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Ester Bond-containing Tea Polyphenols Potently Inhibit Proteasome Activity *in Vitro* and *in Vivo**

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It has been discovered that proteasome inhibitors are able to induce tumor growth arrest or cell death and that tea consumption is correlated with cancer prevention. Here, we show that ester bond-containing tea polyphenols, such as (−)−epigallocatechin-3-gallate (EGCG), potently and specifically inhibit the chymotrypsin-like activity of the proteasome *in vitro* (IC_{50} = 86–194 nM) and *in vivo* (1–10 μM) at the concentrations found in the serum of green tea drinkers. Atomic orbital energy analyses and high performance liquid chromatography suggest that the carbon of the polyphenol ester bond is essential for targeting, thereby inhibiting the proteasome in cancer cells. This inhibition of the proteasome by EGCG in several tumor and transformed cell lines results in the accumulation of two natural proteasome substrates, $p27^{Kip1}$ and IκB-α, an inhibitor of transcription factor NF-κB, followed by growth arrest in the G_1 phase of the cell cycle. Furthermore, compared with their simian virus-transformed counterpart, the parental normal human fibroblasts were much more resistant to EGCG-induced $p27^{Kip1}$ protein accumulation and G_1 arrest. Our study suggests that the proteasome is a cancer-related molecular target of tea polyphenols and that inhibition of the proteasome activity by ester bond-containing polyphenols may contribute to the cancer-preventative effect of tea.

Previous epidemiological studies have suggested that tea consumption may have a protective effect against human cancer (1–4). Recent animal studies have also demonstrated that green tea polyphenols could suppress the formation and growth of human cancers, including skin (5, 6), lung (7), liver (8), esophagus (9), and stomach (10). The major components of green and black tea include epigallocatechin-3-gallate (EGCG)[1], epigallocatechin (EGC), epicatechin-3-gallate (ECG), epicatechin (EC), and their epimers (see Fig. 1*A*). EGCG among those polyphenols has been most extensively examined because of its relative abundance and strong cancer-preventive properties (1, 11). EGCG has been shown to inhibit several cancer-related proteins, including urokinase (12), nitric-oxide synthase (13), teromerase (14), and tumor necrosis factor-α (15). However, nonphysiological concentrations of EGCG (*i.e.*, concentrations higher than those found in human serum after tea consumption) were used in some earlier studies. Whether one or more of these proteins are the real molecular targets of EGCG and other tea polyphenols under physiological conditions needs further investigations.

The 20S proteasome, a multicatalytic complex (700 kDa), constitutes the catalytic key component of the ubiquitous proteolytic machinery 26S proteasome (16–20). There are three major proteasomal activities: chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide hydrolyzing (PGPH) activities (16, 21). The ubiquitin-proteasome system plays a critical role in the specific degradation of cellular proteins (22), and two of the proteasome functions are to allow tumor cell cycle progression and to protect tumor cells against apoptosis (23). The chymotrypsin-like but not trypsin-like activity of the proteasome is associated with tumor cell survival (24, 25). Many cell cycle and cell death regulators have been identified as targets of the ubiquitin-proteasome-mediated degradation pathway. These proteins include p53 (26), pRB (27), p21 (28), $p27^{Kip1}$ (29), IκB-α (30), and Bax (31).

Here, we report for the first time that ester bond-containing tea polyphenols potently and selectively inhibit the proteasomal chymotrypsin-like but not trypsin-like activity *in vitro* and *in vivo*. Among the tea polyphenols examined, EGCG showed the strongest inhibitory activity against purified 20S proteasome, 26S proteasome of tumor cell extracts, and 26S proteasome in intact tumor cells. Furthermore, the inhibition of the proteasome *in vivo* was able to accumulate the natural proteasome substrates $p27^{Kip1}$ and IκB-α as well as induce the arrest of tumor cells in the G_1 phase. Finally, normal human WI-38 fibroblasts were more resistant to the EGCG treatment than their SV40-transformed counterpart.

EXPERIMENTAL PROCEDURES

Materials—Highly purified tea polyphenols EGCG (>95%), ECG (>98%), EGC (>98%), EC (>98%), GCG (>98%), GC (>97%), CG (>98%), and C (>98%) were purchased from Sigma and used directly without further purification. A green tea extract was a gift from the Lipton Company (Englewood Cliffs, NJ) that contained 51.5% EGCG, 14.7% ECG, 8.3% EGC, 8.5% EC, 4.4% GCG, 2.4% GC, 1.6% C, and 1.6% caffeine. A black tea extract was also a gift from Lipton that contained 19.7% EGCG, 14.9% ECG, 0.9% EGC, 4.8% EC, 0.0% GCG, 0.5% GC, 2.0% C, and 1.2% caffeine. Purified 20S proteasome (*Methanosarcina thermophile*, Recombinant, *Escherichia coli*) and purified calpain I (human erythrocytes) were purchased from Calbiochem. Fluorogenic peptide substrates Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity), benzyloxycarbonyl (Z)-Leu-Leu-Glu-AMC (for the proteasomal PGPH activity), Suc-Leu-Tyr-AMC (for the calpain I

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[1] The abbreviations used are: EGCG, (−)−epigallocatechin-3-gallate; EGC, (−)−epigallocatechin; ECG, (−)−epicatechin-3-gallate; EC, (−)−epicatechin; GCG, (−)−gallocatechin-3-gallate; GC, (−)−gallocatechin; CG, (−)−catechin-3-gallate; C, (−)−catechin; AMC, 7-amido-4-methyl-coumarin; PGPH, peptidyl-glutamyl peptide-hydrolyzing; HPLC, high performance liquid chromatography; IκB-α, inhibitor of transciption factor NF-κB; p70, 70 kDa; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Z, benzyloxycarbonyl.

activity), and Ac-Asp-Glu-Val-Asp-AMC (for the caspase-3 activity) were also obtained from Calbiochem, and Z-Gly-Arg-AMC (for the proteasomal trypsin-like activity) was from Bachem (King of Prussia, PA). The specific calpain inhibitor calpeptin and the specific caspase-3 inhibitor Ac-DEVD-CHO were obtained from Calbiochem. Monoclonal antibody to $p27^{Kip}$ was purchased from PharMingen (San Diego, CA), polyclonal antibodies to IκB-α and actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and polyclonal antibodies to ubiquitin were from Sigma.

Cell Culture and Cell Extract Preparation—Human Jurkat T and prostate cancer (LNCaP, PC-3) cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml streptomycin. Human breast cancer MCF7 cells and normal (WI-38) and SV40-transformed (VA-13) human fibroblasts were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin, and streptomycin. All cells were maintained in a 5% CO_2 atmosphere at 37 °C. A whole cell extract was prepared as described previously (24). Cells were harvested, washed with phosphate-buffered saline twice, and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4 °C. Afterward, the lysates were centrifuged at 14,000 × *g* for 30 min, and the supernatants were collected as whole cell extracts.

Inhibition of Purified 20S Proteasome Activity by Tea Polyphenols—The chymotrypsin-like activity of purified 20S proteasome was measured as follows. 0.5 μg of purified 20S proteasome was incubated with 20 μM fluorogenic peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity), for 30 min at 37 °C in 100 μl of assay buffer (20 mM Tris-HCl, pH 8.0) with or without a tea polyphenol or tea extract. After incubation, the reaction mixture was diluted to 200 μl with the assay buffer followed by a measurement of the hydrolyzed 7-amido-4-methyl-coumarin (AMC) groups using a VersaFluor™ Fluorometer with an excitation filter of 380 nm and an emission filter of 460 nm (Bio-Rad).

Inhibition of the Proteasome Activity in Whole Cell Extracts by Tea Polyphenols—A whole cell extract (3.5 μg) of Jurkat T cells was incubated for 90 min at 37 °C with 20 μM fluorogenic peptide substrates for various activities of the proteasomes, Suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Leu-Glu-AMC, and Z-Gly-Gly-Arg-AMC, in 100 μl of the assay buffer with or without EGCG or EGC. The hydrolyzed AMCs were quantified as described earlier.

Inhibition of the Proteasome Activity in Intact Tumor Cells by Tea Polyphenols—To measure the inhibition of proteasome activity in living tumor cells, Jurkat T (1×10^5 cells/ml/well), MCF-7, or PC-3 cells (1×10^4 cells/ml/well) were cultured in 24-well plates. These cells were first incubated for 12 h with various concentrations of EGCG, EGC, β-lactone, or LLnL followed by an additional 2-h incubation with the fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC or Z-Gly-Gly-Arg-AMC. Afterward, the cell medium (200 μl/sample) was collected and used for measurement of free AMCs.

Assays for Calpain I and Caspase-3 Activities—To measure the activity of calpain I, 3 μg of purified calpain I was incubated with 40 μM fluorogenic peptide calpain substrate, Suc-Leu-Tyr-AMC, for 30 min at 37 °C in 100 μl of assay buffer (50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 5 mM $CaCl_2$, and 0.1% CHAPS) with or without EGCG or the specific calpain inhibitor calpeptin. For caspase-3 activity assay, a Jurkat T cell extract (3.5 μg) was incubated for 90 min at 37 °C with 20 μM fluorogenic peptide substrate, Ac-Asp-Glu-Val-Asp-AMC, with or without EGCG or the specific caspase-3 inhibitor Ac-DEVD-CHO. After incubation, the reaction mixture was diluted to 200 μl with the assay buffer, and the hydrolyzed AMCs were quantified as described above.

Atomic Orbital Energy Analysis—The electron density surface colored by nucleophilic susceptibility was created using the Cache Worksystem version 3.2 (Oxford Molecular Ltd.). After geometrical optimization (using augmented MM3), the electron distribution between the highest occupied molecular orbital and the lowest unoccupied molecular orbital was evaluated, and a three-dimensional isosurface of susceptibility to nucleophilic attack (generated by an extended Huckel wave function) was calculated and superimposed over the molecule. A colored "bullseye" with a white center is characteristic of atoms that are highly susceptible to nucleophilic attack.

Western Blot Analysis—Jurkat T, LNCaP, WI-38, or VA-13 cells were treated with various concentrations of EGCG or EGC for indicated hours (see figure legends). To measure the changes in protein stability, Jurkat T cells were pretreated with 10 μg/ml cycloheximide for 2 h (to inhibit translation) followed by coincubation with EGCG or the proteasome inhibitor LLnV (for a positive control). This was followed by the preparation of whole cell extracts. The enhanced chemiluminescence Western blot analysis was then performed using specific antibodies to $p27^{Kip}$, IκB-α, ubiquitin, or actin as described previously (24).

Flow Cytometry—Cell cycle analysis based on DNA content was performed as follows. At each time point, cells were harvested, counted, and washed twice with phosphate-buffered saline. Cells (5×10^6) were suspended in 0.5 ml of phosphate-buffered saline, fixed in 5 ml of 70% ethanol for at least 2 h at −20 °C, centrifuged, resuspended again in 1 ml of propidium iodide staining solution (50 μg of propidium iodide, 100 units of RNase A, and 1 mg of glucose/ml of phosphate-buffered saline), and incubated at room temperature for 30 min. The cells were then analyzed with FACScan (Becton Dickinson Immunocytometry, San Jose, CA) and ModFit LT cell cycle analysis software (Verity Software, Topsham, ME). The cell cycle distribution is presented as the percentage of cells containing G_1, S, G, S, and G_2/M DNA content as judged by propidium iodide staining.

High Performance Liquid Chromatography (HPLC) Analysis—EGCG or EGC (1 mM) was incubated with either the purified 20S proteasome (45 μg) or its buffer for the indicated hours at 37 °C in 100 μl of a reaction buffer (20 mM Tris-HCl, 1 mM dithiothreitol, pH 7.2). After each reaction, the sample was filtered with a 0.45 μm of nylon syringe filter (Nalge Co., Rochester, NY), and 20 μl of filtered sample was injected to HPLC equipped with a C-18 reverse phase column (0.46 × 25 cm, Separation Group, Hesperia, CA). The solvent system was 12% acetonitrile, 2% ethyl acetate, and 0.05% phosphoric acid, the flow rate was 1 ml/min, and the proteasome cleavage products were monitored at 280 nm. The standard controls also included gallic acid without incubation and the purified proteasome alone.

RESULTS AND DISCUSSION

Inhibition of Chymotrypsin-like Activity of Purified 20S Proteasome by Ester Bond-containing Tea Polyphenols—It has been reported that lactacystin, when converted to its active form *clasto*-lactacystin β-lactone (β-lactone), is a highly specific and irreversible inhibitor of the proteasome (32–34). This β-lactone contains an ester bond (Fig. 1*A*) that is responsible for interacting with and inhibiting the proteasome (32–34). We noticed a similar ester bond present in several tea polyphenols including EGCG, ECG, GCG, and CG (Fig. 1*A*). We hypothesized that tea polyphenols containing ester bonds would inhibit the proteasome activity, whereas tea polyphenols without ester bonds would not. We tested this hypothesis by performing a cell-free proteasome activity assay in the presence of tea polyphenols. The chymotrypsin-like activity of purified 20S proteasome (the catalytic core of 26S proteasome) (22) was significantly inhibited by EGCG (Fig. 1*B*) whose IC_{50} value was calculated to be 86 nM (Fig. 1*A*). In contrast, EGC (IC_{50} = 1.2 mM) and gallic acid (IC_{50} = 7.1 mM), the two moieties of EGCG linked by an ester bond, were 14,000- and 83,000-fold less potent than EGCG, respectively (Fig. 1, *A* and *B*). As a positive control, β-lactone also potently inhibited the proteasomal chymotrypsin activity (IC_{50} = 600 nM in Fig. 1, *A* and *B*) (34). The shape of the inhibition curve of EGCG was similar to that of β-lactone (Fig. 1*B*).

Three other ester bond-containing tea polyphenols, ECG, GCG, and CG (Fig. 1*A*), were also found to be strong inhibitors of the chymotrypsin-like activity of the purified 20S proteasome (IC_{50} values were 194, 187, and 124 nM, respectively). In contrast, all the corresponding polyphenols that do not contain ester bonds, EC, GC, and C (Fig. 1*A*), could not inhibit the proteasomal chymotrypsin-like activity. These results indicate that the ester bonds contained in tea polyphenols are essential for potent inhibition of the proteasomal chymotrypsin-like activity. Furthermore, a green or black tea extract, which contains significant portions of EGCG (51.5 and 19.7%, respectively) and ECG (14.7 and 14.9%, respectively, see under "Experimental Procedures"), also strongly inhibited the chymotrypsin-like activity of the 20S proteasome (IC_{50} values were 0.1 and 0.3 μg/ml, respectively).

The electrophilic ester bond carbon of β-lactone is responsible for its biological inhibition of the proteasome (32–34), sup-

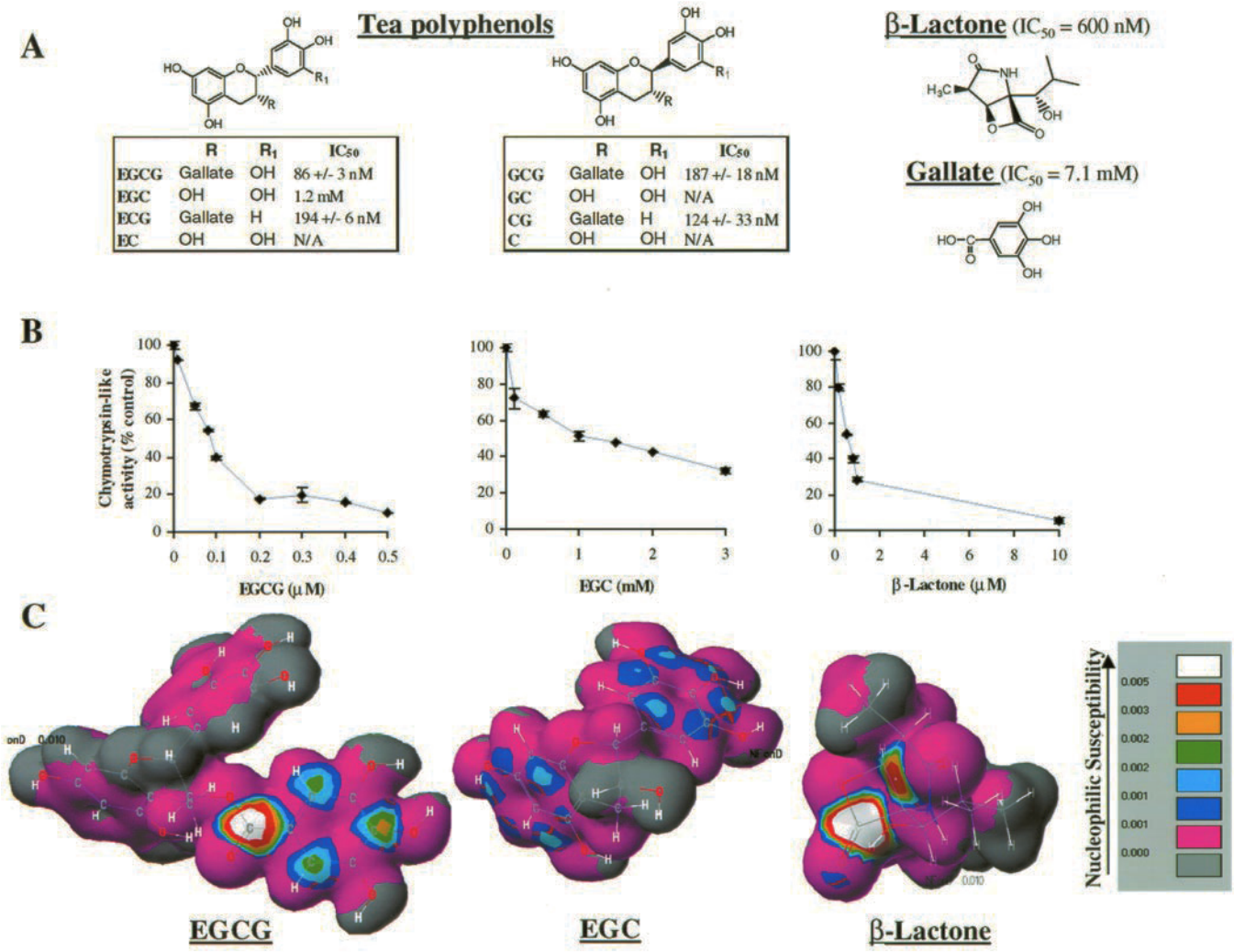

FIG. 1. **Structure-activity relationships of tea polyphenols.** *A*, the structure and potency of polyphenols. IC_{50} of each tea polyphenol toward the chymotrypsin-like activity of the purified 20S proteasome was measured as described under "Experimental Procedures." *N/A* indicates that the inhibitory activity of the corresponding polyphenol at 50 μM was <10%. *B*, concentration-dependent inhibition of the chymotrypsin-like activity of the purified 20S proteasome by EGCG, EGC, and β-lactone. *C*, the susceptibility of EGCG, EGC, and β-lactone to a nucleophilic attack was calculated as described under "Experimental Procedures."

ported by previous studies using x-ray crystallography (16). When the atomic orbital energy was analyzed, the ester bond carbon of β-lactone showed a high susceptibility toward a nucleophilic attack with an arbitrary value of 1.1 (Fig. 1*C*). We then determined if the levels of nucleophilic susceptibility found in tea polyphenols correlate with their proteasome inhibitory activities. The ester bond carbon of EGCG was found to have the highest susceptibility toward a nucleophilic attack among all the other atoms with a value of 0.7, whereas the carbon with the highest nucleophilic susceptibility on EGC was found to have a low value of 0.2 (Fig. 1*C*). Similarly, a high nucleophilic susceptibility was found in other ester bond-containing polyphenols, ECG, GCG, and CG (all with values of 0.7), whereas low nucleophilic susceptibility was found in non-ester bond-containing polyphenols, EC, GC, and C (with values of 0.3, 0.2, and 0.3, respectively). Thus, the nucleophilic susceptibility of tea polyphenols correlated with their ability to inhibit the proteasome chymotrypsin-like activity. These data support the essential role of polyphenol ester bonds in the inhibition of the proteasome activity.

Inhibition of the Proteasomal Chymotrypsin-like Activity in Tumor Cell Extracts and Intact Tumor Cells by EGCG—We then tested if EGCG or EGC could inhibit the 26S proteasome activity in a tumor cell extract. We found that 10 μM EGCG inhibited ~70% of the proteasomal chymotrypsin-like activity in a Jurkat T cell extract, whereas EGC at the same concentration had little effect (Fig. 2*A*). The addition of EGCG to the Jurkat cell extract also potently inhibited another proteasomal activity, the PGPH activity, but did not affect the proteasomal trypsin-like activity (Fig. 2*A*). To investigate whether EGCG specifically inhibits the proteasome activity, its effects on other protease activities were examined. The activity of purified calpain I enzyme was inhibited by the specific calpain inhibitor calpeptin (35) but not EGCG (Fig. 2*B*). Similarly, a caspase-3-like activity in Jurkat T cell extract was blocked by the specific caspase-3 inhibitor Ac-DEVD-CMK (36) but not EGCG (Fig. 2*C*). It appears that EGCG selectively inhibits the proteasomal chymotrypsin (and PGPH) activity over other protease activities.

To determine whether EGCG could also inhibit the living cell proteasomal activity, Jurkat T cells were first incubated with various concentrations of EGCG or EGC followed by an additional incubation with a fluorogenic proteasome peptide substrate. Afterward, the cell medium was collected for the measurement of hydrolyzed products (free AMCs). By performing this assay, we found that EGCG significantly inhibited the proteasomal chymotrypsin-like activity in intact Jurkat cells in a concentration-dependent manner (IC_{50} = 18 μM), whereas

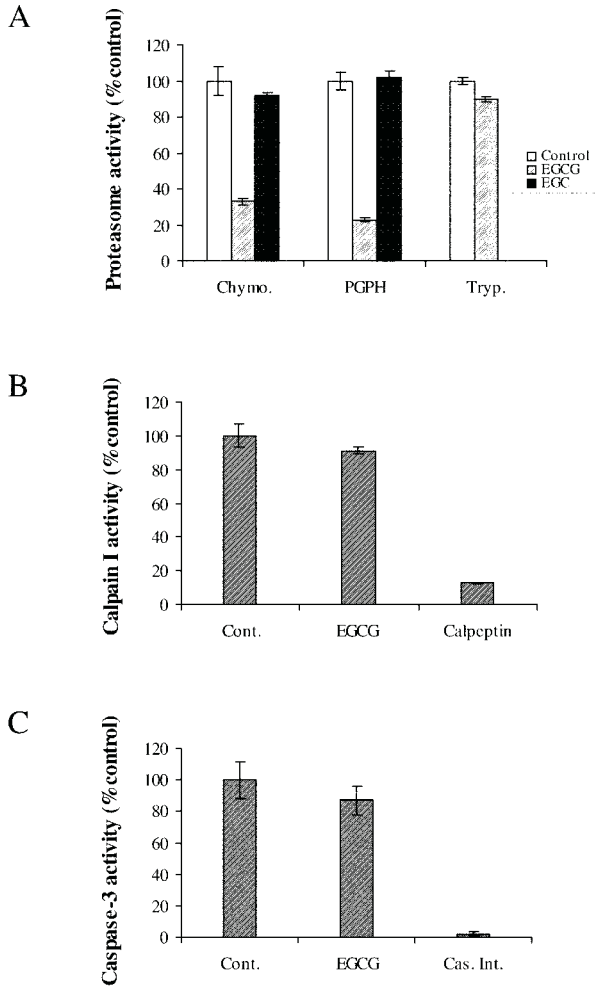

FIG. 2. **Specific inhibition of the proteasome activity by EGCG in Jurkat cell extracts.** A Jurkat cell extract (3.5 μg/reaction) was incubated for 90 min with various fluorogenic peptide substrates for the proteasomal chymotrypsin-like, PGPH, trypsin-like activity (*A*), or caspase-3 activity (*C*), or a purified calpain I (3 μg) was incubated for 90 min with a fluorogenic calpain substrate (*B*) in the absence or presence of EGCG (10 μM), EGC (10 μM), calpeptin (1 μM), or Ac-DEVD-CHO (10 μM) as indicated followed by the measurement of free AMC groups as described under "Experimental Procedures" ($p < 0.05$).

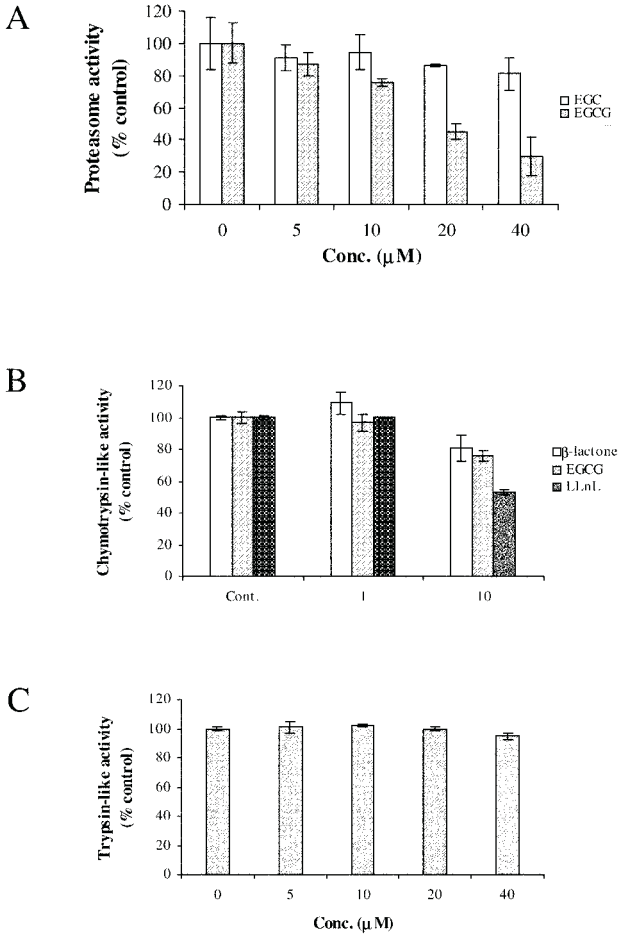

FIG. 3. **Inhibition of the proteasome activity by EGCG but not EGC in intact Jurkat cells.** Human Jurkat T cells were preincubated for 12 h with either the solvent (indicated by 0 or *cont.* for control) or EGCG, EGC, β-lactone, or LLnL at the indicated concentrations followed by an additional 2-h incubation with the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC (for the chymotrypsin-like activity of the proteasome) or Z-Gly-Gly-Arg-AMC (for the trypsin-like activity of the proteasome) (*A* and *B*). *C*, the medium was collected, and the free AMC groups were measured as described under "Experimental Procedures." Most of the data from *A* were derived from three independent experiments, except for EGCG and EGC at 10 μM derived from six independent experiments to most accurately determine the validity of this important concentration.

EGC had a much less effect (Fig. 3*A*).

We noticed that the concentrations of EGCG needed to inhibit the proteasome activity in Jurkat cell extracts (Fig. 2*A*), and intact Jurkat cells (Fig. 3*A*) were much higher than were needed for the inhibition of purified 20S proteasome activity (Fig. 1*A*). We suspected that higher concentrations of other proteasome inhibitors might be needed to reach their *in vivo* cellular target, the proteasome. If true, a specific authentic proteasome inhibitor should display differential potencies between purified proteasome and living cell proteasome activity. To test this idea, the effects of β-lactone, LLnL, and EGCG were measured on inhibition of the proteasomal chymotrypsin-like activity in intact Jurkat T cells. Fig. 3*B* demonstrates that β-lactone, LLnL, and EGCG at 10 μM inhibited 20, 40, and 24% of the proteasomal chymotrypsin-like activity in living Jurkat cells, respectively, with the assay system used. The IC_{50} value of β-lactone to inhibit the chymotrypsin-like activity of a purified 20S proteasome was 0.6 μM under our conditions (Fig. 1*A*) and 0.1–0.2 μM under other conditions (34), and the IC_{50} value of LLnL to inhibit the 20S proteasome chymotrypsin-like activity was 0.14 μM (37). Therefore, it appears that even for a specific proteasome inhibitor, higher concentrations are necessary for the inhibition of the living cell proteasome activity. Because both β-lactone and EGCG showed greater potencies to purified 20S proteasome (IC_{50} values were 600 and 86 nM, respectively, Fig. 1*A*) than to intact cellular proteasome activity (20 and 24% inhibition at 10 μM, respectively, Fig. 3*B*), EGCG seemed to be able to target, thereby inhibiting the proteasome in Jurkat T cells.

We also found that EGCG inhibited the proteasomal chymotrypsin-like activity in intact breast (MCF-7) and prostate (PC-3 and LNCaP) cancer cells (Fig. 4*D* and data not shown). However, EGCG did not inhibit the proteasomal trypsin-like activity in living Jurkat T cells (Fig. 3*C*). Taken together, our data suggest that EGCG but not EGC can selectively inhibit the chymotrypsin-like activity of purified 20S proteasome, 26S proteasome of tumor cell extracts, and 26S proteasome of living tumor cells.

To determine the molecular target(s) responsible for the cancer-preventative effects of green tea, one must adhere to the concentrations of the molecules, which are found physiologically in green tea drinkers. Previous studies indicate that EGCG or other catechins are present in low micromolar ranges

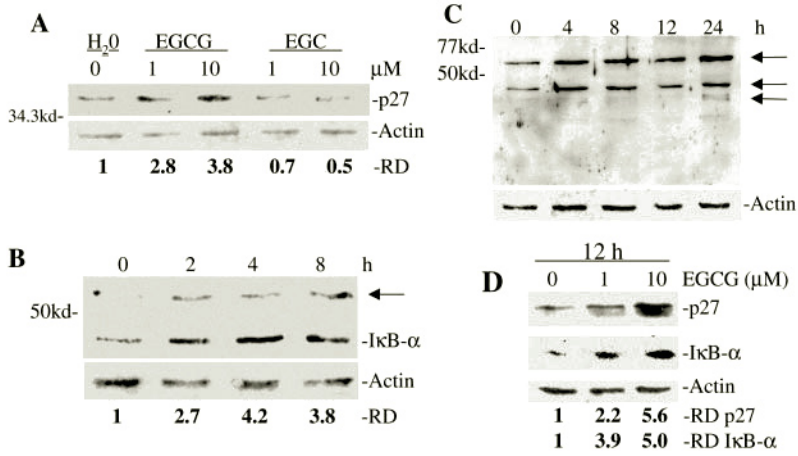

FIG. 4. **Accumulation of p27, IκB-α, and ubiquitinated proteins by EGCG.** *A–C, lane 1,* Jurkat T cells were treated with solvent, 1 or 10 μM EGCG or EGC for 12 h (*A*), or 25 μM EGCG for the indicated hours (*B* and *C*), or prostate cancer LNCaP cells were treated with solvent (*lane 1* in *A–D*), 1 or 10 μM EGCG, or EGC for 12 h (*D*), followed by a Western blot assay using specific antibodies to p27, IκB-α, actin, or ubiquitin. Molecular masses of IκB-α and actin are 40 and 43 kDa, respectively. The band of 56 kDa, indicated by an *arrow* in *B* might be an IκB-α-related protein. The bands indicated in *C* are ubiquitin-containing proteins. Relative density (*RD*) values are normalized ratios of the intensities of p27 or IκB-α band to the corresponding actin band.

in the plasma and saliva of human volunteers (3, 38) and in mice that had been fed with tea (38). Here we found that EGCG in low micromolar ranges acts as a potent proteasome inhibitor *in vitro* and *in vivo* (Figs. 1–3), indicating that EGCG at physiological levels could inhibit the proteasomal chymotrypsin-like activity in intact cancer cells and bring about the resultant tumor growth arrest (see below).

Accumulation of the Proteasome Target Proteins p27^{Kip1} and IκB-α in Tumor Cells Treated with EGCG—To further confirm that EGCG inhibits the proteasome activity *in vivo*, Jurkat T cells were treated with various concentrations of EGCG or EGC for different hours followed by measuring levels of the cyclin-dependent kinase inhibitor p27^{Kip1} and IκB-α, two well known target proteins of the proteasome (29, 30). A 12-h treatment of Jurkat cells with 1 μM EGCG increased p27 levels by ~3-fold (Fig. 4*A*, *lane 2 versus lane 1*), and the same treatment with 10 μM EGCG increased p27 expression by ~4-fold (*lane 3 versus lane 1*). In contrast, EGC at the same concentrations had no such effect (Fig. 4*A*). EGCG treatment also increased IκB-α levels by 2.7-fold after a 2-h treatment and by ~ 4-fold after 4–8 h of treatment (Fig. 4*B*). A band of 56 kDa, detectable by the anti-IκB-α antibody used, was increased significantly during EGCG treatment (indicated by an *arrow*, Fig. 4*B*), suggesting that it might be an IκB-α-related protein.

Because most of the proteasome-mediated protein degradation pathways require ubiquitination (22), we expected that the inhibition of proteasome activity by EGCG should increase the levels of polyubiquitinated proteins. Indeed, when lysates of EGCG-treated Jurkat T cells were immunoblotted with an antiserum to ubiquitin, increased levels of several ubiquitinated proteins were detected (Fig. 4*C*).

To determine whether other cancer cell lines are also responsive to EGCG treatment, human prostate cancer LNCaP cells were treated with EGCG at 1 or 10 μM for 12 h. Again, EGCG at 1 μM increased the levels of p27 and IκB-α proteins by 2.2- and 3.9-fold, respectively, and EGCG at 10 μM increased p27 and IκB-α expression by 5.6- and 5.0-fold, respectively, in these prostate cancer cells (Fig. 4*D*). Therefore, an accumulation of p27 and IκB-α proteins by EGCG treatment is time-dependent and concentration-dependent.

To rule out possible stimulatory effects of EGCG on the syntheses of p27 and IκB-α proteins, Jurkat T cells were preincubated with the protein synthesis inhibitor cycloheximide for 2 h followed by additional incubation with or without EGCG (in the presence of cycloheximide) to determine whether the stability of p27 and IκB-α proteins is increased by EGCG treatment. Incubation with cycloheximide alone significantly decreased the levels of both p27 and IκB-α proteins (Fig. 5, *A* and *B*, *lanes 2 versus lanes 1*). This decrease should be the result of degradation of these proteins in the absence of new protein synthesis. When the cycloheximide-pretreated cells were coincubated with 10 μM EGCG, the levels of p27 protein were increased by 3-fold with respect to cycloheximide treatment alone (Fig. 5*A*, *lane 4 versus lane 2*). This increase should be the result of the inhibition of p27 degradation by EGCG but not due to increased p27 synthesis because of the presence of cycloheximide. In addition, the appearance of a band of ~70 kDa (p70) was significantly increased by this treatment (Fig. 5*A*, *lane 4 versus lane 2*). The p70 may contain ubiquitinated p27, because a similar p70 containing ubiquitinated p27 was found in proteasome inhibitor-treated human osteosarcoma MG-63 cells (29) and breast cancer MDA-MB-231 cells (24). In fact, the sum of the levels of both p27 and p70 was increased by 7-fold in cells cotreated with cycloheximide and EGCG (Fig. 5*A*, *lane 4 versus lane 2*).

Coincubation of the cycloheximide-pretreated cells with 10 μM EGCG also greatly increased the levels of IκB-α protein 4-fold higher than that of the cells treated with cycloheximide alone (Fig. 5*B*, *lane 4 versus lane 2*). The increase in IκB-α expression by EGCG was even greater than that by the proteasome inhibitor LLnV at the same concentration (4- *versus* 2-fold, Fig. 5*B*, *lane 4 versus lane 3*). Therefore, the inhibition of the proteasomal chymotrypsin-like activity in intact tumor cells (Fig. 3) correlates well with the accumulation of p27, IκB-α, and some ubiquitinated proteins (Figs. 4 and 5).

The following arguments support that inhibition of the proteasome activity by EGCG is responsible for the accumulation of p27 and IκB-α proteins in tumor (Figs. 4 and 5) and transformed (for review see Fig. 7) cells. First, as shown in Figs. 1–3, EGCG is a relatively potent specific proteasome inhibitor *in vitro* and *in vivo*. In addition, the accumulation of both p27 and IκB-α proteins was observed in an EGCG concentration-dependent (Figs. 4*A* and *D* and 5*A*) and time-dependent manner (Figs. 4*B* and 7). Furthermore, after EGCG treatment, the anti-p27 antibody detected a p70 band (Fig. 5*A*), which may contain ubiquitinated p27 (24, 29). Finally, the coincubation of cycloheximide-pretreated cells with EGCG demonstrated an almost complete inhibition of p27 and IκB-α protein degradation by EGCG (Fig. 5).

EGCG Induces Tumor Cell Growth Arrest in G_1 Phase of the Cell Cycle—It has been well documented that overexpression of either p27 (39, 40) or IκB-α (41, 42) suppresses the G_1-to-S phase transition. If EGCG-accumulated p27 and IκB-α proteins (Fig. 4) were functional, the treated tumor cells should exhibit some growth arrest at G_1. To test this possibility, Jurkat T or LNCaP cells were treated with EGCG under the similar conditions described in Fig. 4 and harvested for analysis of cell cycle distribution. A 12-h treatment of Jurkat T cells with 10

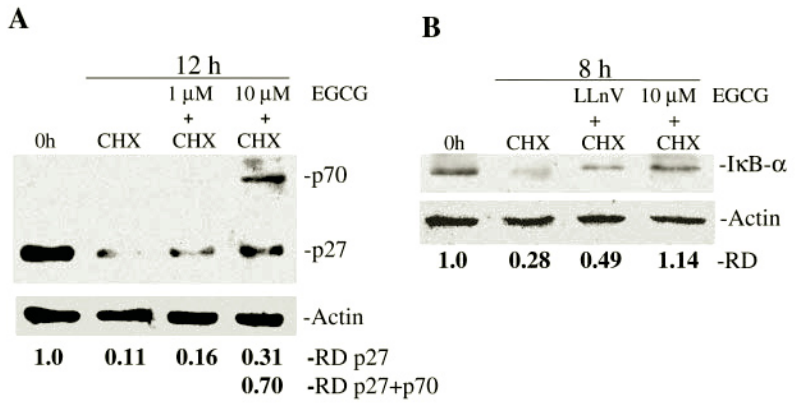

FIG. 5. **Accumulation of p27 and IκB-α proteins by EGCG in cycloheximide-pretreated cells.** Jurkat T cells were pretreated with 10 μg/ml cycloheximide for 2 h followed by coincubation with 1 or 10 μM EGCG or 10 μM LLnV for 8 or 12 h as indicated. This was followed by a Western blot assay using specific antibodies to p27, IκB-α, and actin. The p70 is a putative ubiquitinated p27-containing complex (29, 24). Relative density (*RD*) values are normalized ratios of intensities of p27 (or p27 plus p70) or IκB-α band to the corresponding actin band.

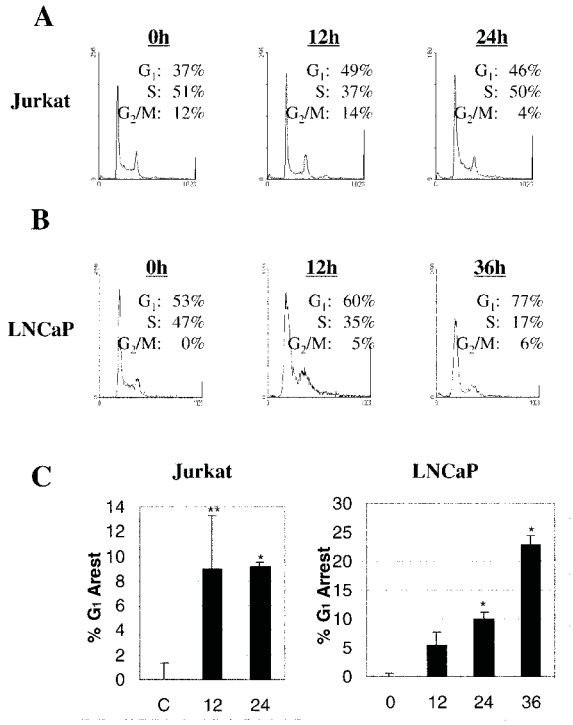

FIG. 6. **EGCG induces G[1] arrest in Jurkat T and LNCaP cancer cells.** Asynchronous (0 h) Jurkat (*A*) or LNCaP tumor cells (*B*) were treated with 10 μM EGCG for indicated hours. At each time point, cells were harvested and analyzed by flow cytometry. Growth arrest is determined by the increase in the percentage of the G_1 population. *C,* statistical analysis. The results were derived from 3–5 independent experiments, and *p* values were calculated as indicated (*, $p < 0.01$ as compared with respective 0 h; **, $p < 0.05$ as compared with respective 0 h).

μM EGCG increased G_1 population by 12% (Fig. 6*A*), consistent with the accumulation of p27 and IκB-α proteins under the same conditions (Fig. 4*A*). A 24-h treatment with EGCG remained an ~10% increase in G_1 population (Fig. 6*A*). The EGCG-induced Jurkat cell G_1 arrest was detected in several independent experiments that showed statistical significance (Fig. 6*C*, *left*).

Exposure of LNCaP prostate cancer cells to 10 μM EGCG for 12 h initiated G_1 arrest by a 7% increase (Fig. 6*B*), which was correlated with p27 and IκB-α accumulation at this time (Fig. 4*D*). EGCG treatment of LNCaP cells for 24 and 36 h increased the G_1 population by 12 and 24%, respectively (Fig. 6*B,* and *C, right*). Again, the EGCG-mediated G_1 arrest of LNCaP prostate cancer cells was observed in multiple independent experiments ($p < 0.01$, Fig. 6*C*, *right*). These results support the functional significance of inhibition of the proteasome activity *in vivo* (Fig. 3) and the accumulation of p27 and IκB-α proteins by EGCG (Figs. 4 and 5). Our study is also consistent with previous reports that overexpression of p27 or IκB-α causes cell arrest in G_1 (39–42).

Normal Human WI-38 Fibroblasts Are More Resistant to EGCG-induced p27 Accumulation and G_1 Arrest Than Their SV40-transformed Counterpart—Previously, we reported that proteasome inhibitors selectively accumulated p27 protein and induced apoptosis in tumor and transformed abnormal human cells (24). To investigate whether EGCG has any differential effects on transformed and normal cells, the normal human fibroblast cell line WI-38 and its SV40-transformed derivative (VA-13) were treated with 10 μM EGCG followed by the measurement of p27 and IκB-α protein levels and cell cycle distribution.

Similar to Jurkat T and LNCaP tumor cells (Fig. 4), the treatment of the transformed VA-13 cells with 10 μM EGCG significantly increased p27 levels (Fig. 7*A*). A 12-h treatment with EGCG increased p27 expression by 2.8-fold; after 36 or 48 h, p27 levels were further increased by 7.6- and 9.2-fold, respectively (Fig. 7*A*). In contrast, the treatment of normal WI-38 cells with 10 μM EGCG for up to 48 h did not increase p27 levels (Fig. 7*A*).

EGCG treatment of VA-13 cells also increased levels of IκB-α protein: 2.7-fold at 12 h, 8.9-fold at 36 h, and 4.2-fold at 48 h (Fig. 7*B*). Between 36 and 48 h, the levels of a p56 band associated with a decrease in IκB-α expression were increased in these transformed cells (Fig. 7*B*, indicated by an *arrow*), again suggesting that p56 is related to IκB-α (also see Fig. 4*B*). Although levels of IκB-α protein were low in the untreated normal WI-38 cells (0 h), a similar p56 protein was highly expressed (Fig. 7*B*). A 12-h treatment with EGCG increased the levels of IκB-α by 4.2-fold without affecting the p56 levels (Fig. 7*B*). EGCG treatment of WI-38 cells for 36–48 h did not further increase the levels of IκB-α, although under these conditions, the levels of p56 were decreased (Fig. 7*B*). As a control, actin levels were relatively unchanged during EGCG treatment in both VA-13 and WI-38 cells (Fig. 7*C*).

Correlated with the selective p27 accumulation in the transformed cells over normal WI-38 cells by EGCG (Fig. 7*A*), VA-13 cells were found to be more sensitive to EGCG-induced G_1 arrest than WI-38 cells. After a 12-h treatment with EGCG, the G_1 population of VA-13 cells was increased by 22% (Fig. 8, *A* and *C*). In contrast, no apparent G_1 arrest was observed in WI-38 cells under this condition (Fig. 8, *B* and *C*). At 24 h, the G_1 population of the transformed cells was further increased (by ~25%); the WI-38 G_1 population began to increase (by <5%) (Fig. 8*C*). After a 36-h treatment, the VA-13 G_1 population continued to increase (by 33%, Fig. 8, *A* and *C*); only at this time, a 16% increase in the G_1 population of WI-38 cells was also detected (Fig. 8, *B* and *C*). The results from several inde-

pendent experiments confirmed that the transformed VA-13 cells were more sensitive to EGCG-induced G_1 arrest than the normal WI-38 cells (Fig. 8*C*). It appears that the delayed EGCG-induced G_1 arrest in WI-38 cells is associated with the lack of p27 accumulation and the partial induction of IκB-α expression in these normal human fibroblasts (Fig. 7, *A* and *B*). Previously, other researchers reported that EGCG has a pronounced growth inhibitory effect on cancerous but not on their normal counterparts (43). Our study has extended their observation by providing a molecular mechanism for such a selectivity of EGCG.

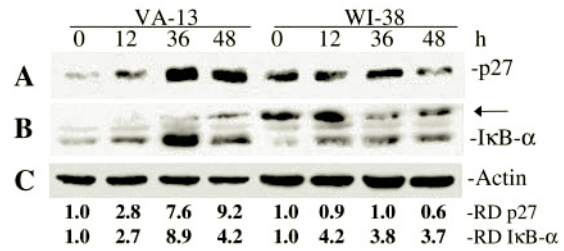

FIG. 7. **Preferable accumulation of p27 protein by EGCG in the transformed fibroblasts over the normal human fibroblasts.** Normal (*WI-38*) and SV40-transformed (*VA-13*) human fibroblasts were treated with 10 μM EGCG for the indicated hours followed by a Western blot assay using specific antibodies to p27 (*A*), IκB-α (*B*), and actin (*C*) as described in the legend of Fig. 4. The band of 56 kDa, indicated by an *arrow* in *B*, might be an IκB-α-related protein.

HPLC Analysis of EGCG After Reaction with Purified Proteasome—The ester bond carbon in β-lactone can be attacked by the strong nucleophilic hydroxyl group of N-terminal threonine residue of the proteasome, forming a covalent complex (32, 33). We hypothesized that the ester bond of EGCG would be attacked by the N-terminal threonine residue of the proteasome, forming a covalent (or tight) EGCG-proteasome complex, thus inactivating the proteasome. If so, EGCG should be quickly lost, associated with production of no (or little) cleavage products of EGCG such as gallic acid and EGC (for review see Fig. 1*A*).

To test this hypothesis, a highly purified EGCG (for review see Fig. 9*D*) was incubated with purified 20S proteasome for various hours followed by HPLC analysis. After a 2-h incubation, a gallic acid-like peak (retention time 4.78) associated with a 40% decrease in the level of EGCG was detected in the HPLC chromatogram whose level was corresponded to a concentration of <5% EGCG (Fig. 9, *A* and *C* and Fig. 10). The gallic acid-like peak was not produced from EGCG in the absence of the proteasome (Fig. 9*D*). The incubation of EGCG with purified proteasome for 4 h resulted in the disappearance

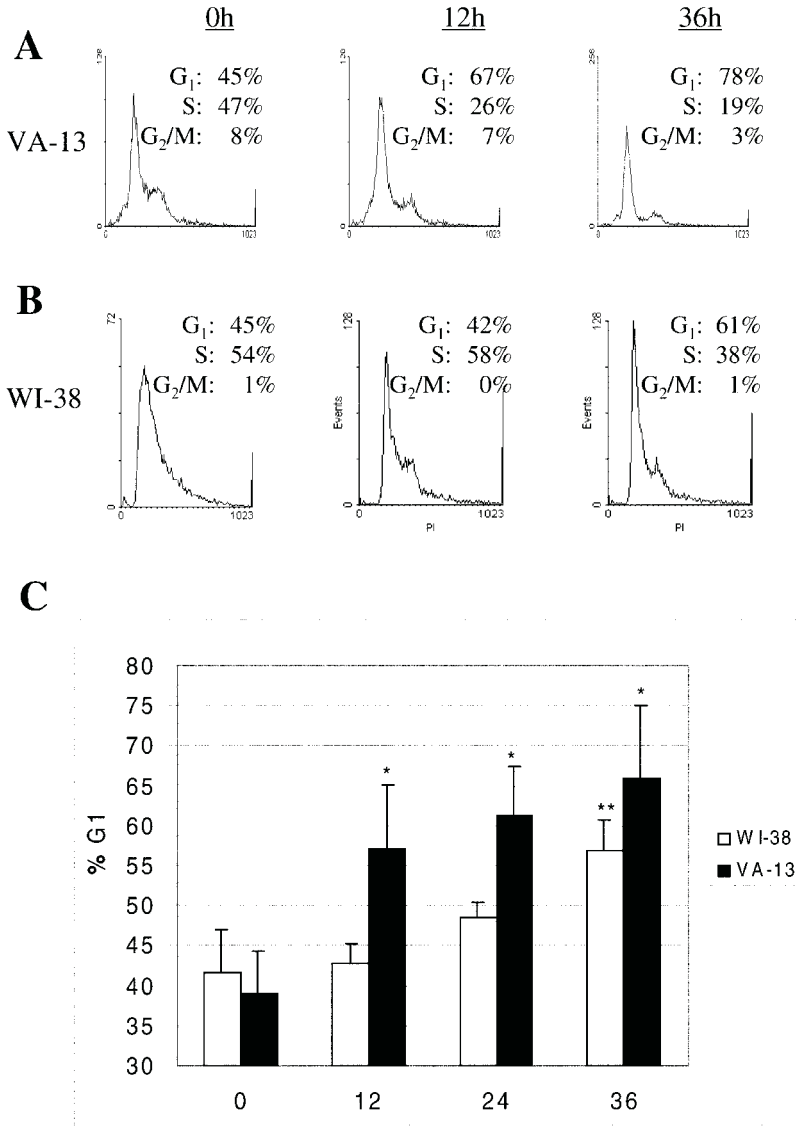

FIG. 8. **Differential sensitivity of the transformed fibroblasts and the normal human fibroblasts to EGCG-induced G^1 arrest.** Normal (*WI-380*) (*B*) and SV40-transformed (*VA-13*) (*A*) human fibroblasts were treated with 10 μM EGCG for the indicated hours followed by flow cytometry analysis. Growth arrest is determined by the increase in the percentage of G_1 population. *C*, statistical analysis. The results were derived from five independent experiments, and *p* values were calculated as indicated (*, $p < 0.01$ as compared with respective 0 h; **, $p < 0.05$ as compared with respective 0 h).

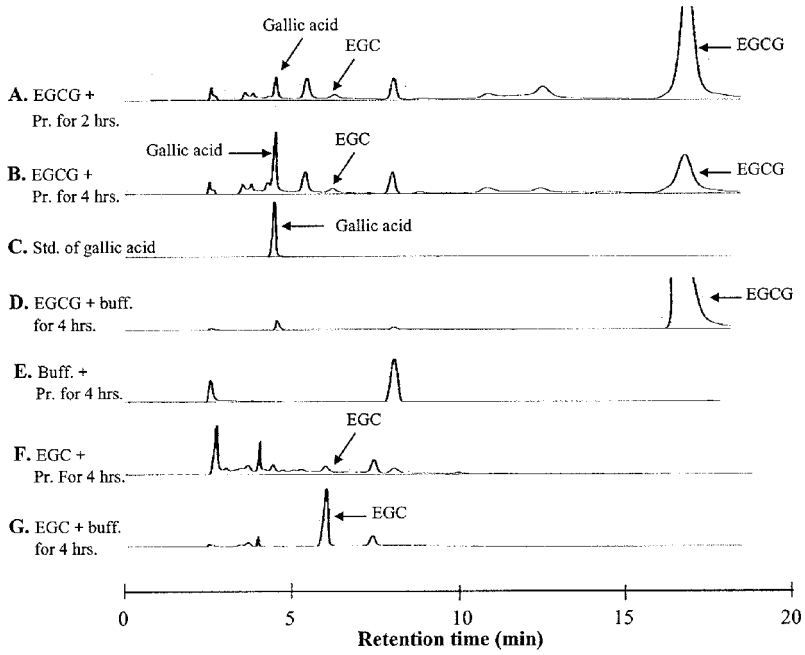

FIG. 9. **HPLC chromatograms of EGCG after incubation with purified 20S proteasome.** EGCG or EGC was incubated with either a purified 20S proteasome (*Pr.*) or the buffer (*buff.*) for the indicated hours followed by HPLC analysis. The retention times of reaction products were confirmed by using tea polyphenol standards (*Std.*). For details, see under "Experimental Procedures."

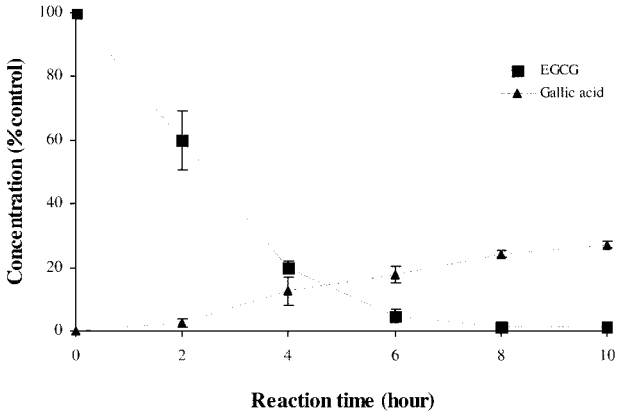

FIG. 10. **The disappearance of EGCG occurs prior to the appearance of gallic acid-like product after incubation with purified 20S proteasome.** EGCG was incubated with purified 20S proteasome for indicated hours followed by HPLC analysis. The levels of EGCG and gallic acid-like product were measured and plotted.

of an ~80% EGCG, associated with an increase in the level of the gallic acid-like peak that is equivalent to a concentration of 13% EGCG (Fig. 9, *B versus A* and Fig. 10). After a 6–10 h incubation with the proteasome, >95% EGCG disappeared, whereas the level of the gallic acid-like product linearly increased to a level equivalent to 20–25% EGCG (Fig. 10).

The incubation of EGCG with the 20S proteasome also resulted in the appearance of an EGC-like product (retention time 6.62 min), although its level was very low (Fig. 9, *A* and *B versus G*), suggesting that the produced EGC could be further degraded by the proteasome. Indeed, purified EGC was degraded almost completely by purified 20S proteasome (Fig. 9, *F versus G*). Several unknown products of EGCG, including one with a retention time of 5.75 min between gallic acid and EGC peaks, were observed (Fig. 9, *A* and *B*). Another unknown peak at a retention time of ~8 min resulted from the mixture of the buffer and purified proteasome (*compare Fig. 9 A* and *B* with *E*). Taken together, the disappearance of most of the free EGCG prior to the appearance of low levels of gallic acid-like product suggests that EGCG might form a tight complex with the proteasome. This hypothesis is consistent with the observed potency of EGCG as a proteasome inhibitor *in vitro* (Figs. 1 and 2) and *in vivo* (Figs. 3–5 and 7). The HPLC data also suggest that the proteasome-bound EGCG could be slowly cleaved at one or more places including the ester bond, which leads to the production of gallic acid, EGC, and other products. Finally, a complete cleavage of EGC by the purified proteasome (Fig. 9*F*) is consistent with failure of EGC to inhibit the purified proteasome activity (Fig. 1*A*) and the proteasome activity in Jurkat cell extracts (Fig. 2*A*) and intact Jurkat T cells (Figs. 3*A* and 4*A*).

Based on our current study, we propose the following molecular mechanisms by which EGCG inhibits the proteasome. The two nucleophilic electrons located on the N-terminal threonine hydroxyl group of the proteasome subunit X (32–34) could attack the ester bond carbon of EGCG after binding to the proteasome active site. A tight EGCG-proteasome complex could be generated, thereby inactivating the proteasome. This complex would slowly disassociate to free EGC and gallic acid. Further studies are needed to understand the nature of ester bond-containing polyphenols as a potent proteasome inhibitor.

In summary, for many years it has been shown through epidemiological studies that green tea is a cancer-preventative agent (1–4). It has also been shown that the proteasome plays an important role in the development and progression of cancer (22, 23). Our study has demonstrated for the first time that the compounds found in tea and in the bodies of green tea drinkers can inhibit the proteasome at or near physiological concentrations. Our results also indicate that the inhibition of the proteasome activity by EGCG can selectively control the growth of tumor and transformed cells. We suggest that the cancer-preventative properties of green tea could be attributed, at least in part, to its ability to inhibit the proteasome activity. Our finding along with the low toxicity of EGCG also implicates the role of tea in a potential clinical therapy in combination with current anticancer drugs.

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