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Curcumin-induced suppression of cell proliferation correlates with down-regulation of cyclin D1 expression and CDK4-mediated retinoblastoma protein phosphorylation

Asok Mukhopadhyay[1], Sanjeev Banerjee[1], Lewis Joe Stafford[2], Chunzhi Xia[2], Mingyao Liu[2] and Bharat B Aggarwal*[,1]

[1]*Cytokine Research Laboratory, Department of Bioimmunotherapy, The University of Texas MD Anderson Cancer Center, Box 143, 1515 Holcombe Boulevard, Houston, Texas, TX 77030, USA;* [2]*Center for Cancer Biology and Nutrition, Institute of Biosciences and Technology, and Department of Medical Biochemistry and Genetics, Texas A&M University System Health Sciences Center, Houston, Texas, TX 77030, USA*

Cyclin D1 is a proto-oncogene that is overexpressed in many cancers including breast and prostate. It plays a role in cell proliferation through activation of cyclin-dependent kinases. Curcumin, a diferuloylmethane, is a chemopreventive agent known to inhibit the proliferation of several breast and prostate cancer cell lines. It is possible that the effect of curcumin is mediated through the regulation of cyclin D1. In the present report we show that inhibition of the proliferation of various prostate, breast and squamous cell carcinoma cell lines by curcumin correlated with the down-regulation of the expression of cyclin D1 protein. In comparison, the down-regulation by curcumin of cyclin D2 and cyclin D3 was found only in selective cell lines. The suppression of cyclin D1 by curcumin led to inhibition of CDK4-mediated phosphorylation of retinoblastoma protein. We found that curcumin-induced down-regulation of cyclin D1 was inhibited by lactacystin, an inhibitor of 26S proteosome, suggesting that curcumin represses cyclin D1 expression by promoting proteolysis. We found that curcumin also down-regulated mRNA expression, thus suggesting transcriptional regulation. Curcumin also inhibited the activity of the cyclin D1 promoter-dependent reporter gene expression. Overall our results suggest that curcumin down-regulates cyclin D1 expression through activation of both transcriptional and post-transcriptional mechanisms, and this may contribute to the antiproliferative effects of curcumin against various cell types.
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Introduction

Cyclins, which are essential for cell division work by activating their partners, the cyclin-dependent kinases, and directing their enzymes to specific substrates. One of the subgroup of cyclins, called cyclin D, consists of three known subtypes, D1, D2, and D3, all of which collectively control cell cycle progression by activating their cyclin-dependent kinase partners, CDK4 and CDK6. These kinases then phosphorylate the retinoblastoma protein and thus advance through the G1 phase of the cell cycle (Sherr, 1995), leading to stimulation of DNA synthesis. Cyclin D1 is a nuclear protein required for cell cycle progression from G1 to S phase (Baldin *et al*., 1993).

Cyclin D1 is a proto-oncogene that is overexpressed as a result of gene amplification or translocation in many cancers, including breast, esophagus, lung, head and neck, colon and prostate (Gillett *et al*., 1994; Bartkova *et al*., 1994a,b, 1995; Dickson *et al*., 1995; Adelaide *et al*., 1995; Caputi *et al*., 1999; Nishida *et al*., 1994; Gumbiner *et al*., 1999; Drobnjak *et al*., 2000; Arber *et al*., 1996). For instance, the *cyclin D1* gene is amplified in 20–50% of squamous cell carcinoma (SCC), and its protein is overexpressed in up to 80% of SCCs (Bartkova *et al*., 1995). Overexpression of cyclin D1 is also associated with metastatic prostate cancer to bone (Gumbiner *et al*., 1999; Drobnjak *et al*., 2000). The *cyclin D1* gene is also amplified in up to 20% of human breast cancers (Dickson *et al*., 1995), while cyclin D1 protein is overexpressed in some 50% of human breast cancers (Gillett *et al*., 1994; Bartkova *et al*., 1994b) and is required for the proliferation of breast cancer cells in culture (Harari and Yarden, 2000; Wang *et al*., 1994). Transgenic mice engineered to overexpress cyclin D1 in mammary glands develop breast cancer, thus suggesting a causative role for this protein (Wang *et al*., 1994). Cyclin D1 also has been associated with aggressive forms of hepatocellular carcinoma (Nishida *et al*., 1994; Deane *et al*., 2001).

The genetic deletion of cyclin D1 in mice impaired breast development during pregnancy (Sicinski and Weinberg, 1997; Fantl *et al*., 1995, 2000; Yu *et al*., 2001), and these mice were found resistant to breast cancers induced by *ras*, *rac* and *neu* oncogenes (Albanese *et al*., 1995; Joyce *et al*., 1999; Lee *et al*., 2000). In comparison, genetic deletion of cyclin D2 and

*Correspondence: BB Aggarwal; E-mail: aggarwal@mdanderson.org
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D3 had no effect. Thus tumor cell proliferation by *ras* and *neu* in breast cancer cells is mediated through cyclin D1 only. Furthermore, *ras* and *neu* have been shown to regulate the promoter region of the cyclin D1 gene (Albanese *et al*., 1995; Lee *et al*., 2000). Thus drugs that can down-regulate cyclin D1 have potential as treatments for breast cancer and other tumors. Antisense to cyclin D1 has been used to down-regulate cyclin D1 and shown to induce apoptosis and tumor shrinkage in SCC (Sauter *et al*., 1999).

Recently it has been shown that the expression of cyclin D1 is regulated by activation of NF-κB (Guttridge *et al*., 1999; Henry *et al*., 2000), which has been shown to control cell growth and the G0/G1-to-S-phase transition (Hinz *et al*., 1999). Ras-dependent regulation of cyclin D1 transcription has been shown to occur through a NF-κB activation (Joyce *et al*., 1999). We and others have previously shown that curcumin (diferuloylmethane), a chemopreventive agent, is a potent down-regulator of NF-κB activation, most likely through the suppression of IκBα kinase (IKK) needed for NF-κB activation and it inhibits NF-κB-mediated gene expression (Singh and Aggarwal, 1995; Kumar *et al*., 1998; Plummer *et al*., 1999; Jobin *et al*., 1999). Curcumin is also known to suppress the proliferation of a wide variety of tumor cells, including breast and prostate cancer cells (Mehta *et al*., 1997; Anto *et al*., 2002; Kuo *et al*., 1996; Jiang *et al*., 1996; Ranjan *et al*., 1999; Piwocka *et al*., 1999; Han *et al*., 1999; Simon *et al*., 1998; Ramachandran and You, 1999; Bhaumik *et al*., 1999), through a mechanism which is incompletely understood. We hypothesized that curcumin must mediate its antiproliferative effects through down-regulation of cyclin D1. We demonstrate in this report that curcumin can indeed down-regulate the expression of both cyclin D1 protein and mRNA in both breast and prostate cancer lines. This leads to a decrease in cyclin D1-dependent kinase (cdk4) activity.

Results

In this report we examined the effect of curcumin on the expression of cyclin D1 in prostate and breast tumor cell lines. We used a prostate cancer cell line (LNCaP), two squamous cell carcinoma cell lines (JMAR and TU167), and two estrogen receptor (ER) positive breast cancer cell lines (MCF-7 and ZR-75-1) and three ER-negative breast cancer cell lines (MDA-MB-436, MDA-MB-231, BT-20, T47D, SKBr3, MB-468 and BT-549).

Curcumin inhibits the proliferation of breast, prostate and SCC cancer cell lines

Exposure of cells to 100 μM curcumin inhibited the proliferation of all breast tumor cancer cell lines when assayed by the MTT method (Figure 1a). The effect of curcumin was dose- and time-dependent (data not shown).

We also examined the effect of curcumin on DNA synthesis by the thymidine incorporation method. Cells were exposed to different concentrations of curcumin for 24 h and during the last 6 h were pulse treated with thymidine. As shown in Figure 1b, curcumin inhibited thymidine incorporation in a dose-dependent manner. Curcumin also inhibited the growth of SCC cell line TU167 in a dose-dependent manner (Figure 1c). Cells were found to be more sensitive to curcumin by the thymidine incorporation method than by the MTT method. The latter is known to measure mitochondrial activity. These results clearly suggest that curcumin exhibits antiproliferative effects against different tumor cells.

Curcumin down-regulates the expression of cyclin D1 in prostate cancer cell lines

The transition of cells from G1 to S is in part regulated by cyclin D1. Whether curcumin manifests its antiproliferative effects through the regulation of cyclin D1 was examined. LNCaP cells were treated with various concentrations of curcumin for 24 h and then examined for expression of cyclin D1 by Western blot analysis. Results shown in Figure 2a demonstrate that curcumin can down-regulate the expression of cyclin D1 in a dose-dependent manner, achieving maximum effect at 25 μM curcumin. When examined for the optimum time required for the down-regulation of cyclin D1, it was found that a 180 min exposure to curcumin was sufficient for maximum suppression of cyclin D1 (Figure 2b). Under these conditions, the levels of β-actin protein were unaffected, suggesting that the down-regulation was specific to cyclin D1 protein.

Curcumin inhibits the expression of cyclin D1 in breast and SCC cancer cell lines

Several reports suggest that cyclin D1 is overexpressed in breast cancer cells and plays a major role in tumorigenesis (Gillett *et al*., 1994; Bartkova *et al*., 1994a,b; Dickson *et al*., 1995), so we also investigated the effect of curcumin on cyclin D1 levels in various breast cancer lines. Breast cancer cells were exposed to 50 μM curcumin for 3 h, and then the cell extracts were prepared and were examined for cyclin D1 levels. All seven breast cancer lines expressed cyclin D1 protein, but MCF-7, T-47D, SK-BR3, MDA-MB-231 and ZR-75-1 cells expressed higher levels than BT-549, and MDA-MB-436 (Figure 3a). The treatment of cells with curcumin down-regulated the expression of cyclin D1 protein in all the cell lines (Figure 3b,c). Indeed, under identical conditions, no cyclin D1 protein could be detected in T-47D, ZR-751, BT-549, BT-20 and SK-BR3 cells after curcumin treatment. Under these conditions, the levels of β-actin protein were unaffected, suggesting that the down-regulation was specific to cyclin D1 protein. Similarly, both the SCC cell lines expressed cyclin D1 protein and the treatment with curcumin down-regulated the expression in both the cell lines (Figure 3d). The levels of β-actin protein were again unaffected.

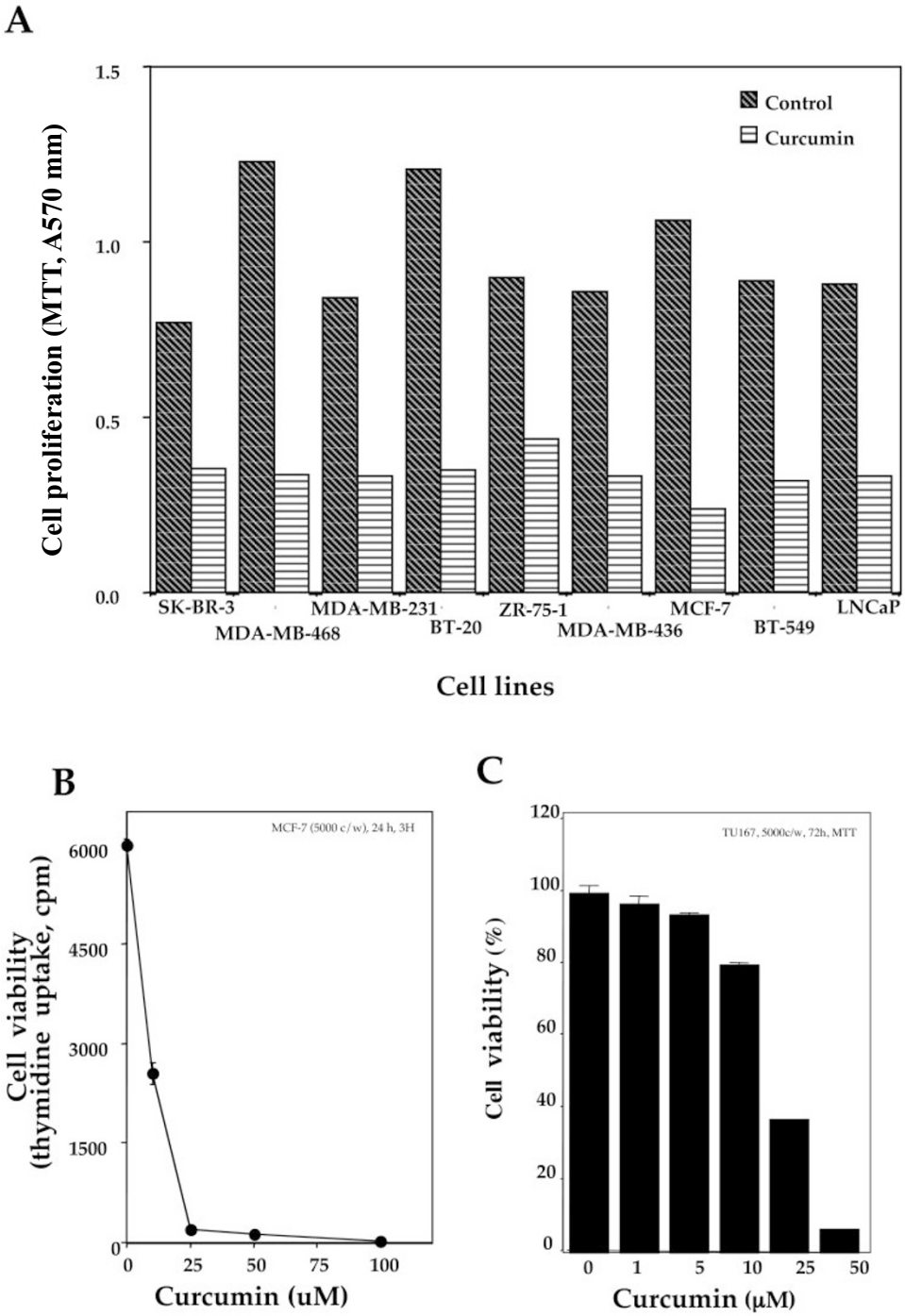

Figure 1 (**a**) Curcumin inhibits the proliferation of breast and prostate cancer cells. 1×10^4 cells were in plated in triplicate in 0.1 ml medium overnight in 96-well plates and then treated with the 100 μM curcumin. After 24 (prostate) or 72 h (breast), cell viability was determined by the MTT method as described in Materials and methods. (**b**) Curcumin inhibits thymidine incorporation in breast cancer cells. 5×10^3 MCF-7 breast cancer cells were plated in triplicate in 0.1 ml medium overnight in 96-well plates and then treated with the indicated concentrations of curcumin. After 18 h, cells were pulse treated with tritiated thymidine for 6 h and then harvested and counted as described in Materials and methods. (**c**) Curcumin inhibits the proliferation of human squamous cell carcinoma cells. TU 167 cells (5000 cells/0.1 ml) were seeded in triplicate for 24 h; then the medium was replaced with 0.2 ml fresh medium containing indicated concentration of curcumin. Cells were incubated for 72 h and then cell viability examined by the MTT method

Curcumin-induced down-regulation of cyclin D1 is not due to its effects on cell viability

It is possible that the down-regulation of cyclin D1 is due to the cytotoxic effects of curcumin. To determine this, LNCaP and MCF-7 cells were exposed to different concentrations of curcumin for either 3 or 24 h and then examined for cell viability either by the MTT method or by Trypan blue dye-exclusion method. As

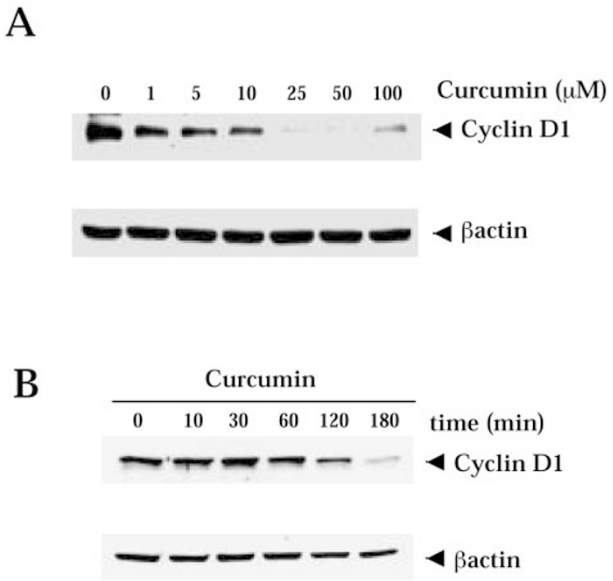

Figure 2 (**a**) Dose-dependent down-regulation by curcumin of the expression of cyclin D1 protein in prostate cancer cells. 5×10^5 cells were plated in 1 ml medium overnight in 6-well plates and treated with the indicated concentrations of curcumin. After 24 h, whole cell extracts were prepared, resolved on 10% SDS–PAGE, electrotransferred, and probed with anti-CyclinD1 antibodies (1 : 1000 dilution) for 2 h as described in Materials and methods. (**b**) Time-dependent down-regulation by curcumin of the expression of cyclin D1 protein in prostate cancer cells. 5×10^5 cells were in plated 1 ml medium for overnight in 6-well plates and treated with 25 μM curcumin for the indicated times. Then whole cell extracts were prepared, resolved on 10% SDS–PAGE, electrotransferred, and probed with anti-CyclinD1 antibodies (1 : 1000 dilution) for 2 h as described in Materials and methods

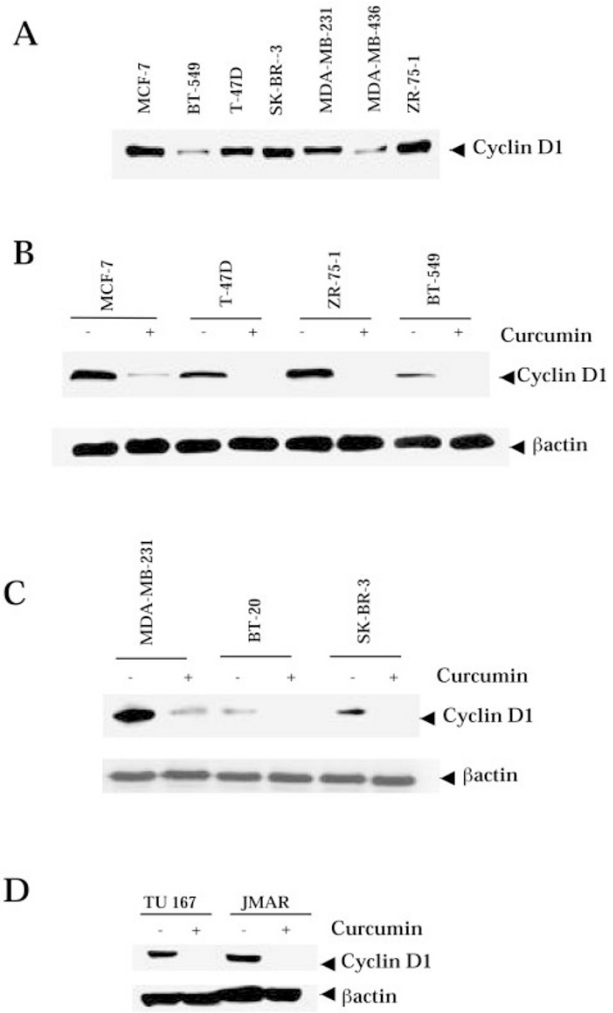

Figure 3 Expression of cyclin D1 protein in various human breast cancer cell lines. (**a**) 1×10^6 cells were plated in 1 ml medium overnight in 6-well plates. After 3 h, whole-cell extracts were prepared, resolved in 10% SDS–PAGE, electrotransferred and probed with anti-Cyclin D1 antibodies (1 : 1000 dilution) for 2 h as described in Materials and methods. (**b**–**d**) Curcumin down-regulates the expression of cyclin D1 protein in various human breast cancer (**b** and **c**) and in SCC (**d**) cell lines. 1×10^6 cells were plated in 1 ml medium overnight in 6-well plates and then treated with 50 μM curcumin. After 3 h, whole-cell extracts were prepared, resolved on 10% SDS–PAGE, electrotransferred and probed with anti-Cyclin D1 antibodies (1 : 1000 dilution) for 2 h as described in Materials and methods

shown in Table 1, LNCaP and MCF-7 cells showed greater than 95% cell viability by either method when exposed to 50 μM curcumin for 3 h. Treatment with curcumin for 24 h showed 20–30% decrease in cell viability with either of the cell lines and by either of the methods. Since a decrease of cyclin D1 occurs in most cells on treatment with 25–50 μM curcumin for 3 h, these results indicate that the effect of curcumin on cyclin D1 is not due to its cytotoxic effects.

Curcumin down-regulates the expression of cyclin D2 and cyclin D3 in selective breast, colon, prostate and SCC cancer cell lines

Whether curcumin down-regulates the expression of only cyclin D1 or also of cyclin D2 and cyclin D3, was also investigated. All seven breast cancer cell lines examined in our study were found to be negative for the expression of cyclin D2 (Figure 4a and data not shown). These results are in agreement with a previous report (Lukas *et al*., 1995; Evron *et al*., 2001). Similarly, both prostate and SCC cell lines showed lack of expression of cyclin D2 (Figure 4a and data not shown). A colon cancer cell line LoVo36 showed a significant expression of cyclin D2 (Figure 4b). These results are in agreement with previous report (Lukas *et al*., 1995). The treatment of LoVo36 with curcumin down-regulated the expression of cyclin D2 (Figure 4b).

We found that all the breast, prostate and SCC cell lines expressed cyclin D3 protein (Figure 4c–e). Treatment of cells with curcumin down-regulated the expression of cyclin D3 in prostate cancer LNCaP cells (Figure 4c). Out of seven breast cancer cell lines examined, curcumin down-regulated the expression of cyclin D3 only in T47D cells (Figure 4d). Among the SCC cell lines examined, curcumin significantly down-regulated the expression of cyclin D3 in JMAR cell line (Figure 4e). Under these conditions, the levels of β-

Table 1 Effect of different concentrations of curcumin on the viability of human prostate cancer LNCaP and human breast adenocarcinoma MCF-7 cells

	Cell lines			
	LNCaP		*MCF-7*	
Curcumin (μM)	*3 h*	*24 h*	*3 h*	*24 h*
MTT method[a]				
0	0.53±0.01 (100)	1.12±0.01 (100)	0.46±0.01 (100)	0.80±0.01 (100)
10	0.61±0.00 (114)	1.04±0.02 (93)	0.46±0.02 (101)	0.81±0.01 (101)
25	0.55±0.00 (102)	0.87±0.00 (77)	0.47±0.00 (103)	0.76±0.02 (95)
50	0.51±0.01 (96)	0.83±0.02 (74)	0.45±0.00 (98)	0.61±0.00 (77)
Trypan blue dye exclusion method[b]				
0	0.97 (100)	1.34 (100)	1.00 (100)	1.12 (100)
10	0.94 (100)	1.25 (94)	1.01 (100)	1.04 (93)
25	0.95 (98)	1.09 (81)	0.98 (98)	1.09 (98)
50	0.93 (96)	0.89 (66)	0.95 (95)	0.85 (77)

[a]10 000 cells were incubated in triplicate with the indicated dose of curcumin for the indicated time and then examined for cell viability by the MTT method. The results shown are mean of triplicate±s.d. [b]One million cells were incubated with indicated dose of curcumin for the indicated time and then examined for cell viability by the Trypan blue dye-exclusion method. The results shown are multiplied by 10^6. Numbers in parenthesis are per cent

actin protein were unaffected. Thus these results suggest that in comparison to cyclin D1 protein, the down-regulation of cyclin D2 and cyclin D3 by curcumin occurs in selective cell lines.

Curcumin down-regulates the activity of cyclin D1-dependent kinase (CDK4)

Cyclin D1 is known to regulate the activity of CDK4 which is known to phosphorylate Rb protein. Therefore we examined the effect of curcumin on CDK4-mediated Rb phosphorylation. Cells were treated with 50 μM curcumin for 30, 60 and 120 min and the CDK4 activity examined by immunecomplex kinase assays using Rb as a substrate. As shown in Figure 5, extracts from untreated cells phosphorylated Rb whereas those from curcumin-treated cells did not. A treatment as short as 30 min was sufficient to significantly inhibit the Rb phosphorylation. These results indicate that curcumin inhibits the activity of CDK4.

Lactacystin blocks curcumin-induced down-regulation of the cyclin D1 protein

Curcumin could down-regulate cyclin D1 expression either by enhancing its degradation or by suppressing its synthesis. We first explored the possibility that curcumin may enhance the rate of degradation of cyclin D1. Inasmuch as cyclinD1 undergoes ubiquitin-dependent proteosomal degradation (Diehl *et al*., 1997), and lactacystin inhibits the 26 S proteosome (Vrana and Grant, 2001), we examined the ability of lactacystin to block curcumin-induced degradation of cyclin D1 in LNCaP cell. Cells were pretreated with 10 μM lactacystin for 2 h and then exposed to 50 μM curcumin for 3 h. Thereafter, whole-cell extracts were prepared and analysed for cyclin D1 protein. As shown in Figure 6a, lactacystin alone had no effect, curcumin induced degradation of cyclin D1, and lactacystin prevented the degradation. It seems that lactacystin alone slightly enhanced the constitutive levels of cyclin D1, most likely through suppression of constitutive proteases. The effect of lactacystin was not specific to LNCaP cells, as it also suppressed the curcumin-induced degradation of cyclin D1 in breast cancer MCF7 cells (Figure 6b). These results thus clearly suggest that one of the mechanisms by which curcumin down-regulates cyclin D1 is through enhancement of proteolysis.

Curcumin down-regulates the cyclin D1 mRNA

To determine if curcumin also down-regulates cyclin D1 expression by affecting its synthesis, LNCaP cells were treated with 50 μM curcumin for indicated times, and then the mRNA was isolated and probed with cyclin D1 cDNA. As shown in Figure 7a, curcumin also down-regulated expression of cyclin D1 mRNA. About 60 min was sufficient to completely down-regulate cyclin D1 mRNA, thus suggesting that curcumin also affects the synthesis of cyclin D1. The effect of curcumin on the mRNA for cyclin D1 was not specific to LNCaP cells, as it also observed in breast cancer MCF-7 cells (Figure 7b).

Curcumin down-regulates cyclin D1 promoter activity

Previously it has been shown that the promoter of the cyclin D1 gene contains an NF-κB binding site needed for expression of cyclin D1 (Guttridge *et al*., 1999; Henry *et al*., 2000; Hinz *et al*., 1999). Because NF-κB is known to be down-regulated by curcumin (Singh and Aggarwal, 1995; Kumar *et al*., 1998; Plummer *et al*., 1999; Jobin *et al*., 1999), it is possible that curcumin affects cyclin D1 expression by affecting the cyclin D1 promoter activity. To determine this, cells were transfected with the cyclin D1 promoter-linked to luciferase reporter, treated with 50 μM curcumin, and assayed for the luciferase reporter activity. As shown in Figure 8, the luciferase reporter was fully active in the untreated cells but when cells were treated with curcumin, the activity was inhibited in a time/dose-dependent manner. These results thus suggest

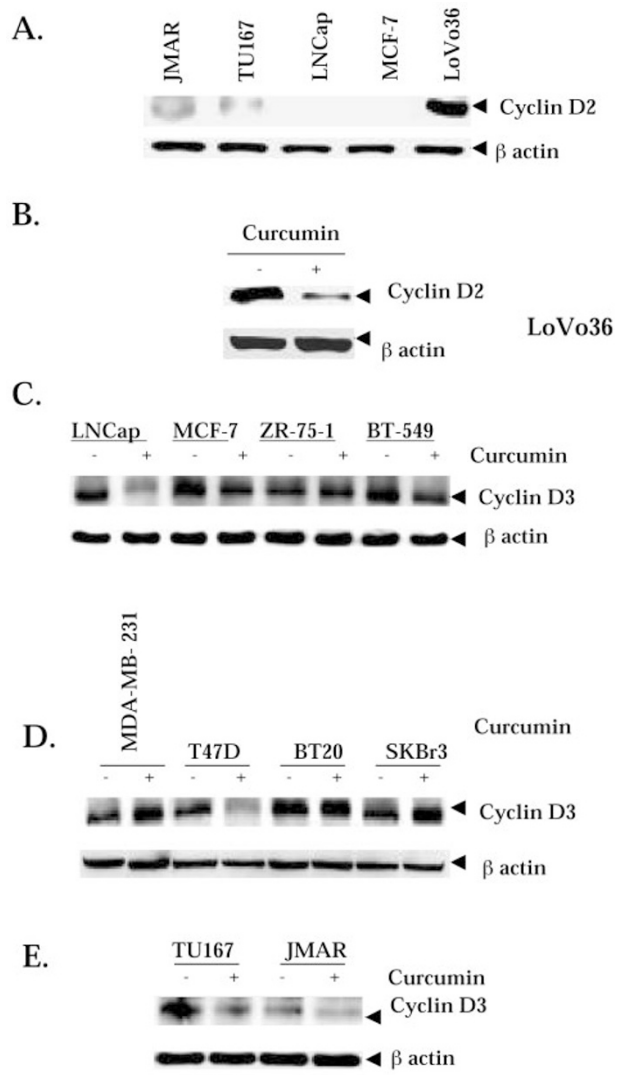

Figure 4 Effect of curcumin on cyclin D2 and cyclin D3 protein in various human cancer cell lines. (**a**) 1×10^6 cells were plated in 1 ml medium overnight in 6-well plates. After 3 h, whole-cell extracts were prepared, 60 μg protein was resolved on 12% SDS–PAGE, electrotransferred and probed with anti-Cyclin D2 antibodies (1 : 1000 dilution) for 2 h as described in Materials and methods. (**b**–**e**) Curcumin down-regulates the expression of cyclin D2 and cyclin D3 protein in human colon cancer (**b**), prostate and breast (**c**), in breast (**d**) and SCC (**e**) cell lines. 1×10^6 cells were plated in 1 ml medium overnight in 6-well plates and then treated with 50 μM curcumin. After 3 h, whole-cell extracts were prepared, 60 μg protein was resolved on 12% SDS–PAGE, electrotransferred and probed with either anti-Cyclin D2 antibodies or anti-Cyclin D3 antibodies (1 : 1000 dilution) for 2 h as described in Materials and methods

that curcumin also down-regulates cyclin D1 promoter activity.

Discussion

The results presented in this report indicate that curcumin, a chemopreventive agent, inhibits the proliferation of various cancer cell lines and this

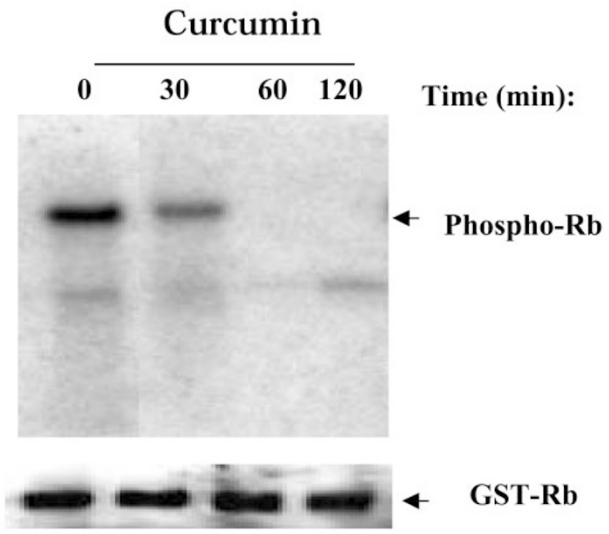

Figure 5 Curcumin inhibits CDK4-catalyzed Rb phosphorylation. LNCAP cells were incubated with 10 μM curcumin for the indicated times, and then whole-cell extracts were prepared. Thereafter, 250 μg of protein was immunoprecipitated with the CDK 4 antibody. A/G agrose beads were added to the IP and allowed to mix. Ten ml of beads was added to the kinase reaction and allowed to phosphorylate the GST-Rb protein (1 μg). Samples were then separated on a 10% SDS–PAGE gel, dried, and exposed to X-ray film. The same experiment was performed at least three times. The decrease in Rb phosphorylation was seen in all experiments. Similar results were seen in all experiments

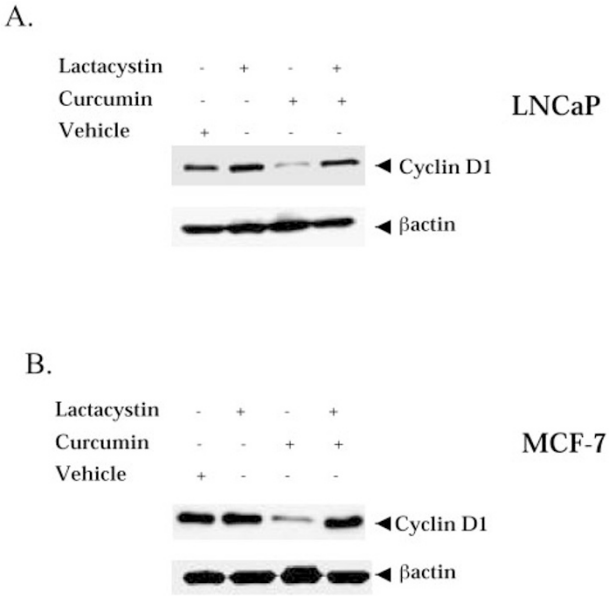

Figure 6 Lactacystin blocks curcumin-induced down-regulation of the cyclin D1 protein in prostate cancer LNCaP (**a**) and in breast cancer MCF-7 (**b**) cells. 1×10^6 cells were plated in 1 ml medium overnight in 6-well plates. After 24 h, cells were pretreated with 10 μM lactacystin for 2 h and then exposed to 50 μM curcumin for 3 h. Thereafter, whole cell extracts were prepared, resolved in 10% SDS–PAGE, electrotransferred, and probed with anti-Cyclin D1 antibodies (1 : 1000 dilution) for 2 h as described in Materials and methods

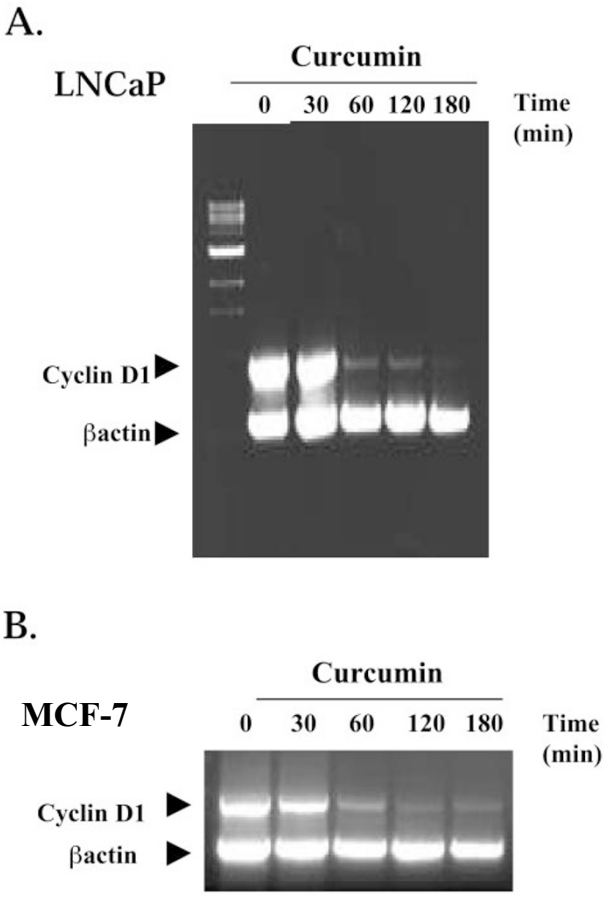

Figure 7 Curcumin down-regulates the expression of cyclin D1 mRNA in prostate cancer LNCaP (**a**) and breast cancer MCF-7 (**b**) cells. 5×10^5 cells were plated in 1 ml medium overnight in 6-well plates and treated with 50 μM curcumin for indicated times. Thereafter, mRNA was isolated and transcribed to cDNA. The cDNA was then used for PCR reactions. The PCR product was resolved on 2% agarose gels and probed with Cyclin D1 cDNA for 2 h as described in Materials and methods. Actin was used as an internal control for equal amount of template. The experiments were performed at least three times and similar results were obtained each time

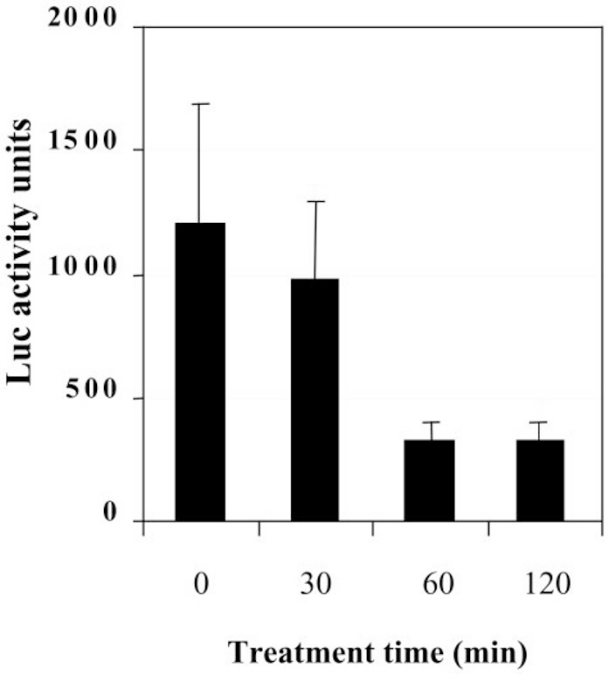

Figure 8 Curcumin down-regulates the cyclin D1 promoter activity in prostate cancer LNCaP cells. LNCaP cells were transiently transfected with 1 μg of cyclin D1 luciferase reporter. Cells were then incubated with 10 μM curcumin at the indicated times. Cells were lysed and measured for luciferase activity. The values represent the means $\pm$ s.e. from three different experiments

inhibition correlates with the down-regulation of the expression of cyclin D1 protein in most cells. In selective cell lines, curcumin also down-regulated the expression of cyclin D2 and cyclin D3. The suppression of cyclin D1 by curcumin led to inhibition of CDK4-mediated phosphorylation of Rb. Lactacystin inhibited the curcumin-induced down-regulation of cyclin D1 expression, and curcumin down-regulated the mRNA expression and inhibited the activity of cyclin D1 promoter-dependent reporter gene expression.

Our results indicate that curcumin does block the proliferation of prostate and breast cancer cells in culture. These results are in agreement with previous reports that showed that curcumin suppresses the proliferation of a wide variety of tumor cells (Mehta *et al*., 1997; Anto *et al*., 2002; Kuo *et al*., 1996; Jiang *et al*., 1996; Ranjan *et al*., 1999; Piwocka *et al*., 1999; Han *et al*., 1999; Simon *et al*., 1998; Ramachandran and You, 1999; Bhaumik *et al*., 1999), including both B cell and T cell leukemia, colon carcinoma, breast carcinoma, prostate cancer cells and other tumor cell types. How curcumin suppresses the proliferation of tumor cells is not well understood. Our results indicate that antiproliferative effects of curcumin correlate with the down-regulation of cyclin D1. The latter has been shown to be overexpressed in many cancers including breast, esophagus, head and neck, and prostate (Gillett *et al*., 1994; Bartkova *et al*., 1994a,b, 1995; Dickson *et al*., 1995; Adelaide *et al*., 1995; Caputi *et al*., 1999; Nishida *et al*., 1994; Gumbiner *et al*., 1999; Drobnjak *et al*., 2000; Arber *et al*., 1996). In some selective cell lines curcumin also down-regulated the expression of cyclin D2 and cyclin D3 but this did not correlate with inhibition of cell proliferation.

It is possible that the antiproliferative effects of curcumin are due to inhibition of cyclin D1 expression. Like curcumin, nitric oxide-induced cytostasis and cell cycle arrest of a human breast cancer cell line (MDA-MB-231) was also found to be mediated through the down-regulation of cyclin D1 (Pervin *et al*., 2001). The cyclin D1 is required for the activity of CDK4, which phosphorylates Rb protein, thus releasing E2F to mediate the G1 to S transition, in turn leading to DNA synthesis and cell cycle progression (Sherr, 1995). Our results indicate that both Rb phosphorylation and DNA synthesis as determined by thymidine incorporation were inhibited by curcumin, possibly through down-regulation of cyclin D1.

Our results also indicate that there are multiple mechanisms by which curcumin down-regulates cyclin D1 expression. The first mechanism involves the activation of proteases. It has been shown that cyclinD1 is degraded through the ubiquitin-dependent proteosomal pathway (Diehl *et al*., 1997). Since lactacystin, a proteosomal inhibitor (Vrana and Grant, 2001), suppressed the curcumin induced down-regulation of cyclin D1, curcumin must induce down-regulation of cyclin D1 through the activation of proteases. These results are similar to those previously reported with retinoic acid, another chemopreventive agent, which down-regulates cyclin D1 by proteolysis (Pervin *et al*., 2001). The second mechanism by which curcumin could down-regulate cyclin D1 expression is through suppression of its biosynthesis. Our results indicate that the expression of mRNA for cyclin D1 was down-regulated by curcumin. Our results are similar to those for nitric oxide, which has been shown to down-regulate cyclin D1 by decreasing its mRNA (Langenfeld *et al*., 1997). How curcumin down-regulates the mRNA for cyclin D1, however, is not clear. It may involve either destabilization of the mRNA or inhibition of its biosynthesis.

Curcumin's ability to down-regulate cyclin D1 promoter activity, as determined by the reporter gene expression, suggests that the drug suppresses the biosynthesis of the mRNA for cyclin D1. How curcumin inhibits the cyclinD1 promoter activity is also not clear. The promoter of cyclin D1 is regulated by NF-κB (Guttridge *et al*., 1999; Henry *et al*., 2000; Hinz *et al*., 1999), and we and others have previously shown that curcumin inhibits NF-κB activation (Singh and Aggarwal, 1995; Kumar *et al*., 1998; Plummer *et al*., 1999; Jobin *et al*., 1999). Thus it is possible that the effect of curcumin on the cyclin D1 mRNA is due to its ability to suppress NF-κB activation. Curcumin has been shown to inhibit the cells at G1/S phase of the cell cycle progression (Mehta *et al*., 1997; Chen and Huang, 1998, Chen *et al*., 1999), and NF-κB has also been implicated in the regulation of the cell cycle (Duckett *et al*., 1995). Another report indicated that NF-κB functions in growth control through the regulation of cyclin D1 expression and the G0/G1-to-S-phase transition (Hinz *et al*., 1999). Thus it is quite possible that curcumin manifests its effects on cell proliferation by affecting NF-κB regulated cyclin D1 expression.

Overall our results indicate that curcumin is a potent inhibitor of cyclin D1 expression. The down-regulation of cyclin D1 by curcumin in tumors that overexpress this proto-oncogene may suppress the growth of those tumors. Although the antiproliferation of these tumors cells by curcumin has been established *in vitro*, our results provide further rationale for testing *in vivo*.

Materials and methods

Materials

Penicillin, streptomycin, RPMI 1640 medium and FBS were obtained from Life Technologies (Grand Island, NY, USA). Tris, glycine, NaCl, SDS, PMA and BSA were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The polyclonal antibodies anti-cyclin D1 against amino acids 1–295, which represents full-length cyclin D1 of human origin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against cyclin D2 and cyclin D3 was obtained from Neomarkers (Union City, CA, USA). Cyclin D2 is a mouse monoclonal and cyclin D3 is rabbit polyclonal antibody.

Cell lines

An androgen-independent prostate cancer cell (LNCaP), breast cancer cell lines (MCF-7, BT-549, T-47D, MDA-MB-231, MDA-MB-436, ZR-75-1, and SK-BR-3) were obtained from American Type Culture Collection. The SCC cell lines (JMAR and TU167) were kindly supplied by Dr Jeffrey Myers of MD Anderson Cancer Center (Houston, TX, USA). Colon cancer cell line LoVo36 was kindly supplied by Ajay Goel (University of California, San Diego, CA, USA). T-47D, MDA-MB-468, MCF-7, BT-549, BT-20, and ZR-75-1 cells were grown in RPMI-1640 medium; and JMAR, TU 167, SK-BR3, MDA-MB231, and MDA-MB436 were grown in minimum essential medium (MEM). All these media were supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. LoVo36 cell line was grown in Iscove's modified Dulbecco's medium containing 10% FBS. TU-167 and JMAR cells were grown in Dulbecco's modified Eagle's medium (high glucose supplemented with 10% FBS, L-glutamine, nonessential amino acids, penicillin, streptomycin, sodium pyruvate and vitamins.

Antiproliferative assays

Cell growth assays were carried out essentially according to the procedure described (Haridas *et al*., 1998). Briefly, cells (5×10^3/well) were plated in 0.1 ml of the medium containing 10% FBS in 96-well Corning 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 the 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 the extraction buffer (20% sodium dodecyl sulphate, 50% dimethyl formamide) was added. After an overnight incubation at 37°C, the optical densities at 570 nm were measured using a 96-well multiscanner autoreader (Dynatech MR 5000), with the extraction buffer used as a blank.

Thymidine incorporation assay

For [^{3}H]TdR incorporation, cells were cultured and treated with curcumin as indicated above. During the last 6 h, [^{3}H]TdR (6.7 Ci/mmole; New England Nuclear, Boston, MA, USA) was added to each well (0.5 μCi/well). Thereafter, the culture medium was removed, the wells were washed twice with PBS, and the cells were detached by the addition of a solution of Trypsin (0.5%) with EDTA (5.3 mM). The cell suspension was then harvested with the aid of a Filtermate 196 cell harvester (Packard Instruments, Canberra, Australia) and lysed by washing with distilled water. Radioactivity bound to the filter was measured directly by Direct Beta Counter Matrix 9600 (Model 1600 TR; Packard Co., Meriden, CT, USA). All determinations were made in triplicate.

Cyclin D1, D2 and D3 protein expression

To determine the expression of cyclins protein, whole cell-extracts were prepared from 1×10^6 cells in lysis buffer (20 mM Tris pH 7.4, 250 mM sodium chloride, 0.1% Triton X-100, 2 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 4 mM sodium orthovanadate and 1 mM DTT), and 60 μg of the protein was resolved on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to nitrocellulose filters, probed with rabbit polyclonal antibodies against cyclin D1, and detected by chemiluminescence (ECL, Amersham). To assure equal loading, gels were stripped and reprobed with antibodies against β-actin.

Cyclin-D-dependent protein kinase (CDK4) assay

The activity of CDK4 was measured by the immune complex kinase assay (Matsushime *et al*., 1994). Kinase assays for cyclin D-Cdk4 were performed using GST-Rb (kindly provided by Dr Mong Hong Lee of University of Texas MD Anderson Cancer Center, Houston, TX, USA) as a substrate as described previously (Matsushime *et al*., 1994). Briefly, 2×10^6 cells of LNCAP cells were induced with 10 μM curcumin at the indicated times. Cells were harvested and lysed in RIPA buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 10% glycerol and 1 mM DTT) and sonicated three times for 15 s each. Cellular debris was pelleted at 14 000 *g* for 3 min, and 250 μg of protein was used for immunoprecipitation with 5 ml of anti-CDK4 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). This was allowed to mix at 4°C for 3 h. A 20 μl batch of A/G agarose beads was added and allowed to incubate an additional 3 h. The beads were pelleted at 14 000 *g* for 3 min at 4°C and washed five times in RIPA buffer. The phosphorylation reaction of GST-Rb was initiated using 10 μl of beads in the kinase buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 2.5 mM EDTA, 1 mM DTT, 0.1 mM SO and 1 mM NaF) by adding ATP mix (1 ml of [γ-^{32}P]ATP and 9 μl of unlabeled ATP). The reaction was incubated at 30°C for 30 min. Proteins were separated by 10% SDS–PAGE. Phosphorylation of Rb protein was visualized by autoradiography.

Cyclin D1-dependent luciferase reporter gene transcription

To measure the activity of cyclin D1 promoter, LNCAP cells were plated at 5×10^5 cells/well prior to transfection. The cells were transfected using lipofectamine as previously described (Liu and Simon, 1996). Briefly, LNCAP cells were seeded in 24-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS a day before transfection. Cyclin D1 luciferase (kindly provided by Dr X-H Feng of the Baylor College of Medicine, Houston, TX, USA), and cDNAs encoding cyclin D1 or *LacZ*, were added to each well in 250 μl of DMEM without FBS. The total amount of cDNA in all transfections was 1 μg per well. The cytomegalovirus vector pCIS, which encodes β-galactosidase, was used to maintain a constant amount of DNA and equivalent amounts of a particular cDNA in each set of experiments. Cells were induced with 10 μM curcumin at the indicated times and then washed two times with PBS. Luciferase assays were performed as recommended by the manufacturer (Promega, Madison, WI, USA). The data presented are the means of three individually-transfected wells, and the experiments were performed at least three times.

Polymerase chain reaction of cyclin D1

To determine the effect of curcumin on cyclin D1 mRNA levels, cells were treated with curcumin, the mRNA was extracted, and equal amounts were transcribed to cDNA from a kit (Promega). The cDNA was taken from samples at various times and used as the DNA templates for PCR. Primers used for cyclin D1 were 5′-CGGGATCCCCAGC-CATGGAACACCAGC-3′ and 5′-CGGAATTCGCGCCCT-CAGATGTCCACG. For a control, β-actin was used in the reaction. Primers for β-actin were 5′-CTCCTTAATGT-CACGCACGATTTC-3′ and 5′-GTGGGGCGCCCCAGG-CACCA. For each cDNA, 2 μl was used as the template and 1 μl of each primer was used. The samples went through 30 rounds of PCR. Samples were separated on a 1% agrose gel and visualized by ethidium bromide.

Abbreviations

IκB, inhibitory subunit of NF-κB; CDK, cyclin-dependent kinase; SCC, squamous cell carcinoma; IKK, IκBα kinase; MTT, tetrazolium salt- 3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl-tetrazolium bromide; luc, luciferase

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References

Adelaide J, Monges G, Derderian C, Seitz JF and Birnbaum D. (1995). *Br. J. Cancer.,* **71,** 64–68.

Albanese C, Johnson J, Watanabe G, Eklund N, Vu D, Arnold A and Pestell RG. (1995). *J. Biol. Chem.,* **270,** 23589–23597.

Anto RJ, Mukhopadhyay A, Denning K and Aggarwal BB. (2002). *Carcinogenesis,* **23,** 143–150.

Arber N, Hibshoosh H, Moss SF, Sutter T, Zhang Y, Begg M, Wang S, Weinstein IB and Holt PR. (1996). *Gastroenterology,* **110,** 669–674.

Baldin V, Lukas J, Marcote MJ, Pagano M and Draetta G. (1993). *Genes Dev.,* **7,** 812–821.

Bartkova J, Lukas J, Muller H, Lutzhoft D, Strauss M and Bartek J. (1994a). *Intl. J. Cancer,* **57,** 353–361.

Bartkova J, Lukas J, Strauss M and Bartek J. (1994b). *Intl. J. Cancer,* **58,** 568–573.

Bartkova J, Lukas J, Muller H, Strauss M, Gusterson B and Bartek J. (1995). *Cancer Res.,* **55,** 949–956.

Bhaumik S, Anjum R, Rangaraj N, Pardhasaradhi BVV and Khar A. (1999). *FEBS Lett.,* **456,** 311–314.

Caputi M, Groeger AM, Esposito V, Dean C, De Luca A, Pacilio C, Muller MR, Giordano GG, Baldi F, Wolner E and Giordano A. (1999). *Am. J. Respir. Cell Mol. Biol.,* **20,** 746–750.
Chen HW and Huang HC. (1998). *Br. J. Pharmacol.,* **124,** 1029–1040.
Chen H, Zhang ZS, Zhang YL and Zhou DY. (1999). *Anticancer Res.,* **19,** 3675–3680.
Deane NG, Parker MA, Aramandla R, Diehl L, Lee WJ, Washington MK, Nanney LB, Shyr Y and Beauchamp RD. (2001). *Cancer Res.,* **61,** 5389–5395.
Dickson C, Fantl V, Gillett C, Brookes S, Bartek J, Smith R, Fisher C, Barnes D and Peters G. (1995). *Cancer Letts.,* **90,** 43–50.
Diehl JA, Zindy F and Sherr CJ. (1997). *Genes Dev.,* **11,** 957–972.
Drobnjak M, Osman I, Scher HI, Fazzari M and Cordon-Cardo C. (2000). *Clin.Cancer Res.,,* **6,** 1891–1895.
Duckett CS, Perkins ND, Leung K, Agranoff AB and Nabel GJ. (1995). *J. Biol. Chem.,* **270,** 18836–18840.
Evron E, Umbricht CB, Korz D, Raman V, Loeb DM, Niranjan B, Buluwela L, Weitzman SA, Marks J and Sukumar S. (2001). *Cancer Res.,* **61,** 2782–2787.
Fantl V, Creer A, Dillon C, Bresnick J, Jackson D, Edwards P, Rosewell I and Dickson C. (2000). *Adv. Exp. Med Biol.,* **480,** 1–7.
Fantl V, Stamp G, Andrews A, Rosewell I and Dickson C. (1995). *Genes Dev.,* **9,** 2364–2372.
Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, Barnes D and Peters G. (1994). *Cancer Res.,* **54,** 1812–1817.
Gumbiner LM, Gumerlock PH, Mack PC, Chi SG, deVere WhiteRW, Mohler JL, Pretlow TG and Tricoli JV. (1999). *Prostate,* **38,** 40–45.
Guttridge DC, Albanese C, Reuther JY, Pestell RG and Baldwin Jr AS. (1999). *Mol. Cell. Biol.,* **19,** 5785–5799.
Han SS, Chung ST, Robertson DA, Ranjan D and Bondada S. (1999). *Clin. Immunol.,* **93,** 152–161.
Harari D and Yarden Y. (2000). *Oncogene,* **19,** 6102–6114.
Haridas V, Darnay BG, Natarajan K, Heller R and Aggarwal BB. (1998). *J. Immunol.,* **160,** 3152–3162.
Henry DO, Moskalenko SA, Kaur KJ, Fu M, Pestell RG, Camonis JH and White MA. (2000). *Mol. Cell. Biol.,* **20,** 8084–8092.
Hinz M, Krappmann D, Eichten A, Heder A, Scheidereit C and Strauss M. (1999). *Mol. Cell. Biol.,* **19,** 2690–2698.
Jiang MC, Yang-Yen HF, Yen JJ and Lin JK *et al.* (1996). *Nutr. Cancer,* **26,** 111–120.
Jobin C, Bradham CA, Russo MP, Juma B, Narula AS, Brenner DA and Sartor RB. (1999). *J. Immunol.,* **163,** 3474–3483.
Joyce D, Bouzahzah B, Fu M, Albanese C, d'Amico M, Steer J, Klein JU, Lee RJ, Segall JE, Westwick JK, Der CJ and Pestell RG. (1999). *J. Biol. Chem.,* **274,** 25245–25249.
Kumar A, Dhawan S, Hardegen NJ and Aggarwal BB. (1998). *Biochem. Pharmacol.,* **55,** 775–783.
Kuo ML, Huang TS and Lin JK. (1996). *Biochim. Biophys. Acta,* **1317,** 95–100.
Langenfeld J, Kiyokawa H, Sekula D, Boyle J and Dmitrovsky E. (1997). *Proc. Natl. Acad. Sci. USA,* **9,** 12070–12074.
Lee RJ, Albanese C, Fu M, d'Amico M, Lin B, Watanabe G, Haines IIIGK, Siegel PM, Hung MC, Yarden Y, Horowitz JM, Muller WJ and Pestell RG. (2000). *Mol. Cell. Biol.,* **20,** 672–683.
Liu M and Simon MI. (1996). *Nature,* **382,** 83–87.
Lukas J, Bartkova J, Welcker M, Petersen OW, Peters G, Strauss M and Bartek J. (1995). *Oncogene,* **10,** 2125–2134.
Matsushime H, Quelle DE, Shurtleff SA, Shibuya M, Sherr CJ and Kato JY. (1994). *Mol. Cell. Biol.,* **14,** 2066–2076.
Mehta K, Pantazis P, McQueen T and Aggarwal BB. (1997). *Anticancer Drugs,* **8,** 470–481.
Nishida N, Fukuda Y, Komeda T, Kita R, Sando T, Furukawa M, Amenomori M, Shibagaki I, Nakao K and Ikenaga M. (1994). *Cancer Res.,* **54,** 3107–3110.
Pervin S, Singh R and Chaudhuri G. (2001). *Proc. Natl. Acad. Sci. USA,* **98,** 3583–3588.
Piwocka K, Zablocki K, Wieckowski MR and Sikora E. (1999). *Exp. Cell. Res.,* **249,** 299–307.
Plummer SM, Holloway KA, Manson MM, Munks RJ, Kaptein A, Farrow S and Howells L. (1999). *Oncogene,* **18,** 6013–6020.
Ramachandran C and You W. (1999). *Breast Cancer Res. Treat.,* **54,** 269–278.
Ranjan D, Johnston TD, Reddy KS, Wu G, Bondada S and Chen C. (1999). *J. Surg. Res.,* **87,** 1–5.
Sauter ER, Nesbit M, Litwin S, Klein-Szanto AJ, Cheffetz S and Herlyn M. (1999). *Cancer Res.,* **59,** 4876–4881.
Sherr CJ. (1995). *Trends Biochem. Sci.,* **20,** 187–190.
Sicinski P and Weinberg RA. (1997). *J. Mamm. Gland Biol. Neoplasia,* **2,** 335–342.
Simon A, Allais DP, Duroux JL, Basly JP, Durand-Fontanier S and Delage C. (1998). *Cancer Lett.,* **129,** 111–116.
Singh S and Aggarwal BB. (1995). *J. Biol. Chem.,* **270,** 24995.
Vrana JA and Grant S. (2001). *Blood,* **97,** 2105–2114.
Wang TC, Cardiff RD, Zukerberg L, Lees E, Arnold A and Schmidt EV. (1994). *Nature,* **369,** 669–671.
Yu Q, Geng Y and Sicinski P. (2001). *Nature,* **411,** 1017–1021.