2. (E) Flow cytometric analysis of annexin V-FITC stained cells after treatment with different concentrations of curcumin. SP-53 cells were incubated alone or with indicated concentrations of curcumin for 24 h; thereafter either cells were left unstained or stained with annexin V-FITC.

proliferation of MCL is linked to the suppression of NF-κB, we used two distinct and specific NF-κB blockers. We used NBD-peptide that has been shown to block the interaction between NEMO and IKKα and IKKβ [26]. For cell-permeabilization, the NBD-peptide was conjugated to a small sequence from the antennapedia homeodomain. We also used a PTD-p65-P1 peptide that is derived from the p65 subunit of NF-κB amino acid residues 271–282 that has been shown to be a specific inhibitor of NF-κB activation [35]. This peptide also required linking with a PTD derived from the third helix sequence of antennapedia. These peptides specifically suppress NF-κB activation. The peptide without the antennapedia homeodomain protein sequence and the PTD sequence alone were used as a control.

Treatment of SP-53 cells with NEMO-control peptide had no effect on the constitutive NF-κB activation, but NBD-peptide suppressed the constitutive NF-κB in a time-dependent manner, with complete suppression occurring at 4 h (Fig. 6A). Suppression of NF-κB by NBD-peptide also

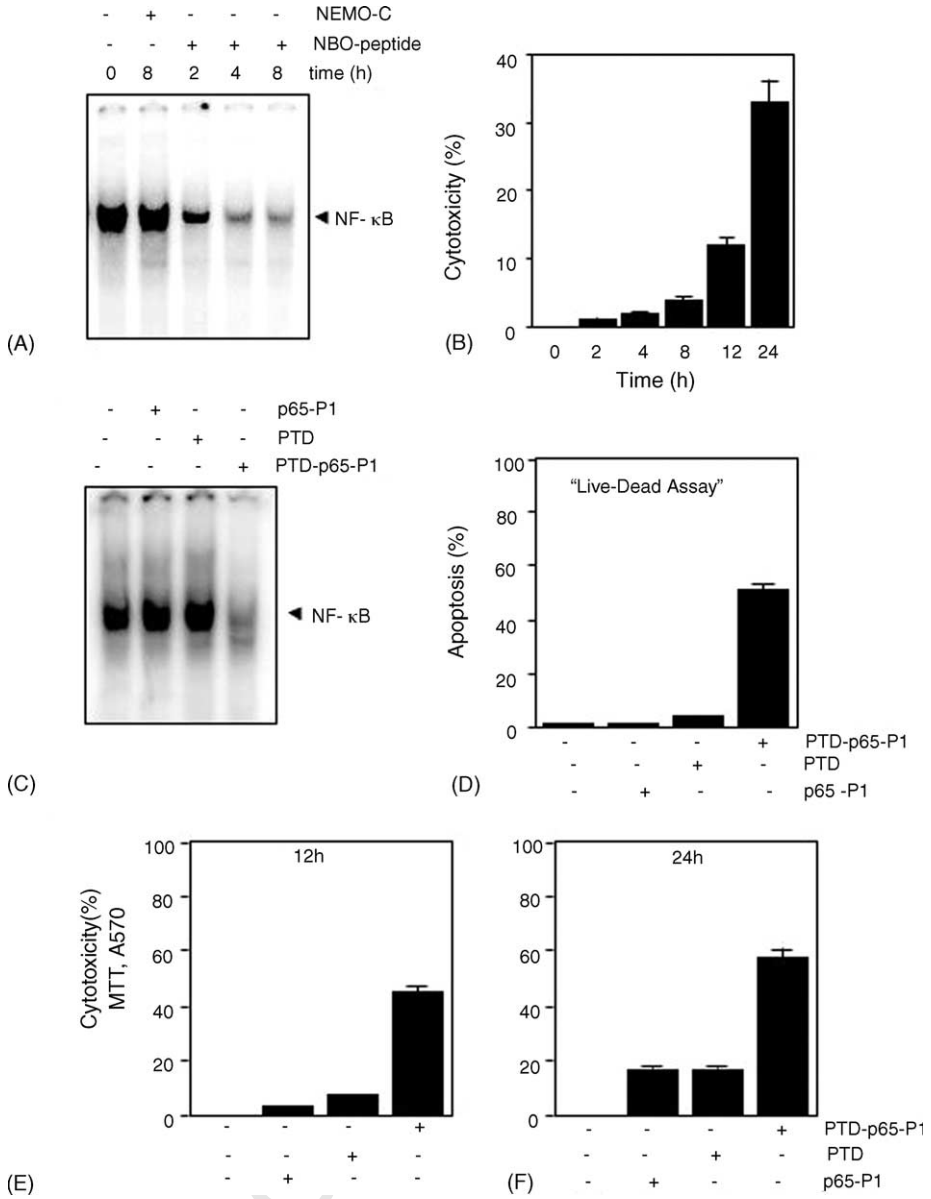

Fig. 6. NEMO-binding domain (NBD) peptide inhibits constitutive NF-κB and induces cytotoxicity in MCL cells. (A) SP-53 cells (2×10^6 cells/ml) were treated with indicated concentrations of NEMO-control or NBD-peptide (100 μM) for indicated times. Nuclear extracts were checked for the presence of NF-κB DNA-binding activity by EMSA. (B) SP-53 cells (5×10^3 cells/0.1 ml) were treated with indicated concentrations of NEMO-control or NBD-peptide (100 μM) for indicated time periods, and then cell viability was monitored by the trypan blue dye exclusion method. PTD-p65-P1 peptide inhibits constitutive NF-κB activation and inhibits the proliferation of MCL cells. (C) SP-53 cells (2×10^6 ml^{-1}) were treated with p65-P1 peptide (150 μM), protein transduction domain (PTD, 150 μM) or PTD-p65-P1 peptide (150 μM) for 4 h. Nuclear extracts were examined for the presence of NF-κB DNA-binding activity by EMSA. (D) SP-53 cells (5×10^3 cells/0.1 ml) were treated with above indicated concentrations of p65-P1, PTD or PTD-p65-P1 for 12 h and then the cell viability was examined by live-dead assay. (E) SP-53 cells (5×10^3 cells/0.1 ml) were treated with 150 μM each p65-P1, PTD or PTD-p65-P1 for 12 and 24 h and then cell viability was monitored by the trypan blue dye exclusion method.

led to inhibition of proliferation of SP-53 cells. Approximately 34% suppression of cell growth was observed after NBD treatment for 24 h (Fig. 6B). Similarly, PTD-P65-P1 inhibitory peptide but not the control peptides alone inhibited the constitutive NF-κB activity (Fig. 6C). Inhibition of NF-κB by PTD-P65-P1 led to about 40% inhibition of proliferation in 12 h and 60% in 24 h (Fig. 6D and E). These results thus suggest that the suppression of NF-κB is linked to the antiproliferative effects of curcumin.

3.11. MCL cell lines express TNF and curcumin inhibits the TNF expression

Among the cytokines, TNF and RANKL are the most potent activator of NF-κB. Whether constitutive activation of NF-κB in MCL cells is due to autocrine expression of TNF was examined. The level of TNF mRNA expression in MCL cell lines was examined by RT-PCR. The results showed that all the four MCL cell lines constitutively expressed TNF mRNA (Fig. 7A). We next examined the effect of curcumin on the expression of TNF mRNA and TNF protein expression in MCL cells. We found that curcumin inhibited the expression of both TNF mRNA (Fig. 7B) and TNF protein (Fig. 7C) in a time-dependent manner.

3.12. Suppression of TNF leads to inhibition of NF-κB activation and proliferation of MCL

To determine whether the constitutively active NF-κB in MCL is due to expression of TNF, cells were treated with anti-TNF antibody and the NF-κB expression was examined. We found that neutralization of TNF led to the suppression of constitutively active NF-κB (Fig. 7D), thus suggesting that TNF plays a major role in activation of NF-κB in MCL.

To determine whether the proliferation of MCL is due to expression of TNF, cells were treated with anti-TNF antibody and the proliferation was examined by thymidine incorporation. We found that neutralization of TNF led to

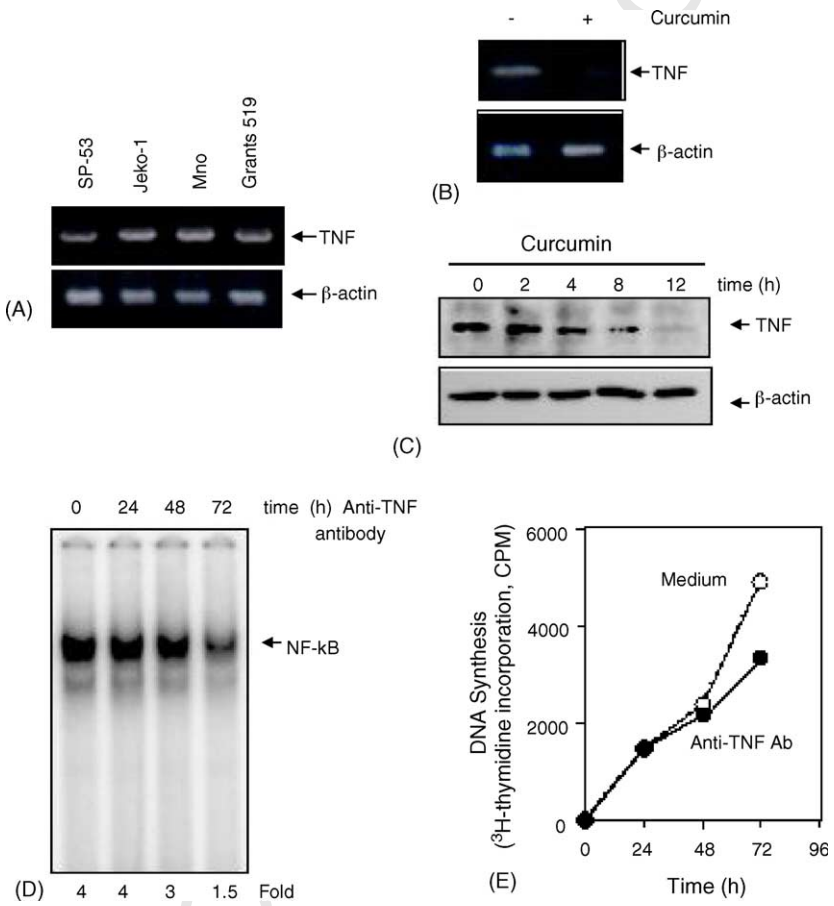

Fig. 7. TNF is an autocrine growh factor for MCL. (A) MCL express TNF mRNA. Two million cells/ml were tested for TNF by RT-PCR. β-actin was used as a loading control. (B) Curcumin inhibits expression of TNF mRNA. Five million Granta 519 cells were treated with curcumin (50 μM) for 3 h, and tested for TNF by RT-PCR. (C) Curcumin inhibits expression of TNF protein. Two million SP-53 cells were treated with curcumin (50 μM) for indicated time points, and tested for TNF by Western blot. (D) Anti-TNF antibody inhibits constitutive NF-κB activation. Two million SP-53 cells were treated with anti-TNF antibody (50 ng/ml) for indicated times, then nuclear extract was prepared and examined for NF-κB. (E) Anti-TNF antibody inhibits proliferation of MCL cells. SP-53 cells (5000/0.1 ml) were incubated at 37 °C with anti-TNF antibody (50 ng/ml) for indicated time points, and then cell proliferation was determined using ^{3}H-thymidine incorporation. The results are shown as the mean ± S.D. of triplicates.

the inhibition of proliferation of cells (Fig. 7E), thus suggesting the role of TNF-induced NF-κB activation in proliferation of MCL cells.

4. Discussion

In this study, we examined the effect of curcumin on the constitutive NF-κB activation in MCL cells. Our results show that NF-κB is constitutively expressed in all the four MCL cell lines that we examined. All these cells showed constitutive activation of IKK, constitutive phosphorylation of p65, and IκBα and overexpression of mRNAs for TNF, IL-6, RANK and RANKL. MCL cells also overexpressed IκBα, Bcl-2, Bcl-XL and COX2 that are known to be regulated by NF-κB. Treatment of MCL cells with curcumin downregulated the constitutively active NF-κB and IKK, inhibited the phosphorylation of IκBα and p65; suppressed the expression of IκBα, Bcl-2, Bcl-xL, cIAP-1, xIAP, TRAF-1, survivin, cyclin D1, TNF, IL-6, and COX2; and this led to the induction G1/S arrest, suppression of proliferation and induction of apoptosis in MCL cells. Treatment of MCL cells with anti-TNF antibody also downregulated the constitutively active NF-κB and proliferation of MCL cells.

Our finding that NF-κB was constitutively active in all four MCL cell lines (SP-53, Jeko-1, Mino and Granta-519), is in agreement with another recent report [36]. Why NF-kB is constitutively active in MCL, however, has not been reported. We found for the first time that all the MCL cell lines expressed mRNA for TNF, the most potent inducers of NF-κB activation. Curcumin inhibited the expression of both TNF mRNA and the TNF protein in MCL. We also found that neutralization of TNF secretion by an anti-TNF antibody led to the suppression of NF-κB in MCL.

Our results indicate that NF-κB is responsible for proliferation of cells, as suppression of NF-κB either by curcumin, NF-κB specific peptide or by anti-TNF-antibody, suppressed the proliferation of cells. Our results also suggest that TNF is an autocrine growth factor or MCL. The molecular signature of mantle cell lymphoma reveals multiple signals favoring cell survival [14]. TNF has been described to be abnormally increased in patients affected by malignant lymphomas, particularly non-Hodgkins lymphoma (NHL) [37].

IKK, the kinase required for NF-κB activation [28] is constitutively phosphorylated in MCL. We found that curcumin treatment abrogated the constitutive NF-κB activation through the inhibition of IKK. Inhibition of IKK resulted in the suppression of constitutive phosphorylation of IκBα and p65. Our results are in agreement with earlier reports where curcumin has been shown to suppress NF-κB activation in colon cancer cells, macrophages and multiple myeloma cells through the suppression of IKK [26,38,39]. Akt, NIK, mitogen-activated protein kinase kinase kinase 1, and atypical protein kinase C have also been linked to IKK [40]. Our study shows that MCL expressed constitutive active Akt and curcumin inhibits the Akt activation. Suppression of Akt activation could lead to inhibition of IKK activation. Akt has been described as a cell survival kinase [41], and inhibition of this kinase could also mediate the suppression of proliferation of MCL.

We found that the NF-κB-regulated genes, Bcl-2, Bcl-XL, cIAP-1, xIAP, TRAF-1, survivin, COX2 and cyclin D1 [15,42,43] were overexpressed in MCL cell lines and suppression of NF-κB by curcumin inhibited the expression of these genes. The deregulation of Bcl-2 and Bcl-XL results in increased resistance to cell death. A previous report implicated the activation of NF-κB in the deregulated overexpression of Bcl-2 in follicular lymphoma [44]. In our study curcumin downregulated Bcl-2 and Bcl-xL [45] expression, the proteins that have been implicated in the survival of MCL cells [13,46]. Curcumin suppressed the protein expression of IκBα that is an NF-κB-regulated gene. As IκBα is required to keep NF-κB in a resting stage, therefore, it is expected that this could lead to further activation of NF-κB. Interestingly, however, curcumin suppressed the activation of NF-κB.

To determine whether induction of apoptosis of MCL is linked to the suppression of NF-κB, we used two distinct and specific NF-κB blockers, viz; NBD-peptide that inhibits IKK activation [26], and a PTD-p65-P1 peptide that inhibits binding of p65 to the DNA [35]. Both approaches suggest that the suppression of NF-κB is linked to the cytotoxic effects of curcumin. The cytotoxic effects of curcumin in MCL cells is in agreement with previous report that curcumin-induced suppression of NF-κB leads to inhibition of cellular proliferation of cutaneous T-cell lymphoma [47] and acute myelogenous leukemia [48].

Cyclin D1, another NF-κB-regulated gene, is overexpressed in MCL as a result of a t(11;14) chromosomal translocation. We found that the expression of cyclin D1 is also downregulated by curcumin. Cyclin D1 plays a role in cell proliferation through activation of cyclin-dependent kinases. In the present report we show that inhibition of proliferation of MCL correlated with the down-regulation of the expression of cyclin D1 protein. An earlier report from our laboratory has shown that curcumin blocks the proliferation of various prostate, breast and squamous cell carcinoma cell lines by down-regulating the expression of cyclin D1 protein [49]. The suppression of cyclin D1 by curcumin resulted in the cell cycle arrest at G1/S phase because cyclin D1 is needed for cells to advance from the G1 to S phase of the cell cycle.

Our studies show that TNF, IL-6, RANK, and RANKL mRNA were constitutively expressed in MCL. Although all these cytokines are known to be regulated by NF-κB, TNF and RANKL are potent activators of NF-κB. These cytokines are produced in NHL and cooperate in vivo to increase NHL cell proliferation [50]. IL-6 has been shown to play a role in the clinical aggressiveness of human NHL

by stimulating the expression of matrix metalloproteinases [51]. Overexpression of RANKL, which correlated with bone resorption was observed in multiple myeloma [52,53] and adult T-cell leukemia [54]. We found that curcumin inhibited the expression of these cytokines through the inhibition of NF-κB.

Because NF-κB is known to mediate antiapoptotic effects [16,55], we examined whether suppression of NF-κB by curcumin could lead to apoptosis. We found that curcumin activated caspases 9, 7, and 3 and induced the cleavage of PARP in MCL. These results are in agreement with reports indicating that curcumin induces apoptosis in AML and prostate cancer cells [56,57].

Overall our results show that curcumin can block the constitutive expression of cytokines that are known to activate NF-κB, inhibits IKK activation, suppresses the proliferation of MCL and leads the MCL cells to apoptosis through the inhibition of NF-κB regulated anti-apoptosis genes as well as through the activation of caspases. In addition to these multiple mechanisms by which curcumin inhibits the growth and proliferation of MCL, it also is a pharmacologically safe compound with no known side effects even at doses as high as 8 g per day [58]. Therefore, the suppression of constitutive NF-κB by curcumin may prove useful in the treatment of MCL.

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