Ketoconazole Induces G0/G1 Arrest in Human Colorectal and Hepatocellular Carcinoma Cell Lines

Rong-Jane Chen, Wen-Sen Lee,* † Yu-Chih Liang,‡ Jen-Kun Lin,‡ Ying-Jan Wang,§ Chien-Huang Lin,† Jui-Ying Hsieh,‡ Chiu-Chin Chaing,§ and Yuan-Soon Ho‡

*Graduate Institute of Medical Sciences, Taipei Medical College, Taipei, Taiwan; † Department of Physiology, School of Medicine, Taipei Medical College, Taipei, Taiwan; ‡ Institute of Biochemistry, College of Medicine, National Taiwan University, Taipei, Taiwan; § Department of Environmental and Occupational Health, National Cheng Kung University Medical College, Taipei, Taiwan; and ¶ Institute of Biomedical Technology, Taipei Medical College, Taipei, Taiwan

Received April 21, 2000; accepted July 24, 2000

Ketoconazole is an oral-antifungal agent that has been used worldwide in the treatment of some hormone-dependent human cancer. In this study, we demonstrated that ketoconazole (20 μM) induced various types of human cancer cell growth arrest in the G0/G1 phase. Our results revealed that ketoconazole-induced growth arrest was more profound in COLO 205 and Hep G2 (with wild-type p53) than in HT 29 (p53 His175 mutant) and Hep 3B (with deleted p53) cells. The protein levels of p53, p21/Cip1, and p27/Kip1 were significantly elevated by ketoconazole (10 μM) treatment in COLO 205 but not in HT 29 cells. The ketoconazole-induced G0/G1 phase arrest in COLO 205 cells was attenuated by p53-specific antisense oligodeoxyribonucleotides (20 μM) treatment. These results suggested that the p53-associated signaling pathway is involved in the regulation of ketoconazole-induced cancer cell growth arrest. By Western blot analysis, we demonstrated that cyclin D3 and CDK4 protein but not other G0/G1 phase regulatory protein levels were decreased by ketoconazole-treatment in both COLO 205 and HT 29 cells. Our study provides the basis of molecular mechanisms for ketoconazole in growth inhibition of human cancer cells and such results may have significant applications for cancer chemotherapy.

Key Words: G0; G1 arrest; ketoconazole; p53; p21; Cip1.

Abbreviations used: CDK, cyclin-dependent kinase; CKIs, CDK inhibitors; IP, immunoprecipitation; FCS, fetal calf serum; KT, ketoconazole; ODN, oligonucleotide.

Tinea capitis and disseminated tinea corporis require systemic antifungal treatment. Ketoconazole (KT)¹ has been registered for therapy of adults with dermatophyte infections of the skin and nails (Gupta et al., 1999). In a previous report, KT was used as a treatment for women with acne and/or hirsutism (Venturoli et al., 1990). KT is also effective as an initial treatment for Leishmania braziliensis (Saenz et al., 1990). KT has been demonstrated to block steroidogenesis (Loose et al., 1983a) by inhibition of cholesterol side-chain cleavage (Kraemer and Spilman, 1986) and thereby has been used in the treatment of the hormone-dependent prostate cancer (Trachtenberg, 1984; Heyns et al., 1985; Mahler et al., 1993). In addition to its indirect effect on hormone-dependent cancers, ketoconazole has been reported to inhibit hepatic metastasis from a human pancreatic adenocarcinoma in the nude mouse model (Tzanakakis et al., 1990) and to reduce the incidence of pulmonary metastases in a mouse melanoma model (Nardone et al., 1988). Moreover, ketoconazole has been found to exert a cytotoxic effect in various cancer cell lines as indicated by clonogenicity assay in soft agar (Rochlitz et al., 1988) and to potentiate the antitumoral effect of interleukin 1α on murine RIF tumors (Braunschweiger et al., 1990). However, the exact mechanisms of KT action in the hormone-independent cell lines have not been identified. KT inhibits the activities of cytochrome P450 enzymes (Capdevila et al., 1988; Maurice et al., 1992) and arachidonic acid lipoxygenase (Beetens et al., 1986) and has an antiglucocorticoid property (Loose et al., 1983b). On the other hand, we have recently demonstrated that KT induced apoptosis in various types of human cancer cells (Ho et al., 1998), suggesting that other processes (such as caspase-induced apoptosis) were participating in the KT-induced repression of growth in human cancer cell lines. These processes may be indispensable for mitogenic signaling or for cell cycle progression.

Previous studies have demonstrated that the progression of the mammalian cell cycle is facilitated by the presence of multiple CDKs as well as cyclins (Gu et al., 1992; Solomon et al., 1992; Kato et al., 1994; Ohtsubo et al., 1994). The progression of the cell cycle from G0/G1 to S phase involves the activation of cyclin D/CDK4, cyclin D/CDK6, cyclin E/CDK2, and cyclin A/CDK2, whereas G2/M transition requires the activation of cyclinB/cdc2. The activity of CDKs is regulated by association with an activated cyclin, phosphorylated CDK, at a Thr residue by the CDK-
activating kinase and the dephosphorylation of Tyr residues by the phosphatase cdc 25 (Matsuoka et al., 1994). In addition, negative p34cdc2 kinase inhibitor, Wee1, has been identified to be essential for the control of p34cdc2 kinase activity in response to extra- or intracellular signals (McGowan and Russell, 1993).

In this study, we demonstrated that KT induced growth inhibition in various human cancer cells through G0/G1 cell cycle arrest. The specific aim of this study was to evaluate the molecular mechanisms of the G0/G1 cell cycle arrest induced by KT so that further mechanistic investigations for growth inhibition in human cancer cells could be performed. The protein levels of p53, CDK inhibitors (CKIs, such as p21/Cip1 and p27/Kip1), PCNA, and cyclin D, E, A, and B were determined in KT-treated human cancer cells. The CDK2 or CDK4 kinase complex was immunoprecipitated from KT-treated and untreated control cells, and the kinase activity was also investigated. As described above, KT has been widely applied in therapy of patients with dermatophyte infections as well as some types of hormone-dependent prostate cancer (Trachtenberg, 1984; Heyns et al., 1985). Our study provides further evidences that KT may have significant applications for cancer chemotherapy.

FIG. 1. Growth inhibition of different human cancer cells by KT. (A) HT 29, (B) COLO 205, (C) Hep 3B, and (D) Hep G2 cells were treated with various concentrations of KT (2 to 80 μM). The medium containing various dosages of KT was renewed everyday and the total cell number was counted at the indicated time points. In all cases, plots are the mean of triplicate experiments; Bars, SE. *p < 0.05, ** p < 0.001, all versus control group by Student’s t test.

MATERIALS AND METHODS

Cell lines and cell culture. The HT 29 and COLO 205 cell lines were isolated from human colon adenocarcinoma (American Type Culture Collection [ATCC] HTB-38 and CCL-222) (Semple et al., 1978). Hep 3B and Hep G2 cell lines were derived from human hepatocellular carcinoma (ATCC HB-8064 and HB-8065) (Knowles et al., 1980). The p53 gene in the COLO 205 and Hep G2 cells was wild-type (Bressac et al., 1990; Ho et al., 1996). In contrast, the p53 gene is mutated in codon 273 in HT 29 cells (Niewolik et al., 1995). The p53 gene was found to be partially deleted (7 kb) in Hep 3B cells (Darlington et al., 1987). Cell lines were grown at 37°C in a 5% carbon dioxide atmosphere in Eagle’s minimal essential medium (for Hep 3B and Hep G2 cells) or RPMI 1640 (for COLO 205 and HT 29 cell) supplemented with 10% fetal calf serum (FCS), 50 μg/mL gentamycin, and 0.3 mg/mL glutamine. The p53-specific antisense (5'-CGGCTCCTCCATGGCAGT-3') and sense (5'-ACTGCCATGGAGGAGCCG-3') phosphothioates (S-oligos) were synthesized and purified using high-performance liquid chromatography by Genset.

Determination of cell growth curve. Human colon cancer (5 × 10^4) and hepatoma (20 × 10^4) cells were plated in 35-mm petri dishes. The next day, the medium was changed and KT (2–80 μM) was added. Control cells were treated with DMSO in a final concentration of 0.05% (v/v). The incubation medium was renewed every day during the experiment. At the end of incubation, cells were harvested for cell count with a hemocytometer.

Cell synchronization, drug treatment, and flow cytometry analysis. At 24 h after plating of cells, medium was removed. Cells were washed three times with phosphate buffer saline and then incubated with medium containing 0.04% FCS for 24 h. Under these conditions, cells were arrested in G0/G1 as
**RESULTS**

**KT Induces G0/G1 Cell Cycle Arrest in Different Human Cancer Cells with Various p53 Status**

In this study, we demonstrated that KT induced significant growth inhibition of human cancer cells. As shown in Fig. 1, the growth rates of COLO 205 and Hep G2 cells (with wild-type p53) were significantly inhibited by KT (2–80 μM) treatment (Figs. 1B and 1D). Compared to COLO 205 and HepG2, the KT-induced growth inhibitions of HT 29 (p53 His273 mutant) and Hep 3B (with deleted p53) were less profound (Figs. 1A and 1C). In order to determine whether KT has a cell cycle arrest effect in human cancer cells, HT 29 and COLO 205 cells were synchronized with 0.04% serum starvation, the low serum (0.04% FCS) medium was removed and the cells were then stimulated by the addition of medium containing 10% FCS. KT solutions were prepared by dissolving this compound in a final concentration of 0.05% DMSO. The stages of cell cycle in KT- and mock-treated groups were measured by flow cytometry analysis. After serum starvation, the low serum (0.04% FCS) medium was removed and the cells were then stimulated by the addition of medium containing 10% FCS. KT solutions were prepared by dissolving this compound in a final concentration of 0.05% DMSO. The stages of cell cycle in KT- and mock-treated groups were measured by flow cytometry analysis. After serum starvation, the low serum (0.04% FCS) medium was removed and the cells were then stimulated by the addition of medium containing 10% FCS. KT solutions were prepared by dissolving this compound in a final concentration of 0.05% DMSO. The stages of cell cycle in KT- and mock-treated groups were measured by flow cytometry analysis.

**Western analysis.** Treated and untreated cells were rinsed three times with ice-cold phosphate-buffered saline, pelleted at 800g for 5 min, and lysed in 500 μl of freshly prepared extraction buffer (10 mM Tris—HCl, pH 7.4, 140 mM sodium chloride, 3 mM magnesium chloride, 0.5% [w/v] NP-40, 2 mM phenylmethylsulfonyl fluoride, 1% [w/v] aprotinin, and 5 mM dithiothreitol) for 20 min on ice. The extracts were centrifuged for 30 min at 10,000g. Proteins were loaded at 50 μg/lane on 12% (w/v) SD–PAGE, blotted, and probed using antibodies including cyclin E (Santa Cruz, Inc. CA), p53, p21/Cip1, p27/Kip1, cyclin A, cyclin D1, cyclin D3, PARP, cyclin B1, CDK2, and CDK4 kinase. (Transduction Laboratories, Lexington, KY). Immunoreactive bands were visualized by incubating with the colorogenic substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma Chemical Co., St. Louis, MO). The expression of GAPDH was used as the control for equal protein loading.

**Immunoprecipitation and CDK kinase activity assay.** The p21-associated CDK-2 kinase activity was determined as described by Wu et al. (1996). Briefly, using anti-CDK2 antibody (2 μg) and protein A agarose beads (20 μl), the protein complexes were precipitated from 200 μg of protein lysate per sample as described above. The beads were washed three times with lysis buffer and then once with kinase assay buffer (50 mM Tris—HCl, pH 7.4, 10 mM MgCl2, and 1 mM DTT). Phosphorylation of histone H1 was measured by incubating the beads with 40 μl of “hot” kinase solution (0.25 μl [2.5 μg] of histone H1, 0.5 μl of [γ-32P]ATP, 0.5 μl of 0.1 mM ATP, and 38.75 μl of kinase buffer) for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed by 12% SDS–PAGE, and the gel was dried and subjected to autoradiography.

Similarly, the p21-associated CDK4 kinase activity was also determined as described by Wu et al. (1996) with some modifications. Briefly, KT-treated cells were lysed in Rb lysis buffer (50 mM Hepes—KOH, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 80 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupetin and aprotinin) and immunoprecipitated with anti-CDK4 antibody (2 μg). The protein complexes in beads were washed twice with Rb lysis buffer and then once with Rb kinase assay buffer (50 mM Hepes—KOH, pH 7.5, containing 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM MgCl2, and 1 mM DTT). Phosphorylation of Rb was measured by incubating the beads with 40 μl of hot Rb kinase solution (0.25 μl [2 μg] of Rb–GST fusion protein, 0.5 μl of [γ-32P]ATP, 0.5 μl of 0.1 mM ATP, and 38.75 μl of Rb kinase buffer) for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed by 12% SDS–PAGE, and the gel was dried and subjected to autoradiography.

**FIG. 2.** Time-dependent response of KT-induced G0/G1 phase arrest in HT 29 and COLO 205 cells. HT 29 (left) and COLO 205 (right) cells were synchronized with 0.04% FCS for 24 h as described under Materials and Methods. After synchronization, cells were then released into complete medium (10% FCS) containing 0.05% DMSO with or without KT (40 μM). Different phases of cell cycle were measured by flow cytometry at the indicated time points after exposure to KT. In all cases, plots are the means ± SE of triplicate experiments and all of the time points (KT-treated versus control group) were selected for statistical analysis by Student’s paired t test. Significance was observed in DMSO-treated cells after challenged with 40 μM KT for 20 min on ice. The extracts were centrifuged for 30 min at 10,000g. Proteins were loaded at 50 μg/lane on 12% (w/v) SD–PAGE, blotted, and probed using antibodies including cyclin E (Santa Cruz, Inc. CA), p53, p21/Cip1, p27/Kip1, cyclin A, cyclin D1, cyclin D3, PARP, cyclin B1, CDK2, and CDK4 kinase. (Transduction Laboratories, Lexington, KY). Immunoreactive bands were visualized by incubating with the colorogenic substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma Chemical Co., St. Louis, MO). The expression of GAPDH was used as the control for equal protein loading.
COLO 205 Cells Were More Sensitive to KT-Induced G0/G1 Arrest

Figure 2 shows that the biggest difference of G0/G1 cell population between KT-treated and control group was at 15 h after replacing with complete medium. Accordingly, this time point (15 h) was selected for studying the dose-dependent effect of KT and the minimal dose of KT required for induction of G0/G1 arrest was determined with flow cytometry analysis (Figs. 3A and 3B). Our results revealed that the minimal dose of KT inducing a significant G0/G1 arrest in HT 29 cell was 20 μM (Fig. 3A). In contrast, the dose of KT required for the induction of a significant G0/G1 arrest was as low as 0.2 μM in COLO 205 cells (Fig. 3B). With a higher dose of KT (60 μM) exposure, more than 90% of cells were arrested in G0/G1 phase in both cell lines. Our previous report indicated that p53 was involved in KT-induced apoptosis in COLO 205 cells (Ho et al., 1998). The present study further demonstrated that COLO 205 cells were more sensitive to KT-induced G0/G1 arrest compared with HT 29 cells, suggesting that p53 might be involved in KT-induced G0/G1 arrest.

KT Induces G0/G1 Arrest through Elevation of p21/Cip1, p27/Kip1, and Inhibition of Cyclin D3 and CDK4 Protein Expression

We chose HT 29 cells to investigate the general molecular mechanisms of KT-induced G0/G1 phase arrest. After serum starvation, HT 29 cells were synchronized at the G0/G1 phase as described above. Changes of G0/G1 regulatory protein levels in HT 29 cells in response to KT (60 μM) treatment were then determined (Fig. 4). In this study, the time points that we selected for drug treatment were according to Fig. 2: 0 h (representing the G0/G1 phase), 15 h (representing the S phase), 18 h (representing the G2/M phase), and 24 h (representing the second G0/G1 phase). The expression of p21/Cip1, a key regulator of cell entry into mitosis, was first monitored by immunoblotting analysis. Our results revealed that p21/Cip1 expression was elevated initially at 18 h and was persistent for at least 24 h by KT treatment (Fig. 4, right). Our results also showed that the levels of cyclin D3 and CDK4 in KT-treated HT 29 cells were significantly downregulated at 15 h by KT treatment whereas cyclin D1 and PCNA levels were not (Fig. 4).
The other cell cycle regulatory protein p27, an inhibitor of cyclin-dependent kinase (CDK), has been indicated to play a role in (i) prognosis in breast and colon cancer (Loda et al., 1997; Erlanson et al., 1998), (ii) induction of apoptosis in cancer cells (Hiromura et al., 1999), and (iii) resistance to cancer chemotherapy (St. Croix et al., 1996; Naumann, 1999).

A previous study demonstrated that the level of p27 protein was increased in quiescent cells and rapidly decreased after stimulated with serum (Coats et al., 1996). A similar result was observed in Fig. 4, showing that the p27 protein level increased dramatically in HT 29 cells after 24 h of serum starvation (Fig. 4, left, lane 2) and then decreased after challenge with 10% FCS (Fig. 4, left, lanes 3–6). In contrast, 10% FCS treatment failed to decrease the p27 protein levels in the KT-treated cells.

The expression levels of CDK2 protein were inhibited in a lesser extent than CDK4 in the KT-treated group (Fig. 4). Previous studies demonstrated that two types of cyclin A (A1, 66 kDa, and A2, 58 kDa) existed in mammalian cells. Cyclin A1 differs from other cyclins in its highly restricted expression pattern. Besides its expression during spermatogenesis (Ravnik and Woglemuth, 1999; Liu et al., 1998), cyclin A1 is also expressed in hematopoietic progenitor cells and in acute myeloid leukemia (Muller et al., 1999). Cyclin A2 is the major regulator of the cell cycle progression and its synthesis is required for progression to S phase (Howe et al., 1995; Desdouets et al., 1999). In this study, the faster migration form of cyclin A2 (58 kDa) and cyclin B, which promote cells entry from G0/G1 into S and from S into G2/M phase, respectively, were also downregulated in KT-treated HT 29 cells (Fig. 4). As described above, the protein level of CDK2 was not significantly changed in KT-treated cells. Another cell cycle regulator, cyclin E, which associates with CDK2, was determined. The cyclin E protein slightly accumulated in both control and KT-treated cells in a time-dependent manner (Fig. 4). However, dose-dependent experiments revealed that the expression of cyclin E did not change in either KT-treated or control cells until 18 h of drug treatment (Fig. 5).

p53, p21/Cip1, and p27/Kip1 Were the Key Regulators in KT-Induced G0/G1 Arrest

Previous studies have demonstrated that the p53 protein is a potent transcription factor, which is activated and accumulated in response to different DNA-damaging agents (Kastan et al., 1991; Kern et al., 1991; Haffiner and Oren, 1995), leading to cell cycle arrest or apoptosis occurrence (Ko and Prives, 1996; Levine, 1997). Our previous report (Ho et al., 1998) demonstrated that KT induced p53 protein expression in a dose-dependent manner. The p53 and p21/Cip1 proteins were elevated significantly by 10 μM of KT treatment in COLO 205 cells (Fig. 5, right). However, a higher dose of KT (20 μM) is required to induce the p53 protein expression in HT 29 cells (Fig. 5, left). The cyclin D3 and CDK 4 expression were downregulated concomitant with the p21/Cip1 induction in both cells. These results suggest that the expression of p53 and p21/Cip1 proteins is critical for KT-mediated G0/G1 arrest.

FIG. 4. Time-dependent response of KT-induced G0/G1 phase regulatory protein levels in HT 29 cells. HT 29 cells were synchronized with 0.04% FCS for 24 h as described under Materials and Methods. After synchronization, cells were then released into complete medium (10% FCS) containing KT (60 μM) for 0, 15, 18, 21, and 24 h. Control was HT 29 cells treated with DMSO (0.05%, v/v). Protein extracts (50 μg/lane) were separated by SDS–PAGE, immunoblotted with antibodies, and detected using the NBT/BCIP system. The expression of GAPDH was used as the control for equal protein loading.

FIG. 5. Dose-dependent response of KT-induced G0/G1 phase regulatory protein levels in HT 29 and COLO 205 cells. HT 29 and COLO 205 cells were synchronized with 0.04% FCS for 24 h as described under Materials and Methods. After synchronization, cells were then released into complete medium (10% FCS) containing KT (1–60 μM) for 15 h. Control group was cells treated with DMSO (0.05%, v/v). Protein extracts (50 μg/lane) were separated by SDS–PAGE, immunoblotted with antibodies, and detected using the NBT/BCIP system. The expression of GAPDH was used as the control for equal protein loading.
KT-Induced G0/G1 Arrest Was through p21/Cip1-Mediated CDK2 and CDK4 Kinase Inhibition

As described previously, p21/Cip1 was a potent inhibitor of the cyclin-dependent kinase. We suggest that KT treatment may result in the inhibition of CDK4 and CDK2 activity that is necessary for cells to overcome the restriction point in the G0/G1 phase of the cell cycle. As shown in Fig. 6, the protein levels of both p53- and CDKs-associated p21/Cip1 were induced, whereas the kinase activities of CDK2 and CDK4 were inhibited in 10 μM of KT-treated cells compared with control groups in both HT 29 and COLO 205 cells. These analyses revealed that the decreased CDK4 activity was concomitant with increased binding of p21/Cip1 to CDK4 in the cells treated with KT (Fig. 6). Collectively, these results indicate that the G0/G1 arrest induced by KT was due to decreased kinase activities of CDK4 and CDK2 mediated by an increase of p21/Cip1–CDK association.

To investigate the role of p53 protein played in KT-induced G0/G1 arrest in human cancer cells, COLO 205 cells (with wild-type p53) were pretreated with 20 μM of p53-specific antisense oligonucleotides (ODNs) for 16 h prior to addition of KT (60 μM) for another 15 h. Our results revealed that KT-induced G0/G1 arrest was attenuated by the p53 specific antisense ODNs, but not sense ODNs (Fig. 7A). To further examine whether the p53-regulated p21 protein induction was reduced by the p53-specific antisense ODNs, the change of p53 and p21 protein level was determined by Western blot analysis (Figs. 7B and 7D). Indeed, the KT-induced increase of p21 protein level was effectively inhibited by treatment with p53-specific antisense ODN, but not sense ODN (Fig. 7D). The CDK4 kinase activity inhibited by KT treatment was attenuated by p53-specific antisense ODN (Fig. 7D). In order to determine the extent of p53 and p21 level in response to KT treatment, the p53 and p21 protein expression in COLO 205 cells treated with mitomycin C was measured as a positive control group (Fig. 7C). These results suggest that p53 protein indeed play a critical role in the KT-induced G0/G1 cell cycle arrest.

DISCUSSION

KT is an orally active broad-spectrum antifungal drug especially active in patients with histoplasmosis or nonmeningeal cryptococcosis (Dismukes, 1983). Recent studies have demonstrated that KT is an active agent against various malignant cell lines in vitro (Rochlitz et al., 1988) and in vivo (Trachtenberg, 1984; Heyns et al., 1985; Tzanzakakis et al., 1990; Nardone et al., 1988). Previous results from a human study showed that the serum levels of KT after administration of single doses of 200 and 400 mg were 3.6 and 6.5 μg/ml, respectively (Van Tyle, 1984). Heyns et al. (1985) demonstrated that nine patients with prostate cancer treated with high dose of KT (400 mg every 8 h) were detected to have serum levels of KT at a range of 2.0–9.1 μg/ml (means of 12 measurements recorded during 1 year of continuous treatment). Different human cancer cell lines treated with KT showed similar IC50 values from 7.3 to 10.0 μg/ml (Rochlitz et al., 1988). Our recent study demonstrated that the minimal dose of KT inducing apoptosis in various human cancer cell lines was 5 μM (Ho et al., 1998). As shown in this study, the G0/G1 arrest in human COLO 205 cells was clearly induced by KT at a concentration of 1 μM. Our results described above indicate that some other mechanisms such as apoptosis induction and G0/G1 cell cycle arrest could be involved in suppression of human cancer cells growth by KT treatment.

The tumor suppressor, p53, has been implicated in a variety...
of cellular processes (Greenblant et al., 1994; Bates and Vousden, 1996). However, the undisputed roles of p53 are the induction of cell growth arrest and apoptosis (El-Deiry et al., 1994). Our previous study has shown that KT-induced apoptosis occurs in various type of human cancer cell lines (Ho et al., 1998). The apoptosis regulatory proteins such as Bax and Bcl-2 were changed, apparently by KT (5 μM), in cells with wild-type p53 (Hep G2 and COLO 205). In this study, we found that cells containing wild-type p53 (COLO 205 and Hep G2) were more sensitive to KT exposure, in terms of proliferation inhibition. We further demonstrated that the process of G0/G1 cell cycle arrest induced by KT is correlated with the induction of the p53-associated signaling pathway, as evidenced by the p53 specific antisense oligonucleotide experiment. These results

FIG. 7. The p53-specific antisense ODNs attenuated the KT-induced G0/G1 arrest through p21/Cip1-regulated CDK4 kinase activity. COLO 205 cells were pretreated with 20 μM of p53-specific antisense oligonucleotides for 16 h prior to addition of KT (60 μM). (A) The G0/G1 phase cell cycle arrest in COLO 205 cells was measured by flow cytometry at 15 h after exposure to KT. (B) The p53 protein level in COLO 205 cells treated KT with or without antisense ODNs was measured. (C) The p53 and p21 protein levels in COLO 205 cells treated either with 5 μM mitomycin C (positive control) or with KT (60 μM) for 15 h were determined by Western blot. (D) The CDK4-associated p21/Cip1 protein was immunoprecipitated with CDK4-specific antibody and then detected with p21/Cip1 antibody by Western blot. On the other hand, the p21/Cip1 antibody-immunoprecipitated CDK4 kinase activity from lysates of COLO 205 cells was measured as described under Materials and Methods. Each blot is representative of three similar experiments. Mit C, mitomycin C; AS, antisense; S, sense.
support the hypothesis that the p53-signaling pathway is involved in mediating KT-induced apoptosis and growth arrest in human cancer cells.

The p21/Cip1 is induced by the p53 tumor suppressor upon DNA damage (Gartel et al., 1996). Previous studies demonstrated that the cell growth arrest was mediated by transcriptional activation of p21/Cip1, which binds and inactivates the cyclin-dependent kinases required for cell cycle progression (El-Deiry et al., 1993, 1994). A variety of recent data suggest that p21/Cip1 does have tumor suppressor properties. P21/Cip1 mutations are found at a low frequency in several human tumors (Malkowicz et al., 1996), and a p21/Cip1 mutation, which was demonstrated to specifically abrogate its binding to CDKs, was identified in a primary breast tumor (Balbin et al., 1996). The human papilloma virus E7 transforming protein interacts with p21/Cip1, disrupting its interaction with CDKs (Funk et al., 1997; Jones et al., 1997). Keratinocytes from p21/Cip1-null mice are more highly tumorigenic than their wild-type counterparts following expression of an activated ras allele (Missero et al., 1996), and highly-intensified Raf signaling is opposed by p21/Cip1 (Sewing et al., 1997). Finally, mice doubly nullizygous for p21 and pRB are more tumor prone than those are null for pRB alone (Brugarolas et al., 1998). The later finding also reinforces evidence that p21 does not act solely through its effects on pRB.

Another CKI is p27/Kip1, which mediates growth arrest and is thought to play a critical role in negative regulation of cell division in vivo (Sherr, 1996). In contrast to p21, mice null for the p27 gene demonstrated increased body size, female sterility, and a high incidence of spontaneous pituitary tumors (Fero et al., 1996). Recently, overexpression of p27 has been shown to induce apoptosis and inhibition of tumor formation in vitro and in vivo (Wang et al., 1997). In addition, a p23 fragment was detected from the proteolysis of p27/Kip1 by a caspase-3-like protease. The p23 was exclusively located in the cytosol and was a true inhibitor of CDK2 activity in intact cells (Loubat et al., 1999). The outcome of CKIs induction in most cells is cessation of cell proliferation, differentiation, or even cell death. Since an inhibition of CKIs activity is one of the factors causing uncontrolled proliferation of tumor cells, one possible strategy to control the cancer cell proliferation is to induce CKIs expression, which would lead to G0/G1 arrest and stop tumor cell growth. In this study, we observed that KT can cease cell proliferation in a wide variety of tumor cells with different p53 status, and treatment of cells with KT led to G0/G1 arrest through the induction of p21/Cip1 and p27/Kip1. We also provide evidence that KT inhibited not only the increase of CKIs (p21/Cip1 and p27/Kip1) levels but also the decrease of cyclin D3/CDK4 kinase activity. The consequent reduction of CDK4 activity by CKIs is most likely responsible for the KT-induced G0/G1 arrest in tumor cells. The present studies suggest that KT may have chemotherapeutic properties by inducing the inhibitory activity of the negative regulators of the cell cycle. Therefore, it is quite pertinent to investigate the direct mechanism by which KT activates the CKIs in the cells. Our study provides the basis of molecular mechanisms for KT in cancer treatment. The universality and the p53-dependent action of KT in the inhibition of cancer cell proliferation would make it a very attractive agent for cancer chemotherapy.

ACKNOWLEDGMENTS

This study was supported by the National Science Council Grant NSC 89–2320-B-038–032.

REFERENCES


Fero, M. L., Rivkin, M., Tasch, M., Porter, P., Carow, C. E., Firpo, E., Polyak,
and lymphoid cell lines from a patient with carcinoma of the colon for a cytotoxic model. *Cancer Res.* **38**, 1345–1355.


