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 (IC₅₀ = 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, p27Kip1 and p18INK4c, an inhibitor of transcription factor NF-κB, followed by growth arrest in the G₁ phase of the cell cycle. Furthermore, compared with their simian virus-transformed counterparts, the parental normal human fibroblasts were much more resistant to EGCG-induced p27Kip1 protein accumulation and G₁ 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 (ECC), and epicatechin (EC), and their epimers (see Fig. 1A). 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 ubiquitin-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), p27Kip1 (29), Ib-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 p27Kip1 and Ib-α as well as induce the arrest of tumor cells in the G₁ phase. Finally, normal human WI-38 fibroblasts were more resistant to EGCG treatment than their SV40-transformed counterpart.

EXPATIMENTAL PROCEDURES

Materials—Highly purified tea polyphenols EGCG (>95%), ECG (>98%), EGC (>98%), GC (>98%), EC (>98%), GCG (>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% EGC, 8.3% GCC, 8.5% EC, 4.4% GCC, 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% EGC, 9.0% GCC, 4.8% EC, 0.0% GCG, 0.5% GC, 2.0% C, and 1.2% caffeine. Purified 20S proteasome (Methanococcus thermophila, B.combinans, 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), benzoylxy carbonyl (Z)-Leu-Leu-Glu-AMC (for the proteasomal PGPH activity), Suc-Leu-Tyr-AMC (for the calpain I...
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activity, and Ac-Asp-Glu-Val-Asp-AMC (for the caspase-3 activity) were also obtained from Calbiochem, and Z-Gly-Aryl-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 antibodies against Bcl-x

were from Sigma.

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 proteosomes, Suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Leu-Glu-AMC, and Z-Gly-Gly-Aryl-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 × 10⁶ cells/ml/well), MCF-7, or PC-3 cells (1 × 10⁶ 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 LLcL, followed by an additional 2-h incubation with the fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC or Z-Gly-Gly-Aryl-AMC. Afterward, the cell medium (200 µl/sample) was collected and used for measurement of free AMCs.

Assays for Caspase 1 and Caspase 3 Activities—To measure the activity of caspase 1, 1 µg of purified caspase 1 was incubated with 40 µM fluorogenic peptide substrate and 0.4% CHAPS buffer for 3 h at 37 °C (20 mM Tris-HCl, pH 7.5, containing 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 5 mM CaCl₂, and 0.1% CHAPS) with or without EGCG or EGC or the specific caspase 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 Workstation version 3.2 (Oxford Molecular Ltd.). After geometric 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 “bulge” on a sphere 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 washed twice with phosphate-buffered saline. Cells (5 × 10⁶) 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 iodine 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 washed again with PBS and resuspended in 1 ml of propidium iodide staining solution. The cell cycle distribution is presented as the percent of cells containing G1, S, G2, G1/S, and G2/M DNA content as judged by the specific caspase-3 inhibitor Ac-DEVD-CHO. After incubation, 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% methanol, 88% water (9.5% acetic acid, 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. 1A) 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, EGC, CG, and CG (Fig. 1A). 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. 1B) whose IC₅₀ value was calculated to be 86 nM (Fig. 1A). In contrast, EGC (IC₅₀ = 1.2 mM) and gallic acid (IC₅₀ = 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-like activity (IC₅₀ = 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. 1B).

Three other ester bond-containing tea polyphenols, EGC, CG, and CG (Fig. 1A), were also found to be strong inhibitors of the chymotrypsin-like activity of the purified 20S proteasome (IC₅₀ 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. 1A), 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. Further, a greater inhibitory effect than the total tea extract, which contains significant portion of EGCG (51.5 and 29.7%, respectively) and EGC (14.7 and 14.9%, respectively, see under “Experimental Procedures”), also strongly inhibited the chymotrypsin-like activity of the 20S proteasome (IC₅₀ 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-
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. 1C). 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. 1C). Similarly, a high nucleophilic susceptibility was found in other ester bond-containing polyphenols, EGC, 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 20S 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. 2A). 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. 2A). 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. 2B). 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. 2C). 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...
EGC had a much less effect (Fig. 3A).

We noticed that the concentrations of EGCG needed to inhibit the proteasome activity in Jurkat cell extracts (Fig. 2A), and intact Jurkat cells (Fig. 3A) were much higher than were needed for the inhibition of purified 20S proteasome activity (Fig. 1A). 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. 3B demonstrates that β-lactone, LLnL, and EGCG at 10 μM inhibited 20, 40, and 24% of the proteasomal chymotrypsin-like activity in Jurkat cells, respectively, with the assay system used. The IC50 value of β-lactone to inhibit the chymotrypsin-like activity of a purified 20S proteasome was 0.6 μM under our conditions (Fig. 1A) and 0.1–0.2 μM under other conditions (34), and the IC50 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 (IC50 values were 600 and 86 nM, respectively, Fig. 1A) than to intact cellular proteasome activity (20 and 24% inhibition at 10 μM, respectively, Fig. 3B), 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. 4D and data not shown). However, EGCG did not inhibit the proteasomal trypsin-like activity in living Jurkat T cells (Fig. 3C). 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...
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 p27Kip1 and IxB-a 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 p27Kip1 and IxB-a, 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. 4A, 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, EGCG at the same concentrations had no such effect (Fig. 4A). EGCG treatment also increased IxB-a levels by 2.7-fold after a 2-h treatment and by ~4-fold after 4–8 h of treatment (Fig. 4B). A band of 56 kDa, detectable by the anti-IxB-a antibody used, was increased significantly during EGCG treatment (indicated by an arrow, Fig. 4B), suggesting that it might be an IxB-a-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. 4C).

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 IxB-a proteins by 2.2- and 3.9-fold, respectively, and EGCG at 10 μM increased p27 and IxB-a expression by 5.6- and 5.0-fold, respectively, in these prostate cancer cells (Fig. 4D). Therefore, an accumulation of p27 and IxB-a proteins by EGCG treatment is time-dependent and concentration-dependent.

To rule out possible stimulatory effects of EGCG on the syntheses of p27 and IxB-a 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 IxB-a proteins is increased by EGCG treatment. Incubation with cycloheximide alone significantly decreased the levels of both p27 and IxB-a 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. 5A, 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. 5A, 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. 5A, lane 4 versus lane 2).

Coincubation of the cycloheximide-pretreated cells with 10 μM EGCG also greatly increased the levels of IxB-a protein 4-fold higher than that of the cells treated with cycloheximide alone (Fig. 5B, lane 4 versus lane 2). The increase in IxB-a expression by EGCG was even greater than that by the proteasome inhibitor L66N at the same concentration (4- versus 2-fold, Fig. 5B, 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, IxB-a, 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 IxB-a 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 IxB-a proteins was observed in an EGCG concentration-dependent (Figs. 4A and D and 5A) and time-dependent manner (Figs. 4B and 7). Furthermore, after EGCG treatment, the anti-p27 antibody detected a p70 band (Fig. 5A), which may contain ubiquitinated p27 (24, 29). Finally, the coincubation of cycloheximide-pretreated cells with EGCG demonstrated an almost complete inhibition of p27 and IxB-a protein degradation by EGCG (Fig. 5).

EGCG Induces Tumor Cell Growth Arrest in G1, Phase of the Cell Cycle—It has been well documented that overexpression of either p27 (39, 40) or IxB-a (41, 42) suppresses the G1-to-S phase transition. If EGCG-accumulated p27 and IxB-a proteins (Fig. 4) were functional, the treated tumor cells should exhibit some growth arrest at G1. 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
the G1 population by 12 and 24%, respectively (Fig. 6D). EGCG treatment of LNCaP cells for 24 and 36 h increased k with the accumulation of p27 and I
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.

**Normal Human WI-38 Fibroblasts Are More Resistant to EGCG-induced p27 Accumulation and G1 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. 7A). 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. 7A). In contrast, the treatment of normal WI-38 cells with 10 μM EGCG for up to 48 h did not increase p27 levels (Fig. 7A).

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. 7B). 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. 7B, indicated by an arrow), again suggesting that p56 is related to IκB-α (also see Fig. 4B). 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. 7B). A 12-h treatment with EGCG increased the levels of IκB-α by 4.2-fold without affecting the p56 levels (Fig. 7B). 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. 7B). As a control, actin levels were relatively unchanged during EGCG treatment in both VA-13 and WI-38 cells (Fig. 7C).

Correlated with the selective p27 accumulation in the transformed cells over normal WI-38 cells by EGCG (Fig. 7A), VA-13 cells were found to be more sensitive to EGCG-induced G1 arrest than WI-38 cells. After a 12-h treatment with EGCG, the G1 population of VA-13 cells was increased by 22% (Fig. 8, A and C). In contrast, no apparent G1 arrest was observed in WI-38 cells under this condition (Fig. 8, B and C). At 24 h, the G1 population of the transformed cells was further increased (by ~25%); the WI-38 G1 population began to increase (by <5%) (Fig. 8C). After a 36-h treatment, the VA-13 G1 population continued to increase (by 33%, Fig. 8, A and C); only at this time, a 16% increase in the G1 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 G₁ arrest than the normal WI-38 cells (Fig. 8C). It appears that the delayed EGCG-induced G₁ 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.

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. 1A).

To test this hypothesis, a highly purified EGCG (for review see Fig. 9D) 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. 9D). The incubation of EGCG with purified proteasome for 4 h resulted in the disappearance 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.

FIG. 8. Differential sensitivity of the transformed fibroblasts and the normal human fibroblasts to EGCG-induced G₁ arrest. Normal (WI-38) (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 G₁ 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).
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, A versus B 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 EGCG by the purified proteasome (Fig. 9F) is consistent with failure of EGCG to inhibit the purified proteasome activity (Fig. 1A) and the proteasome activity in Jurkat cell extracts (Fig. 2A) and intact Jurkat T cells (Figs. 3A and 4A).

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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