BIOCHEMICAL PHARMACOLOGY 73 (2007) 1024–1032

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Curcumin induces the degradation of cyclin E expression through ubiquitin-dependent pathway and up-regulates cyclin-dependent kinase inhibitors p21 and p27 in multiple human tumor cell lines

Bharat B. Aggarwal, Sanjeev Banerjee, Uddalak Bharadwaj, Bokyung Sung, Shishir Shishodia, Gautam Sethi*

Cytokine Research Laboratory, Department of Experimental Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, TX, United States

ARTICLE INFO

Article history:
Received 25 October 2006
Accepted 11 December 2006

Keywords:
Curcumin
Cyclin E
Proteasome
p21
p27
p53

ABSTRACT

Curcumin, a well-known chemopreventive agent, has been shown to suppress the proliferation of a wide variety of tumor cells through a mechanism that is not fully understood. Cyclin E, a proto-oncogene that is overexpressed in many human cancers, mediates the G_1 to S transition, is a potential target of curcumin. We demonstrate in this report a dose- and time-dependent down-regulation of expression of cyclin E by curcumin that correlates with the decrease in the proliferation of human prostate and breast cancer cells. The suppression of cyclin E expression was not cell type dependent as down-regulation occurred in estrogen-positive and -negative breast cancer cells, androgen-dependent and -independent prostate cancer cells, leukemia and lymphoma cells, head and neck carcinoma cells, and lung cancer cells. Curcumin-induced down-regulation of cyclin E was reversed by proteasome inhibitors, lactacystin and *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal, suggesting the role of ubiquitin-dependent proteasomal pathway. We found that curcumin enhanced the expression of tumor cyclin-dependent kinase (CDK) inhibitors p21 and p27 as well as tumor suppressor protein p53 but suppressed the expression of retinoblastoma protein. Curcumin also induced the accumulation of the cells in G1 phase of the cell cycle. Overall, our results suggest that proteasome-mediated down-regulation of cyclin E and up-regulation of CDK inhibitors may contribute to the antiproliferative effects of curcumin against various tumors.

1. Introduction

Curcumin, a diferuloylmethane present in *Curcuma longa* (also called turmeric or yellow coloring agent in curry powder), has been shown to inhibit cell proliferation in a wide variety of human cancer cell lines *in vitro* [1] and in various xenotransplant and orthotopic models of human cancer in rodents [1,2]. Curcumin is currently in clinical trials as a treatment for numerous cancers including multiple myeloma, pancreatic cancer, and colon cancer [1]. How curcumin mediates its

* *Corresponding author*. Tel.: +1 713 792 3503/6459; fax: +1 713 794 1613.
E-mail address: aggarwal@mdanderson.org (B.B. Aggarwal).

Abbreviations: CDK, cyclin-dependent kinase; ALLN, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal; Rb, retinoblastoma; FBS, fetal bovine serum

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doi:10.1016/j.bcp.2006.12.010

antiproliferative effects is incompletely understood, but the role of AKT [3], NF-κB [4,5], AP-1 [6], cyclin D1 [7], and PPAR-γ [8], has been reported. Numerous studies indicate that curcumin can regulate cell cycle [9–15], but just how it does this is unclear.

One possible mediator of curcumin's effects on the cell cycle is cyclin E, a member of a cell cycle regulatory family. First discovered in 1991, cyclins are essential for cell division and mediate their effects by interacting with the cyclin-dependent kinases (CDK) and directing their enzymes to specific substrates [16,17]. Cyclin E is one of the two types of cyclin-CDKs that regulate the transit of mammalian cells from quiescence into S phase: the D-type cyclins activate CDK4/6, whereas cyclin E activates CDK2. Cyclin E is a nuclear protein that associates with CDK2 and forms an active complex in late G_1 and directs entry into S phase. Activity of the cyclin E-Cdk2 complex is highest in G1-S cells and lowest in quiescent cells [18,19]. The complex is often deregulated in cancer cells, including breast cancer [20], colon cancer [21], gastric cancer [22], non-small cell lung cancer [23], acute myleogenous leukemia [24], and adult acute lymphoblastic leukemia cells [25], among others [26]. Furthermore, cyclin E expression has also been found to be prognostic marker for breast cancer [27], colorectal cancer [28], ovarian cancer [29] and gastric cancer [30]. Its over expression has recently been linked with diminished survival of patients with breast cancer [31]. Deregulated cyclin induces malignant transformation [32] and chromosome instability [33], predicts metastasis and survival [23] and modulates infiltrative behavior of breast cancer [34]. These activities suggest that cyclin E is a potential therapeutic target for cancer therapy [35].

Based on all these considerations, we hypothesized that curcumin exercises its antiproliferative activity at least partly through cyclin E. The present study investigates the effects of curcumin on cyclin E and CDK inhibitors *in vitro*. The results indicate that curcumin is potent regulator of cyclin E expression. It induces the degradation of cyclin E and up-regulates the expression of p21 and p27, which are key cell cycle regulatory proteins.

2. Materials and methods

2.1. Reagents

Curcumin, with purity ≥95%, was obtained from LKT (St. Paul, MN). Penicillin, streptomycin, RPMI1640 medium, and FBS were obtained from Life Technologies (Grand Island, NY, USA). Tris, glycine, sodium chloride, sodium dodecyl sulfate (SDS), BSA, and lactacystin, were obtained from Sigma Chemical Company (St. Louis, MO). N-ALLN was obtained from Calbiochem (San Diego, CA). The polyclonal antibody anti-cyclin E against amino acids I-295, which represents full-length cyclin E of human origin, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), as were antibodies against p21, p27, p53 and retinoblastoma (Rb) protein.

2.2. Cell lines

An androgen-independent prostate cancer cell line (LNCaP), two androgen-dependent prostate cancer cell lines (DU145 and PC-3), ER+ breast cancer cell lines (MCF-7, BT-20, T-47D, ZR-75-1), and ER− breast cancer cell lines (MDA-MB-231, MDA-MB-435, MDA-MB-436, MDA-MB-468), leukemia cell lines (HDMYZ, HDLM2, L428, KMH2, RPMI-7951, MCL, HL-60, K562, KBM-5, MM1, SP-53 and Daudi), head and neck cancer cell lines (LN686, TU167, JMAR, TU686), and lung cancer cell lines (H1299, Calu 6 and U322J) were obtained from American Type Culture Collection. T-47D, MDA-MB-468, MCF-7, BT-20, and ZR-75-1 cells were grown in RPMI-1640 medium; and JMAR, TU 167, TU686, MDA-MB231, and MDA-MB436 were grown in minimum essential medium (MEM). The medium were supplemented with 10% FBS, 100 U/ml penicillin, and 100 g/ml streptomycin. The KBM5, K562 and HL-60 cell line was grown in Iscove's modified Dulbecco's medium containing 10% FBS.

2.3. Anti-proliferative assay

Cell growth assays were carried out essentially according to the procedure described [7]. Briefly, cells (5×10^3 per well) were plated in 0.1 ml medium containing 10% FBS in 96 well plates; after 24 h medium was removed and replaced with 0.2 ml medium containing the indicated concentrations of curcumin for different times. At the end of incubation, proliferation was measured by the modified tetrazolium salt-3-(4-5-dimethylthiozol-2-yl)2-5-diphenyl-tetrazolium bromide (MTT) assay. For this 0.025 ml of MTT solution (5 mg/ml in PBS) was added to each well. After a 2-h incubation at 37 °C, 0.1 mL of lysis buffer (20% SDS, 50% dimethylformamide) was added, incubation was continued overnight at 37 °C and the optical densities were measured using a 96-well multiscanner auto reader (Dynatech MR 5000), with the extraction buffer used as a blank.

2.4. Western blot analysis

To determine the expression of different proteins, whole-cell extracts were prepared from untreated or curcumin treated (2×10^6 cells/ml) in lysis buffer (20 mM Tris pH 7.4, 250 nM sodium chloride, 0.1% NP-40, 2 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 4 mM sodium orthovanadate and 1 mM dithiothreitol (DTT)), and 30 μg of the protein was resolved on 10% SDS-polyacrylamide gels. After electrophoresis, proteins were electrotransferred to nitrocellulose filters and incubated with specific antibodies against cyclin E, p21, p27, p53, and retinoblastoma (Rb) proteins. Proteins were detected via incubation with horseradish peroxidase-conjugated secondary antibodies and visualized using the enhanced chemiluminescence detection system (ECL, Amersham). To assure equal loading, gels were stripped and reprobed with antibody against β-actin.

2.5. Flow cytometric analysis

To determine the effect of curcumin on the cell cycle, MCF-7 cells were treated with 25 μM curcumin for 36 h, 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 Cell Quest acquisition and analysis programs (Becton Dickinson, San Jose, CA). Gating was set to exclude cell debris, cell doublets, and cell clumps.

3. Results

In the present study, we examined the effect of curcumin on regulation of proliferation through modulation of expression of cyclin E in a wide variety of human tumor cell lines. For most studies, we used the androgen-independent prostate cancer cell line LNCaP and the estrogen receptor positive breast cancer cell line MCF-7 as representative cell types.

3.1. *Curcumin inhibits the proliferation of human breast, prostate, and head and neck cancer cell lines*

Human breast (MCF-7), prostate (LNCaP), and head and neck (Tu-167, and JMAR) cancer cells were exposed to different concentrations of curcumin and their proliferation determined over 2, 4 and 6 days after exposure to the drug. Curcumin inhibited the proliferation of all the four cell lines (Fig. 1). The anti-proliferative effects of curcumin were both dose and time dependent.

3.2. *Curcumin down-regulates the expression of cyclin E in breast and prostate cancer cell lines*

Whether curcumin manifests its antiproliferative effects through the regulation of cyclin E was examined. MCF-7 and LNCaP cells were treated with concentrations of curcumin ranging from 0 to 100 μM for 3 h and then examined for

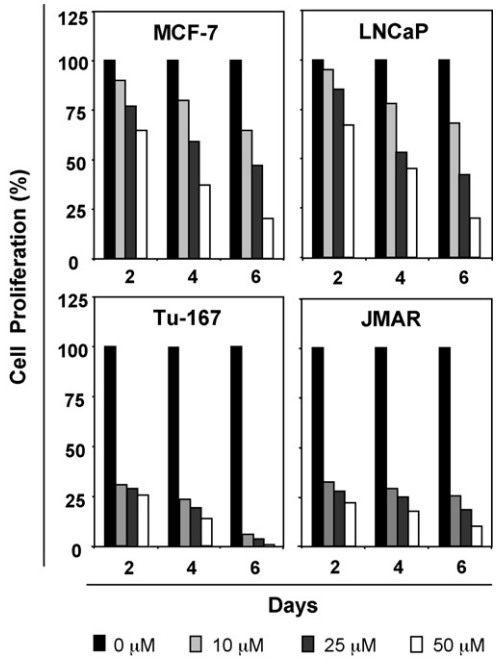

Fig. 1 – Curcumin inhibits the proliferation of human breast MCF-7, prostate LNCaP, and head and neck Tu-167, and JMAR cancer cells. 2×10^3 cells were plated in triplicate in 0.1 ml medium overnight in 96-well plates; after 12 h the indicated concentrations of curcumin were added. Cell proliferation was assessed on days 2, 4 and 6 by MTT method as described under Section 2. The variation between the triplicates was less than 10%.

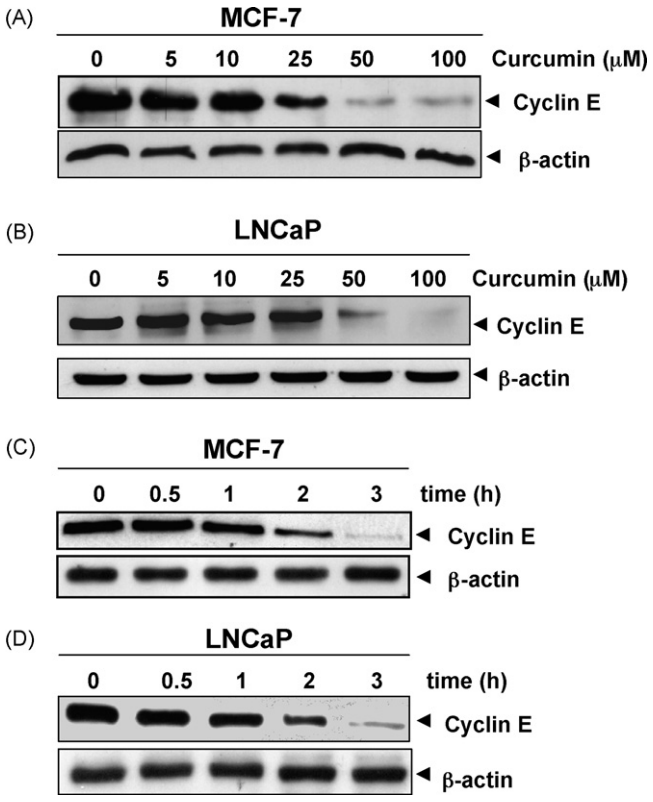

Fig. 2 – Dose-dependent down-regulation of cyclin E in MCF-7 (A) and LNCaP (B) cells. 2×10^6 cells/ml were incubated with different concentrations of curcumin for 3 h and whole-cell extract was prepared. Thirty micrograms of protein was resolved on 10% SDS-PAGE, electrotransferred and probed with anti-cyclin E antibody as described in Section 2. Time-dependent down-regulation of cyclin E in MCF-7 (C) and LNCaP (D), cells (2×10^6 cells/ml) were incubated with 50 μM of curcumin for indicated times and whole-cell extract was prepared. Thirty micrograms of protein was resolved on 10% SDS-PAGE, electrotransferred, and probed with anti-cyclin E antibody as described in Section 2.

expression of cyclin E by Western blot analysis. The results shown in Fig. 2A and B demonstrate that curcumin down-regulates the expression of cyclin E in a dose-dependent manner, with maximum effect at a concentration of 50–100 μM curcumin.

We then examined the optimum time required for the down-regulation of cyclin E and found that 3 h incubation with curcumin was sufficient for maximum suppression of cyclin E (Fig. 2C and D). β-actin controls showed that the levels of protein were unaffected under these conditions.

3.3. *Curcumin inhibits the expression of cyclin E in both ER+ and ER− breast cancer cell lines*

Some reports suggest that cyclin E expression is connected with ER status [36], so we tested whether curcumin modulated cyclin E levels in ER+ (MCF-7, BT-20, T-47D,

ZR-75-1) and ER– (MDA-MB-231, MDA-MB-435, MDA-MB-436, MDA-MB-436) breast cancer cell lines. All cells were exposed to 50 μM curcumin for 3 h, and then whole-cell extracts were prepared and examined for cyclin E levels. All eight breast cancer cell lines expressed cyclin E proteins and BT20 expressed the lowest level. Curcumin down-regulated the expression of cyclin E protein in almost all these breast cancer cells to variable degrees: Under identical conditions, no cyclin E protein could be detected in MCF-7, ZR-75-1, and T47D cells, and MDA-MB-231 cells after curcumin treatment (Fig. 3A). Again, β-actin levels were unaffected.

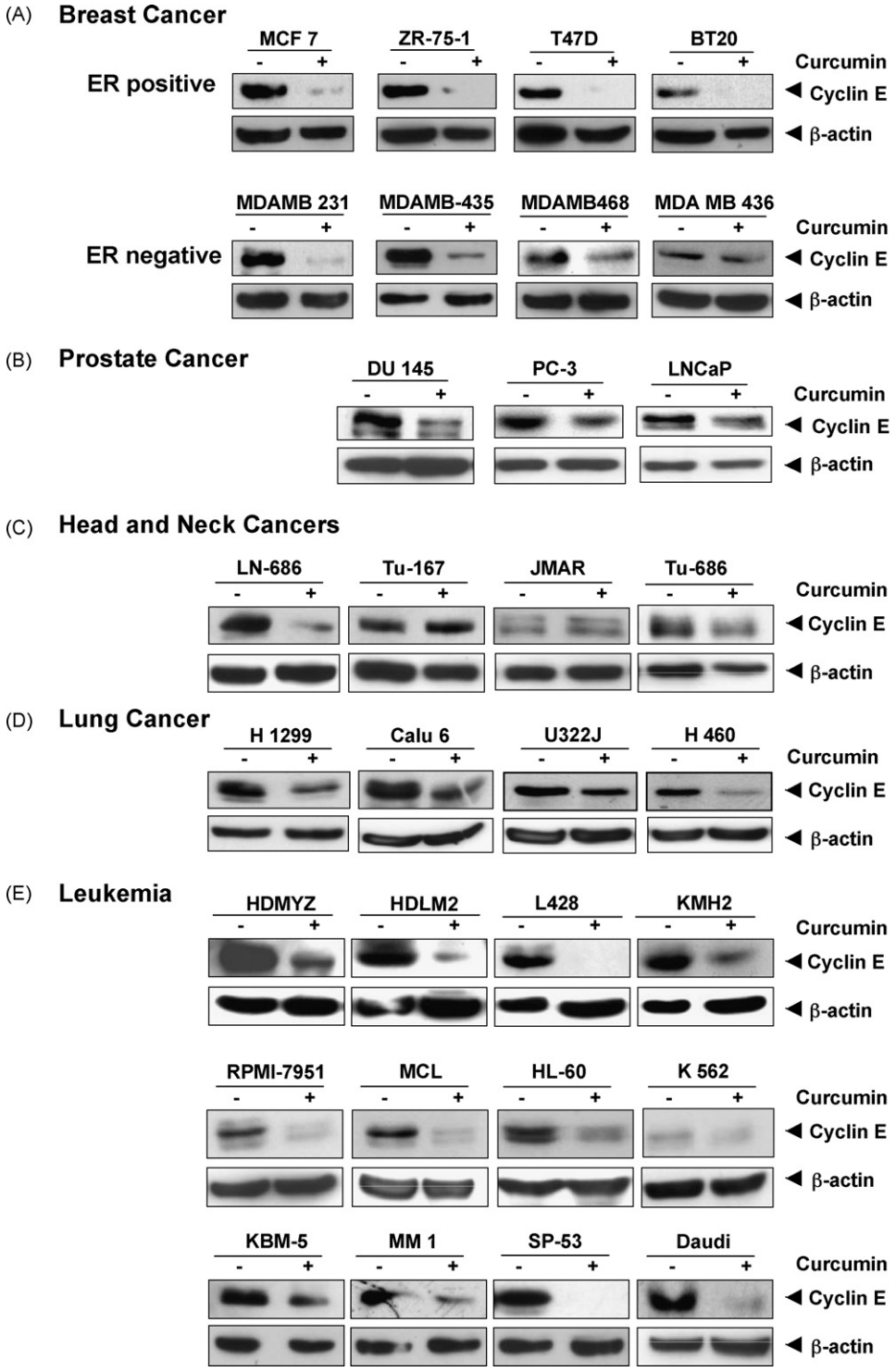

Fig. 3 – Effect of curcumin on expression of cyclin E protein in various human cancer cell lines. 2×10^6 cells/ml were plated, and after 12 h were incubated with 50 μM concentrations of curcumin. After 3 h whole-cell extracts were prepared and resolved on 10% SDS-PAGE, electrotransferred, and probed with anti-cyclin E antibody as described in Section 2.

3.4. *Curcumin inhibits the expression of cyclin E in prostate cancer cell lines*

Whether curcumin modulates the cyclin E expression in both androgen-dependent (DU145 and PC-3) and androgen-independent (LNCaP) prostate cancer cells was examined. DU 145, PC-3, and LNCap showed constitutive expression of cyclin E. Treatment with curcumin significantly inhibited the basal level of cyclin E expression in all three (Fig. 3B).

3.5. *Curcumin inhibits the expression of cyclin E in head and neck cancer cell lines*

Variable levels of constitutive cyclin E expression were evident in the human head and neck cancer cells. Curcumin effectively abrogated the basal level of cyclin E expression in both LN686 and TU-686 cells. In JMAR cells the basal level of cyclin E expression was unaffected by curcumin treatment whereas in TU-167 no substantial changes were noticeable (Fig. 3C).

3.6. *Curcumin inhibits the expression of cyclin E in lung cancer cell lines*

Cyclin E was also found to be constitutively active in all the four human lung cancer cell lines examined. Curcumin suppressed the basal level of cyclin E expression in H1299, Calu6, H322J and H460, most dramatically in H1299 and H460 (Fig. 3D).

3.7. *Curcumin inhibits the expression of cyclin E in lymphoma, myeloma and leukemic cell lines*

The efficacy of curcumin in down-regulating the expression of cyclin E in wide variety of human lymphoma, myeloma and leukemic cells (including T- and B-cell derived) was also investigated. Curcumin treatment at a dose of 50 μM for 3 h down-regulated the cyclin E to non-detectable levels in eight (HDLM2, L428, RPMI-7951, HL-60, MM1, SP-53 and Daudi cells) out of 12 cell lines investigated. With the exception of K562, where no cyclin E could be detected in the first place, curcumin significantly down-regulated the basal expression of cyclin E (Fig. 3E).

3.8. *Lactacystin and N-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN) block curcumin induced down-regulation of the cyclin E protein*

How curcumin modulates the levels of cyclin E, was further investigated. Curcumin could down-regulate cyclin E expression either by enhancing its degradation or by suppressing its synthesis. We first explored the possibility that curcumin enhances the rate of degradation. Cyclin E undergoes ubiquitin-dependent proteasomal degradation [37,38] and lactacystin and ALLN inhibits the 26S proteasome, so we examined the ability of lactacystin and ALLN to block curcumin-induced degradation of cyclin E in MCF-7 and LNCaP cells. Cells were pretreated with lactacystin for 2 h before being exposed to curcumin for 3 h. As shown in Fig. 4A, lactacystin prevented curcumin-induced degradation of cyclin E, Furthermore, curcumin's down-regulation of cyclin E through the enhancement of proteolysis was also noted when MCF-7 cells were treated with ALLN (Fig. 4B). Similar observations were made for prostate cancer LNCaP cells (Fig. 4C).

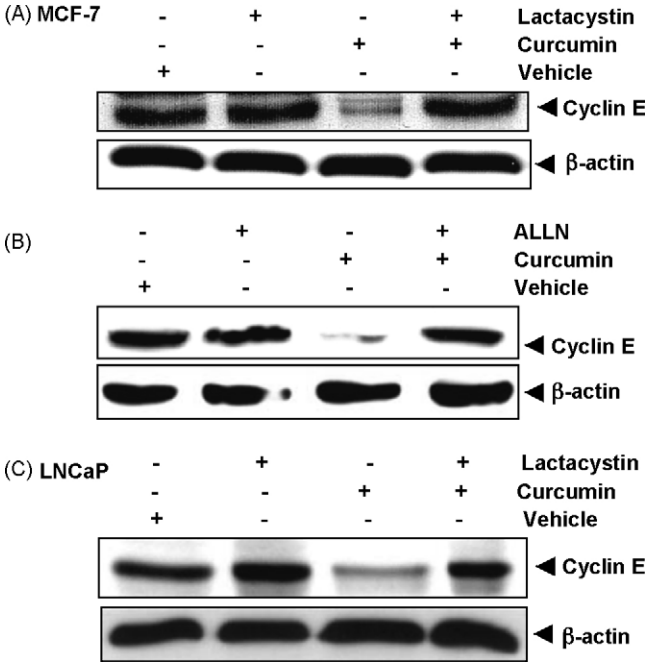

Fig. 4 – A. Proteasome inhibitors lactacystin (A) and ALLN (B) block curcumin-induced down-regulation of cyclin E protein in MCF-7 cells. 2×10^6 cells/ml were incubated with 10 μM of lactacystin or 25 μM ALLN for 2 h and then exposed to 50 μM curcumin for 3 h. Thereafter, whole-cell extracts were prepared, and 50 μg of protein was resolved on 10% PAGE, electrotransferred, and probed with anti-cyclin E antibody as described in Section 2. (C) Proteasome inhibitor lactacystin blocks curcumin-induced down-regulation of cyclin E protein in LNCaP cells. LNCaP cells (2×10^6 cells/ml) were incubated with 10 μM of lactacystin for 2 h and then exposed to 50 μM curcumin for 3 h. Thereafter, whole-cell extracts were prepared, and 30 μg of protein was resolved on 10% SDS-PAGE, electrotransferred, and probed with anti-cyclin E antibody as described in Section 2.

3.9. *Curcumin arrests cells at the G1 phase of the cell cycle*

We determined in which phase of the cell cycle curcumin arrests the cells. Flow cytometric analysis of the DNA from curcumin-treated cells showed a significant increase in the percentage of cells in the G1 phase, from 54 to 76%, and a decrease in the percentage of cells in the S phase, from 17 to 8%, within 36 h of curcumin treatment (Fig. 5). These results clearly show that curcumin also induces G1 arrest of the breast cancer cells.

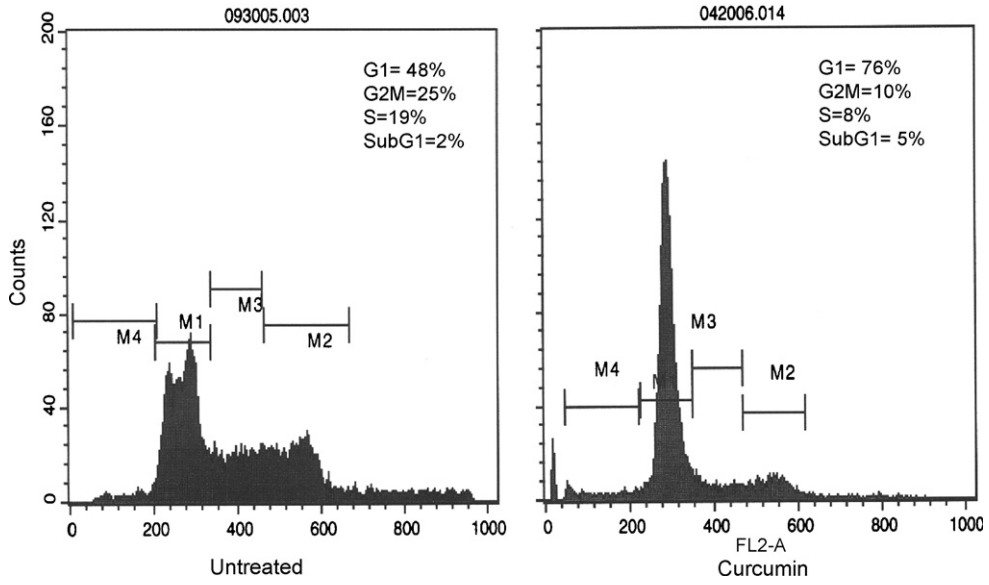

Fig. 5 – Curcumin arrests the cells at S phase of the cell cycle. Serum-starved MCF-7 (1×10^6 cells/ml) were incubated in the absence or in presence of different doses of curcumin for 36 h. Thereafter, the cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry as described in Section 2.

3.10. *Curcumin enhances the expression of p21, p27 and p53 but suppresses the expression of Rb protein in MCF-7 breast cancer cells*

We next examined the effect of curcumin on other cell cycle regulatory proteins and observed that curcumin enhanced the expression of cyclin-dependent kinase inhibitors p21 and p27 as well of tumor suppressor protein p53. Curcumin, however, inhibited the expression of Rb protein in MCF-7 cells (Fig. 6).

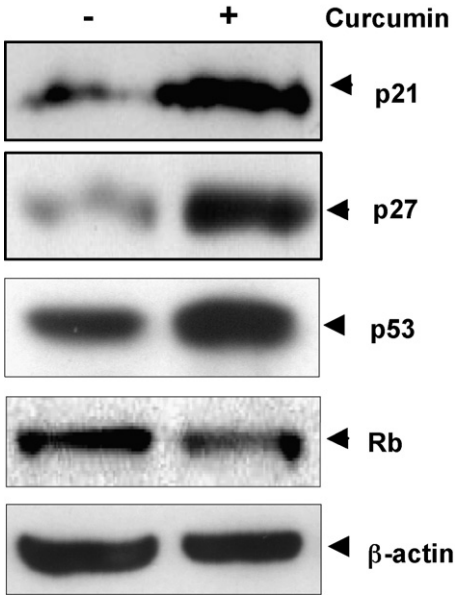

Fig. 6 – Effect of curcumin on the expression profile of p21, p27, p53 and Rb proteins in MCF-7 cells. 2×10^6 cells/ml were plated overnight and then incubated with 50 μM curcumin for 3 h. Thereafter, whole-cell extracts were prepared, and 30 μg protein was resolved on 10% PAGE, electrotransferred, and probed with anti-p21, anti-p27, anti-p53, and anti-Rb antibodies as described in Section 2.

4. Discussion

The current study was designed to investigate the mechanism by which curcumin mediates its antiproliferative effects. We specifically focused on proteins that regulate the cell cycle. Our results show that curcumin's ability to inhibit the proliferation of human prostate and breast cancer cells correlated with down-regulation of expression of cyclin E. The suppression of cyclin E expression was not cell type dependent. Curcumin-induced down-regulation of cyclin E was reversed by proteasome inhibitors lactacystin and ALLN, suggesting the role of ubiquitin-dependent proteasomal pathway. We found that curcumin enhanced the expression of CDK inhibitors p21 and p27. It also induced the expression of p53 but down-regulated the expression of Rb protein. Curcumin induced the accumulation of the cells in G1 phase of the cell cycle. In sum, these results suggest that down-regulation of cyclin E and up-regulation of CDK inhibitors contribute to the antiproliferative effects of curcumin.

This is first report to show that curcumin can induce the degradation of cyclin E. The down-regulation occurred in a wide variety of tumor cell lines. The down-regulation of cyclin E expression by both caffeine [39] and retinoic acid [40] have been reported. Previously we have shown that curcumin can also down-regulate cyclin D expression, which is mediated through both translational and post-translational modifications [7]. Cyclin D1 is regulated by NF-κB [41], and curcumin regulates the expression of cyclin D1 through suppression of NF-κB. Curcumin has also been reported to induce the degradation of $p185^{ErbB2}$ [42,43], c-Jun [44], C/EBPα and C/EBPβ [45].

How curcumin induces the degradation of cyclin E was also investigated. The pathway for the degradation of cyclin E is very well established [37,38]. Reversal of the effect of curcumin on cyclin E by proteasome inhibitors suggests the role of ubiquitin-dependent proteasome pathway. Qin et al. also showed that caffeine-induced degradation of cyclin E occurred through the ubiquitin/proteasome pathway [39]. Our results are in agreement with a recent report which showed that curcumin decreases the expression of CCAAT/enhancer binding protein (C/EBP) and this suppression could be reversed by the proteasome inhibitor MG132 [45]. Similarly, Marcu et al. found that curcumin promoted proteasome-dependent degradation of histone acetyltransferases (HAT) p300 and the closely related CBP protein without affecting the HATs PCAF or GCN5 [46].

Curcumin has been shown to be an inhibitor of the COP9 signalosome (CSN) [47]. Bech-Otschir et al. showed that inhibition of CSN-mediated phosphorylation, leads to inhibition of E6-dependent p53 degradation and accumulation of p53 in MCF-7 cells [47]. Similarly, curcumin also inhibited the ubiquitin-dependent degradation of Id1 and Id3 through inhibition of the COP9 signalosome [48]. Interestingly, Pollman et al. demonstrated that inhibition of CSN kinase activity by 50 μM curcumin for 2 h decreases the cellular c-Jun concentration, resulting in a reduction of VEGF production by approximately 75% [49]. Our results also differ from another report, which showed that apoptosis of neuronal cells is mediated through inhibition of the proteasomal pathway by curcumin [50]. How curcumin induces proteasomal activity in some systems and inhibits it in others, is not clear. Perhaps the cause is its biphasic response; at low doses (up to 1 μM for 24 h) it increases chymotrypsin-like activity but at higher concentrations (10 μM) it decreases the proteasome activity [51]. This mechanism, however, is unlikely in our system as the down-regulation of cyclin E, was observed at both low and high doses.

We found that curcumin up-regulated the expression of tumor suppressor gene p53 in MCF-7 cells. This up-regulation may be mediated through the inhibition of CSN-mediated phosphorylation, E6-dependent p53 degradation and accumulation of p53, as described previously [47]. We also found that the levels of CDK inhibitors p21 and p27 were also enhanced by curcumin. The levels of Rb, however, were decreased similar to cyclin E. Thus it is possible that curcumin activates the proteasomal pathway under some conditions and inhibits under other conditions. Moreover, deregulated expression of cyclin E has also been correlated with malignant transformation [32], chromosome instability [33], tumor progression [29], and patient survival [31]. Cyclin E has been shown to be over expressed in many cancers including breast, head and neck, prostate, lung and leukemic cell lines [20–31]. Our results with MCF-7 breast cancer cells further strengthen the link between cyclin E overexpression and curcumin-mediated inhibition of cell proliferation. These results corroborate the previous reports that curcumin suppresses the proliferation of a wide variety of tumor cells [52–56]. It is possible that the antiproliferative effect of curcumin may be attributed to dual effects on the inhibition of cyclin E and cyclin D expression; as well up-regulation of p21 and p27. Our previous published work demonstrated that curcumin indeed inhibits Rb protein phosphorylation [7].

Overall, our results provide an additional mechanism through which curcumin may mediate its antiproliferative effects, specifically the down-regulation of cyclin E and the up-regulation of p53, p21 and p27. This may lead to cell cycle arrest at G1 phase. These results suggest that curcumin, combined with its pharmacological safety, can be used to target cyclin E for cancer treatment.

Acknowledgements

We would like to thank Walter Pagel for carefully proofreading the manuscript and providing valuable comments. Dr. Aggarwal is a Ransom Horne Jr., Professor of Cancer Research. This work was supported by a grant from the Clayton Foundation for Research (to B.B.A.), National Institutes of Health PO1 grant CA91844 on lung chemoprevention (to B.B.A.), and National Institutes of Health P50 Head and Neck SPORE grant P50CA97007 (to B.B.A.).

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