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Molecular mechanism of curcumin induced cytotoxicity in human cervical carcinoma cells

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Abstract Cervical cancer is the most common cancer in Indian females and is associated with infection with high risk Human papillomaviruses (HPVs). Curcumin (Diferuloyl methane), a chemopreventive agent, is a natural compound extracted from *Curcuma longa* that allows suppression of carcinogenesis. The objective of this study was to identify the molecular mechanism of curcumin induced apoptosis in HPV positive cervical cancer HeLa, SiHa and Ca Ski cells. Curcumin causes distinct inhibition of human telomerase reverse transcriptase (hTERT) the catalytic core of telomerase thereby reducing proliferation of cancer cells. Curcumin mediated apoptosis in these cells appears to be due to upregulation of proapoptotic Bax, AIF, release of cytochrome c and down regulation of antiapoptotic Bcl-2, Bcl-XL in HeLa and SiHa. This was accompanied by an increase in caspase-3 and -9 activity, suggesting the role of mitochondria in curcumin mediated apoptotic cell death. Curcumin acts as an anti-inflammatory and anti-proliferative agent by causing down regulation of COX-2, iNOS and cyclin D1 in all the three cell lines but to different extent.

Keywords Cervical cancer · Curcumin · Telomerase · Bcl-2 · AIF · Apoptosis

Introduction

Curcumin is a natural compound extracted from *Curcuma longa* that allows suppression, retardation and inversion of carcinogenesis [1]. Curcumin [1,7-bis-(4-Hydroxy-3-methoxyphenyl)-1,6 heptadiene-3,5-dione] is a major constituent of turmeric powder extracted from the rhizome of the plant *Curcuma longa* found in South and Southeast tropical Asia. Curcumin has been shown to be a potent chemo preventive agent inhibiting tumor progression against skin, oral, intestinal, breast, colon and prostate cancer [2]. However the efficacy of curcumin in cervical carcinoma cell lines has not been fully determined.

The anticancer properties of curcumin have been attributed to modulation of various molecular pathways. Curcumin inhibits the transcription factor NF-κB by inhibition of κB kinase and subsequent IkB$\acute{\alpha}$ phosphorylation [3]. As a result curcumin downregulates the expression of NFκB regulated gene product in cell survival pathway [4]. Curcumin has been shown to inhibit AKT/mTOR pathway and phosphorylation of p70 ribosomal protein S6 kinase (p70S6k) and eukaryotic initiator factor 4E binding protein resulting in inhibition of proliferation and induction of apoptosis [5]. Curcumin has also been shown to induce downregulation of transcription factor AP 1 and Egr-1 [4].

The human papillomaviruses (HPVs) associated with cervical cancer are designated as the "high risk" subgroup of HPVs (e.g. HPV-16 and -18) [6] and encode viral proteins E6 and E7, which interfere with functioning of cellular tumor suppressor proteins p53 and retinoblastoma susceptibility gene product Rb, respectively. The E6 protein binds to cellular protein p53 and promotes its ubiquitin dependent degradation. E7 associates with Rb and interferes with its binding to E2F, resulting in impaired Rb cell cycle control functions [7]. It has been shown that the expression of E6 in primary human keratinocytes leads to activation of telomerase [8] an enzyme capable of preventing the shortening of telomeres during DNA replication [9]. Telomerase has thus emerged as an attractive target for arresting cancer cell growth in various cancers.

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In the present study, the anticancer effect of curcumin was investigated on HPV 18 positive HeLa and HPV 16 positive SiHa and Ca Ski cervical cancer cell lines. Curcumin induced apoptosis in all the three cell lines, which increased in a dose dependent manner with Ca Ski being most sensitive to curcumin. Curcumin efficiently repressed the activity of telomerase in a dose dependent manner in all the three cell lines. Curcumin induced the down regulation of a variety of cellular proteins involved in cell survival pathway and lead to upregulation of apoptosis inducing factor (AIF), activation of proapoptotic Bax, release of cytochrome c in case of HeLa, SiHa but not Ca Ski which results in induction of both caspase-3 and -9, thereby suggesting the role of mitochondrial pathway in curcumin induced apoptosis. Inhibition of caspase activity did not result in complete inhibition of apoptosis thereby suggesting that AIF is also involved in the observed apoptosis. It was observed that curcumin at higher doses induced repression of both p53 and p73 in all the three cell lines.

Materials and methods

Cell culture and chemicals

HeLa, SiHa and Ca Ski cells were obtained from National centre for cell sciences, Pune and were maintained in Dulbecco's modified Eagle's medium and RPMI1640 (Sigma, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone), antibiotics, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Antibodies against p53, p73, pERK, pJNK, Bcl-XL, Bax, Ras, c-Myc, Cyclin D1, AIF, iNOS, COX-2, cytochrome c and Hsp 70 as well as secondary AP conjugated antibodies were obtained from Santa Cruz, USA. Curcumin was obtained from Sigma, USA.

Flow cytometery

Cells (1×10^4 cells) were treated with 50 and 100 μM curcumin for 24 h and then harvested. Cells were fixed in 70% ethanol and left overnight at −20°C. Cells were then washed with PBS and incubated in staining solution (20 μg/ml propidium iodide, 50 μg/ml RNase, 0.1% Triton X-100 and 0.1 mM EDTA) for 2 h at 4°C in dark. The DNA content of the cells was measured by flow cytometer (Becton Dickenson, USA) using Diva software.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

Apoptotic cells were visualized by the Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique using the Dead End Colorimetric Cell Death Detection kit (Promega Inc, USA) as described earlier [10]. The apoptotic index (AI) was determined by microscopic examination of randomly selected fields containing at least 500 cells.

Assay of telomerase activity

This was measured using the PCR-ELISA kit (Roche Molecular Biochemicals, Germany). The samples were lysed and an aliquot containing 2 μg protein was used for the assay. Telomerase positive embryonic kidney cell line (HEK-293) was used as positive control while heat inactivated HeLa extract was used as negative control. The telomerase activity was detected and expressed as relative units (RU) [11].

Western blot analysis

The level of expression of various proteins was determined in control and treated cells by Western blotting as described previously [12]. Briefly, cells were washed twice in PBS and lysed in RIPA lysis buffer. Total protein was determined by the Bradford assay. Equal amount of protein was loaded and run on 10–15% SDS-polyacrylamide gel and the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA, followed by hybridization with respective primary and secondary antibody. Final detection was performed with BCIP/NBT substrate (Promega, USA). The bands were analyzed and quantified using α image scanner densitometer (Alpha innotech, USA) and normalized with β actin control. The density of control was taken as 1 and results of treatment were expressed in relation to the control as relative unit (RU).

Mitochondrial cytochrome c release

For cytochrome c determination, cytosolic and mitochondrial fractions were obtained using differential centrifugation. Fractions were loaded on 12% SDS PAGE gel and cytochrome c quantification in each fraction was done by Western blotting as described earlier [12].

Assay of caspase-3, -9 activities

Caspases-3 and -9 activity were measured by the direct assay for caspase enzyme activity in the cell lysate using synthetic fluorogenic substrate (Ac-DEVD-AFC; substrate for caspase 3; MBL Bioscience, USA; Ac-LEHD-AFC, substrate for caspase 9; Genotech, USA) as described by the manufacturer. Amount of fluorogenic AMC/AFC moiety released was measured using a spectrofluorimeter

(ex. 380 nm, em. 420–460 nm for caspase-3; ex. 400 nm, em. 490–520 nm for caspase-9). The results are expressed in arbitrary fluorescence units/mg protein [12].

Caspase-3 and -9 inhibition assay

Caspase-3 and -9 inhibition was done to check the activation of AIF under these conditions. Caspase-3 (Z-DEVD-FMK) and -9 (Z-LEHD-FMK) inhibitors were used and caspase-3 and -9 activity assay was performed according to the manufacturer's instructions (G Biosciences, USA). Briefly, cells were plated in a six well plate and incubated for 1 h in the respective media containing caspase-3 and -9 inhibitor, and then treated with respective doses of curcumin. Cells were processed for caspase assay, Flow cytometery and Western blotting as described above.

Statistical analysis

Results are expressed as mean of three individual experiments ± standard deviation (SD) was calculated using Microsoft excel.

Results

Curcumin induces apoptosis in cervical cancer cells

Apoptosis was measured using Flow cytometery and TUNEL assay. We observed that treatment of HeLa, SiHa and Ca Ski cells with 50 μM and 100 μM curcumin resulted in 19.40%, 33.40%; 21.50%, 36.90% and 22.80%, 37.80% apoptosis as measured by flow cytometery, respectively after 24 h (Fig. 1). The TUNEL data was in agreement with the Flow cytometry data. Apoptosis was observed using light phase contrast microscopy (Olympus CWX59) at 20× magnification (Fig. 2).

Curcumin causes distinct suppression in activity of telomerase

Telomerase has been implicated as a potential biomarker in many cancers because of its intricate relation to cell proliferation. Curcumin caused a substantial decrease in activity of telomerase in all the three cell lines as measured using PCR-ELISA. Treatment with 50 and 100 μM curcumin resulted in 47% and 62% decrease in Telomerase activity in HeLa, Treatment with 50 and 100 μM curcumin resulted in 4% and 38% decrease in Telomerase activity in SiHa while there was 44% and 81% decrease in telomerase activity after treatment with 50 and 100 μM curcumin in Ca Ski after 24 h (Fig. 3).

Curcumin induces suppression of ERK, but activation of JNK in all the three cell lines but nearly no change of Ras in SiHa and Ca Ski

It is known that increase in JNK and ERK protein expression plays an important role in apoptosis. Hence, we studied their expression by Western blotting using a phospho specific ERK and JNK antibody. Treatment of cervical cancer cells with 50 and 100 μM curcumin for 24 h resulted in a marked dose dependent decrease in phosphorylation pattern of ERK but surprisingly it caused marginal increase in JNK (Fig. 4), suggesting their involvement in curcumin mediated apoptosis. Treatment with 50 and 100 μM curcumin after 24 h resulted in 21% and 40% decrease in ERK in HeLa, Treatment with 50 and 100 μM curcumin for 24 h resulted in 32% and 39% decrease in ERK in SiHa while there was 35% and 68% decrease in ERK after treatment with 50 and 100 μM curcumin in Ca Ski after 24 h (Fig. 4). Treatment with 50 and 100 μM curcumin after 24 h resulted in 14% and 20% increase in JNK in HeLa; treatment with 50 and 100 μM curcumin for 24 h resulted in 14% and 41% increase in JNK activity in SiHa while there was 21% and 42% increase in JNK after treatment with 50 and 100 μM curcumin in Ca Ski after 24 h (Fig. 4).

Ras has been implicated as an important oncoprotein in tumor initiation and progression. We observed that curcumin caused 11% and 14% decrease in Ras on treatment with 50 and 100 μM curcumin in HeLa but nearly no change in its expression in case of SiHa while no expression of Ras was seen in Ca Ski (Fig. 4).

Curcumin causes downregulation of Cyclin D1 and Hsp 70

Cyclin D1 is an important protooncogene which plays an important role in progression from G1 to S phase. Its overexpression has been linked to many types of malignancies; curcumin effectively inhibited cyclin D1 at higher dose. Treatment with 50 and 100 μM curcumin after 24 h resulted in 6% and 30% decrease in cyclin D1 in HeLa; treatment with 50 and 100 μM curcumin for 24 h resulted in 9% and 13% decrease of Cyclin D1 in SiHa while there was 16% and 24% decrease in cyclin D1 after treatment with 50 and 100 μM curcumin in Ca Ski after 24 h (Fig. 4).

Overexpression of Hsp 70 protein has been linked with many malignancies. Here we observed that treatment with 50 and 100 μM curcumin after 24 h resulted in 8% and 15% decrease in Hsp 70 in HeLa; treatment with 50 and 100 μM curcumin for 24 h resulted in 5% and 11% decrease of Hsp 70 in SiHa while there was 27% and 41% decrease in Hsp 70 after treatment with 50 and 100 μM curcumin in Ca Ski after 24 h (Fig. 4).

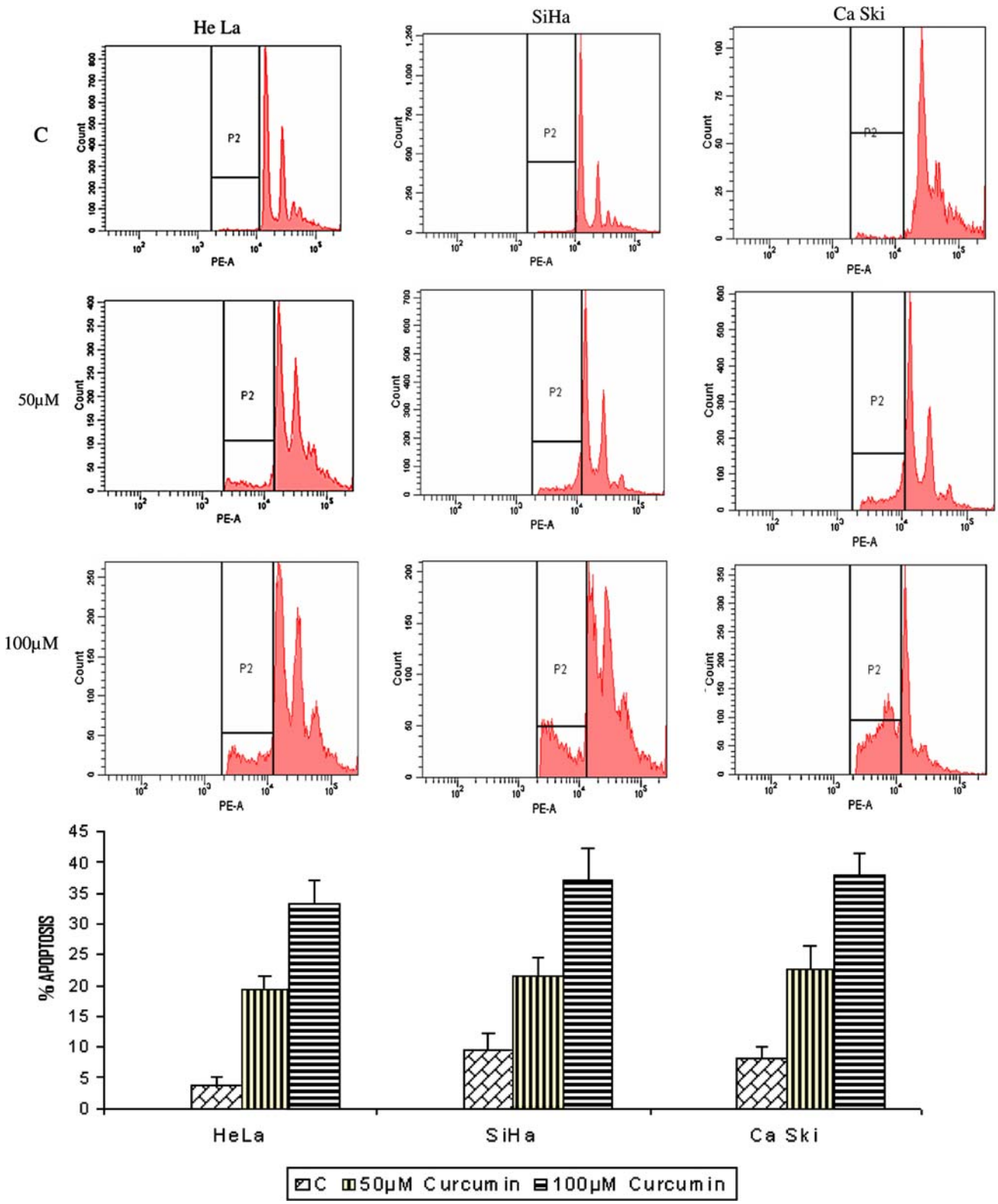

Fig. 1 Flow cytometric analysis of apoptosis in HeLa, SiHa and Ca Ski cells on treatment with 50 and 100 μM curcumin for 24 h. The percentage apoptosis shown in the bar diagram is mean ± SD of three individual experiments

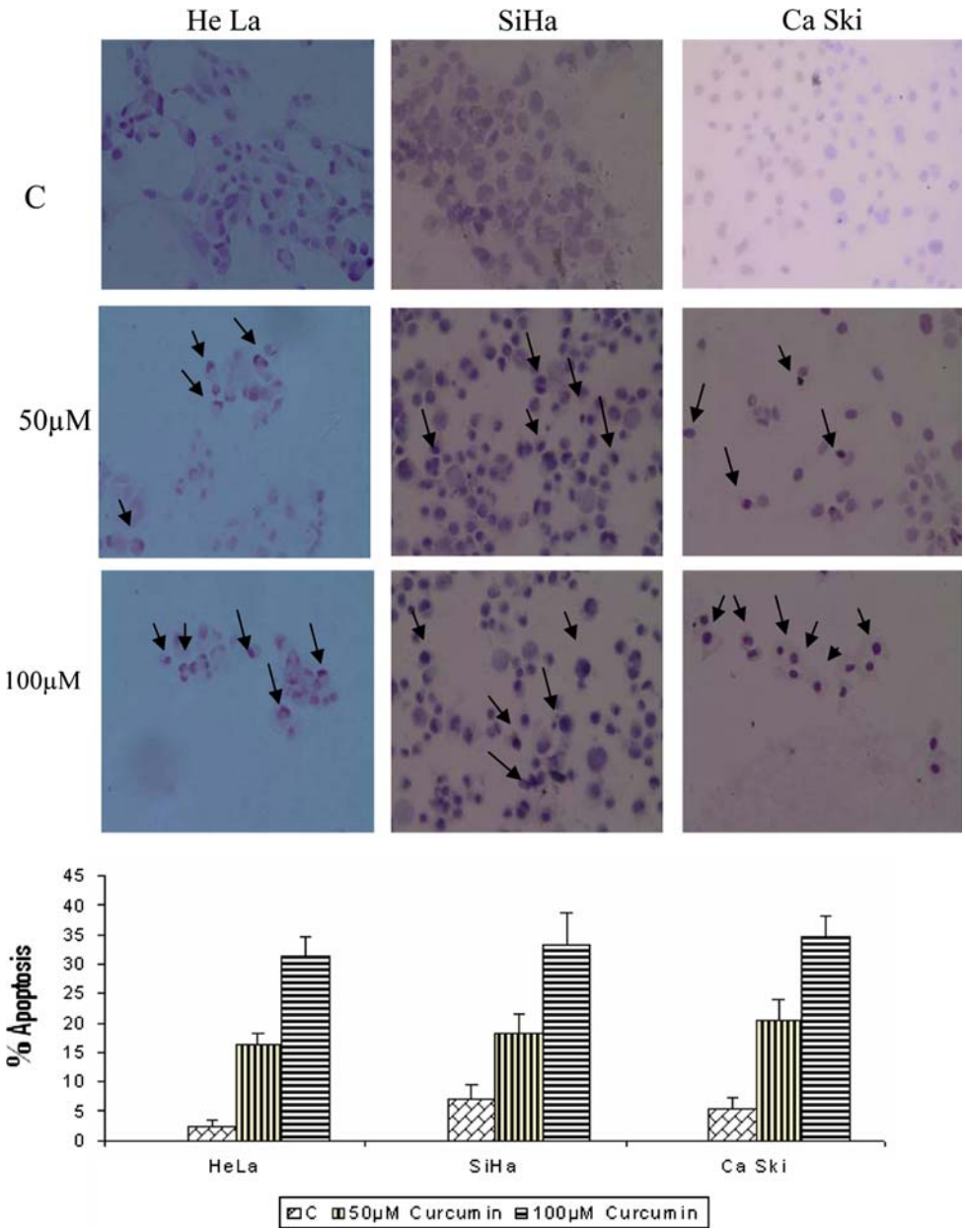

Fig. 2 TUNEL analysis of apoptosis in HeLa, SiHa and Ca Ski cells on treatment with 50 and 100 μM curcumin for 24 h. The percentage apoptosis shown in the bar diagram is mean ± SD of three individual experiments

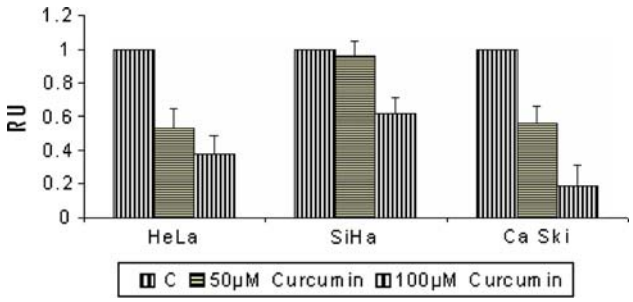

Fig. 3 Effect of 50 and 100 μM curcumin on telomerase activity in HeLa, SiHa and Ca Ski cells. The results shown are mean ± SD of three individual experiments

Curcumin resulted in dowregulation of inflammatory proteins

COX-2 and inducible form of NOS (iNOS) play an important role in carcinogenesis by increasing ROS. Curcumin effectively inhibited both these proteins at higher doses. Treatment with 50 and 100 μM curcumin after 24 h resulted in 16% and 42% decrease in COX-2 in HeLa; treatment with 50 and 100 μM curcumin for 24 h resulted in 15% and 27% decrease of COX-2 in SiHa while there was 24% and 30% decrease in COX-2 on treatment with 50 and 100 μM curcumin in Ca Ski after 24 h (Fig. 4).

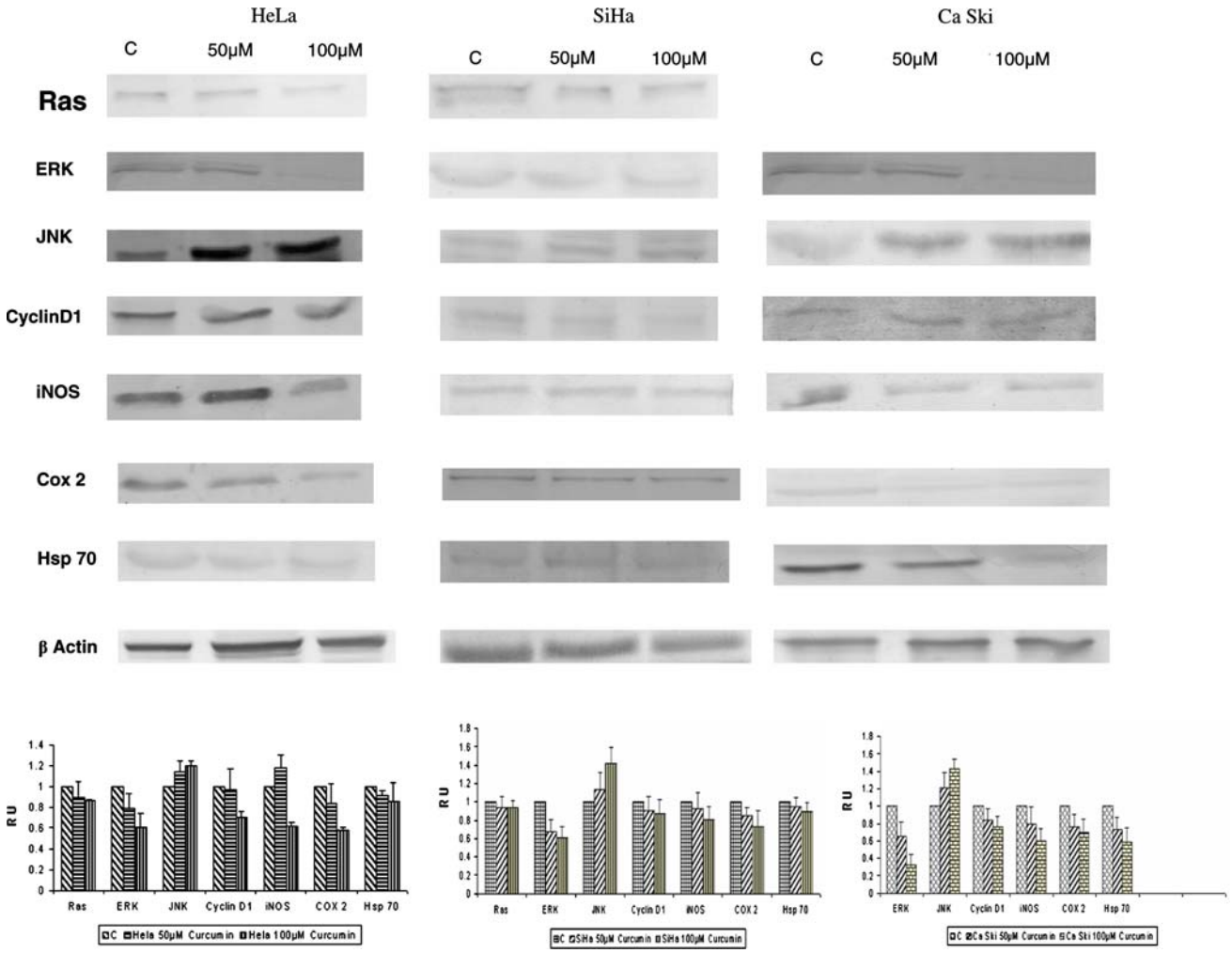

Fig. 4 Effect of 50 and 100 μM curcumin on the expression of various proteins in HeLa, SiHa and Ca Ski cells. *Lane* 1 control, *lane* 2 50 μM curcumin for 24 h, *lane* 3 100 μM curcumin for 24 h. The results shown are mean ± SD of three individual experiments

There was an initial 18% up regulation of iNOS after treatment with 50 μM curcumin followed by 38% down regulation after treatment with 100 μM curcumin in HeLa while there was a 7%, 19% and 20%, 40% decrease in expression of iNOS in case of SiHa and Ca Ski after treatment with 50 μM, 100 μM curcumin after 24 h (Fig. 4).

Curcumin decreases the p53 family of protein

The p53 family plays a central role in apoptosis with p53, p63 and p73 acting as stress sensors of the cell and triggering the activation of various pro-apoptotic genes. Western blotting was done to check the expression of p53 and p73 in response to curcumin. Surprisingly, curcumin induced the downregulation of p53 family of proteins. As shown in Fig. 5 we obtained a decrease in p53 and p73 protein expression after treatment with 50 and 100 μM curcumin after 24 h. Curcumin treatment resulted in 18%, 27% decrease in p53 expression in HeLa while in case of SiHa and Ca Ski there was an initial 45% and 32% increase in expression on treatment with 50 μM curcumin but at 100 μM dose there was 24% decrease in expression of p53 in SiHa. In case of p73, we obtained a 9%, 24% and 33%, 55% decrease in expression of p73 in SiHa and Ca Ski after treatment with 50 and 100 μM curcumin while nearly no change in expression of p73 was obtained in HeLa (Fig. 5).

Modulation of Bax, Bcl-2 and Bcl-XL in curcumin induced apoptosis in HeLa cells

We investigated the effect of curcumin on various pro-apoptotic and anti-apoptotic genes whose interplay decides the fate of cell. We monitored the protein expression of Bax, a pro-apoptotic member, Bcl-2 and Bcl-XL which are anti-apoptotic members of Bcl-2 family, following treatment of cervical cancer cells with curcumin. Curcumin treatment resulted in increase in Bax expression in a dose

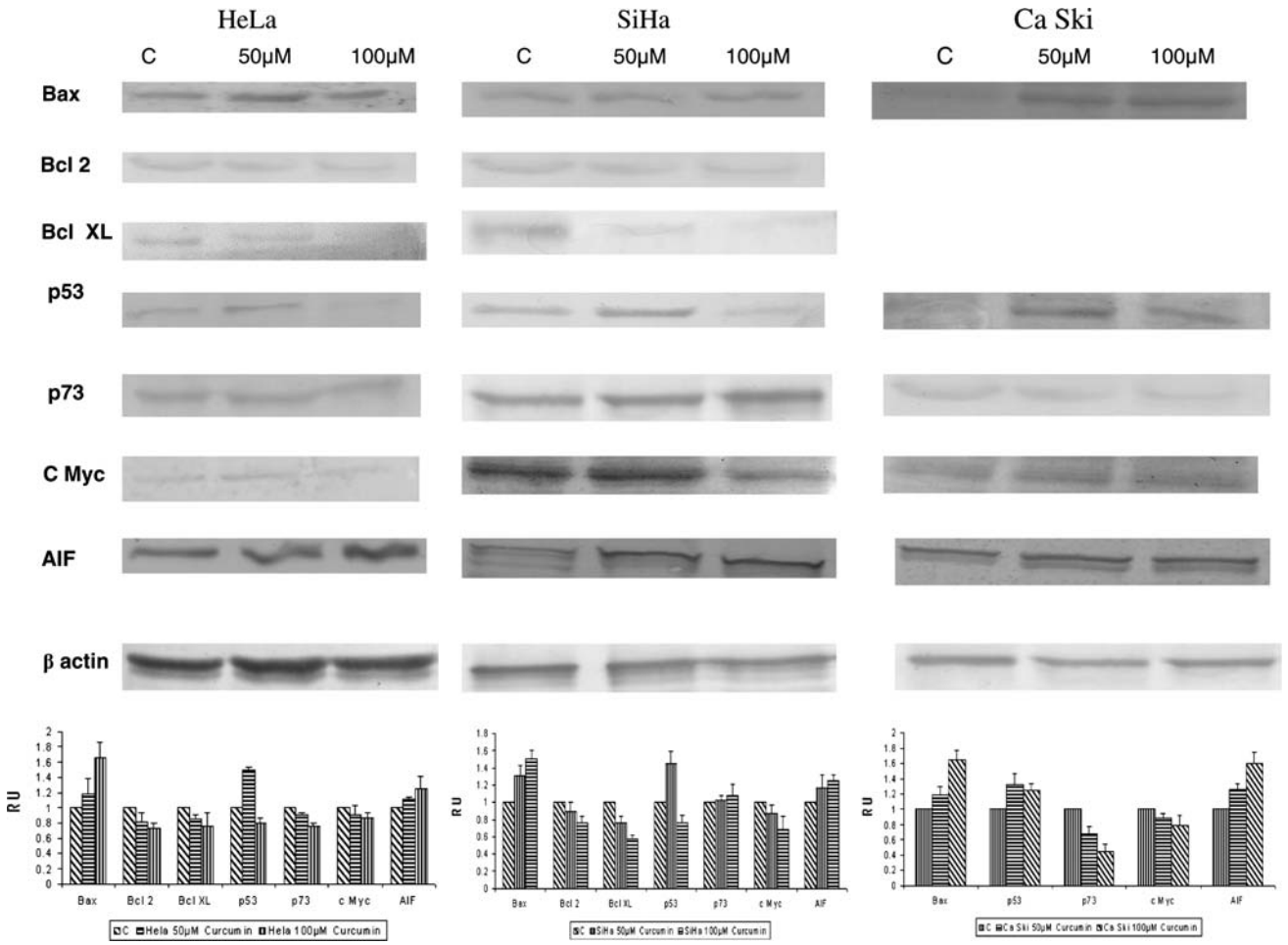

Fig. 5 Effect of 50 and 100 μM curcumin on the expression of various proteins in HeLa, SiHa and Ca Ski cells. *Lane* 1 control, *Lane* 2 50 μM curcumin for 24 h, *Lane* 3 100 μM curcumin for 24 h. The results shown are mean ± SD of three individual experiments

dependent manner in all the three cell lines. This was accompanied by a simultaneous decrease in anti-apoptotic Bcl-2 and Bcl-XL in all the three cell lines (Fig. 5).The finding indicates the involvement of Bax, Bcl-2 and Bcl-XL proteins in curcumin-induced apoptosis in HeLa, SiHa and Ca Ski cells. Treatment with 50 and 100 μM curcumin after 24 h resulted in 18% and 65% increase in Bax in HeLa; treatment with 50 μM and 100 μM curcumin for 24 h resulted in 31% and 51% increase of Bax in SiHa while there was 26% and 49% increase in Bax after treatment with 50 μM and 100 μM curcumin in Ca Ski after 24 h (Fig. 5), while a simultaneous 18% and 27% decrease in Bcl-2 expression was seen in HeLa on treatment with 50 and 100 μM curcumin after 24 h, There was an 11% and 24% decrease in expression of Bcl-2 in SiHa after treatment with 50 and 100 μM curcumin (Fig. 5), No change was seen in Ca Ski. A 15%, 24%; 24%, 42% decrease in level of Bcl-XL was obtained in HeLa and SiHa after treatment with 50 and 100 μM curcumin for 24 h again no change in expression of Bcl-XL was obtained in Ca Ski (Fig. 5).

Curcumin decreases the protein expression of c-Myc

Expression of c-Myc transcription factor sensitizes cells to diverse apoptotic stimuli. c-Myc induces apoptosis by cooperating with other proteins like Max, Mad. The role of c-Myc protein has also been implicated in malignant transformation of cells along with another oncoprotein Ras. So we also probed the effect of curcumin on c-Myc. A slight time dependent decrease in c-Myc expression was obtained. HeLa, SiHa and Ca Ski cells which showed a 9%, 13%; 13%, 31% and 11%, 21% decrease in c-Myc expression was obtained after treatment with 50 and 100 μM curcumin for 24 h (Fig. 5).

Curcumin causes activation of AIF in HeLa, SiHa and Ca Ski but release of cytochrome c in HeLa and SiHa

AIF when activated and released from mitochondrion is translocated to nucleus where it causes degradation of DNA. Curcumin causes activation of AIF in a dose

dependent manner. Treatment of cervical cancer cells HeLa, SiHa and Ca Ski with 50 and 100 μM curcumin for 24 h resulted in 11%, 25%; 17%, 25% and 27%, 60% increase in AIF (Fig. 5). Cytochrome c release from mitochondria is a critical step in the apoptotic cascade as this activates downstream caspases. To examine the release of cytochrome c in curcumin treated cervical cancer cells, we conducted Western blotting using anti cytochrome c antibody in both the cytosolic and mitochondrial fractions. These experiments demonstrate that there is release of cytochrome c in the cytosol with 1.1- and 1.5-fold increase in cytochrome c level in cytosol after treatment with 50, 100 μM curcumin for 24 h with a simultaneous 0.48-fold decrease in its level in mitochondrial fraction in HeLa (Fig. 6). In case of SiHa, there was a 1.7-and 1.8-fold increase in cytochrome c level in cytosol after treatment with 50 μM, 100 μM curcumin for 24 h with a simultaneous 0.5-fold decrease in its level in mitochondrial fraction (Fig. 6). However no cytochrome c release in Ca Ski was seen.

Caspases-9 and -3 mediate curcumin induced apoptosis

Activation of caspases is a key event during apoptosis caused by various toxic agents. To confirm whether caspases are involved in apoptosis we measured the activity of caspase-9 and -3. As shown in Fig. 6, a time dependent increase in activity of caspase-9 and caspase-3 was observed in curcumin treated cervical cancer cells. There was a significant increase in caspase-9 and -3 activity after 24 h suggesting that curcumin-induced apoptosis in cervical cancer cells is by activation of caspases. There was a 0.7-, 1.5-fold; 1.7-, 4-fold and 4.7-, 9.8-fold increase in caspase-3 activity after treatment with 50 and 100 μM in HeLa, SiHa and Ca Ski. There was a 0.4-, 0.6-fold; 1.17-, 3.37-fold and 1.24-, 2.34-fold increase in caspase-9 activity after treatment with 50 μM and 100 μM curcumin in HeLa, SiHa and Ca Ski (Fig. 7). There was no change in caspase-8 activity after treatment of cervical cancer cell lines with curcumin.

Inhibition of caspase-3 and -9 did not result in complete inhibition of apoptosis

AIF release has been implicated as a caspase independent mechanism of cell death. Since we observed activation of AIF in response to curcumin, hence we were interested in seeing whether caspase-3 and -9 inhibition would result in inhibition of apoptosis or not. We observed that the treatment of HeLa cells with caspase-3 and -9 inhibitors followed by 50 and 100 μM curcumin treatment for 24 h resulted in 11.2%, 18.50% apoptosis; 9.50%, 15.80% for SiHa and 12.60%, 21.20% for Ca Ski as measured by flow cytometery (Fig. 8). It was observed that the caspase inhibitors were causing inhibition of caspase-3 and -9 activity in all the three cell lines (Fig. 9a). AIF activation was seen under these conditions thereby confirming the involvement of AIF also in curcumin mediated apoptosis (Fig. 9b).

Discussion

Results from this study clearly show that curcumin has selective modulatory effect on proliferation, apoptosis and signaling pathways in HPV positive cervical cancer cell lines HeLa, SiHa and Ca Ski. It induced cytotoxicity in these cells by inhibition of growth and induction of apoptosis. It has been reported previously that curcumin causes suppression of NFκB and viral oncogenes E6 and E7 in cervical carcinoma cell lines [13]. In the present study we investigated the mechanism by which curcumin manifests its effect on telomerase and apoptotic cascades.

Most human cancers have short telomeres and express high levels of telomerase activity as compared to normal tissue [14]. Telomerase has been shown in several studies to be a potentially sensitive biomarker for early cancer screening [9]. Telomerase is an enzyme comprising of template RNA (hTR) and catalytic protein component (hTERT). The catalytic protein of telomerase RNP, hTERT is critical in production of telomerase activity [14]. In the present study, we investigated activity of catalytic core of telomerase using PCR-ELISA. Cervical carcinoma cells have been shown to possess high level of telomerase protein [9]. We demonstrate here that curcumin directly inhibits activity of telomerase in a dose-dependent manner in all the cell lines, though the amount of inhibition varied from cell line to cell line, with Ca Ski showing maximum sensitivity and SiHa showing minimum sensitivity.

The importance of MEK/ERK signaling pathway in cell survival has been demonstrated in a number of cell lines [15]. It appears that for most of these cell lines survival and regrowth after prolonged media starvation was correlated with presence of phospho ERK. In the present study, substantial inhibition of ERK activity was achieved at higher doses of 100 μM curcumin in all the three cell lines with decrease in active phosphorylated form of ERK in a dose-dependent manner with Ca Ski showing maximum inhibition of ERK at 100 μM dose which indicated that curcumin is targeting the MAPK/ERK pathway. An increase in expression of JNK was observed in all the three cell lines. While activation of ERK pathway has generally been linked to proliferation response, the physiological role of JNK is less well defined. Depending on the circumstances JNK has been shown to trigger apoptosis, proliferation, as well as differentiation [16]. Curcumin has been reported to

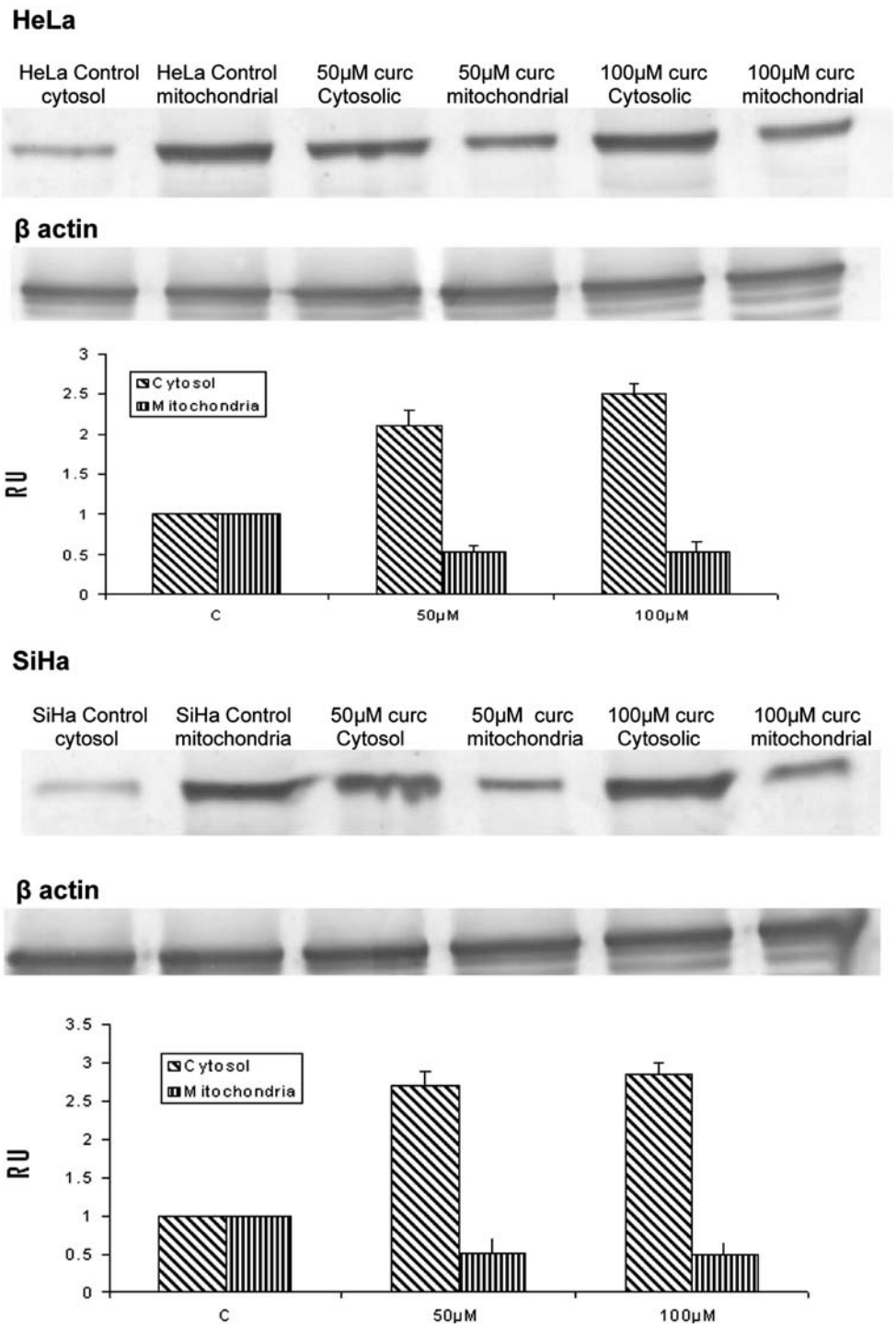

Fig. 6 Effect of 50 and 100 μM curcumin on release of cytochrome c in HeLa and SiHa. The results shown are mean ± SD of three individual experiments

be the inhibitor of JNK pathway in several cell systems [17] but in this study we observed about 50% increase in JNK in SiHa and Ca Ski, however the involvement of JNK in this cell system might not be relevant to the growth arrest and apoptosis observed. Curcumin marginally reduces the activity of Ras protein, an upstream target of Ras/Raf pathway in a dose-dependent manner suggesting a possible cross talk between Ras and MAPK pathway. Curcumin may be downregulating the downstream targets of Ras/Raf pathway via its direct effect on Ras oncoprotein.

The cyclin D1 protooncogene is an important regulator of cell progression from G1 to S phase in many cell types [18]. Together with its binding partner cyclin-dependent kinase 4 and 6 (CDK4 and CDK6) cyclin D1 forms an active complex Cyclin D1 is important for development and progression of several cancers including those of breast, oesophagus, bladder and lung [19]. Overexpression

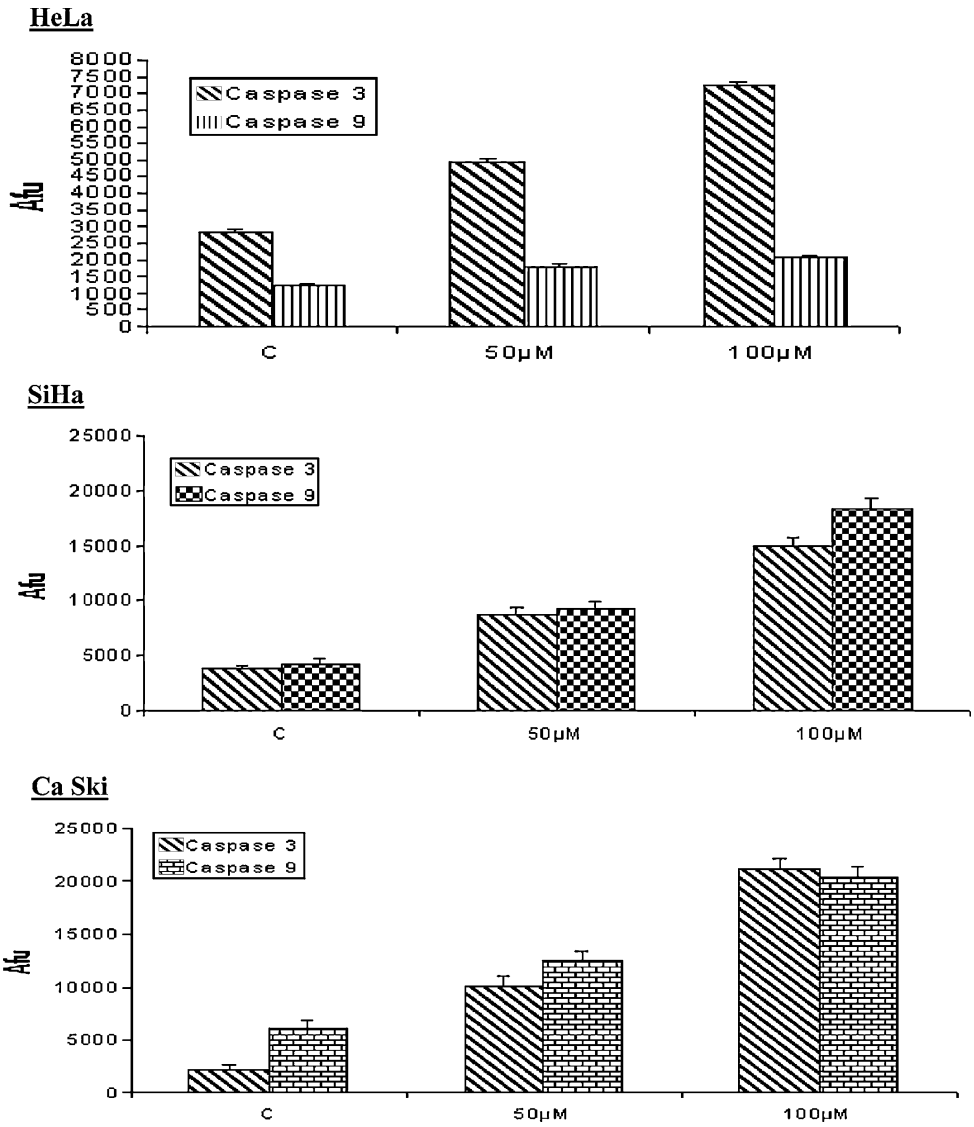

Fig. 7 Caspase-3 and -9 activity after treatment with 50 and 100 μM curcumin (Arbitrary fluorescence unit, Afu) in HeLa SiHa and Ca Ski. The results are mean ± SD of three individual experiments

of cyclin D1 has been linked to the development of endocrine resistance in breast cancer cells [20].Curcumin has been shown to inhibit Cyclin D1 in prostate cancer cells and various breast cancer cell lines [19, 20]. In the present study, curcumin inhibited the activity of cyclin D1 in a dose dependent manner to the same extent in all the three cell lines and this effect is perhaps mediated through the inhibition of Ras/ERK pathway.

c-Myc is able to repress or activate transcription. The proposed target genes of c-Myc are numerous. c-Myc is one of the most important known regulators of cell cycle progression, its expression alone can push the quiescent cell into DNA synthesis phase of cell cycle, thus it has been implicated in malignant transformation [21]. We obtained a dose dependent decrease in c-Myc expression after treatment with curcumin in all the three cell lines thereby suggesting that curcumin efficiently represses the activity of this protooncogene, HPV 16 positive cell lines SiHa and Ca Ski showed greater sensitivity as compared to HPV 18 positive HeLa cells.

Heat shock proteins (Hsp) molecular chaperones are a class of proteins that interact with diverse protein substrates to assist in their folding and play a critical role during cell stress to prevent the appearance of folding intermediates that lead to misfolded or otherwise damaged molecules [22].They are transiently expressed during cell cycle to prevent differentiating cells from undergoing apoptosis. Many tumor cells have constitutively elevated level of Hsp 70 that impart protection against cytotoxic agents and block apoptosis. Curcumin decreases the level of Hsp 70 in all the three cell lines with Ca Ski showing maximum sensitivity to curcumin thereby exposing the cells to programmed cell death and thus these cells are able to escape the protective function of Hsp 70 on treatment with curcumin.

COX-2, the inducible form of cyclooxygenase, catalyzes the rate limiting step in prostaglandin synthesis from

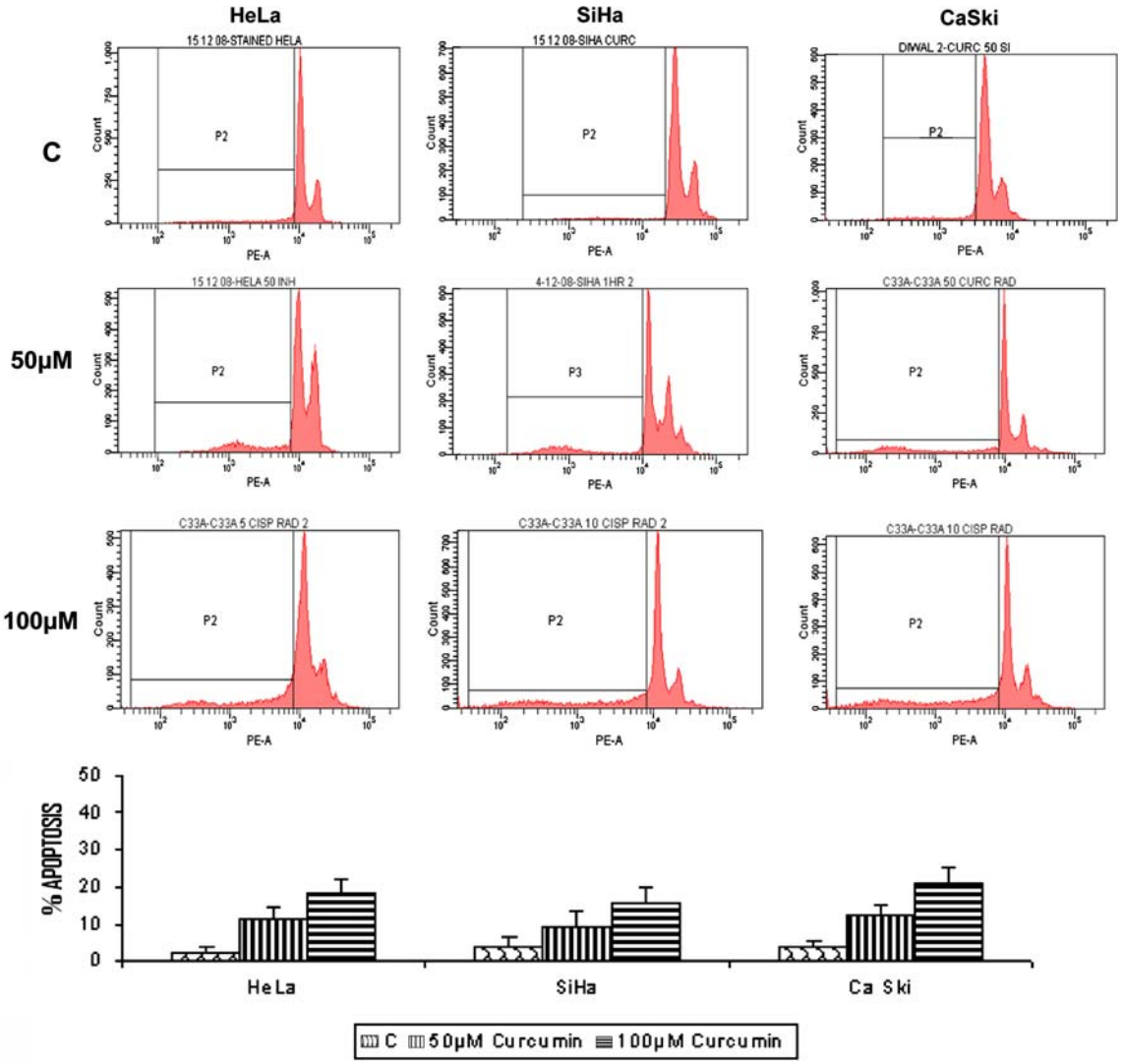

Fig. 8 Flow cytometric analysis of apoptosis in HeLa, SiHa and Ca Ski cells pretreated with caspase-3 and -9 inhibitors for 1 h, followed by treatment with 50 and 100 μM curcumin for 24 h. The percentage apoptosis shown in the *bar diagram* is mean ± SD of three individual experiments

arachidonic acid and plays an important role in cancer. Several lines of evidence suggest critical role of COX-2 in carcinogenesis as a well-established tumor promoter. Overexpression of COX-2 leads to malignant cell proliferation and invasion. This effect can be reversed by non steroidal anti-inflammatory agents, elucidating the importance of COX-2 inhibitors in cancer chemotherapeutics [23]. Curcumin has been shown to decrease the proliferation of cancers of colon, blood, submandibular gland and the liver [24] by down regulating COX-2. Cervical carcinoma is the site of excessive inflammation with COX-2 playing an active role in inflammation. Curcumin efficiently inhibited the activity of COX-2 in all the three cell lines with the level of inhibition varying from one cell line to another. Nitric oxide synthase (NOS) is another enzyme which plays an important role in inflammation due to release of Nitric oxide a highly reactive unstable radical, its inducible form (iNOS) has been implicated in carcinogenesis. Curcumin substantially inhibited the activity of iNOS at the higher dose of 100 μM in all the three cell lines. The collective inhibition of COX-2 and iNOS by curcumin establishes it as an important anti-inflammatory agent and its importance in conditions like cervical cancer which are associated with excessive inflammation.

Mitochondria have been shown to play a critical role in apoptosis. Number of stimuli including chemotherapeutic agents, UV radiation, stress molecules (reactive oxygen species, reactive nitrogen species) and growth factor withdrawal appear to mediate apoptosis via mitochondrial pathway, by release of factors like cytochrome c, AIF which are associated with permeabilization of mitochondrial membrane [25, 26]. Curcumin has been shown to

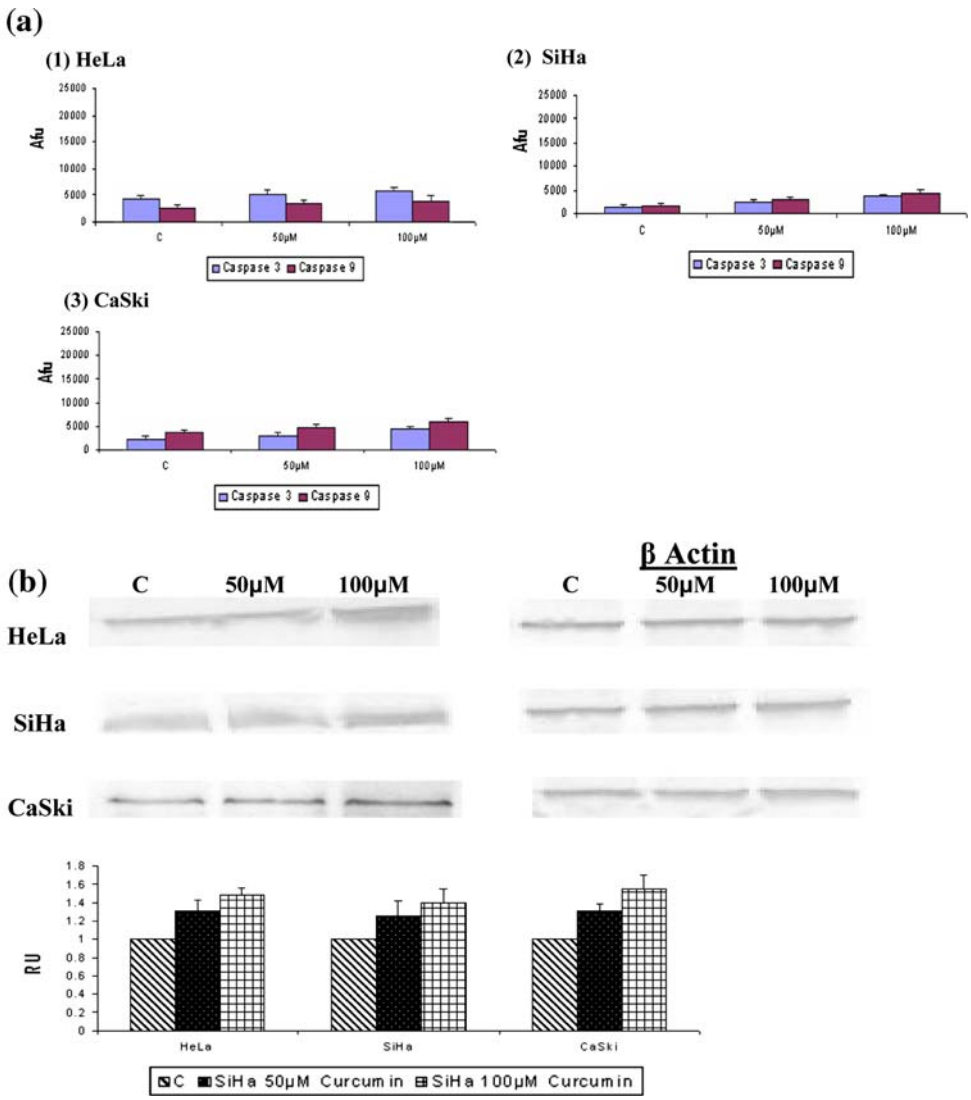

Fig. 9 **a** Effect of caspase-3 and -9 inhibitors on caspase-3 and -9 activity after treatment with 50 and 100 μM curcumin in HeLa, SiHa and Ca Ski cells. **b** Effect of caspase-3 and -9 inhibitiors on the expression of AIF in HeLa, SiHa and Ca Ski cells. *Lane* 1 control, *Lane* 2 treatment with 50 μM curcumin for 24 h, *Lane* 3 treatment with 100 μM curcumin for 24 h. The results shown are mean ± SD of three individual experiments

induce apoptosis via cytochrome c release in caki cells. Our findings that curcumin induced release of AIF in all the three cell lines but of cytochrome c in HeLa, SiHa but not Ca Ski from mitochondria to cytosol provides a direct link between mitochondria and curcumin induced apoptosis in cervical cancer cells. Members of Bcl-2 family of proteins have been associated with the mitochondrial membrane and regulation of its integrity [27] Our findings that anti-apoptotic Bcl-2 and Bcl-XL decreased in response to curcumin, suggests that curcumin decreased the expression of antiapoptotic proteins while simultaneously increasing the activity of proapoptotic protein Bax in all the three cell lines. Bax has been shown to exert its proapoptotic activity by its translocation from cytosol to mitochondria, whereas Bcl-2 exerts its antiapoptotic effect at least in part by inhibiting the translocation of Bax to mitochondria [27]. Apoptosis in cervical cancer cells appears to be mediated by activation of caspase-3 and -9 but not of caspase-8. Inhibition of caspase-3 and -9 activity did not result in complete inhibition of apoptosis in all the three cell lines indicating that cells are undergoing apoptosis via caspase independent mechanism as well. AIF release was seen under these conditions thereby indicating that AIF activation is also a potential mechanism of curcumin induced cytotoxicity in cervical cancer cell lines. Our data suggests that at higher doses curcumin inhibits both the activity of p53 and p73. There have been similar reports in colon cancer cell lines [28].

In a nutshell, our data suggests that the molecular mechanism of curcumin induced cytotoxicity in cervical cancer cells possess multiple targets including inhibition of telomerase, inhibition of Ras, ERK pathway finally resulting in inhibition of cyclin D1, c-Myc, Hsp 70, activation of AIF, release of cytochrome c and triggering apoptosis via mitochondrial pathway. A simultaneous decrease in antiapoptotic proteins and increase in proapoptotic proteins ultimately results in activation of caspase-3 and -9.

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