MOLECULAR CARCINOGENESIS 45:320–332 (2006)

Antitumor Action of Curcumin in Human Papillomavirus Associated Cells Involves Downregulation of Viral Oncogenes, Prevention of NFkB and AP-1 Translocation, and Modulation of Apoptosis

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Curcumin (diferuloyl methane), the major yellow pigment from the rhizomes of turmeric (*Curcuma longa* Linn), has anticancer properties. Infection with high-risk human papillomaviruses (HPV) leads to development of cervical carcinoma, predominantly through the action of viral oncoproteins E6 and E7.The present study aims at analyzing the antitumor and antiviral properties of curcumin, on HPV associated cervical cancer cells. Our findings indicate curcumin to be cytotoxic to cervical cancer cells in a concentration-dependent and time-dependent manner. The cytotoxic activity was selectively more in HPV16 and HPV18 infected cells compared to non-HPV infected cells. Balance between tumor cell proliferation and spontaneous cell death via apoptosis had an important role in regulation of tumor cell growth. Curcumin-induced apoptosis in cervical cancer cells. Morphological hallmarks of apoptosis such as nuclear fragmentation and internucleosomal fragmentation of DNA were observed. Curcumin also selectively inhibited expression of viral oncogenes E6 and E7, evident from RT-PCR and Western blotting data. Electrophoretic mobility shift assay revealed that activation of NFκB-induced by TNFα is downregulated by curcumin. Curcumin blocked IkBα phosphorylation and degradation, leading to abrogation of NFκB activation. Curcumin also downregulated the expression of *COX*-2, a gene regulated by NFκB. Binding of AP-1, an indispensable component for efficient epithelial tissue-specific gene expression of HPV was also selectively downregulated by curcumin. These results provide attractive data for the possible use of curcumin in the management of HPV associated tumors.

Key words: curcumin; HPV; apoptosis

INTRODUCTION

Carcinoma of the uterine cervix is the second most common cancer, accounting for about 15% of all neoplasms. It is the most common cancer among women in India, representing about 26% of female cancers, resulting in about 90 000 women developing the disease annually [1]. Epidemiological studies have strongly implicated human papillomavirus (HPV) as an etiological agent [2]. Current treatment modalities including surgical ablation, radiotherapy, and adjuvant chemotherapy have been less than satisfactory particularly for locally advanced disease. Long-term disease-free treatment consists of cytotoxic chemotherapeutic agents, which kills cancer cells mainly by apoptosis. However, commonly used cytotoxic chemotherapy is largely associated with highly nonspecific cytotoxicity, narrow therapeutic indices, and undesirable effects [3–5].

Natural products have proved to be an infinite source for remedies over the ages and the concept that certain diet-derived substances can be used to prevent cancer or delay its onset has currently elicited considerable interest. Wide arrays of phenolic substances, particularly those present in dietary and medicinal plants, have been reported to possess substantial anticarcinogenic and antimutagenic effects [6]. Curcumin, derived from the root of *Curcuma longa*, is one such compound. Curcumin

Abbreviations: HPV, human papillomavirus; URR, upstream regulatory regions; TUNEL, terminal deoxynucleotidyl transferase-mediated biotin dUTP nick end labeling assay; RT-PCR, reverse-transcription polymerase chain reaction; EMSA, electrophoretic mobility shift assay, TNF-α, tumor necrosis factor-α; EGF, epidermal growth factor receptor.

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Received 20 April 2005; Revised 10 October 2005; Accepted 26 October 2005

DOI 10.1002/mc.20170

blocks tumor initiation induced by benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene [7] and suppresses phorbol ester-induced tumor promotion [8,9]. Animal studies have shown curcumin to suppress carcinogenesis in the skin [9,10], forestomach [11,12], colon [13,14], breast [15,16], and liver in mice [17]. In vitro, curcumin was found to induce apoptosis of a wide variety of tumor cells, including B-cell and T-cell leukemia [18,19], colon carcinoma [20], and breast carcinoma [21,22]. It has been shown to suppress angiogenesis in vivo, abrogate FGF-2-induced angiogenic response [23] matrix metalloprotease expression [24], block expression of adhesion molecules [25], and cyclooxygenase-2 [26].

Mechanisms that suppress tumorigenesis often involve modulation of signal transduction pathways, leading to alterations in gene expression, arrest of cell cycle progression, or apoptosis. Apoptosis is a mode of cell death used by multicellular organisms to eradicate cells in diverse physiological and pathological settings [6]. Several studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents, including cisplatin, camptothecin, etoposide etc. There is accumulating evidence that the efficiency of antitumor agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis [27], a phenomenon that we have established in leukemia [28]. Recent evidence also shows that suppression of apoptosis by tumor promoting agents in preneoplastic cells is thought to be an important mechanism in tumor promotion. Apoptosis inducing ability seems to have become a primary factor in considering the efficiency of chemopreventive agents [29].

Infection with high-risk human papillomaviruses can lead to the development of cervical carcinomas. This process critically depends on the virus encoded E6 and E7 oncoproteins, which stimulate proliferation by manipulating the function of a variety of host key regulatory proteins [30]. A number of transcription factors or survivor molecules have been linked to the development of human tumors. Some E6/E7 regulated products are target genes of NFκB, a dimeric transcription factor involved in the expression of proteins necessary for innate immunity [31], apoptosis, and cell proliferation [32]. This transcription factor is a dimeric complex composed of different members of the Rel/NFκB family of polypeptides [33]. The p50–p65 heterodimer is retained in the cytoplasm by the inhibitory subunit IκBα. On activation of the complex, IκBα sequentially undergoes phosphorylation, ubiquitination, and degradation, thus releasing the p50–p65 heterodimer for translocation to the nucleus [34]. An IκBα kinase, IKK, has been identified that phosphorylates serine residues in IκBα at position 32 and 36 [35].

Most agents that activate NFκB also activate another transcription factor, AP-1, closely linked with proliferation and transformation of tumor cells. AP-1 plays a key role during development of cervical cancer [36], because site-directed mutagenesis of the corresponding AP-1 binding sites within the viral upstream regulatory regions (URR) almost completely abolishes transcription of URR–driven reporter constructs, either under transient transfection conditions[37,38] or in stable differentiation-dependent infection assays in organotypic raft cultures [39,40]. The inappropriate regulation of NFκB and AP-1 has been associated with various pathological conditions including septic shock, inflammatory conditions, acute-phase response, viral replication, radiation damage, and cancer [41]. There have been reports that curcumin can inhibit NFκB and AP-1 activation induced by various agents [42]. This study thus analyzed the effects of curcumin on cervical cancer cell lines (HPV 18, HPV 16, and HPV negative) looking specifically at the cytotoxic activity, apoptosis inducing activity, and effects on NFkB and AP-1 activation as well as COX-2 expression. Furthermore the study also investigated the effects of curcumin on the expression of the viral oncogenes E6 and E7.

MATERIALS AND METHODS

Materials

Curcumin was purchased from Sigma (St. Louis, MO). Stock solution of curcumin was made at 10 mmol/L concentration in dimethyl sulfoxide (DMSO) (Sigma, St. Louis Co.), and diluted with cell culture media and stored at −20°C. The final concentration of DMSO for all treatments was less than 0.1%. Antibodies against HPV 16 and 18 E6, E7 were purchased from Santacruz Biotechnology (Santacruz Biotechnology, Inc., Santacruz, CA) and those against IκB and phospho IκB were obtained from Imgenex (San Diego, CA).

Cell Lines

Human Cervical cancer cell lines, HeLa, SiHa, and C33A were obtained from National Centre for Cell Sciences (NCCS), Pune. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis, MO) containing 10% Fetal Bovine Serum (Sigma, St. Louis, MO) and antibiotics (Streptomycin 0.1g/L and penicillin G 0.06 g/L) in a humidified atmosphere of 5% CO_2 at 37°C. HeLa cells were confirmed to carry the HPV 18 genome and SiHa to contain HPV 16 genome. C33A cells were confirmed to be HPV negative.

Cytotoxicity Assay

Cytotoxic effect of curcumin on cervical cancer cells was detected by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay. Cells were plated into 96-well microtiter plates at 5000 cells/

well, grown for 24 h, and treated with different concentrations of curcumin. After 24-h incubation MTT was added to each well (5 mg/mL final concentration). Cells were incubated for 2 h. The formazan crystals formed by the reduction of MTT by the activity of respiratory enzymes were solubilized in lysis solution (20% SDS in 50% formamide). The colour intensity was measured at 570 nm in a Multiskan MS ELISA reader (Labsystems, Helsinki, Finland). The experiments were performed in triplicate. Cell survival was expressed as percentage over the untreated control.

Acridine Orange–Ethidium Bromide Staining for Morphological Evaluation of Apoptosis

Characteristic apoptotic morphological changes were assessed by fluorescent microscopy, with an acridine orange and ethidium bromide staining method [43]. Briefly, cells were seeded in a 6-well plates at seeding densities of 0.6×10^4 and treated with different concentrations of curcumin for 24 h. Cells were washed with phosphate buffered saline, stained with a mixture (1:1) of Acridine Orange (AO) and ethidium bromide (4 μg/mL each). Cells were visualized under fluorescent microscope (Axioskop 2 plus, Zeiss, Jena).

DNA Fragmentation Assay

Cells were seeded in 90-mm petri dishes at seeding densities of 2.2×10^6 and treated with different concentrations of curcumin for 24 h. Cells were washed with Tris Buffered saline, and lysed by adding extraction buffer (1 M Tris, 5 M NaCl, 0.2 M EDTA, 0.5% SDS). Subsequently, 20 μg/mL RNaseA (Sigma, St. Louis, MO) was added and the mixture incubated for 1 h at 37°C. Then, 0.5 mg/mL Proteinase K (Sigma, St. Louis, MO) was added and the mixture incubated for 1 h at 55°C. Proteinase K was inactivated by incubating the mixture at 65°C for 10 min. The intact chromosomal DNA was removed by salting out using 1 M NaCl. The fragmented DNA was precipitated by isopropanol. The fragments were resolved in a 2% agarose gel.

Terminal Deoxynucleotidyl Transferase-Mediated Biotin dUTP Nick End Labeling Assay (TUNEL)

To detect apoptotic cells, in situ end labeling of the 3′OH end of the DNA fragments generated by apoptosis-associated endonucleases was performed using the Dead End apoptosis detection kit (Promega, Madison, WI). Cells were grown on coverslips and treated with or without curcumin for 24 h. Medium was removed and cells were washed in phosphate buffered saline and fixed in 4% paraformaldehyde in 1 × PBS. Cells were permeabilized with 0.2% Triton X-100 solution in phosphate-buffered saline for 5 min, washed twice in phosphate buffered saline, and then covered with 100 μl of equilibration buffer and kept for 5–10 min. the equilibrated areas were blotted around with tissue paper and 100 μl of Terminal deoxynucleotidyl transferase (TdT) reaction mix was added to the sections on the slide and were then incubated at 37°C for 60 min inside a humidified chamber for the end labeling reaction to occur. Termination of the reaction was performed by immersing the slides in 2 × SSC for 15 min. The slides were washed thrice, by immersing in fresh phosphate buffered saline, for 5 min to remove unincorporated biotinylated nucleotides. The endogenous peroxidase activity was blocked by immersing the slides in 0.3% H_2O_2. After washing, horseradish-peroxidase-labeled streptavidin solution was added and the slides were incubated for 30 min. After incubation, the color was developed with the peroxidase substrate (H_2O_2) and the stable chromogen (diaminobenzidine). The slides were then mounted and examined under a light microscope.

Reverse- Transcription Polymerase Chain Reaction (RT-PCR) for Viral Oncogenes E6 and E7

RNA was extracted from tumor cells (5×10^7) treated with different concentrations of curcumin for 24 h. One milliliter Trizol (Sigma, St. Louis, MO) reagent was added and cells were scraped out. RNA was extracted with 0.2 mL of chloroform. RNA present in the aqueous layer was precipitated with 0.5 mL of isopropanol, washed with 75% ethanol, dried, and dissolved in DEPC-treated water. The integrity of the bands was checked in 1% agarose gel after ethidium bromide staining. The bands were visualized under UV light.

RNA isolated (5 μg) was reverse transcribed to cDNA in a 25 μl reaction mix containing 200 U of MMLV Reverse Transcriptase (Promega), 2 μg of random hexamer (Promega), 6 U of RNA guard (Promega), and 100 μM dNTP mix at 37°C for 1 h. The enzyme was inactivated at 95°C for 5 min and quick chilled. This cDNA was used for PCR amplification (50 μl) containing 2.5 U Taq DNA polymerase, 1.5 mM $MgCl_2$, 2.5 mM dNTP mix, and 25 pmol of sense and antisense primers. The primer sequences used for the amplification of genes and their annealing temperatures are given in Table 1. GAPDH was amplified as a control. RT-PCR products were separated on a 1.5% agarose gel.

Western Blot Analysis for E6, E7, IκB, and p IκB

The total protein from monolayer cells was isolated with RIPA buffer and protein content was quantified by Bradford's method using BSA as the standard. Hundred microliters of aqueous sample was mixed with 1 mL Bradford's reagent. The color developed after 5 min of incubation was measured at 595 nm.

Fifty micrograms of total cell extract was separated by SDS–PAGE. Samples were denatured in a sample

Table 1. Primer Sequences

Name		Sequence	Size (bp)	Annealing temperature (°C)
16 E6	S	5′-TGAGGTATATGACTTTGCTTTTC-3′	297	57
	AS	5′-CAAGACATACATCGACCGGTCC-3′		
16 E7	S	5′-AAATGACAGCTCAGAGGAGGAG-3′	209	59
	AS	5′-GTTTCTGAGAACAGATGGGGCAC-3′		
18 E6	S	5′-AAGCTACCTGATCTGTGCACGG-3′	308	57
	AS	5′-GCTGGATTCAACGGTTTCTGG-3′		
18 E7	S	5′-CAAAATGAAATTCCGGTTGACC-3′	214	57
	AS	5′-GCTGCTGGAATGCTCGAAGG-3′		
COX-2	S	5′-GTGACATCGATGCTGTGGAGC-3′	257	58
	AS	5′-CGAATGAAGTAAAGGGACAGCC-3′		
GAPDH	S	5′-GACCACAGTCCATGCCATCACT-3′	452	59
	AS	5′-TCCACCACCCTGTTGCTGTAG-3′		

buffer. Samples were run at a constant voltage of 80 V through the stacking gel and 100 V through the separating gel. The resolved proteins were transferred to a nitrocellulose sheet as detailed by Towbin et al. [44]. The membrane was washed to remove traces of methanol and blocking was performed with 3% BSA for 1 h. The nitrocellulose membrane was then incubated with primary antibodies against HPV 16 and 18 E6, E7 (Santacruz Biotechnology, Inc., Santacruz, CA) phosphorylated and nonphosphorylated forms of IκB (Imgenex, San Diego, CA). The blots were probed with corresponding secondary antibodies conjugated to alkaline phosphatase. The bands were developed with 5-bromo 4-chloro 3-indoyl phosphate (BCIP)/nitroblue tetrazolium (NBT). The identities of the bands were confirmed with reference to a prestained molecular weight marker (Sigma, St. Louis, MO).

Electrophoretic Mobility Shift Assay (EMSA) for NFκB and AP-1

Monolayer cells were treated with and without Tumor Necrosis Factor (TNF-α) 1 ng/mL for 15 min, Epidermal growth factor receptor (EGF)-1.7 ng/mL, and with 20 μM curcumin for 15, 30, 45 min. Cells were washed, scraped out, and pelleted in cold PBS. Cytosolic components were released to get intact nuclei by treating with Buffer A (10 mM HEPES pH 7.9; 10 mM (KCl), 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF with 2 μg each of aprotinin, leupeptin, and benzamidine) for 30 min on ice. After centrifugation at 12 000*g* for 10 min at 4°C, the pellet was treated with buffer C (10 mM HEPES pH 7.9; 400 mM (NaCl), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF with 2 μg each of aprotinin, leupeptin, and benzamidine) for 2 h on ice to get the nuclear extract. The extract was centrifuged at 12 000*g* to remove the debris and the supernatent was stored at −80°C till use. For gel shift analysis, NFκB and AP-1 oligonucleotides (Santacruz Biotechnology, Inc., Santacruz, CA) was used as the DNA probe following the protocol provided with the product. Oligonucleotides were labeled with [γ-P ^{32}] ATP, using T_4- Polynucleotide kinase (50 000 cpm/ng). Binding reaction mixtures were incubated fro 1 h on ice that contains 0.5 ng of labeled probe and 10 μg nuclear extracts in 10 mM Tris (pH 7.5) buffer with 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol and 1 μg of poly dI-dC (to inhibit nonspecific binding). DNA-protein complexes were resolved by electrophoresis through 4% PAGE containing 2.5% glycerol with 0.25× TBE. The gels were subsequently dried and developed by autoradiography. For supershift assay, the nuclear extracts from TNFα treated cells were incubated with anti-p65 or anti-p50 antibody for 30 min at room temperature and analyzed by EMSA.

RESULTS

Curcumin Inhibits Cell Proliferation

The antiproliferative effects of curcumin on cervical cancer cells were analyzed by MTT assay. Cells were exposed to varying concentrations of curcumin (5 μM, 10 μM, 20 μM, 40 μM, 60 μM) for 24 h (Figure 1A). In HeLa cells, the percentage of viable cells at 5 μM concentration was 91.5 that decreased gradually to 34.4 at 60 μM. In SiHa cells, the values ranged from 94 (5 μM) to 31.4 (60 μM) while in C-33A cells, 88.7% viability was observed at 5 μM concentration and 46.9% at 60 μM concentration. The cell viability was reduced by curcumin in a concentration-dependent manner. The differences among the mean values were analyzed with one-way ANOVA ($P < 0.05$).

MTT assay on three cell types treated with 5 μM curcumin for 24, 48, and 72 h showed a gradual increment in their sensitivity to curcumin with time of incubation even though at 72 h the mean differences between three cell lines were reduced than at 24 and 48 h (Figure 1B). HeLa cells were found to be more sensitive to curcumin. The mean differences in viability between the cell lines at 24, 48, and 72 h were analyzed by one-way ANOVA followed by Tukey's post hoc analysis. Differences

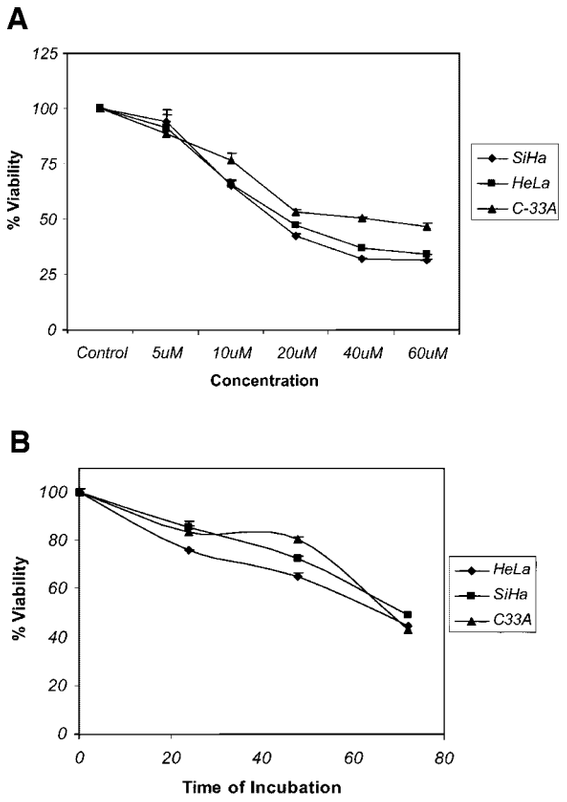

Figure 1. Effect of curcumin on cervical cancer cells (HeLa, SiHa, C33A) was determined by MTT assay. (A) Cells were exposed to various concentrations of curcumin (5 μM, 10 μM, 20 μM, 40 μM, and 60 μM) for 24 h. After drug treatment, cultures were assessed for cell survival as described in Materials and Methods. The experiment was repeated thrice with similar results. The differences among the mean values were analyzed with one-way ANOVA ($P < 0.05$). Bars indicate standard error. (B) Time-dependent effect of curcumin on cervical cancer cells. HeLa, SiHa, and C33A cells were exposed to 5 μM concentration of curcumin for different time periods. Cell viability was examined by the MTT method as described in Materials and Methods. All determinations were made in triplicate. The mean differences in viability between the cell lines at 24, 48, and 72 h were analyzed by one-way ANOVA followed by Tukey's post hoc analysis. Differences between means were considered statistically significant at $P < 0.05$.

between means were considered statistically significant at $P < 0.05$. The data has been plotted using means S.E. of triplicate determinations.

Curcumin Induces Morphological Features of Apoptosis

To check whether curcumin has any role in the regulation of apoptotic morphology, cervical cancer cells were treated with curcumin (5 μM and 20 μM) for 24 h and stained with Acridine orange-Ethidium bromide (Figure 2A). In untreated cells, all the cells were normal as indicated by the green fluorescence. The number of apoptotic cells (yellow) increased as the curcumin concentration increased in HeLa cells. Similar experiment on SiHa cells also revealed a concentration dependent increase in the number of apoptotic cells. In C-33A, which was HPV negative, the number of apoptotic cells was less compared to HPV positive cell lines, SiHa and HeLa. The number of cells with nuclear condensation was quantitated by counting the cells in random fields. Quantitated data is represented in the form of a bar diagram (Figure 2B).

Curcumin Induces DNA Fragmentation

The degradation of DNA into multiple internucleosomal fragments of 180–200 base pairs is a distinct biochemical hallmark for apoptosis. The internucleosomal DNAase activity in apoptotic cells was studied by DNA fragmentation assay. DNA fragmentation pattern was observed in HeLa cells at 5, 10, and 20 μM concentrations of curcumin (Figure 3A). The amount of fragmentation increased with concentration where as there was no fragmentation in untreated cells. In SiHa cells (Figure 3B), the chromosomal DNA fragments were observed at 5 μM, 10 μM, and 20 μM concentration. A faint fragmentation pattern was visible in C-33A cells (Figure 3C).

The quantitation of double stranded DNA breaks upon apoptosis was examined by TUNEL assay. Compared to the untreated cells, HeLa cells treated with 5 μM curcumin showed TUNEL reactivity (Figure 4A). Chromatin condensation was also observed in a few cells. At 10 μM concentration more intense reactivity as well as chromatin condensation was observed in HeLa cells. In SiHa cells (Figure 4B) treated with 5 μM concentration of curcumin, TUNEL reactivity was observed compared to untreated cells. At 10 μM concentration, an intense reactivity was visible along with chromosomal condensation. Successive cell shrinkage was visible at 5 μM and 10 μM concentrations. C-33A cells showed TUNEL reactivity at 5 μM and 10 μM concentrations, however chromatin condensation and cell shrinkage at both these concentrations was not evident (Figure 4C).

Modulation of HPV Viral Oncogene Expression by Curcumin

Total RNA was extracted from HeLa and SiHa cells treated with 5 μM, 10 μM, and 20 μM curcumin for 24 h. RT-PCR was performed using specific primer sets for HPV-16 E6 and E7 and also HPV 18 E6 and E7 along with house keeping gene, GAPDH. Densitometric analysis was performed and the normalized values showed that in untreated SiHa cells HPV-16 E6 and E7 intensity was high (Figure 5A) when compared with treated cells. E6 and E7 showed lower expression in curcumin treated cells when compared with untreated SiHa cells.

HPV-18 E6 and E7 intensity was high (Figure 5B) in untreated HeLa cells compared with treated cells. Lower expression of E6 and E7 genes was found following curcumin treatment. The expression of

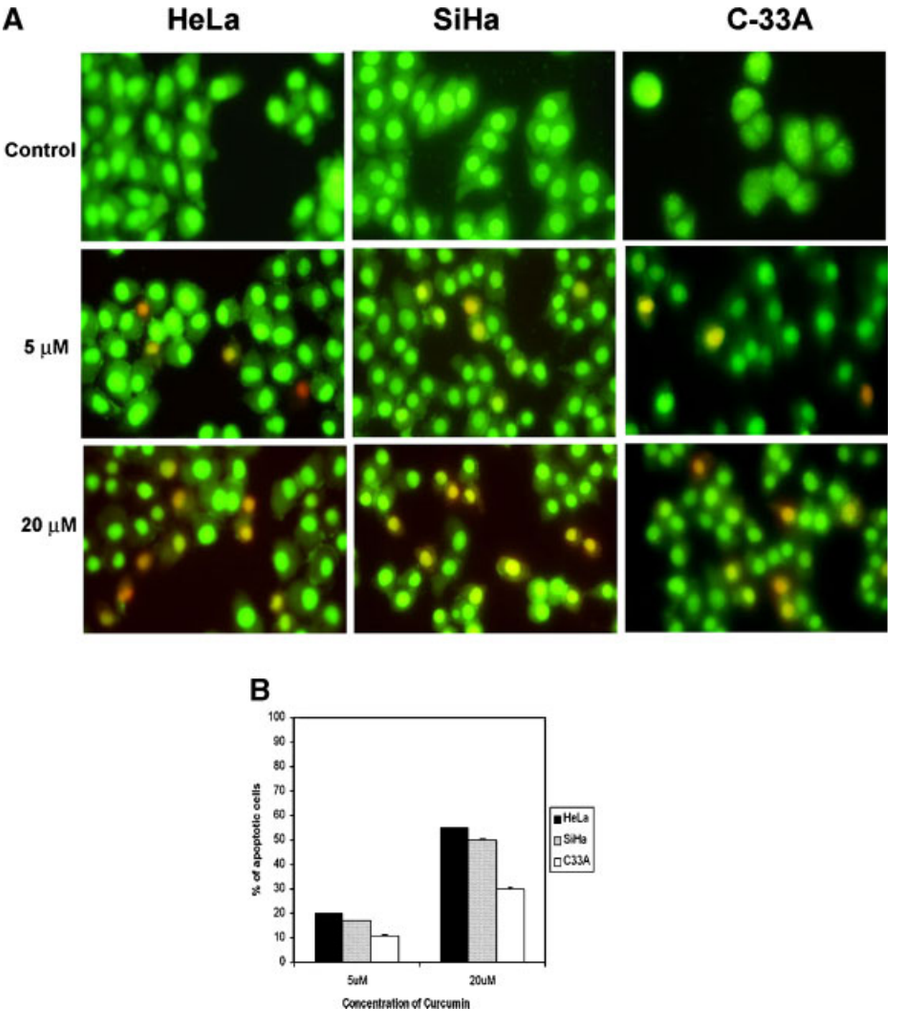

Figure 2. Changes in nuclear morphology-induced by curcumin (A) HeLa, SiHa, and C33A were seeded in 6-well plates and then treated with and without curcumin (5 μM and 20 μM) for 24 h. After washing with phosphate buffered saline, the cells were stained with a mixture of acridine orange-ethidium bromide solutions. Cells were viewed under fluorescent microscope and photographed as described under Materials and Methods. The results were similar when another experiment was performed under the same conditions. (B), Quantitation of nuclear condensation was performed by counting the cells in random fields and was represented as bar diagram. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

HPV 16 and 18 E6, E7 genes relative to untreated control were calculated.

For the detection of oncoproteins, E6 and E7, total protein was isolated from SiHa and HeLa cells treated with 20 μM curcumin for 16, 24, 48, and 72 h. Western blotting was performed for the detection of protein levels of HPV-18 and HPV 16 E6 and E7. Densitometric analysis was performed and normalized with β-actin. In untreated SiHa cells HPV 16 E6 and E7 intensity was high. Curcumin treatment inhibited the expression of HPV 16 E6 and E7 (Figure 6A). In untreated HeLa cells also HPV-18 E6 and E7 intensity was high when compared to curcumin-treated cells (Figure 6B). Expression of E6 and E7 proteins decreased with curcumin treatment. The expression of HPV 16 and 18 E6, E7 proteins relative to untreated control were calculated and the fold change values are represented in the figure itself.

Curcumin Inhibits TNF-α-Induced NFκB Activation

The cells were pretreated with TNF-α for 30 min followed by treatment with curcumin (20 μM) for 45 min and 1 h. Nuclear extracts were prepared and assayed for NFκB by EMSA. As shown in Figure 7A, TNF-α-induced activation of NFκB (lane 4) which was suppressed by curcumin in a time dependent manner (lanes 2, 3, and 5). At 1 h, complete inhibition was observed.

To show that the retarded band (lane 3 in Figure 7B) visualized by EMSA in TNF-treated cells was indeed NFκB, nuclear extracts from TNF treated cells were incubated with antibodies to either p50

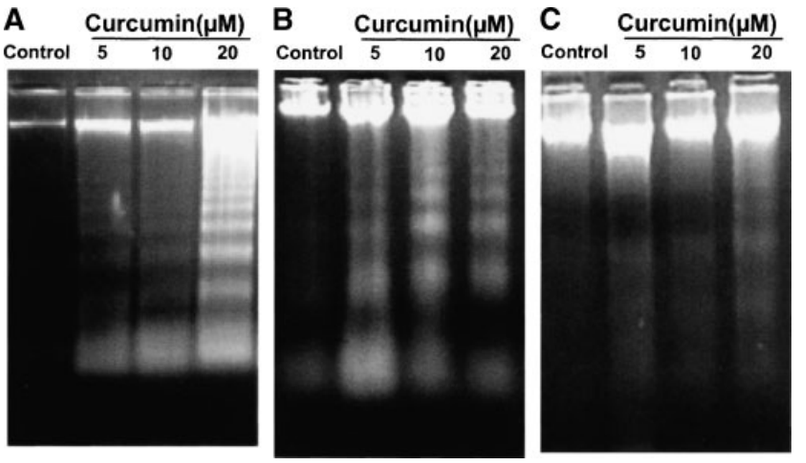

Figure 3. Curcumin-induced DNA fragmentation. HeLa (3A), SiHa (3B), and C33A (3C) grown in 90-mm petri dishes were treated with (5 μM, 10 μM, 20 μM) curcumin for 24 h. Cells were harvested and the oligonucleosomal DNA fragments were isolated, separated by gel electrophoresis, and analyzed as described under Materials and Methods. These results were confirmed in another independent experiment.

or p65 and then analyzed by EMSA (lanes 2 and 4, respectively). Antibodies to either subunit of NFκB shifted the band to higher molecular weight suggesting that the active complex consisted of p50 and p65 subunits.

Activation of NFκB is preceded by phosphorylation and proteolytic degradation of IκBα. To determine whether the inhibitory action of curcumin was because of its effect on the phosphorylation and degradation of IκBα, the cytoplasmic levels of IκBα

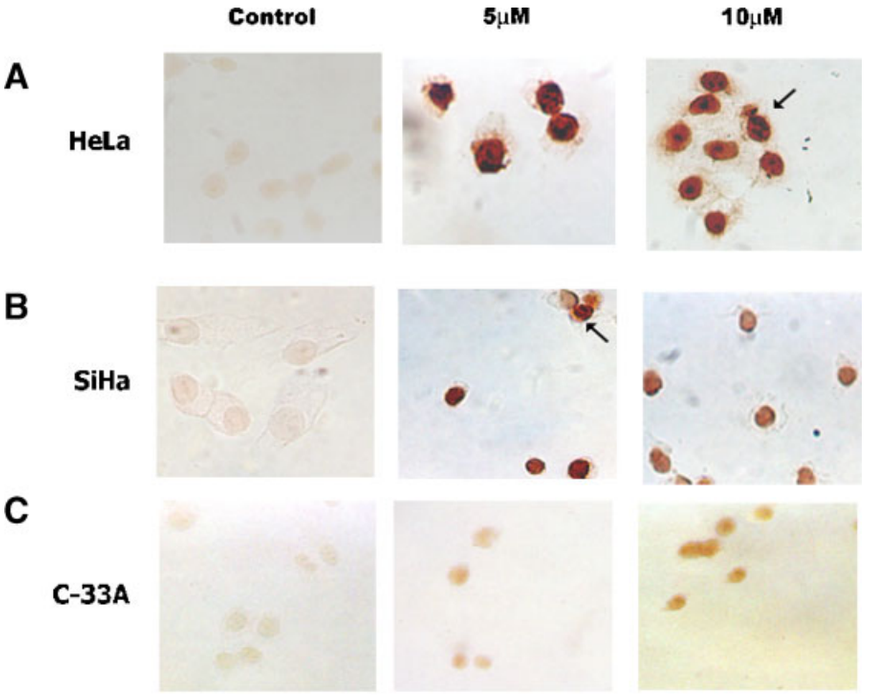

Figure 4. DNA fragmentation-induced by curcumin as determined by TUNEL assay. HeLa (4A), SiHa (4B), and C33A (4C) were grown in coverslips and treated with or without curcumin (5 μM, 10 μM) for 24 h. The cells were fixed, permeabilized with 0.2% Triton X-100, end labeled with Terminal deoxynucleotidyl transferase reaction mix and the TUNEL reactivity was visualized as described in Materials and Methods. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

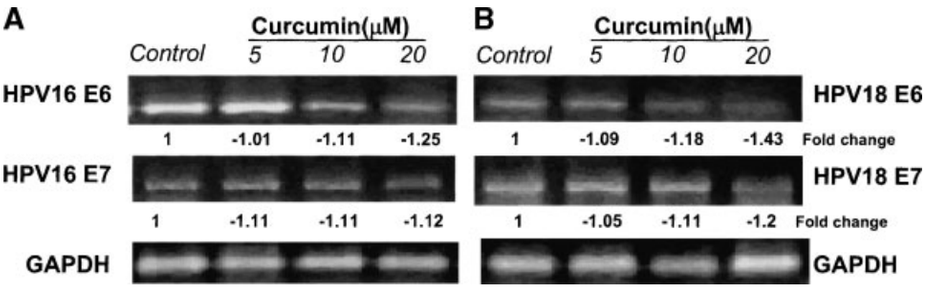

Figure 5. RT-PCR analysis of mRNA expression of HPV 16,18; *E6* and *E7* gene in curcumin-treated cells. Total RNA was extracted from cells incubated with 5 μM, 10 μM and 20 μM curcumin for 24 h. RT-PCR assay was performed with 5 μg total RNA using gene specific primers.

proteins were examined by Western blot analysis. As shown in Figure 8, upon TNF treatment for 15 min, the serine phosphorylated IκBα protein increased as detected by Ser 32-phospho-specific IκBα antibody. Curcumin inhibited TNF-induced IκBα phosphorylation and degradation in these cells. As loading controls, these blots were reprobed with anti-actin antibody. These bands were quantified by densitometry, and the values were normalized with respect to actin expression. The fold changes, as compared with control were calculated.

Curcumin Downregulates the Expression of Cycloxygenase-2

Since our data showed that curcumin suppressed activation of NFκB by inhibiting the phosphorylation and degradation of IκBα, we also looked to understand whether curcumin-induced inhibition

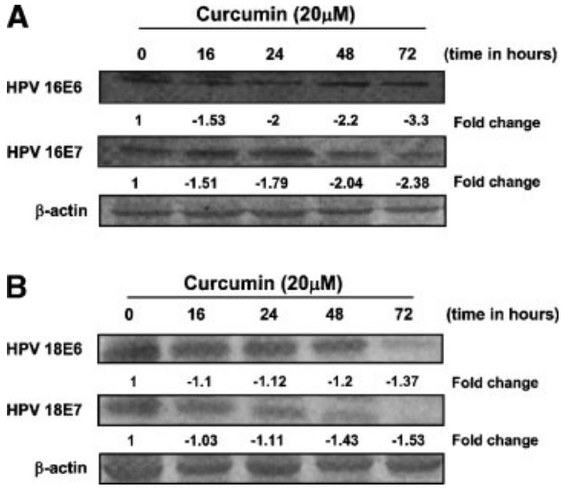

Figure 6. Expression of HPV oncogenes. Cells were plated in 60 mm dishes and treated with 20 μM curcumin for 16, 24, 48, and 72 h. Cells were harvested and whole cell lysates prepared. Fifty micrograms of the cell lysate were subjected to SDS-PAGE (12.5%) and HPV oncoproteins were detected using antibodies specific against each protein as described under Experimental procedures. The same blot was reprobed with β-actin antibody. Bands were analysed by densitometry and normalized to actin expression. The expression of proteins relative to untreated control was calculated.

of COX-2. Total RNA was isolated from SiHa, HeLa, and C33A cells treated with 5 μM, 10 μM, and 20 μM curcumin for 24 h. RT-PCR was performed using specific primer sets COX-2 along with house keeping gene, GAPDH. As is evident from Figure 9 shows that curcumin downregulated the expression of COX-2 in a dose dependent manner.

Inhibition of EGF-Induced AP-1 Activation by Curcumin

Since AP-1 is an indispensable key regulator for epithelial tissue specificity and transcriptional activity for various HPV types, we analyzed the effect of curcumin on AP-1 DNA binding activity (Figure 10). Cells were treated with Epidermal Growth factor (EGF) followed by treatment with curcumin (20 μM) for 45 min and 1 h. Nuclear extracts from EGF-stimulated cervical cancer cells formed a prominent DNA- protein complex with the AP-1 site (lane 2). Curcumin treatment at 45 min demonstrated a decrease in DNA binding activity (lane 3) and at 1 h the AP-1 DNA binding activity was completely inhibited (lane 4). The activation of AP-1 was completely inhibited in a time-dependent manner, with maximum suppression occurring at 1 h.

DISCUSSION

Cancer of the uterine cervix is the single largest female malignancy in India. With nearly half a million women developing the disease annually, the malignancy continues to pose an unresolved health problem. Although different chemotherapeutic agents are in clinical use, the situation demands a better drug, which can either independently or in combination with other drugs induce cell death or apoptosis selectively in cancer cells in an efficient manner. Several plant-derived components are currently successfully employed in cancer therapy. This study selected curcumin to study its role as an effective inducer of apoptosis in cervical cancer cells and also to investigate whether it can modulate HPV viral oncogene expression.

The effects of curcumin on three different cervical cancer cell lines were analyzed. Curcumin induces cell death in HeLa, SiHa, and C33A cells in a

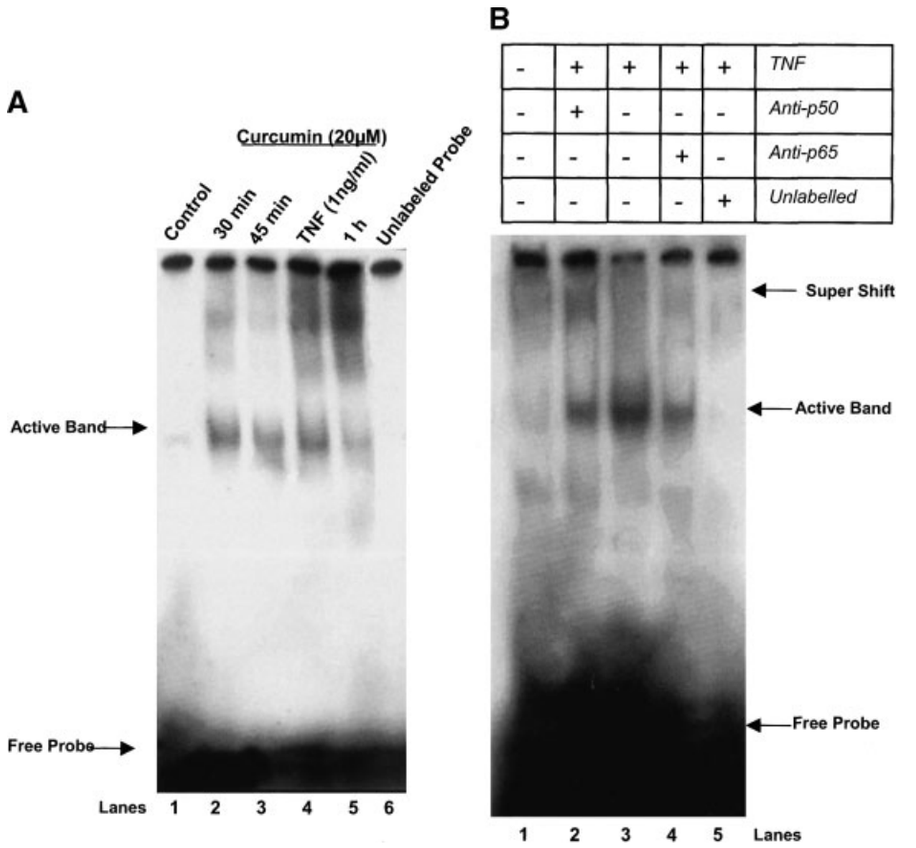

Figure 7. Suppression of NFκB activation by curcumin. (A), Electrophoretic mobility shift assay. SiHa cells were stimulated with TNFα (1 ng/mL) for 15 min and treated with 20 μM curcumin for 30 min, 45 min and 1 h. The arrow indicates the NFκB specific band. TNFα treatment (lane 4) induces NFκB binding compared with treated cells (lane 1). Curcumin inhibits TNFα-induced NFκB DNA binding (lanes 2,3,5 respectively). (B), Supershift assay. The nuclear extracts from TNFα treated cells were incubated with anti-p65 or anti-p50 antibodies and analysed by EMSA. Lane 1, without nuclear extract; Lane 2, with p50 antibody; Lane 3, TNF alone; Lane 4, with p65 antibody; Lane 5, unlabeled probe.

concentration- and time-dependent manner. Among the three cell lines, the HPV 18 positive HeLa cells, and HPV 16 positive SiHa cells were more sensitive to increased concentrations of curcumin compared to HPV negative C-33A cells. Previous studies have shown a dose-dependent inhibition of proliferation by curcumin in Lovo cells, a colon cancer cell line [20].

Apoptosis plays a pivotal role in the regulation of the development and homeostasis of multicellular

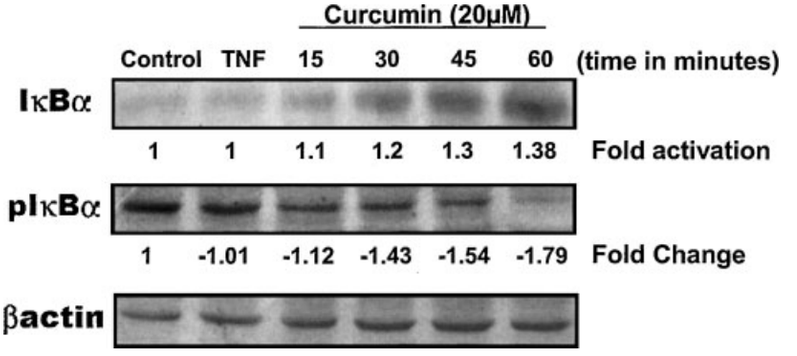

Figure 8. Curcumin blocks TNF-α-induced IκBα phosphorylation. SiHa cells were treated with TNFα (1 ng/mL) for 15 min and treated with 20 μM curcumin for 15, 30, 45, and 60 min. The cells were lysed, and the lysates were used for western blot analysis using anti phospho IkBα antibody. The blots were reprobed with anti IκBα or anti-actin antibody. The blots were analysed densitometrically, and the values are normalized to actin. The fold changes were calculated. Phosphorylation of IκBα occurs at 15 min in TNF-α treated cells and curcumin blocks TNF-α-induced phosphorylation and degradation.

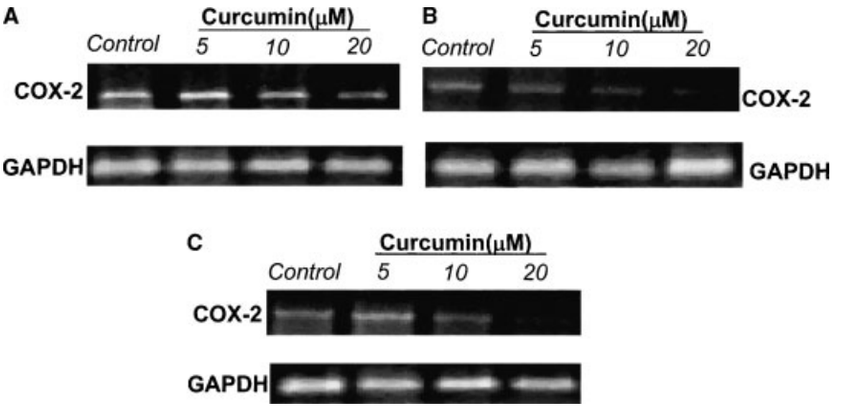

Figure 9. RT-PCR analysis of mRNA expression of *COX-2* gene in curcumin treated SiHa (Figure 9A) HeLa (Figure 9B) and C33A (Figure 9C) cell lines. Total RNA was extracted from cells incubated with 5 μM, 10 μM and 20 μM curcumin for 24 h. RT-PCR assay was performed with 5 μg total RNA using gene specific primers.

organisms [45]. Balance between tumor cell proliferation and spontaneous cell death via apoptosis has an important role in the regulation of tumor cell growth [46]. Anticancer drugs also function by inducing tumor cell death via induction of apoptosis in sensitive cells [47–49]. In this study, we demonstrate that curcumin is capable of inducing apoptosis in cervical cancer cells. This was demonstrated by time and dose dependent decrease in tumor cell number. The morphological hallmarks of apoptosis such as nuclear fragmentation and internucleosomal fragmentation of DNA were also observed. Previous studies have shown apoptosis inducing effects of curcumin in colon cancer [50], breast cancer [22,29], and ovarian tumor cells [51].

Many experimental studies have demonstrated that expression of the high-risk *HPV E6* and *E7* genes is required for cell transformation and for maintenance of the transformed state [52]. These two viral oncoproteins exert cellular immortalization and transformation by interfering with the function of two tumor suppressor proteins, p53 and Rb, respectively [53]. Our results indicate that curcumin downregulates the expression of HPV-18 E6 and E7 and HPV 16 E6 and E7. Several reports point out that the viral oncogene products E6 and E7 can modulate cellular proliferation and apoptosis [52]. Indeed suppression of E6 by an antisense approach in Caski cells restored the apoptotic potential of the cell line [54]. Further by Western blot, we also confirmed that curcumin could inhibit the expression of HPV 16 and 18 E6, E7 oncogenes. Downregulation of E6 and E7 expression in cancer cells results in loss of some transforming phenotypes [55,56] and cessation of cellular growth [57]. Several studies underline the importance of HPV oncoprotein expression in malignant cervical carcinoma cells. Reversible expression of E6/E7 expression in the HPV 18 positive cervical carcinoma cell line SW756 by dexamethasone blocks their malignant phenotype [58].

Because proliferative, proinflammatory and proangiogenic factors associated with aggressive tumor growth are regulated by nuclear factor NFκB, agents that can suppress NFκB activation have the potential as anticancer agents [59]. Our studies indicate that treatment with curcumin inhibited activation of NFκB as indicated by EMSA. These results are thus in agreement with previous reports [60–62]. We confirmed that the inhibitory action of curcumin was because of its effect on IκBα degradation. Previous studies have shown that activated NFκB suppresses apoptosis in a wide variety of tumor cells [63,64] and it has been implicated in chemoresistance [65]. We have shown that curcumin inhibited the activation of NFκB by preventing the phosphorylation and degradation of IκBα.

Cox-2 is another gene that has been implicated in carcinogenic processes, and its overexpression in malignant cells has been shown to enhance cellular invasion, induce angiogenesis, regulate antiapoptotic cellular defenses, and augment immunlogical resistance through production of prostaglandin E2 [66]. Treatment of cervical cancer cells with curcumin downregulated the expression of *Cox-2*, a gene regulated by NFκB. It has been demonstrated that Cox-2 is overexpressed in patients with cervical cancer, lung cancer, head and neck cancer, and breast cancer [67–70]. Inhibition of Cox-2 expression has been suggested as a therapeutic and chemopreventive approach [71].

Although E6 and E7 themselves possess intrinsic trans-activation capacity on their homologous promoters [72,73] constitutive expression of E6 or E7 in immortalized or malignantly transformed human keratinocytes is mainly dependent on the availability of a defined set of transcription factors derived from the infected host cell. AP-1, for

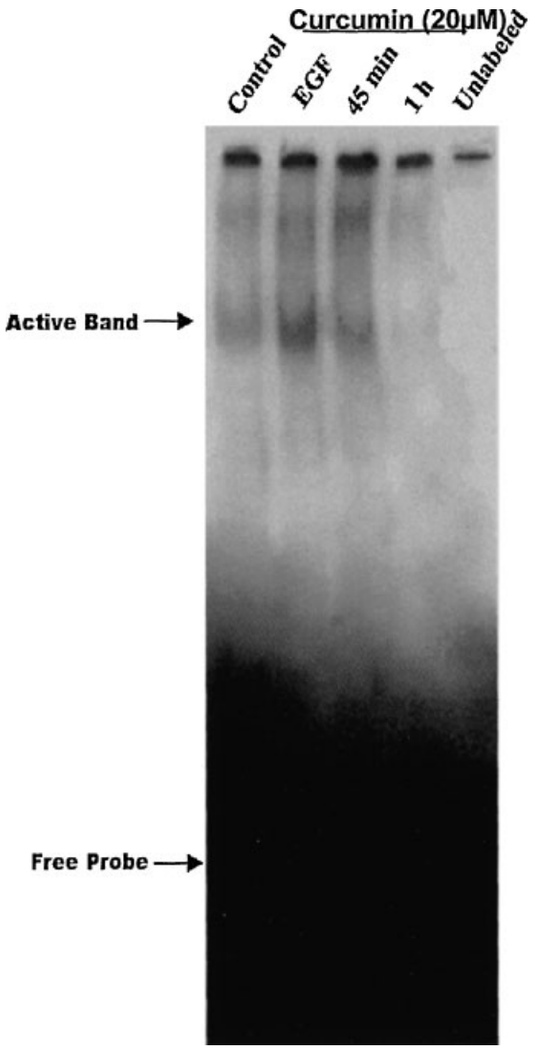

Figure 10. Electrophoretic mobility shift assay. SiHa cells were treated with epidermal growth factor receptor (EGF) (1.7 ng/mL) for 30 min and then treated with 20 μM curcumin for 45min and 1h. the position of the retarded bands is indicated. *Lane 1*, untreated cells; *Lane 2*, EGF treated cells; *Lanes 3–4*, 45 min and 1 h of incubation in presence of 20 μM curcumin respectively. Note that the binding activity of AP-1 diminishes by 45 min and it completely disappears by 1 h.

example, normally consisting of a heterodimer between c-Fos and c-*Jun* seems to play a central role in transcriptional regulation of viral oncogene expression, because point mutations of the corresponding consensus sequences within the upstream regulatory region (URR) of HPV-16 or HPV-18 almost completely abolish the expression of URR-driven reporter plasmids in transient transfection assays [37,38]. To determine whether curcumin suppresses HPV viral gene expression by preventing AP-1 DNA binding, we examined the bindings of nuclear proteins to oligonucleotides containing sequences of the AP-1 site. Nuclear extracts from EGF- stimulated cervical cancer cells formed a prominent DNA-protein complex with the AP-1 site, and curcumin suppressed the AP-1 binding activity observed in EGF- stimulated cervical cancer cells.

Many anticancer drugs exert their action via apoptosis. The selective induction of tumor cell apoptosis without affecting healthy cells and thus reducing the side effects of therapeutic regimens is a major goal for the development of new therapeutic techniques [74]. Cervical cancer is the result of sustained oncogenic activity of high-risk HPV types predominantly through the action of the *E6* and *E7* genes [1]. Curcumin in vitro down regulates the expression of these genes at both RNA and protein levels. Curcumin also blocks the activation of NFκB and prevents AP-1 binding. These actions are reflected in the downregulation of COX-2 and increased tumor cell apoptosis. Hence these preliminary results indicate the potential of curcumin being designed as an anticancer and anti HPV compound. Previous studies [75] have shown curcumin to be nontoxic even at high doses. A local delivery system would make the feasibility even better. Considering the large number of cervical cancer cases and the even larger number of women infected with HPV, a low cost broad spectrum anti tumor and anti HPV agent would be a top priority.

ACKNOWLEDGMENTS

This study was supported by a research grant from the Council of Scientific and Industrial Research (No. 27(0126)/03/EMR-II), Government of India. M.Radhakrishna Pillai is supported by a Career Development Grant (No. BT/HRD/34/01/03) from the Department of Biotechnology, Government of India. The authors thank Dr. Tessy Maliekal for assistance in standardization of experimental protocols.

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