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ARTICLES

Inhibition of Cyclin-Dependent Kinases 2 and 4 Activities as Well as Induction of Cdk Inhibitors p21 and p27 During Growth Arrest of Human Breast Carcinoma Cells by (−)-Epigallocatechin-3-Gallate

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Abstract (−)-Epigallocatechin-3-gallate (EGCG) potently inhibits cell proliferation and suppresses tumor growth both in vitro and vivo, but little is known regarding the cell cycle regulatory proteins mediating these effects. This study investigated the effects of EGCG and other catechins on the cell cycle progression. DNA flow cytometric analysis indicated that 30 μM of EGCG blocked cell cycle progression at G1 phase in asynchronous MCF-7 cells. In addition, cells exposed to 30 μM of EGCG remained in the G1 phase after release from aphidicolin block. Over a 24-h exposure to EGCG, the Rb protein changed from hyper- to hypophosphorylated form and G1 arrest developed. The protein expression of cyclin D1, and E reduced slightly under the same conditions. Immunocomplex kinase experiments showed that EGCG inhibited the activities of cyclin-dependent kinase 2 (Cdk2) and 4 (Cdk4) in a dose-dependent manner in the cell-free system. As the cells were exposed to EGCG (30 μM) over 24 h a gradual loss of both Cdk2 and Cdk4 kinase activities occurred. EGCG also induced the expression of the Cdk inhibitor p21 protein and this effect correlated with the increase in p53 levels. The level of p21 mRNA also increased under the same conditions. In addition, EGCG also increased the expression of the Cdk inhibitor p27 protein within 6 h after EGCG treatment. These results suggest that EGCG either exerts its growth-inhibitory effects through modulation of the activities of several key G1 regulatory proteins such as Cdk2 and Cdk4 or mediates the induction of Cdk inhibitor p21 and p27. J. Cell. Biochem. 75:1–12, 1999.

Key words: (−)-epigallocatechin-3-gallate; cell cycle; cyclin; cyclin-dependent kinase; retinoblastoma protein

Tea has been used as a daily beverage and crude medicine in China for several thousand years. Tea is thought to exert a possible inhibitory effect against tumorigenesis and tumor growth due to the biologic activities of its polyphenols [Anonymous et al., 1996; Stoner and Mukhtar, 1995; Yamane et al., 1995; Shi et al., 1994; Katiyar et al., 1993; Ho et al., 1992]. The green tea polyphenols comprise (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), (−)-epicatechin (EC), (+)-gallocatechin (GC), and catechin (C). EGCG is the major polyphenol component of green tea. Tea polyphenols are natural plant flavonoids present in the leaves and stem of tea plants, especially in young shoots. We previously reported that EGCG could reduce the autophosphorylation levels of EGF-R induced by EGF and block EGF binding to its receptor [Liang et al., 1997]. Another recent study from our laboratory used lipopolysaccharide (LPS)-activated peritoneal macrophages to demonstrate that EGCG could prevent the binding of nuclear factor-κB (NF-κB) to the inducible nitric oxide synthase (iNOS) promoter, thereby inhibiting transcription and transla-

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tion of iNOS, and decreasing the activity of iNOS [Lin and Lin, 1997]. These results suggest that EGCG and other catechins may play an important role in preventing carcinogenesis and anti-inflammation.

Previous studies have shown that many flavonoids exhibit potent antitumor activity against several rodent and human cancer cell lines both in vitro and in vivo [Lin et al., 1996; Traganos et al., 1992; Kaur et al., 1992; Wei et al., 1990; Yoshida et al., 1990]. The antitumor properties of some flavonoids have been studied with respect to apoptosis and the cell cycle. Genistein is known to cause a G2/M arrest in several human and murine cell lines and to cause apoptosis in several myeloid and lymphoid cell lines [Spinozzi et al., 1994; Matsukawa et al., 1993; Traganos et al., 1992].

Quercetin has been shown to block G1 into the S phase in a human gastric cancer cell line and also to cause apoptosis in several cell lines [Wei et al., 1994; Yoshida et al., 1990]. Apigenin causes G2/M arrest in keratinocytes and induces differentiation in rat neuronal cells [Lepley et al., 1996; Sato et al., 1994]. Flavopiridol is a synthetic flavone, which strongly inhibits the cyclin-dependent kinases and can potentially cause inhibition of cell cycle progression in G1 and G2 by multiple mechanisms [Sedlacek et al., 1996]. The molecular mechanisms of cell cycle arrest by flavonoids remain largely unclear but appear to involve modulation of multiple cell cycle regulatory proteins. The eukaryotic cell cycle is regulated through the sequential activation and inactivation of cyclin-dependent kinases (Cdks) that drive cell cycle progression through phosphorylation [Morgan, 1995] and dephosphorylation of several regulatory proteins.

In normal cells, Cdks exist predominantly in quaternary complexes consisting of a Cdk, a cyclin, a proliferating cell nuclear antigen (PCNA), and a 21-kDa protein (p21) [Xiong et al., 1992]. Cdk activation requires cyclin binding and phosphorylation of conserved threonine residue by Cdk-activating kinase (CAK). The activated Cdk-cyclin complexes can be changed to an inactive state by phosphorylation of a conserved threonine-tyrosine pair or binding to Cdk inhibitory subunits (CKIs). Progression from G1 to S phase in mammalian cells is regulated by the accumulation of cyclins D, E, and A, which bind to and activate different Cdk catalytic subunits. Activation of the Cdk4-cyclin D and/or Cdk6-cyclin D complex is necessary for transition from early to mid-G1 phase. Transition through mid G1 to S phase is regulated by activation of the Cdk2-cyclin E complex. Progression through late G1 to S phase also requires the presence of Cdk2-cyclin A complex [Sherr, 1996]. The retinoblastoma tumor suppressor protein (Rb) is a critical target protein that is phosphorylated by these Cdk-cyclin complexes [Weinberg, 1995]. Rb control gene expression is mediated by a family of heterodimeric transcriptional regulators, collectively termed the E2F, which can transactivate genes whose products are important for transition from G1 to S phase [Botz, 1996]. Phosphorylation of Rb frees these regulators, enabling them to transactivate the target genes. Therefore, the hypophosphorylated forms of Rb are found predominantly in G0–G1 phase, but the hyperphosphorylated forms of Rb are required during S and G2-M phase [Weinberg, 1995].

Recent studies show that Cdk regulation involves a diverse family of proteins, termed the CKIs (Cdk inhibitors), that bind and inactivate Cdk-cyclin complexes. In mammalian cells, CKIs fall into two classes: (1) p21 (Cip1/Waf1/Cap20/Sdi1/Pic1), p27 (Kip1), and p57 (Kip2), which are related proteins with a preference for Cdk2- and Cdk4-cyclin complexes; and (2) p16INK4, p15INK4B, p18INK4C, and p19INK4D, which are closely related CKIs specific for Cdk4- and Cdk6-cyclin complexes [Sherr and Roberts, 1995]. Most in vivo studies suggest that the inhibitory effect of p21 is largely exerted during the G1 phase of the cell cycle, with preferential binding to Cdk4- and Cdk2-containing complexes, and that it either inhibits their kinase activities or prevents their activation by CAK [Aprelikova et al., 1995]. In addition, the regulation of p21 is largely dependent on the presence of functional p53, a transcriptional regulator that mediates cell cycle arrest after DNA damage [El-Deiry, 1994] and in senescence [Noda et al., 1994]. However, the expression of p21 in a variety of tissues from p53 null mice suggests that it is also regulated by a p53-independent mechanism [Parker et al., 1995].

In this study, we examined the effects of EGCG and other catechins on the growth of breast carcinoma MCF-7 cells, expression of G1 cyclins, phosphorylation state of Rb, and kinase activities of Cdk2 and Cdk4 in a cell-free system and in cultured cells. The effects of these

catechins on the protein and mRNA levels of CKIs were also investigated.

EXPERIMENTAL PROCEDURES

Materials

EGCG, EGC, and ECG were purified from Chinese tea (Longjing tea, *Camellia sinensis*), using the method of Nonaka et al. [1983], with some modifications as described in our previous report [Lin et al., 1996]. Their purity was 97%, 95%, and 93%, respectively. EC, catechin (C), gallic acid (GA), caffeine, and all protease inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO). The antibodies of p27, cyclins A, D1, E, and Cdk2 were obtained from Santa Cruz Company (CA); anti-p21 monoclonal antibody was from Transduction Lab. (Lexington, KY); anti-Rb monoclonal antibody was kindly provided by Prof. W.-H. Lee; Cdk4 from Upstate Biotechnology (Lake Placid, NY); and p53 from Oncogene Research Products (Cambridge, MA). Polynucleotide kinase and oligo (dT) 18 were obtained from Pharmacia (Piscataway, NJ).

Cell Culture and Cell Growth Assays

MCF-7 breast carcinoma cells was generously provided by Dr. M.-T. Lee (Taiwan University) and MDA-MB 231 breast carcinoma cells by Prof. W.-K. Yang (National Health Research Institutes, Taiwan). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS) (Gibco-BRL, Grand Island, NY) and were incubated at 37°C in humidified 5% CO_2. Cell growth assays were performed by counting trypan blue dye-excluding cells in a hemacytometer [Lin et al., 1996].

Cell Synchrony Methods and Flow Cytometric Cell Analysis

Cells were synchronized at the beginning of the S phase by the addition of the reversible DNA polymerase inhibitor aphidicolin (4 μg/ml; Sigma) for 12 h [Pedrali-Noy et al., 1980]. For synchronization in the M phase, cells were incubated with 0.4 μg/ml nocodazole (Sigma) for 12 h [Zieve et al., 1980]. To release the block, cells were washed twice in DMEM and replated in fresh medium with or without EGCG.

Cell cycle distribution was analyzed by flow cytometry as described previously [Chen et al., 1996]. Briefly, cells were trypsinized, washed once with phosphate-buffered saline (PBS), and fixed in 100% ethanol for 1 h at −20°C. Fixed cells were washed with PBS, incubated with 0.5 ml PBS containing 0.05% RNase and 0.5% Triton X-100 for 30 min at 37°C, and stained with propidium iodide. The stained cells were analyzed using a FACScan laser flow cytometer (Becton Dickinson, San Jose, CA).

Western Blot Analysis

Equal amounts of total cellular proteins (50 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham, Arlington, IL), and then probed with primary antibody followed by secondary antibody conjugated with horseradish peroxidase or alkaline phosphatase. The immunocomplexes were visualized using enhanced chemiluminescence (ECL) kits (Rb, Cdk2, Cdk4, p21, p27, p53, and cyclin D1) or by NBT/BCIP substrates (Rb, cyclin A, and cyclin E).

Generation of Gst-Rb Fusion Protein

cDNA encoding full-length human Rb was kindly provided by Prof. W.-H. Lee [Hensey et al., 1994]. To generate the vector encoding pGEX-Rb, the Rb cDNA encoding amino acids 769–921 were amplified by polymerase chain reaction (PCR). Each 5′ amplimer contained a *Bam*HI site, and each 3′ amplimer contained an *Eco*RI site in addition to a TGA stop codon, such that the resulting PCR product, upon digestion with *Bam*HI/*Eco*RI, could be ligated in frame into the unique *Bam*HI/*Eco*RI site present within the pGEX-2T (Pharmacia) polylinker. pGEX-2T was linearized with *Bam*HI and *Eco*RI (BioLabs, Beverly, MA) and ligated to the above *Bam*HI/*Eco*RI-digested PCR products, using standard techniques. PCR was carried out in a 100-μl reaction volume containing approximately 0.5 ng of DNA template, 0.6 μg of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate, and 2.5 U of Super Taq polymerase. Thermal cycle conditions were as follows: 30 cycles at 95°C for 1 min, 50°C for 1.5 min, and 72°C for 30 s. The following primers were used: 5′-TGGATCCTTGCAGTATGCTTCCACC-3′ and 5′-AGAATTCTCAATCCATGCTATCATTCAT-3′.

For preparation of the probe substrate, *Escherichia coli* transformed with pGEX-Rb (769–921) was grown to saturation overnight, diluted

1:10 in Luria-Bertani (LB) medium, and incubated at 37°C for 1 h with shaking. Glutathione S-transferase-pRb fusion protein was induced by the addition of 0.5 mM isopropylthioglycoside to the culture for 3 h at 30°C. Cells were recovered by centrifugation (5,000*g* for 10 min) at 4°C and resuspended in 1/5 vol NETN (100 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.5% Nonidet P-40 [NP-40]). The bacteria were then lysed on ice by mild sonication and centrifuged at 10,000*g* for 10 min at 4°C. Cleared bacterial supernatant was rocked for 18 h at 4°C with glutathione-Sepharose 4B (Pharmacia). The glutathione-Sepharose beads were then washed three times with NETN buffer and twice with kinase buffer, and the pRb fusion proteins were released at 4°C by incubation in kinase buffer containing 2.5 mM reduced glutathione. The concentration and purity of soluble pRb fusion protein were estimated by Coomassie blue staining of electrophoretically separated proteins on SDS-polyacrylamide gels, in comparison with bovine serum albumin (BSA) standards of known concentration.

In Vitro and Cell Culture Cdks Kinase Assay

For in vitro Cdk kinase assay, exponentially growing MCF-7 cells were washed with cold PBS and lysed with gold lysis buffer [10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris-HCl pH 7.9, 100 µM β-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin] for 30 min at 4°C. The cell lysate was clarified by centrifugation at 12,000*g* for 10 min at 4°C. A total of 4 mg of protein was incubated with anti-CDK2 or anti-CDK4 antibody and protein A/G plus agarose (Santa Cruz) for 18 h at 4°C. The immunoprecipitate was washed 3× with immunoprecipitate buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM PMSF, 0.5% NP-40) and 3× with kinase buffer (50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 2.5 mM EDTA, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM DTT for Cdk4; 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 2.5 mM EDTA, 1 mM DTT for Cdk2) 3× and separated into 6–8 tubes [Akiyama et al., 1997]. The kinase reactions were carried out in a final volume of 40 µl containing 5 µg histone H1 (Calbiochem, San Diego, CA) or 10 µg Gst-Rb, 20 µM cold ATP, 5 µCi [r-32p] ATP (5,000 Ci/mmol, Amersham) and incubated for 20 min at 25°C. Each sample was mixed with 10 µl of 5× Laemmli's loading buffer, to stop the reaction, heated for 10 min at 100°C, and subjected to SDS-PAGE. The gels were dried, visualized by autoradiography, and quantitated by densitometry (IS-1000 Digital Imaging System).

For cell culture Cdks kinase assay, cells (3×10^5/dish) were precultured in 100-mm plastic dishes for 2 days and treated for various periods. Cell lysis and immunoprecipitation were performed as described above. Kinase assay was carried out in 50 µl of kinase buffer with Cdk2 or Cdk4-immunoprecipitate from 500 µg of protein of the lysate. The kinase reaction was performed as described above.

RT-PCR

Total RNA was isolated from control or EGCG treatment cultured cells and cDNA was prepared as previously described [Lin and Lin, 1997]. The amplification of p21 cDNA was performed by incubating 20-ng equivalents of cDNA in 100 mM Tris-HCl buffer, pH 8.3, containing 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin, 200 µM concentration of each dNTP, and 50 U/ml Super *Taq* DNA polymerase with the following oligonucleotide primers: 5′-AGGAGGCCCGTGAGCGAGCGATGGAAC-3′ and 5′-ACAAGTGGGGAGGAAGTAGC-3′. The cDNA sequence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also amplified as a control in the same method, using the following primers: 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′. Thermal cycle conditions were as follows: 1 cycle at 94°C for 5 min, 30 cycles at 94°C for 1 min (p21) or 45 s (GAPDH), 59°C for 1 min (p21) or 65°C (GAPDH) for 45 s, 72°C for 1 min (p21) or 2 min (GAPDH), and 1 cycle at 72°C for 10 min. PCR products were analyzed on 1.8% agarose gels.

RESULTS

Effects of EGCG on Growth of MCF-7 Cells and Cell Cycle Progression

Previous studies have shown that EGCG inhibits the growth of many cell lines [Liang et al., 1997; Lin et al., 1996; Yang and Wang, 1993]. In this study, MCF-7 cells were exposed to 0.1–50 µM of EGCG, and the cell proliferation was assayed. Exponentially growing MCF-7 cultures rapidly underwent growth inhibition

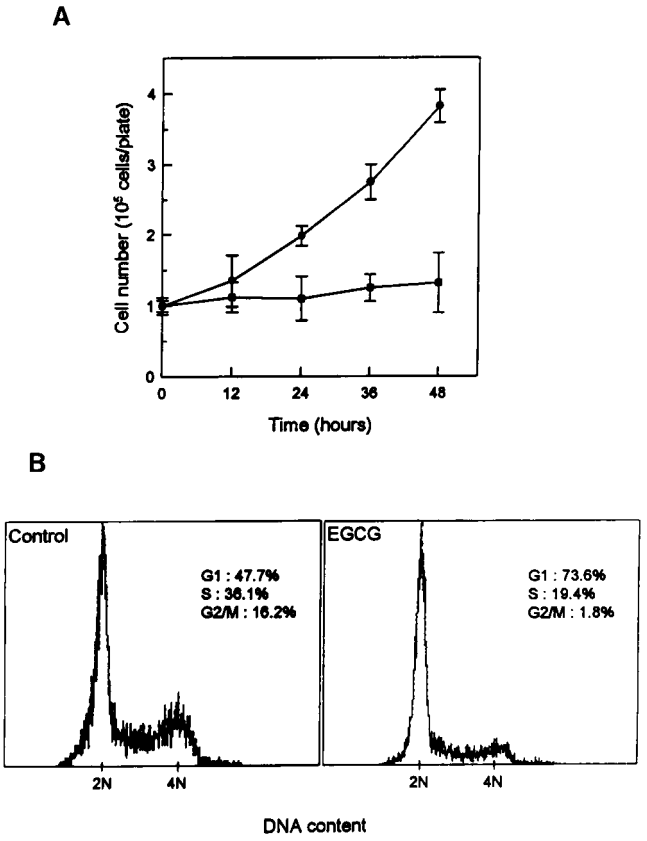

Fig. 1. Effects of epigallocatechin gallate (EGCG) on growth and cell cycle distribution of MCF-7 cells. **A:** Cells were treated without (●) or with (■) 30 μM of EGCG and cell number was determined every 12 h thereafter, using a hemacytometer. **B:** The cells were harvested after 24-h treatment with 30 μM of EGCG or without (control) and FACS analysis as described under Experimental Procedures. DNA histograms and cell cycle phase proportions were analyzed by flow cytometer.

with the addition of 30 μM EGCG, as evidenced by the decrease of cell proliferation over the experimental period (Fig. 1A). Trypan blue dye exclusion assay indicated marginal toxicity under these conditions (>95% viability after 48 h). Treatment with 50 μM EGCG did not cause cell death within the first 12 h of culture, and <10% trypan blue-positive cells had died at 24 h of culture. The DNA content of MCF-7 nuclei was measured by flow cytometry 24 h after the addition of EGCG. As shown in Figure 1B, 24-h treatment with 30 μM EGCG resulted in a higher proportion of cells in G1 phase (73.6%) compared with untreated cells (47.7%). MCF-7 cells were synchronized at G1/S boundary by exposure to 4 μg/ml aphidicolin for 12 h and then restimulated to proliferate by the addition of fresh medium. As shown in Figure 2, control cells readily entered the S and G2/M phase after 12 h of release from aphidicolin block. By contrast, EGCG-treated cells were still in the G1 phase and failed to proceed into the S and G2/M phase at 12 h. These results clearly demonstrate that EGCG can induce G1 arrest in asynchronized cells and prevent entry into the S phase from G1 in aphidicolin-synchronized cells.

Effects of EGCG on pRb Phosphorylation, Cyclins, and Cdk2 and Cdk4 Protein Expression

To elucidate the arrest point of EGCG-treated MCF-7 cells in the G1 phase, we analyzed the phosphorylation state of pRb and the expression of G1 cyclin protein and Cdk2 and Cdk4. The phosphorylation state of pRb was analyzed by SDS-PAGE, followed by immunoblotting. The underphosphorylated or unphosphorylated forms of Rb migrate faster than the multiple phosphorylated forms of Rb. As shown in Figure 3A (top), the Rb protein was changed from a chiefly hyperphosphorylated form at 0 h to a hypophosphorylated form 9–24 h over a 24-h exposure to 30 μM EGCG. MCF-7 cells were treated with different concentrations of EGCG for 24 h and the results are shown in Figure 3A (bottom). In the control exponentially growing cells, Rb protein was predominantly in the fully phosphorylated form. Treatment with 0.1–1 μM EGCG showed no effect on Rb phosphorylation. When cells were treated with 5–20 μM EGCG for 24 h, the Rb protein was distributed between unphosphorylated and phosphorylated states. By contrast, the Rb protein from the 30-μM EGCG-treated cells was predominantly

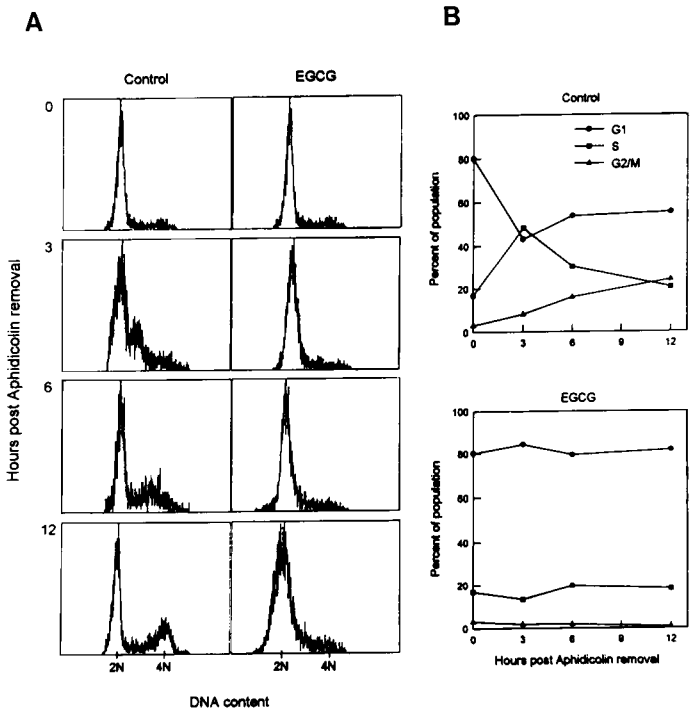

Fig. 2. Cell cycle profiles of synchronized MCF-7 cells in the presence or absence of epigallocatechin gallate (EGCG). MCF-7 cells were synchronized at the beginning of the S phase by exposure to 4 μg/ml aphidicolin for 12 h, washed twice, and then replated in fresh Dulbecco's modified Eagle's medium (DMEM) without (control) or with 30 μM of EGCG. At the times indicated in hours (at left) after release from aphidicolin block and FACS analysis, as described under Experimental Procedures. DNA histogram **(A)** and the percentages of cells in cell cycle phase **(B)** were analyzed by flow cytometer.

in the unphosphorylated state. These phenomena were dose dependent. Over a 24-h exposure to 30-μM EGCG, the levels of cyclins A, D1, and E were analyzed by immunoblotting. As shown in Figure 3B, cyclin A levels did not change with increasing time of EGCG exposure. Thus, EGCG appears to have little effect on the levels of cyclin D1 and E. However, cyclin E protein was markedly increased in the fastest migrating band during 12–24 h of EGCG exposure (Fig. 3B, panel 3, lanes 5–7, arrow). By contrast, there was no change in the protein expression of Cdk2 and Cdk4, which are associated with Rb phosphorylation. However, changes in the levels of cyclins and Cdk2 and Cdk4 were not sufficient to explain the reduction of Rb protein kinase activity. Therefore, we subsequently investigated the effects of EGCG on the activities of Cdk2 and Cdk4 in a cell-free system and in cultured cells, as well as its effects on the expression of Cdk inhibitory subunits (CKIs).

Effects of EGCG on the Activities of Cdk2 and Cdk4 Kinases in a Cell-Free System and in Cultured Cells

To examine whether EGCG would directly inhibit Cdks in MCF-7 cells, we first evaluated the effect of EGCG on the kinase activities of Cdk2 and Cdk4 in cell-free systems. The kinase activities of Cdk2 and 4 were measured after immunoprecipitation from exponentially growing MCF-7 cells using anti-Cdk2 or anti-Cdk4 antibodies; EGCG was then added, with histone H1 (for Cdk2) or Gst-Rb fusion protein (for Cdk4) as substrates. As shown in Figure 4A, 30 μM of EGCG was sufficient to inhibit the kinase activities of Cdk2 and Cdk4 by 86.5% and 80.6%, respectively, in the dose-response tested groups. The in vitro inhibition of Cdk2 and Cdk4 by EGCG was concentration dependent, assuming an IC_{50} value of about 18 μM and 20 μM for Cdk2 and Cdk4, respectively. We then examined the kinase activities of Cdk2 and Cdk4 from EGCG-treated MCF-7 cells. MCF-7 cells were treated with 30 μM EGCG for increasing periods, and the kinase activity was then determined by the immunoprecipitation method described above. As shown in Figure 4B, the kinase activities of both Cdk2 and Cdk4 were inhibited in a time-dependent manner; more than 90% of the activities were inhibited compared with untreated control cells (100%) after 24-h treatment. On the other hand, the inhibition of Cdk2 and Cdk4 kinase activities by EGCG were also concentration-dependent in cultured MCF-7 cells (data not shown). These findings were consistent with the lack of Cdk2 and Cdk4 activities in EGCG-treated cells, the underphosphorylated form of Rb, and the failure of these cells to progress from G1 into S phase.

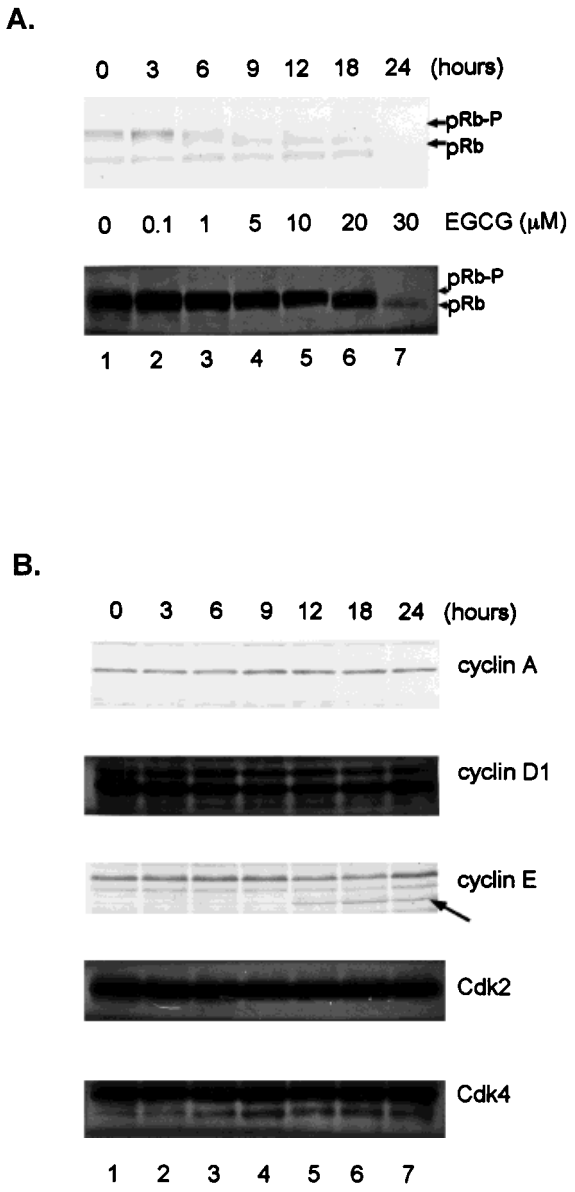

Fig. 3. Effects of epigallocatechin gallate (EGCG) on the Rb protein phosphorylation and the protein expression of G1 cyclins as well as Cdk2 and Cdk4. **A:** MCF-7 cells were treated with 30 μM of EGCG for the time indicated (top), or with various concentrations of EGCG for 24 h (bottom). **B:** MCF-7 cells were treated with 30 μM of EGCG for the time indicated. The cell lysis and Western blotting were performed as described under Experimental Procedures. Faster-migrating (lower) Rb band, unphosphorylated Rb; more slowly migrating Rb bands, phosphorylated Rb.

Effects of EGCG on Protein Expression of Cdk Inhibitors p21 and p27 in Wild-Type p53 and p53-Mutant Cells

The expression of p21 is reported to be regulated by either a p53-dependent or p53-independent mechanism. To determine whether the growth-inhibitory response to EGCG was dependent on the p53 status, we used p53-mutated MDA-MB-231 cells [Cattoretti et al., 1988] and the wild-type p53 cell line, MCF-7. Expression of p21 protein was examined after 30-μM EGCG treatment of the two cell lines. As shown in Figure 5A, EGCG treatment induced a significant increase of p21 in the wild-type p53 MCF-7 cells. Under the same conditions, p53 protein was induced in MCF-7 cells, reaching a maximal level at 6 h. Because p53 can regulate the gene expression of p21, the p21 induction by EGCG appears later than p53 induction in wild-type p53 MCF-7 cells (Fig. 5A). This phenomenon suggests that EGCG might first induce p53 expression and then transcribe the p21 gene via newly synthesized p53. To determine whether EGCG could also induce p21 protein expression in p53-mutant cells, MDA-MB-231 cells were treated with EGCG and this also resulted in growth arrest (data not shown). EGCG also induced the expression of p21 in p53-mutant MDA-MB-231 cells but did not affect the level of p53 protein (Fig. 5B). The mutated p53 protein is more stable than the wild type (Fig. 5). On the basis of these findings, we concluded that EGCG-induced p21 expression might occur through both the p53-dependent and independent pathways. To further examine whether EGCG could induce other members of the Cdk inhibitor protein family, we also investigated the effect of EGCG on the expression of p27 protein in both cells. As with p21 protein, the expression of p27 protein was significantly increased within 6 h after exposure to EGCG (Fig. 5A,B). However, under similar experimental conditions, we could not detect any p16 proteins by Western blotting in MCF-7 cells using ECL kits.

Effects of EGCG on mRNA Level of p21 in p53-Mutant MDA-MB-231 Cells

To determine whether the induction of p21 protein by EGCG was due to an increase of mRNA in the p53-mutant MDA-MB-231 cells, we investigated the effect of EGCG on p21 mRNA by RT-PCR. The expression of p21 mRNA (Fig. 6) was substantially elevated within 3 h after 30-μM EGCG treatment of MDA-MB-231 cells. By contrast, no effect of EGCG was found on the steady-state mRNA level of GAPDH in these cells. These results suggest that the induction of p53 was not an absolute requirement for growth arrest or p21 induction by EGCG and that the increase in p21 mRNA was, in part, a

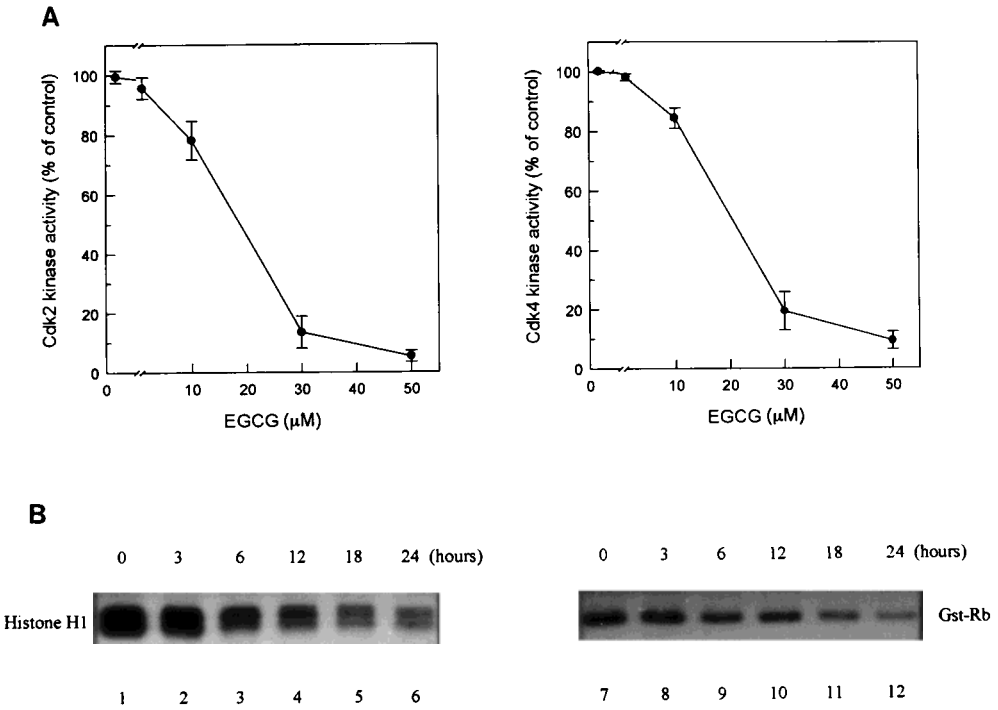

Fig. 4. Effects of epigallocatechin gallate (EGCG) on the activities of Cdk2 and Cdk4 kinases in cell-free system and cultured cells. **A:** Cdk2 and Cdk4-immuno complex were prepared from growing MCF-7 cells and reacted with [γ-^{32}P] ATP, various concentrations of EGCG and substrates (histone H1 for Cdk2, left; Gst-Rb for Cdk4, right) for 20 min at RT as described under Experimental Procedures. Data were representing the mean ±SE of three samples. **B:** MCF-7 cells were treated with 30 μM of EGCG for the increasing periods. Total cell lysates (500 μg) were used for immunoprecipitation, the kinase activities association with the Cdk2 and Cdk4 immunocomplexes were assayed with histone H1 (for Cdk2) or Gst-Rb (for Cdk4) as substrates. The experiments were performed as described under Experimental Procedures and ^{32}P-labeled histone H1 or Gst-Rb are shown.

consequence of transcriptional activation of the gene in MDA-MB-231 cells.

Effects of EGCG and Related Compounds on the Activities of Cdk2 and Cdk4 in a Cell-Free-System and in Cultured Cells

To investigate the structure-activity relationships, the effects of several green tea polyphenols and caffeine on the kinase activities of Cdk2 and Cdk4 were examined by the immunoprecipitation method, as described above. Among these compounds, only EGCG exhibited a rather strong inhibitory activity in the cell-free system, and ECG exhibited moderate inhibitory activity. The other green tea polyphenols and caffeine display limited inhibitory activity (Fig. 7A). In cultured MCF-7 cells, the inhibitory capacities of these compounds on the kinase activities of Cdk2 and Cdk4 were also investigated and similar inhibition patterns were found (Fig. 7B), except for gallic acid (GA), which showed a moderately inhibitory effect on these two kinases.

DISCUSSION

Epidemiological studies have shown that the intake of certain vegetables, fruits, and tea in the daily diet provides effective cancer prevention [Yang and Wang, 1993; Hirayama, 1986]. These effects have been attributed to the flavonoids and related flavanols in these plants. Several studies have also demonstrated that flavonoids and compounds related to flavanols have antitumorigenic properties [Yang and Wang, 1993]. In previous studies, it has been demonstrated that many flavonoids can block cell cycle progression in the G2/M or G1 phase [Matsukawa et al., 1993].

The present study showed that green tea polyphenol-EGCG also arrested cell cycle progression in the G1 phase in human breast carcinoma MCF-7 cells and MDA-MB-231 cells (data not shown). As shown in Figures 1B and 2, 30 μM EGCG blocked cell cycle progression at the G1/S boundary in asynchronized and aphidicolin-synchronized MCF-7 cells. However, when higher concentrations of EGCG (50 μM) were

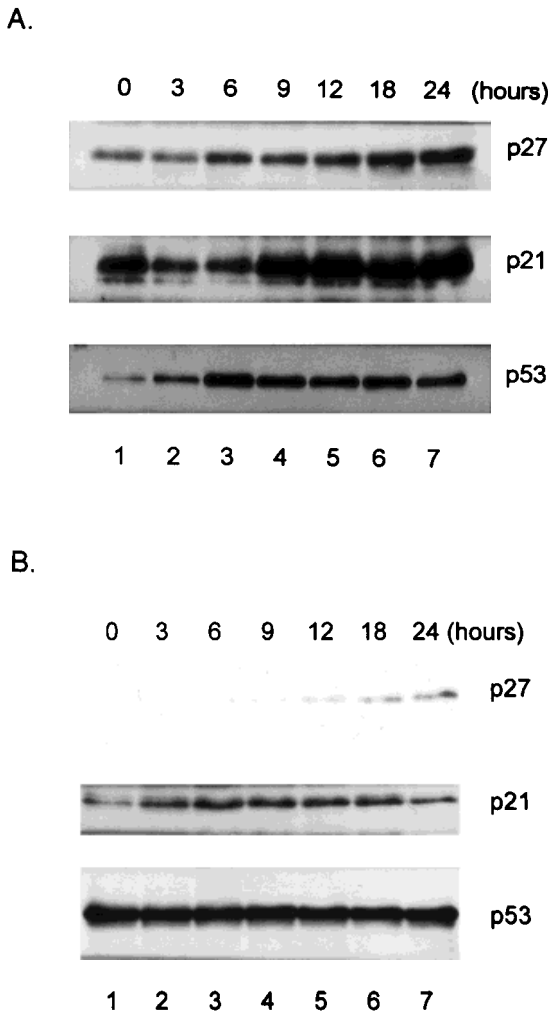

Fig. 5. Effects of epigallocatechin gallate (EGCG) on protein expression of p21, p27, and p53 in wild-type p53 MCF-7 cells and p53-mutant MDA-MB-231 cells. **(A)** MCF-7 cells and **(B)** MDA-MB-231 cells were treated with 30 μM of EGCG for the time indicated. The cell lysis and Western blotting were performed as described under Experimental Procedures.

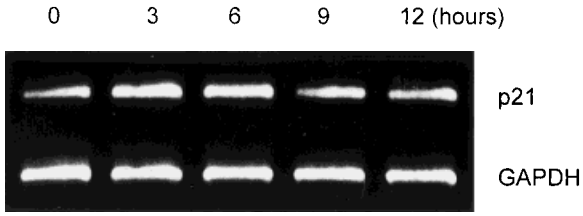

Fig. 6. Effect of epigallocatechin gallate (EGCG) on p21 mRNA expression in p53-mutant MDA-MB-231 cells. Cells were treated with 30 μM of EGCG for the time indicated, total RNA was isolated and the mRNA express were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) as described under Experimental Procedures.

added to nocodazole-arrest MCF-7 cells after removal of nocodazole. EGCG also blocked G2/M progression (data not shown). (Nocodazole is a reversible mitrotubule inhibitor, which synchronizes cells at G2/M boundary [Zieve et al., 1980]). We also examined the effects of various concentrations of EGCG on cell cycle distribution. The results indicated that a higher concentration of EGCG (50 μM) could inhibit phase change in cell cycle progression (data not shown). These results suggest that the effects of EGCG on cell cycle distribution are dependent on concentration, cell cycle states of the cultured cells, and possibly cell types. Moreover, cells arrested at the G1/S or G2/M boundary with either aphidicolin or nocodazole were more sensitive to EGCG treatment than were asynchronously growing cells. Previous studies have showed that many flavonoids, such as apigenin, genistein, and quercetin, which have a structure similar to that of EGCG, induce G2/M arrest in several cell lines [Lepley et al., 1996; Sato et al., 1994; Matsukawa et al., 1993]. On the other hand, many flavonoids also induce G1 arrest [Matsukawa et al., 1993]. These results suggest that arrest of the cell cycle by flavonoids or EGCG is an effect, which is independent of the structural groups of these agents.

Previous studies have shown that both UCN-01 and staurosporine exert inhibitory activity against Cdk2 in cell-free systems [Kawakami et al., 1996; Wang et al., 1995]. EGCG also exhibited a concentration-dependent inhibitory effect not only on Cdk2 but also on Cdk4 obtained from cycling MCF-7 cells, resulting in an IC_{50} value of about 18 μM and 20 μM, respectively (Fig. 4A). The kinase activities of Cdk2 and Cdk4 were inhibited by 30 μM EGCG, but these results were not consistent in the cell-free system and in cultured cells. For example, 30 μM of EGCG inhibited the activity of Cdk4 by about 80% and 93% in the cell-free system and in cultured cells, respectively (Fig. 4). This discrepancy suggests that there might be additional mechanism(s) for the inhibition of Cdk2 and Cdk4 activities by EGCG in the cultured cells. The Cdk2 and Cdk4 inhibition attributable to these direct and indirect actions might also be one of the mechanisms of dephosphorylation of ppRb by EGCG. In addition to these inhibitions, Cdk activation and inhibition also require phosphorylation or dephosphorylation at some conserved amino acids residues [Morgan, 1995]. It is therefore possible that EGCG inhibits the Cdk-activating kinases

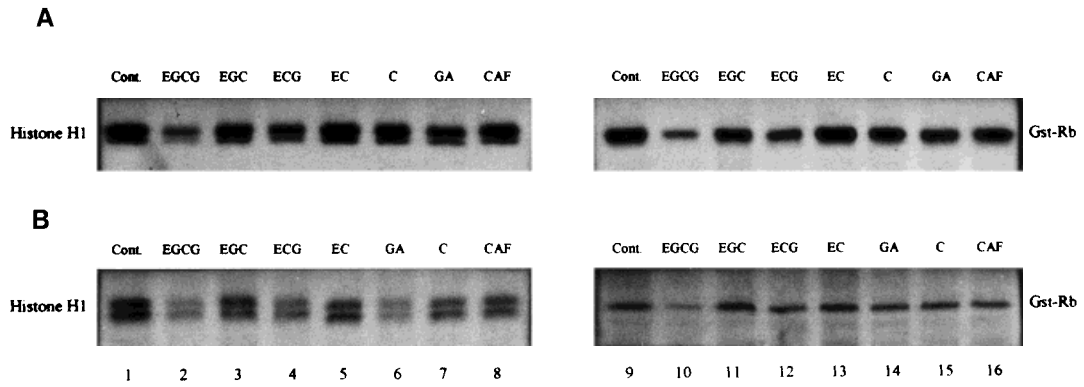

Fig. 7. Effects of epigallocatechin gallate (EGCG) and related compounds on the kinase activities of Cdk2 and Cdk4 in cell-free system and cultured cells. **A:** Cdk2 and Cdk4 immunocomplexes were prepared from growing MCF-7 cells and reacted with [γ-^{32}P]ATP, different kinds of green tea polyphenols and caffeine (30 μM), and substrates (histone H1 for Cdk2, left; Gst-Rb for Cdk4, right) for 20 min at RT as described under Experimental Procedures. **B:** MCF-7 cells were treated without or with 30 μM of green tea polyphenols and caffeine for 24 h and kinase assay as described in Fig. 4B. ^{32}P-labeled histone H1 or Gst-Rb is shown.

(CAKs) or activates the Cdk-inactivating phosphatases, which are regulators for Cdks. Additional studies are needed to determine whether Cdk2 and Cdk4 inhibition contributes to the inhibition of CAKs or activation of Cdk-inactivating phosphatases. However, Cdk6 might phosphorylate pRb in cells and might also be inhibited by EGCG. They have little change on the levels of cyclin D1 and E after 24 h of EGCG exposure (Fig. 3B). But, it seemed that the protein amount of cyclins was not the main event to affect the activities of Cdks in EGCG-treated MCF-7 cells. However, we could not rule out the possibility that EGCG might block cyclin binding to Cdk, inhibiting the kinase complex activities of Cdks.

Inhibition of Cdk activity might also have occurred through induction of p21 by EGCG. Both the wild-type p53 MCF-7 cells and p53-mutant MDA-MB-231 cells induced the expression of p21 by EGCG. However, the expression of p21 was a transient event, reaching a maximal level at 3 h after exposure to EGCG in the MDA-MB-231 cells (Fig. 5B). In the MCF-7 cells, the p21 continued to show a high expression from 9 h to 24 h after exposure to EGCG (Fig. 5A). These results suggest that induction of p21 by EGCG might occur through different pathways in MCF-7 and MDA-MB-231 cells. In the MCF-7 cells, which possess functional p53, the G1 arrest produced by EGCG occurred with an accompanying p21 and p27 accumulation. However, results in the MDA-MB-231 cells, which possess mutant p53 and also induced p21 accumulation by EGCG. The results suggest that the induction of p21 by EGCG were not only dependent on functional p53. RT-PCR showed that the steady-state level of p21 mRNA was upregulated by EGCG in MDA-MB-231 cells (Fig. 6B). However, additional studies are needed to clarify whether transcriptional, translational, or post-translational levels control the activation of the p21 gene by EGCG. Previous studies have indicated that p53-mediated arrest of the cell cycle is associated with DNA damage or with inhibition of DNA or RNA synthesis [Chernora et al., 1995]. It has also been reported that EGCG can inhibit DNA and RNA polymerase [Nakane and Dno, 1990] and extracellular signals [Liang et al., 1997; Okabe et al., 1997], thereby blocking DNA or RNA synthesis. These effects might trigger increased expression of p53 by EGCG. In this study, EGCG also increased the p27 protein level in both cells (Fig. 5), suggesting that p27 is also important for G1 arrest by EGCG.

Both EGCG and ECG were effective at inhibiting the kinase activities of Cdk2 and Cdk4 in both the cell-free system and in cultured cells (Fig. 7). These two compounds share the same structure, while EGCG has one more hydroxyl group in position 3′ than ECG. Because the other green tea polyphenols have different structures, they could not inhibit Cdk2 and Cdk4 activities significantly. However, gallic acid slightly inhibited Cdk2 and Cdk4 kinase activities in cultured cells, but not in the cell-free system. This result suggests that the inhibitory effect of gallic acid on Cdk2 and Cdk4 might not inhibit the kinase activities directly and that it

might block some intracellular mitogenic signals.

In summary, we have shown that the growth-inhibitory response to EGCG, including the inhibition of Cdk2 and Cdk4 kinase activities and the induction of p21 and p27, occurs dependent or independent of p53 activity. The p21 and/or p27 induction by EGCG might play an important role in its antiproliferative activity in cultured cells, in light of our previous results that EGCG could modulate the cellular mitogenic signals through blocking EGF binding to EGF-R [Liang et al., 1997]. These results suggest that EGCG or other green tea polyphenols indeed play an important role in the function of antiproliferation and antitumor promotion, and that they are potential candidates for the development of cancer chemopreventive agents.

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REFERENCES

Anonymous. 1996. Clinical development plan: tea extracts. Green tea polyphenols. Epigallocatechin gallate. J Cell Biochem 26S:236–257.

Aprelikova O, Xiong Y, Liu ET. 1995. Both p16 and p21 families of cyclin-dependent kinase (Cdk) inhibitors block the phosphorylation of cyclin-dependent kinases by the cdk-activating kinase. J Biol Chem 270:18195–18197.

Botz J. 1996. Cell cycle regulation of the murine cyclin E gene depends on an E2F binding site in the promoter. Mol Cell Biol 16:3401–3409.

Cattoretti G, Rilke F, Andreola S, D'Amato L, Delia D. 1988. p53 expression in breast cancer. Int J Cancer 41:178–183.

Chen Y-C, Kuo T-C, Lin-Shiau S-Y, Lin J-K. 1996. Induction of Hsp 70 gene expression by modulation of Ca^{+2} ion and cellular p53 protein by curcumin in colorectal carcinoma cells. Mol Carcinogen 17:224–234.

Chernora OB, Chernov MV, Agarwal ML, Taylor WR, Stark GR. 1995. The role of p53 in regulating genomic stability when DNA synthesis are inhibited. Trends Biochem Sci 20:431–434.

El-Deiry WS. 1994. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res 54:1169–1174.

Hensey CE, Hong F, Durfee T, Qian Y-W, Lee Eva Y-HP, Lee W-H. 1994. Identification of discrete structural domains in the retinoblastoma protein. J Biol Chem 269:1380–1387.

Hirayama T. 1986. A large scale cohort study on cancer risks by diet–with special reference to the risk reducing effects of green-yellow vegetable consumption. In: Diet, nutrition and cancer. Hayashi Y, Nagao M, Sugimura T, Takayama S, Tomatis L, Wattenbery LW, Wogan GN, editors. Tokyo: Japan Scientific Societies Press. p 41–53.

Ho CT, Chen Q, Shi H, Zhang KQ, Rosen RT. 1992. Antioxidative effect of polyphenol extract prepared from various Chinese teas. Prev Med 21:520–525.

Katiyar S, Kagarwal R, Zain MT, Mukntar H. 1993. Protection against N-nitrosodiethylamine and benzo[a]pyrene-induced forestomach and lung tumorigenesis in A/J mice by green tea. Carcinogenesis 14:849–855.

Kaur G, Stetler-Stevenson M, Sebers S, Worland P, Sedlacek H, Myers C, Czech J, Naik R, Sausville E. 1992. Growth inhibition with reversible cell cycle arrest of carcinoma cells by the flavone L86-8275. J Natl Cancer Inst 84:1736–1740.

Kawakami K, Futami H, Takahara J, Yamaguchi K. 1996. UCN-01, 7-hydroxylstaurosporine, inhibit kinase activity of cyclin-dependent kinase and reduces the phosphorylation of the retinoblastoma susceptibility gene product in an A549 human lung carcinoma cell line. Biochem Biophys Res Commun 219:778–783.

Lepley DM, Li B, Birt DF, Pelling JC. 1996. The chemopreventive flavonoid apigenin induces G2/M arrest in keratinocytes. Carcinogenesis 17:2367–2375.

Liang Y-C, Lin-Shiau S-Y, Chen C-F, Lin J-K. 1997. Suppression of extracellular signals and cell proliferation through EGF receptor binding by (−)-epigallocatechin gallate in human A431 epidermal carcinoma cells. J Cell Biochem 67:55–65.

Lin Y-L, Lin J-K. 1997. (−)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lippolysaccharide-induced activity of transcription factor nuclear factor-κB. Mol Pharm 52:465–472.

Lin Y-L, Juan I-M, Chen Y-L, Liang Y-C, Lin J-K. 1996. Composition of polyphenols in fresh tea leaves and associations of their oxygen-radical-absorbing capacity with antiproliferative actions in fibroblast cells. J Agri Food Chem 44:1387–1394.

Matsukawa Y, Marui N, Sakai T, Satomi Y, Yoshida M, Matsumoto K, Nishino H, Aoike A. 1993. Genistein arrests cell cycle progression at G2-M. Cancer Res 53:1328–1331.

Morgan DO. 1995. Principles of CDK regulation. Nature 374:131–134.

Nakane H, Dno K. 1990. Differential inhibitory effects of some catechin derivatives on the activities of human immunodeficiency virus reverse transcriptase and cellular deoxyribonucleic and ribonucleic acid polymerases. Biochemistry 29:2841–2845.

Noda A, Ning Y, Venable SF, Pereira-Smith OM, Smith JR. 1994. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. Exp Cell Res 211: 90–98.

Nonaka GI, Kawahara O, Nishioka I. 1983. Tannins and related compounds XV. A new class of dimeric flavan-3-ol gallates, theasinensins A and B, and proanthocyanidin gallates from green tea leaf. Chem Pharm Bull 31:3906–3914.

Okabe S, Suganuma M, Hayashi M, Sueoka E, Komori A, Fujiki H. 1997. Mechanisms of growth inhibition human lung cancer cell line, PC-9, by Tea polyphenols. Jpn J Cancer Res 88:639–643.

Parker SB, Eichele G, Zhang P, Rawls A, Sands AT, Bradley A, Olson EN, Harper JW, Elledege SJ. 1995. p53-independent expression of p21cip1 in muscle and other terminally differentiating cells. Science 267:1024–1027.

Pedrali-Noy G, Spadari S, Miller-Faures A, Miller AO, Kruppa J, Koch G. 1980. Synchronization of HeLa cell cultures by inhibition of DNA polymerase-α with aphidicolin. Nucleic Acids Res 8:377–387.

Sato F, Matsukawa Y, Matsumoto K, Nishino H, Sakai T. 1994. Apigenin induces morphological differentiation and G2-M arrest in rat neuronal cells. Biochem Biophys Res Commun 204:578–584.

Sedlacek HH, Czech J, Naik R, Kaur G, Worland P, Losiewicz M, Parker B, Carlson B, Smith A, Senderowicz A, Sausville E. 1996. Flavopiridol (L86 8275; NSC 649890), a new kinase inhibitor for tumor therapy. Int J Oncol 9:1143–1168.

Sherr CJ. 1996. Cancer cell cycles. Science 274:1672–1677.

Sherr CJ, Roberts JM. 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev 9:1149–1163.

Shi ST, Wang ZT, Smith TJ, Hong JY, Chen WF, Ho CT, Yang CS. 1994. In vivo enhancement of genomic instability in minisatellite sequences of mouse C3H/10T1/2 cells transformed in vitro by x-ray. Cancer Res 54:4641–4647.

Spinozzi F, Pagliacci MC, Migliorati G, Moraca R, Grignani F, Riccardi C, Nicoletti I. 1994. The natural tyrosine kinase inhibitor genistein products cell cycle arrest and apoptosis in jurkat T-leukemia cells. Leuk Res 18:431–439.

Stoner GD, Mukhtar H. 1995. Polyphenols as cancer chemopreventive agents. J Cell Biochem 22S:169–180.

Traganos F, Ardelt B, Halko N, Bruno S, Darzynkiewicz Z. 1992. Effects of genistein on the growth and cell cycle progression of natural human lymphocytes and human leukemic MOLT-4 and HL-60 cells. Cancer Res 52:6200–6208.

Wang Q, Worlan PJ, Clark JL, Carlson BA, Sausville EA. 1995. Apoptosis in 7-hydroxystaurosporine-treated T lymphoblasts correlates with activation of cyclin-dependent kinase 1 and 2. Cell Growth Diff 6:927–936.

Wei H, Tye L, Bresnick E, Birt DF. 1990. Inhibitory effect of apigenin, a plant flavonoids, an epidermal ornithine decarboxylase and skin tumor promotion in mice. Cancer Res 50:449–502.

Wei Y, Zhao X, Kariya Y, Fukata H, Teshigawara K, Uchida A. 1994. Induction of apoptosis by quercetin: involvement of heat shock protein. Cancer Res 54:4952–4957.

Weinberg RA. 1995. The retinoblastoma protein and cell cycle control. Cell 81:323–330.

Xiong Y, Zhang H, Beach D. 1992. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell 71:505–514.

Yamane T, Takahashi T, Kuwata K, Oya K, Inagake M, Kitao Y, Suganuma M, Fujiki H 1995. Incorporation of 5-bromo-2′-deoxyuridine into colorectal liver metastases and liver in patients receiving a 7-day hepatic arterial infusion. Cancer Res 5:2081–2084.

Yang CS, Wang ZY. 1993. Tea and cancer. J Natl Cancer Inst 85:1038–1049.

Yoshida M, Sakai T, Hosokawa N, Marui N, Matsumoto K, Fujioka A, Nishino H, Aoike A. 1990. The effect of quercetin on cell cycle progression and growth of human gastric cancer cells. FEBS Lett 260:10–13.

Zieve GW, Turnbull D, Mullins JM, McIntoch JR. 1980. Production of large numbers of mitotic mammalian cells by use of the reversible microtubule inhibitor nocodazole. Nocodazole accumulated mitotic cells. Exp Cell Res 126: 397–405.