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Role of Intracellular Calcium in Clotrimazole-Induced Alteration of Cell Cycle Inhibitors, p53 and p27, in HT29 Human Colon Adenocarcinoma Cells

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Abstract – Clotrimazole (CLT), a potent antifungal drug, is known to inhibit tumor cell proliferation. In the present study, we examined the role of intracellular Ca^{2+} in CLT-induced cell cycle arrest of colon adenocarcinoma HT29 cells. CLT inhibited growth of HT29 cells in a concentration-dependent manner, which was associated with inhibition of cell cycle progression at the G(1)-S phase transition and an increase in the expression of cell cycle inhibitor proteins p27 and p53. CLT also suppressed the Ca^{2+} overload by A23187, a calcium ionophore, suggesting its role in modulation of intracellular Ca^{2+} concentration in HT29 cells. The simultaneous application of CLT and A23187 with addition of $CaCl_2$ (1 mM) to the medium significantly reversed CLT-induced p27 and p53 protein level increase and growth suppression. Our results suggest that CLT induces cell cycle arrest of colon adenocarcinoma HT29 cells via induction of p27 and p53, which may, at least in part, be mediated by alteration of intracellular Ca^{2+} level.

Key words □ Intracellular Ca^{2+}, Cell cycle arrest, Clotrimazole, p27, p53

INTRODUCTION

It is widely accepted that the Ca^{2+} is a ubiquitous intracellular signal responsible for controlling numerous cellular processes such as cell growth and cell cycle progression (Cheng *et al.*, 1996, Berridge *et al.*, 2000, Kahl and Means, 2003). The alteration of intracellular Ca^{2+} concentration controls cellular processes such as proliferation, fertilization and development. However, when exceeding its normal spatial and temporal boundaries, Ca^{2+} can result in cell death through both necrosis and apoptosis (Berridge *et al.*, 2000). Therefore, intracellular Ca^{2+} concentration is absolutely required not only for mitosis and cell growth progression through G1 but also for survival (Hazelton *et al.*, 1979, Kahl and Means, 2003).

Clotrimazole (CLT), an antifungal imidazole derivative that has been in clinical use for more than 20 years, inhibits proliferation of variety of human cancer cells originated from breast (Ouadid-Ahidouch *et al.*, 2004), pancreas (Jager *et al.*, 2004) and prostate (Parihar *et al.*, 2003) *in vitro*. More recently, it has been shown that growth inhibitory effect of CLT results from the cell cycle arrest at G1 by reducing the expression of G1 cyclin (Aktas *et al.*, 1998). In an *in vivo* mouse model, CLT is also known to exert inhibitory effect on tumor growth (Khalid *et al.*, 2005; Takahashi *et al.*, 1998) and metastasis (Benzaquen *et al.*, 1995). Unlike the antimycotic effect that is mediated through inhibition of sterol-1,4-demethylase, a cytochrome P-450-dependent enzyme (Yoshida and Aoyama, 1987), anti-cancer effect of CLT is suggested to be mediated by a novel mechanism involving Ca^{2+} store-mediated inhibition of translation initiation in human cancer cells (Benzaquen *et al.*, 1995). Specifically, CLT induces the release of Ca^{2+} from endoplasmic reticulum intracellular stores and blocks the influx of extracellular Ca^{2+} through Ca^{2+} store-regulated Ca^{2+} channels. The sustained depletion of intracellular Ca^{2+} stores activates RNA-dependent protein kinase R, resulting in phosphorylation of eukaryotic translation initiation factor (eIF2α) at serine 51 and its concomitant inactivation. Inactivation of eIF2α inhibits the formation of ternary complex between Met-tRNA, eIF2α, and GTP, a rate-limiting step in translation initiation. This results in an abrogation of synthesis of growth promoting proteins such as cyclin D, E, and A at the level of translation initiation, leading to growth arrest in early G1 (Aktas *et al.*, 1998, Apati *et al.*, 2003). On the other hand, a group of the pro-

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teins that acts on fundamental regulator of the cell cycle transitions is the cyclin-dependent kinases (CDKs) (Morgan, 1995). The family of p21/p27 protein binds CDK complexes to inhibit kinase activity, thereby playing a negative role in cell cycle progression. Furthermore, p53 protein also inhibits cell cycle progression (Kastan *et al.*, 1991). Although CLT is known to inhibit cell cycle progression, its action on cell cycle inhibitors has not been elucidated.

Therefore the purpose of this study was to examine the role of intracellular Ca^{2+} in growth and cell cycle progression via regulation of cell cycle related proteins in HT29 human colon adenocarcinoma cells. In the present study, we examined the expression level of cell cycle inhibitors such as p53 and p27 are associated with alterations in intracellular Ca^{2+} level.

MATERIALS AND METHODS

Chemicals

The liquid RPMI1640 medium, fetal bovine serum and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY, USA). A23187, trypsin-EDTA solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium pyruvate, propidium iodide, ribonuclease A, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), protease inhibitor cocktail, sodium dodecyl sulfate (SDS), nitroblue tetrazolium (NBT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fura-2 AM was purchased from Molecular Probes. CLT was supplied from Korea United Pharm. Inc. (Seoul, Korea).

Cell line and cell culture

The HT29 human colon cancer cell line was purchased from American Type Culture Collection (Rockville, MA, USA), and were grown in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 200 IU/ml penicillin/200 μg/ml streptomycin and 2 mmol/L glutamine, at 37°C under 5% CO_2/95% air. During propagation, the culture medium was changed every other day, and subcultured weekly using Dulbecco's PBS containing 0.25 % trypsin and 1% EDTA.

Cell viability assay (MTT Assay)

Cell viability was measured by the 3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay, which is based on the conversion of MTT into an insoluble purple formazan crystals by mitochondrial dehydrogenases. Cells were plated at a density of 2×10^4 cells/100 μl medium in a 96-well plate and treated with CLT for given time. The medium was incubated with 10 μl of 5 mg/ml of MTT solution for 4 hr at 37°C. Culture medium was then aspirated, and formazan was instantly dissolved by the addition of 200 ml of DMSO. Absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Versa MAX Sunnyvale, CA, USA). The cell viability was expressed as a percent of the control culture.

Cell cycle and apoptosis analysis

For flow cytometry analysis, cells were starved for 24 h and then released into complete medium with different concentrations of CLT for 24 h. The cells were collected and washed twice with a PBS buffer (pH 7.4). After fixing in 80% ethanol for 30 min, cells were then washed twice, and resuspended in a PBS buffer (pH 7.4) containing 0.1% Triton X-100, 100 μg/ml PI and 50 μg/ml ribonuclease A for DNA staining. Cells were then analyzed by a FACScalibur (Becton Dickson, USA). At least 20,000 events per sample were evaluated. All histograms were analyzed using Cell Quest (Becton Dickson, USA) to determine the percentage of cells in the G1, S and G2/M stages of the cell cycle. The percentage of nuclei with hypodiploid content indicative of apoptosis was also analyzed (Hockenbery *et al.*, 1990).

Cellular extraction and Western Blot analysis

Nuclear and total protein extractions were carried out according to previous procedure (Li *et al.*, 2006) with some modifications. Briefly, cells were scraped in 1 ml aliquot of ice-cold PBS, collected by centrifugation (3500 rpm for 5 min, 4°C), and the cell pellet was washed in a 1 ml aliquot of buffer A (10 mM Tris, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl). The cells were then resuspended in five volumes of ice-cold modified buffer A (10 mM Tris, pH 7.9; 1.5 mM $MgCl_2$; 10% NP40; 1 mM DTT and 0.5 mM PMSF) containing 1x protease inhibitor cocktail. The resuspended cells were homogenized and left on ice (4°C) for 15 min. The homogenates were centrifuged at 12000 rpm for 10 min. Supernatant (cytoplasmic fraction) was transferred to a fresh tube and stored at -70°C. To lyse the cell pellet containing nuclear fraction, a 50 μl aliquot of buffer C (Tris 10 mM, pH 7.9, 1.5 mM $MgCl_2$, 25% Glycerol, 5 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.5 mM Na_3VO_3, and 1x protease inhibitor cocktail,

was added. The pellets were vortexed for 1 min, left on ice (4°C) for 40 min, and centrifuged for 10 min (4°C, 12000 rpm) to collect the supernatant (nuclear fraction).

The soluble protein concentrations in cytoplasmic or nuclear lysates were determined by BCA™ protein assay kit (Pierce, Rockford, USA). Western blot analysis was carried out by separating equal amounts of proteins (20 μg) on SDS-PAGE and transferring to a Hybond ECL nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK) at 200 mA for an hour. Subsequently, membranes were blocked for 1 h at room temperature with 5% (w/v) skimmed milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Membranes were then incubated overnight at 4°C with the mouse monoclonal antibody against human p53 (Santa Cruz, CA), rabbit polyclonal antibody against human p27 (Santa Cruz, CA), and anti-human actin antibody (Santa Cruz, CA). After incubation with a corresponding horseradish peroxidase-conjugated anti-IgG antibody (all from Santa Cruz, CA), immunoreactive proteins were visualized with ECL kit (Amersham Life Science, Buckinghamshire, UK). For quantitative analysis, bands were detected by densitometry, using an Image Analyzing System (UVP, upland, USA).

Intracellular Ca^{2+} measurement

Briefly, HT29 cells were seeded at the density of 2×10^5 cells/cm^2 in 6-well plate (Nunc Denmark). After 24 hours of seeding, the monolayer was washed with PBS and added serum free RPMI 1640 media. Fura-2 AM at a final concentration of 3 μM was added to the monolayer, and was incubated for 50 min at 37°C. After being washed with Locke's solution containing 250 μM sulfinpyrazone for 3 times, cells were further incubated for 30 min in serum free media. Fura-2 loaded cells were then incubated with CLT for 10 min at 37°C. These experiments were performed in the presence of 1 mM $CaCl_2$, or EGTA alone. Finally, the monolayers were stimulated by the addition of Ca^{2+} ionophore A23187 at a final concentration of 1 μM. Fura-2 fluorescence was measured using a fluorescence detector, FLUOstar optima (BMG Labtech, Germany) with an excitation wavelength at 340/380 nm and the emission wavelength at 510 nm.

Data analysis

The data were expressed as the mean ± standard error of the mean (SEM) and analyzed using one-way analysis of variance (ANOVA) and Student-Newman-Keul's test for individual comparisons. P values less than 0.05 were considered statistically significant.

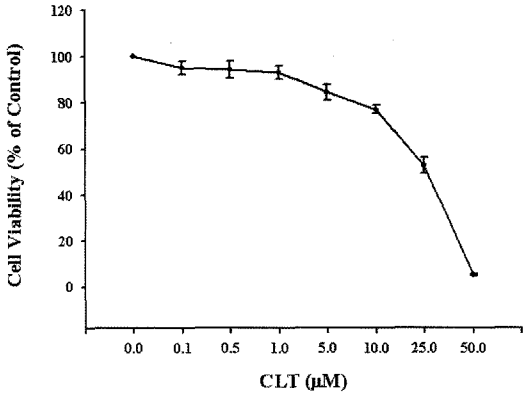

Fig. 1. Effects of CLT on the viability of HT29 human colon cancer cells. Cells treated with CLT for 48 h were analyzed for the viability by MTT assay. The data represent the mean values of four replications with bars indicating S.E.M.

RESULTS

CLT inhibits cell cycle progression and induces apoptosis in HT29 human colon cancer cells

It has been known that CLT inhibits cell proliferation in

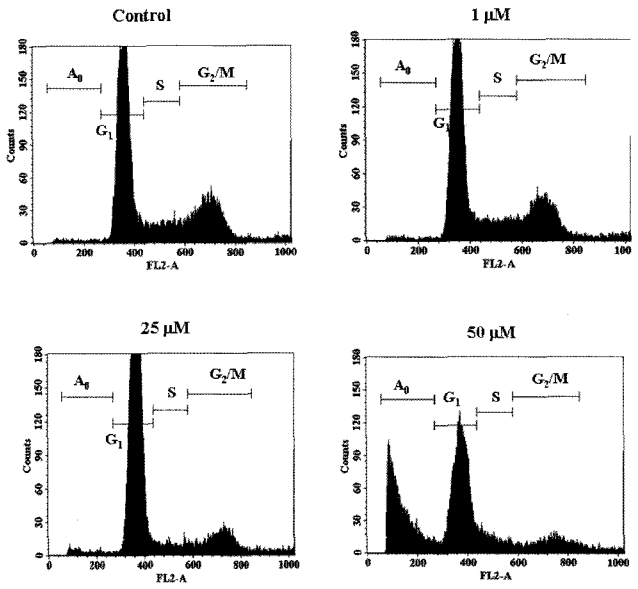

Fig. 2. Effect of CLT on cell cycle arrest in HT29 cells. Cells were starved serum (1% FBS) for 24 hr, and then, stimulated with 10% FBS in the absence or presence of CLT. After 24 hr, the cells were collected and then stained with propidium iodide for the cell cycle analysis using flow cytometry.

Table I. CLT-induced cell cycle arrest in G_1 phase.

CLT (μM)	A_0 (%)	G_1 (%)	S (%)	G_2/M (%)
0	1.14±0.04	63.7±0.58	11.49±0.27	23.53±0.35
1	1.25±0.09	62.5±0.42	11.95±0.09	24.03±0.39
25	2.25±0.08*	81.0±0.10*	5.71±0.17*	10.81±0.28*
50	39.44±1.93*	48.8±1.17*	7.35±0.45*	6.35±0.30*

HT29 cells were synchronized in the medium without FBS for 24 hr and then released into complete medium containing 0.1% DMSO or different concentrations of CLT. After incubation for 24 h, the cells were harvested, stained with propidium iodide, and analyzed for DNA contents by using flow cytometry. A_0 represents the percentage of nuclei with hypodiploid content indicative of apoptosis. *P<0.05 compared to vehicle-treated control.

various cancer cell lines. However, the effect of CLT in colon cancer has not been reported. We first examined the effect of CLT on the viability of HT29 human colon cancer cells. As shown in Fig. 1, treatment with CLT for 48 h decreased cell viability in a dose-dependent manner with an IC_{50} of 25 μM. To further examine the effect of CLT on the cell cycle progression, serum starved cells were then treated with increasing concentrations of CLT along with complete medium. As shown in Fig. 2 and Table I, the population of cells in the S phase was 11.49± 0.27% in the presence of 10% serum. CLT (25 μM) decreased the S phase population to 5.71±0.17%, while increasing the G1 phase population from 63.7±0.58% to 81.0±0.10%. Higher concentration of CLT (50 μM) decreased the population of cells in the S phase from 11.49±0.27% to 7.35±0.45% and G1 phase from 63.7±0.58% to 48.8±1.17%, but significantly increased cells in sub-G1 phase from 1.14±0.04% to 39.44± 1.93%.

Effects of CLT on the cell cycle-related proteins

In order to understand the exact mechanism of CLT-induced cell cycle arrest in HT29 cells, we examined the effect of CLT on the level of cell cycle inhibitor p27 and p53 proteins. As depicted in Fig. 3, CLT (25 μM) significantly increased the expression level of p27 time-dependently up to 24 h, thereafter, decreased at 48 hr. In addition, CLT increased the expression of p53 slightly up to 24 h, and tremendously at 48 h.

Intracellular Ca^{2+} involvement in the CLT-induced cell cycle arrest

Since CLT is known to modulate intracellular Ca^{2+} concentration and Ca^{2+}-dependent processes, we measured CLT-induced changes in the intracellular Ca^{2+} level in HT29 cells. The A23187 (1 μM), a known calcium ionophore, was used to induce the basal Ca^{2+} level in the cells. As shown in Fig. 4, the incubation of HT29 cells with A23187 progressively increased the Ca^{2+} level as calculated by a Ca^{2+}-sensitive fluorescence dye, Fura-2. CLT concentration-dependently reduced A23187-induced intracellular Ca^{2+} increase up to a control level.

To explore the involvement of intracellular Ca^{2+} in CLT-

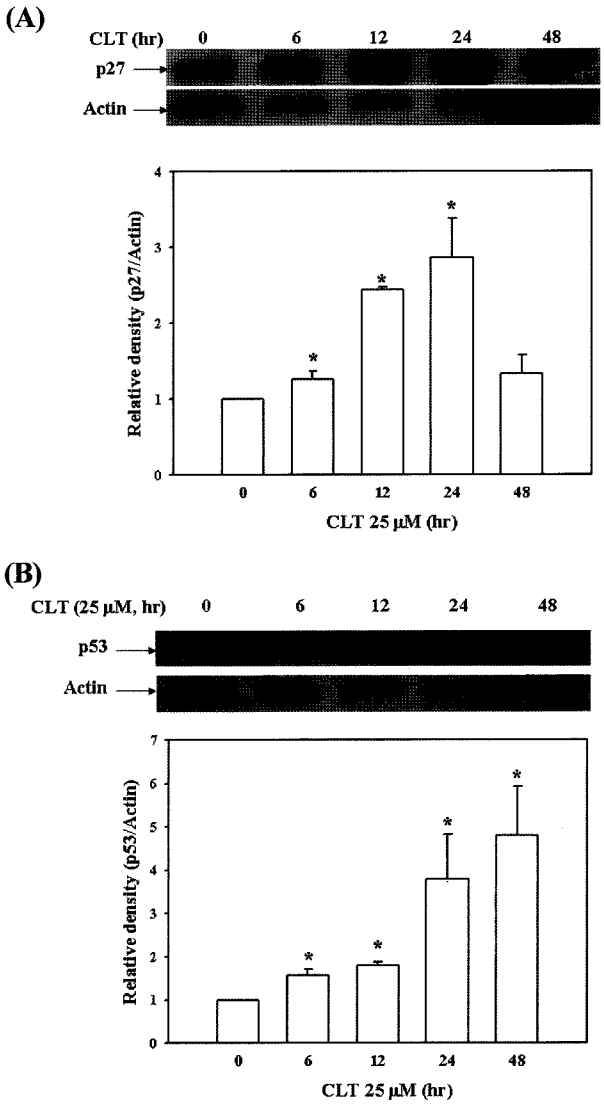

Fig. 3. Effects of CLT on the expression of p27 (A) and p53 (B) in HT29 cells. Cells were treated with CLT (25 μM) at different time points. The control cells received an equal volume of DMSO. In each experiment, nuclear proteins were isolated, and the level of p27 and p53 proteins was detected with anti-human p27 and p53 antibodies, respectively. Equal loading was confirmed by measuring actin proteins. Experi-ments were repeated 3 times and the bar graph represents the band intensity quantitated by densitometry. *P<0.05, compared to vehicle (DMSO)-treated controls.

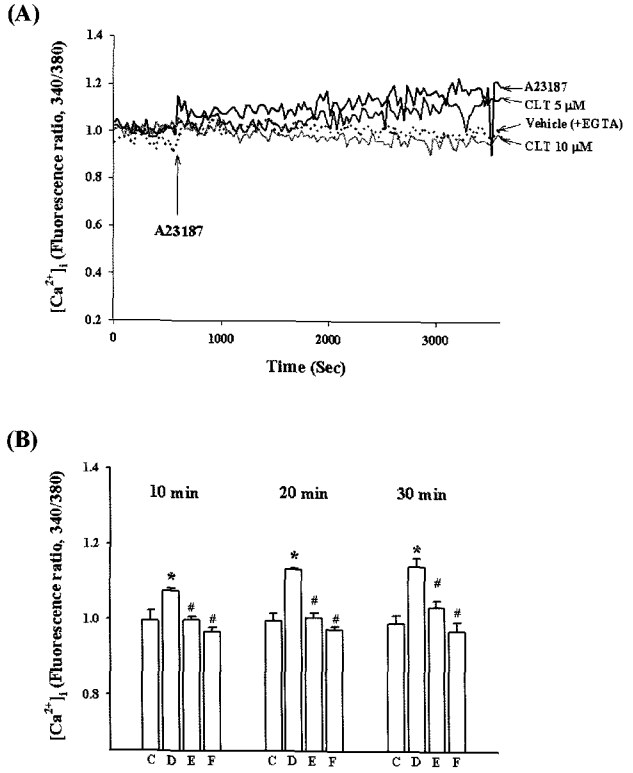

Fig. 4. The effect of CLT on A23187-induced intracellular Ca^{2+} level. The Fura-2-loaded cells were treated with CLT in serum-free RPMI 1640 containing $CaCl_2$ (1 mM). A23187 (1 μM) was added to the medium in the absence or presence of CLT for 10 min. Intracellular Ca^{2+} level was measured by using a fluorescence-detecting microplate reader (FLUOstar Optima, BMG Labtech, Germany) with an excitation wavelength alternating every 30 sec from 340 to 380 nm and the emission wavelength was set at 510 nm. One representative graph of four independent experi-ments is shown in (A). The bar graph (B) represents the mean± SEM at 10, 20 and 30 min of A23187 incubation of four independent experiments. The letters C, D, E, and F represent vehicle-treated control in the presence of EGTA, A23187 alone, A23187 with CLT (5 μM), and A23187 with CLT (10 μM), respectively.

induced cell cycle arrest in HT29 cells, we then examined that co-treatment with A23187 and CLT alters CLT-induced expression of cell cycle inhibitors. As shown in Fig 5, CLT-induced increase in p27 and p53 was suppressed by co-treatment with A23187 (1 μM). Interestingly, addition of 1 mM of $CaCl_2$ to the medium significantly enhanced the effect of A23187 on the CLT-induced increase in p27 and p53.

Similar to the preventing effect of A23187 on CLT-induced changes of cell cycle inhibitors, co-treatment of cells with A23187 in the presence of 1 mM of $CaCl_2$ reversed the CLT-induced inhibition of cell growth (Fig. 6).

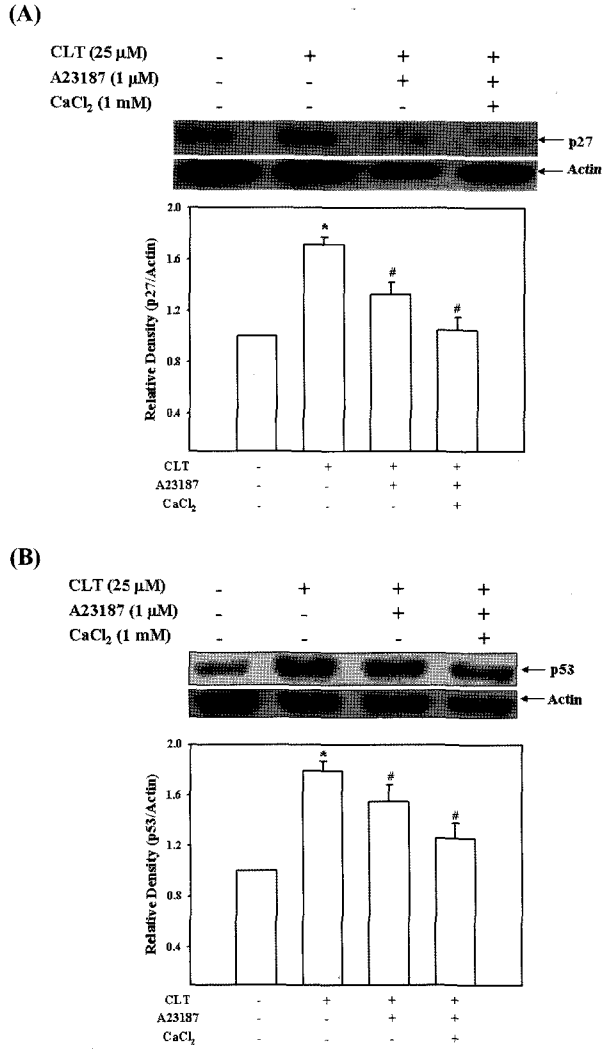

Fig. 5. Effects of calcium ionophore A23187 on CLT-activated p27 and p53. HT29 cells were treated with A23187 (1 μM) and CLT (25 μM) for 12 h concomitantly. Nuclear proteins were isolated, and 27 and p53 proteins were determined by western blot. Equal loading was confirmed by probing with antibodies against actin. Experiments were repeated 3 times and the bar graph represents the band intensity quantitated by densitometry. $^{*}P<0.05$, compared to vehicle (DMSO)-treated controls. $^{\#}P< 0.05$, compared to CLT-treated group.

DISCUSSION

CLT has been suggested as a potential agent for the treatment of cancer. It has been reported that CLT inhibits cell proliferation, and antiproliferative effect of CLT in a number of cancer cells has been suggested by blocking the cell cycle via interfering Ca^{2+} homeostasis. In the present study, we report that CLT inhibited serum-induced growth by halting cell cycle progression at the G(1)-S

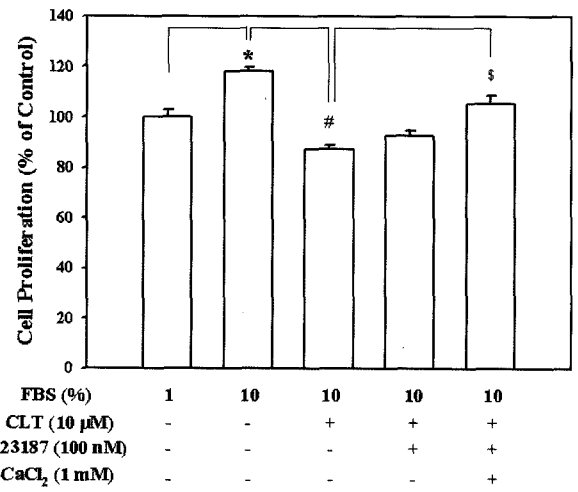

Fig. 6. Preventive Effects of A23187 on CLT-inhibited cell proliferation. Cells were starved serum (1% FBS) for 24 hr, and then, stimulated with 10% FBS in the absence or presence of CLT and A23187. $CaCl_2$ (1 mM) was added to the medium as it was needed. After 24 hr, the cells analyzed for the viable cell number by MTT assay. The data represent the mean values of four replications with bars indicating S.E.M. $^*P<0.05$, compared to serum-starved controls. $^{\#}P<0.05$, compared to 10% FBS-stimulated group. $^{\$}P<0.05$, compared to 10% FBS-stimulation and CLT-treated group.

phase transition through induction of cell cycle inhibitors, p27 and p53 in HT29 colon adenocarcinoma cells.

The cell cycle regulatory pathway is one of the useful targets for drug and gene therapy of cancers (Hunter and Pines, 1994). Cell cycle progression is regulated by sequential formation and activation of a series of cyclin-CDK complexes (Toyoshima and Hunter 1994; Ohtsubo and Roberts, 1993). CLT has been shown to block the cell cycle specifically in G1 by reducing synthesis and expression of G1 cyclins, thereby inhibiting the associated CDK activity required for progression into S phase (Aktas *et al.*, 1998). Similar to that, our results in HT29 cells showed that the growth inhibition by CLT was associated with the arrest of cells in G1 phase. Furthermore, we showed that CLT upregulated cell cycle inhibitors p27 and p53. p27 is one of the important members of *cip/kip* family, which are responsible for halting cell cycle in G1 phase, by binding to, and inactivating, cyclin-CDK complexes. p53, usually triggered by DNA damage, activates p21 and thus plays a significant role to halt cell cycle progression in G1 phase. In addition to cell cycle arrest, high amount of p53 expression induces apoptotic cell death. In the present study, low level of p53 expression was kept at low and short exposure to CLT, which corresponds to cell cycle arrest. In contrast, the p53 level was extensively increased in the cells exposed to high concentration of CLT for longer time period, which corresponded to the significantly increased apoptotic events.

Calcium has long been recognized as an important component in regulating cell growth and cell cycle progression. The alteration of intracellular Ca^{2+} concentration control cellular processes such as fertilization, proliferation and development. It has been reported that anticancer effects of CLT is mediated through depletion of intracellular Ca^{2+} stores, phosphorylation of eIF2alpha, and sustained inhibition of translation initiation mediate the (Aktas *et al.*, 1998). Benzaquen *et al.* (1995) also reported that CLT depletes the intracellular Ca^{2+} stores and prevents the rise in cytosolic Ca^{2+} that normally follows mitogenic stimulation. This was in accordance to our finding that CLT depleted intracellular Ca^{2+} induced by calcium ionophore, A23187. Furthermore, in the search of the action mechanism of CLT that lead to cell cycle arrest in HT29 cells, we showed that our results suggest a link between the Ca^{2+} signal and cell cycle inhibitors. The increased intracellular Ca^{2+} by A23187 reversed the effect of CLT in induction of cell cycle inhibitors. In addition, the recovery effect was more obvious when addition of $CaCl_2$ to the A23187 and CLT co-treated cells. In correlation to the results of intracellular Ca^{2+} and cell cycle related proteins, the antiproliferative effect of CLT was recovered by A23187, suggesting that intracellular Ca^{2+} regulates cell cycle progression, and that the action of CLT may at least in part be mediated by targeting intracellular Ca^{2+} signal for induction of cell cycle related proteins.

In summary, CLT inhibited cell cycle progression in HT29 colon cancer cells by up-regulating the important cell cycle inhibitor proteins, p27 and p53. Importantly, our findings provide a novel crosstalk pathway between intracellular Ca^{2+} and the Ca^{2+} signaling–mediated regulation of the cell cycle related proteins in colon cancer cells. These findings suggest an action mechanism of CLT in HT29 cells and the use of selective intracellular Ca^{2+} modulators like CLT as a valid chemotherapeutic agent in colon caner.

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