(-)-Epigallocatechin Gallate Overcomes Resistance to Etoposide-Induced Cell Death by Targeting the Molecular Chaperone Glucose-Regulated Protein 78

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Abstract

Many beneficial properties have been attributed to (-)-epigallocatechin gallate (EGCG), including chemopreventive, anticarcinogenic, and antioxidant actions. In this study, we investigated the effects of EGCG on the function of glucose-regulated protein 78 (GRP78), which is associated with the multidrug resistance phenotype of many types of cancer cells. Our investigation was directed at elucidating the mechanism of the EGCG and GRP78 interaction and providing evidence about whether EGCG modulates the activity of anticancer drugs through the inhibition of GRP78 function. We found that EGCG directly interacted with GRP78 at the ATP-binding site of protein and regulated its function by competing with ATP binding, resulting in the inhibition of ATPase activity. EGCG binding caused the conversion of GRP78 from its active monomer to the inactive dimer and oligomer forms. Further, we showed that EGCG interfered with the formation of the antiapoptotic GRP78-caspase-7 complex, which resulted in an increased etoposide-induced apoptosis in cancer cells. We also showed that EGCG significantly suppressed the transformed phenotype of breast cancer cells treated with etoposide. Overall, these results strongly suggested that EGCG could prevent the antiapoptotic effect of GRP78, which usually suppresses the caspase-mediated cell death pathways in drug-treated cancer cells, contributing to the development of drug resistance. (Cancer Res 2006; 66(18):9260-9)

Introduction

The resistance of cancer cells to chemotherapeutic drugs remains a major obstacle for effective cancer treatment. The complexity of drug resistance in human cancer strongly suggests that the mechanisms of drug resistance are likely to involve multiple signaling pathways that differ within various cancer types. One important mechanism in cancer involves the aberrant induction of the chaperone protein glucose-regulated protein 78 (GRP78), which is correlated with resistance to chemotherapeutic agents, including doxorubicin, etoposide, and Adriamycin (1-3). Overexpression, antisense, and ribozyme approaches in cell culture systems have directly shown that GRP78 can protect them against cell death and immune attack and also confer drug resistance (16-18). Thus, the induction of GRP78 activity can be an effective defense mechanism to enhance cancer cell survival. (-)-Epigallocatechin gallate (EGCG) is a major component in green tea, and its inhibitory activity against tumorigenesis has been shown (19-22). The mechanisms of action of EGCG explaining its anticancer effects are not very well understood but are being intensively investigated. Previous studies by other investigators have suggested that EGCG is synergistically cytotoxic to human cancer cells through modulating P-glycoprotein and the estrogen receptor (23, 24). On the other hand, the identification of proteins interacting directly with EGCG is a key step in understanding the molecular mechanisms of the anticancer effects of EGCG. Several proteins that can directly bind with EGCG have been identified, including fibronectin, fibrinogen, histidine-rich glycoprotein, laminin (25), Fas (26), matrix metalloproteinase (MMP)-2 and MMP-9 (27), Bcl-2 (28), the 67-kDa laminin receptor (29), and vimentin (30). In the present study, we provide new evidence showing that EGCG prevents the antiapoptotic role of GRP78, a function that is associated with the suppression of GRP78 of the caspase-mediated cell death pathways in drug-treated cells, contributing to the development of drug resistance.
Materials and Methods

Materials. All media were obtained from Invitrogen (Carlsbad, CA); fetal bovine serum (FBS) was from Gemini Bio-Products (Calabasas, CA). CNBr-Sepharose 4B was purchased from Amersham Biosciences (Piscataway, NJ). [3H]EGCG (13 Ci/mmol in ethanol containing 8 mg/mL ascorbic acid) was a gift from Dr. Yukihiko Hara (Food Research Laboratory, Mitsui Norin Co., Ltd., Fujieda, Shizuoka, Japan). Recombinant GRP78 (rGRP78) was purchased from StressGen (Victoria, British Columbia, Canada), and the goat GRP78 polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Etoposide was purchased from Calbiochem (San Diego, CA), thapsigargin was from LC Laboratories (Woburn, MA), and procaspase-7 (human recombinant) was from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). EGCG, (15)–epicatechin (EC), (15)–epicatechin-3-gallate (EGC), and (15)–epigallocatechin (EGC) were generous gifts from Dr. Chi-Tang Ho (Rutgers University, Piscataway, NJ).

Cell culture. The JB6 Cl41 mouse epidermal cell line was grown in MEM supplemented with 5% FBS. The stable T24/83 human bladder carcinoma cell line expressing human GRP78 (T24/83-GRP78) or transfected with the empty expression vector pCDNA3.1 (T24/83-pCDNA) was kindly provided by Dr. Richard C. Austin (McMaster University and Hamilton Health Sciences, Hamilton, Ontario, Canada; ref. 31). These cell lines were maintained in 199 medium supplemented with 10% FBS containing 1% penicillin and 200 μg/mL G418. The MDA-MB-231 human breast cancer cell line was maintained in DMEM supplemented with 10% FBS. The T-47D human breast cancer cell line was maintained in RPMI 1640 adjusted to contain 0.2 unit/mL of bovine insulin and supplemented with 10% FBS.

Affinity chromatography, two-dimensional gel electrophoresis, and matrix-assisted laser desorption/ionization-time of flight analysis. EGCG was first coupled to the CNBr-activated Sepharose 4B matrix, and the binding between GRP78 and EGCG was examined by affinity chromatography according to the manufacturer’s instructions, which corresponded to a method described previously (30). Two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis were done as reported previously (30).

Construction of GRP78 plasmids. The eukaryotic expression plasmid pCDNA3 for human GRP78 was generously provided by Dr. Richard C. Austin. A cDNA encoding GRP78 was generated by PCR and subcloned into the BamHI/XhoI sites of the pGEX-5X-1 vector (Amersham Biosciences) to produce the glutathione S-transferase (GST)-GRP78 fusion protein. The serial truncated deletion mutants of GRP78 were generated from pGEX-5X-1-GRP78 wild-type and also inserted in-frame into the pGEX-5X-1 vector according to the manufacturer’s instructions, which corresponded to a method described previously (30). Two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis were done as reported previously (30).

Results

EGCG interacts with GRP78. To identify novel proteins that interact with EGCG, we did affinity chromatography using EGCG-Sepharose 4B beads and JB6 Cl41 cell lysate proteins. Fractions containing proteins bound with EGCG were analyzed by two-dimensional electrophoresis (Fig. 1A), and MALDI-TOF mass spectrometry was used to identify proteins that directly bind with...
EGCG. A search of the National Center for Biotechnology Information database revealed that two spots corresponded to GRP78 (Fig. 1A, circled spots).

The interaction of GRP78 and EGCG was assessed in an EGCG-Sepharose 4B affinity chromatography pull-down experiment and with subsequent detection by immunoblotting with anti_GRP78 (Fig. 1B, arrow). Further results also indicated not only in vitro binding of EGCG with rGRP78 (Fig. 1C, left, lane 1) but also in vivo binding of EGCG and GRP78 in JB6 Cl41 cell lysates (Fig. 1C, right, lane 2).

To characterize the physical binding between EGCG and GRP78, we determined the binding affinity ($K_d$) of this complex using a GST pull-down assay with radiolabeled EGCG. The $K_d$ value of GRP78 and EGCG was found to be 0.7 $\mu$mol/L (Fig. 1D). Taken together, these data suggested that the interaction between EGCG and GRP78 was a direct and specific interaction.

**Identification of the EGCG-binding site of GRP78.** The GRP78 molecule contains an ATPase domain at the NH$_2$-terminal region, which catalyzes the hydrolysis of ATP to ADP, and a peptide-binding domain at the COOH-terminal region, which binds polypeptides with specific heptapeptide sequences (36, 37). To determine the specific binding region of GRP78 for EGCG, we first generated two GRP78 deletion constructs, an NH$_2$-terminal (GRP78-N) and COOH-terminal (GRP78-C) deletion mutants (Fig. 2A), and evaluated the binding affinity of the two GRP78 domains using the EGCG-Sepharose 4B pull-down assay. Results indicated that the GST-GRP78-N domain (amino acids 1-405) containing the ATP-binding site (amino acids 175-201; refs. 2, 12) interacted efficiently with EGCG, but the GST-GRP78-C (amino acids 406-664) containing the peptide-binding domain was not detected in the EGCG-Sepharose 4B pull-down assay (Fig. 2B).

The specific region of interaction between EGCG and GST-GRP78 was confirmed by the use of serial truncated GST-GRP78 fusion proteins from the NH$_2$-terminal region. We tested the ability of each NH$_2$-terminal truncated GST-GRP78 fusion protein to interact with EGCG using an EGCG-Sepharose 4B pull-down assay. The domains containing the NH$_2$-terminal ATP-binding region (residues 80-654, 160-654, and 211-654) interacted efficiently with EGCG (Fig. 2C, top). Then, to determine whether ATP binds to a region similar to that which binds EGCG, we examined the binding of ATP and each GST-GRP78 truncated GST-GRP78 fusion protein to interact with EGCG using an ATP-Sepharose 4B pull-down assay. The lines indicated that EGCG bound to the same NH$_2$-terminal regions of GST-GRP78 as did ATP, which is the area containing the ATPase activity (Fig. 2C, middle).

Because EGCG binds with the ATPase catalytic domain of GRP78, it might compete with ATP for binding with free GRP78. rGRP78 and EGCG-Sepharose 4B beads or ATP-Sepharose 4B beads were used to determine whether EGCG could inhibit the binding of ATP to GRP78. Results confirmed that ATP competed with EGCG for binding with GRP78 in a concentration-dependent manner because the binding of EGCG with GRP78 decreased with increasing amounts of ATP (Fig. 2D, left). Likewise, the binding of ATP with GRP78 also decreased with increasing amounts of EGCG (Fig. 2D, right). These results further supported the idea that EGCG binds to the ATPase catalytic domain of GRP78.
EGCG inhibits the ATPase activity of GRP78 and modulates GRP78 through conformational conversion. ATP binding and hydrolysis are essential for the chaperone activity of all HSP70 proteins, suggesting that the protective effect of GRP78 may be dependent on a functional ATP-binding domain (2, 38). To determine whether rGRP78 protein maintained ATPase activity, we measured ATPase activity for rGRP78 within a range of rGRP78 concentrations from 0.5 to 3 μmol/L. The results indicated that rGRP78 maintained ATPase activity (Fig. 3A, left). In a second set of experiments, we determined the effect of EGCG on rGRP78 ATPase activity and results showed that EGCG (5 or 10 μmol/L) had a significant inhibitory effect (56% and 61%, respectively; Fig. 3A, middle). The next question to be answered was whether the EGCG inhibition of ATPase activity of GRP78 was related to the presence of the gallate group. Therefore, we investigated the effect of EGCG analogues, including EC, ECG, and EGC, on ATPase activity of GRP78. The results indicated that, besides EGCG, ECG, which contains the gallate group, also inhibited ATPase activity in a manner similar to EGCG (64%; Fig. 3A, right). Compared with EGCG and ECG, other analogues, EC and EGC, showed a weaker effect on ATPase activity of GRP78.

Figure 2. EGCG-binding site of GRP78. A, deletion mutants of GRP78. B, in vitro interactions of EGCG with full-length GST-GRP78 (GRP78-FL; amino acids 1-654), NH2-terminal GST-GRP78 (amino acids 1-405), or COOH-terminal GST-GRP78 (amino acids 406-654). GST proteins were incubated with EGCG-Sepharose 4B beads overnight at 4°C. Precipitates were analyzed by immunoblotting (IB) using a GST antibody (top) and Coomassie blue staining (bottom). C, in vitro interactions of EGCG or ATP with various deletion mutants of GRP78. Various GST-GRP78 deletion mutant proteins were incubated with EGCG-Sepharose 4B or ATP-agarose 4B beads. Precipitates were analyzed by immunoblotting using a GST antibody (top and middle) and Coomassie blue staining (bottom). D, rGRP78 was incubated with EGCG-Sepharose 4B beads overnight at 4°C. ATP (0.5 or 1 μmol/L) was added to the reaction volume and incubated for 30 minutes at 25°C. Precipitates were analyzed by Western blot analysis using a GRP78 antibody (left, top) and quantified using the ImageJ software program (left, bottom). EGCG (0.5 or 1 μmol/L) was incubated with rGRP78 for 4 hours at 4°C, and then 50 μL of ATP-agarose 4B were added to the reaction volume and incubated overnight at 4°C. Precipitates were analyzed by Western blot using a GRP78 antibody (right, top) and quantified using the ImageJ software program (right, bottom).
inhibition of GRP78 ATPase activity (38% and 53%, respectively; Fig. 3A, right). These results indicated that the gallate moiety might be important for binding with GRP78 and/or inhibiting GRP78 ATPase activity.

In mammalian cells, GRP78 exists in interconvertible oligomeric and monomeric forms (39). The monomeric form is the biologically active species that can bind to unfolded or unassembled proteins (39). The oligomeric forms of GRP78 are converted to the active monomeric form by binding with ATP in vitro (40) and in vivo (41). We first used different doses of ATP to examine the conversion state of rGRP78. Nondenatured gel electrophoresis was used to analyze the migration of monomeric, dimeric, and oligomeric forms of rGRP78, and results confirmed that ATP effectively converted rGRP78 dimers to monomers (Fig. 3B, left). We then investigated the effect of EGCG on the state of oligomerization of rGRP78, and results indicated that the addition of EGCG induced the conversion of the active monomeric form of rGRP78 to the inactive dimeric form of rGRP78 (Fig. 3B, right). This effect was dependent on the concentration of EGCG because the monomeric form of rGRP78 was completely converted to the dimeric form as EGCG increased to 10 μmol/L. To further investigate the effect of EGCG on the conversion of the monomeric form induced by ATP, we conducted a competition assay. Results indicated that, in the presence of EGCG (10 μmol/L), the dissociation of dimers to monomers was almost totally prevented (Fig. 3C, left, lane 5). This observation prompted us to investigate the effect of EGCG analogues on the conformational conversion of GRP78. Results indicated that, besides EGCG, only ECG had similar effects on the state of oligomerization of GRP78 (Fig. 3C, right, lane 4). Both EGCG and ECG contain the gallate group, further confirming that

Figure 3. Effect of EGCG and its analogues on GRP78 ATPase activity and conformation of GRP78. A, ATPase activity of GRP78 was assayed under standard conditions as described in Materials and Methods using 1 mmol/L ATP at pH 7.5 and 37 °C for 1 hour. Aliquots were removed from the supernatant fraction and counted in a liquid scintillation counter to determine the amount of [32P]Pi released. Left, ATP hydrolysis was calculated from measuring [32P]Pi activity and standardized by subtracting a blank reaction mixture containing no GRP78. Middle, inhibition of GRP78 (3 μmol/L) ATPase activity by different concentrations of EGCG (1, 5, or 10 μmol/L). Inhibition of GRP78 (3 μmol/L) ATPase activity by analogues of EGCG. Right, assay was done using 10 μmol/L EGCG, EC, ECG, or EGC. Points, mean of three independent experiments; bars, SD. Asterisk, significant decrease in ATPase activity in EGCG or analogue-treated samples compared with untreated control samples. **, P < 0.005; ***, P < 0.0001. B, in vitro effect of ATP concentration on the conversion of the GRP78 dimer to a monomer. rGRP78 (0.5 μg) and different doses of ATP (1, 10, or 30 μmol/L) were incubated for 30 minutes at 25 °C in a 25 μL reaction mixture [20 mmol/L HEPES (pH 7.0)]. The analysis for GRP78 conformation changes was done using nondenaturing gel electrophoresis (4-15% Tris-HCl gradient gels; Bio-Rad, Hercules, CA). Left, for detection of GRP78, an antibody against GRP78 was used. In vitro effect of EGCG on the oligomerization state of GRP78. Right, assay was done using different doses of EGCG (1, 5, or 10 μmol/L) without ATP. C, effect of EGCG on the ATP-induced conformational changes of GRP78. rGRP78 (0.5 μg) was incubated with ATP (30 μmol/L) and different doses of EGCG (1, 5, or 10 μmol/L) for 24 hours. In vitro effect of EGCG analogues on the oligomerization state of GRP78. D, in vivo effect of EGCG on the oligomerization states of GRP78. JB6 CI41 cells were treated with different doses of EGCG (1, 5, or 10 μmol/L) for 24 hours and analyzed by immunoblotting with the GRP78 antibody. M, monomer; D, dimer; O, oligomer.
this moiety might be critical for interacting with GRP78. We next determined whether similar effects might be observed in vivo by using a JB6 Cl41 cell lysate treated with EGCG. Results showed that the dimeric and/or oligomeric form of GRP78 increased after treatment with increasing concentrations of EGCG (Fig. 3D). For detecting endogenous GRP78, the whole-cell lysate was separated on a nondenaturing gel and subsequent detection was done by immunoblotting with anti-GRP78. Data indicated that GRP78 could exist as monomeric, dimeric, and oligomeric forms in cells. EGCG induced the conversion of the monomeric form to dimeric and oligomeric forms in a dose-dependent manner. Thus, EGCG could also induce a conformational change of GRP78, suggesting that EGCG might be inhibiting the activity of GRP78 in vivo.

**EGCG can prevent the formation of the inactive GRP78 and caspase-7 complex.** One of the interesting questions arising from these studies was whether EGCG binding could block the antiapoptotic function of GRP78 (2, 6). Based on the results of GRP78 binding and inhibition of ATPase activity assays, EGCG might block the association of the GRP78-caspase-7 inhibitory complex. This is based on the finding that the mechanism of ER stress-induced apoptosis involves caspase-7 and requires both the association and dissociation of the inhibitory complex of GRP78 and caspase-7 (2, 6, 42). Thus, to confirm that GRP78 and caspase-7 directly interact physically, NH2-terminal truncated GST-GRP78 fusion proteins were evaluated for their ability to associate with recombinant procaspase-7. Previously, residues 175 to 201 of the GRP78 NH2-terminal ATP-binding domain are important for binding caspase-7 (2). Our results also indicated that caspase-7 bound to the NH2-terminal domain of GRP78 (Fig. 4D), which contains the same regions for binding EGCG (Fig. 2B and C). These results strongly suggested that the binding region of caspase-7 and EGCG is similar. Thus, EGCG might prevent the formation of a complex between caspase-7 and GRP78.

To directly test this possibility, we further investigated the effect of EGCG on the binding of caspase-7 and GRP78 in vitro and in vivo. For the in vitro test, we used the GST pull-down assay with recombinant procaspase-7 and GST-GRP78. Different doses of EGCG were added to the reaction for pretreatment of GRP78, and results showed that the binding of procaspase-7 to GRP78 decreased with increasing concentrations of EGCG (Fig. 4B). Then,
to confirm the interaction of GRP78 and caspase-7 in vivo, a JB6 C141 cell lysate was used for immunoprecipitation with anti-GRP78. Given that conditions of cellular stress, such as heat shock or ER stress, are usually responsible for up-regulation of chaperone proteins, we used increasing concentrations of thapsigargin, an inhibitor of intracellular calcium pumps, for induction of GRP78 (Fig. 4C, top). The results indicated that GRP78 interacted with caspase-7 (Fig. 4C, bottom), which has been reported for in vivo association before and following stress conditions (2, 6). On the other hand, prolonged treatment with thapsigargin (24 hours) could also activate caspase-7 as shown by detection of cleaved caspase-7 (Fig. 4C, bottom, lane 5), which agreed well with a previous report (6).

To further confirm whether EGCG prevents the association of GRP78 and caspase-7, we pretreated JB6 C141 cells with different doses of EGCG followed by treatment with thapsigargin. The results showed that as little as 5 μmol/L EGCG could suppress the binding of GRP78 with caspase-7 and 10 μmol/L EGCG totally prevented the formation of the GRP78 and caspase-7 complex (Fig. 4D, left, middle bottom).

**EGCG prevents the protective function of GRP78 against etoposide-induced cell death.** One of the possible mechanisms for the antiapoptotic effects of GRP78 has been suggested that GRP78 overexpression can cause the formation of many inhibitory complexes, which would suppressing the activation of caspase-7 (2). This could lead to increased cancer progression and drug resistance (3). Therefore, to verify whether EGCG affects caspase-7-mediated cell death induced by etoposide, we used human bladder carcinoma T24/83 cells stably transfected with mock empty vector pcDNA3.1 (T24/83-pcDNA) or with GRP78 (T24/83-GRP78). Activated caspase-7 was detected in both etoposide-treated T24/83-pcDNA and T24/83-GRP78 cells (Fig. 5A). On the other hand, T24/83-GRP78 cells showed a weaker etoposide-induced caspase-7 activation compared with T24/83-pcDNA cells (Fig. 5A, lane 7 versus lane 3). EGCG treatment alone did not induce caspase-7 activation in either T24/83-pcDNA or T24/83-GRP78 cells (Fig. 5A lanes 2 and 6). On the other hand, cotreatment of cells with EGCG and etoposide induced activation of caspase-7 in either T24/83-pcDNA or T24/83-GRP78 cells but more strongly in the T24/83-GRP78 cells (Fig. 5A lane 8 versus lane 4).

To further determine whether EGCG can prevent the antiapoptotic formation of the GRP78-caspase-7 inhibitory complex to suppress apoptosis, T24/83-pcDNA and T24/83-GRP78 cells were treated with etoposide (20 μmol/L), EGCG (10 μmol/L), or EGCG plus etoposide. Apoptosis was assessed by flow cytometry analysis as described in Materials and Methods. Treatment of T24/83-pcDNA cells with etoposide resulted in a 26.3% apoptotic cells (Fig. 5B, top), whereas the percentage of apoptotic T24/83-GRP78 cells increased to only 8.9% (Fig. 5B, bottom). On the other hand, the percentage of apoptotic cells in etoposide/EGCG-treated T24/83-pcDNA cells was similar to etoposide-treated cells (28.2%; Fig. 5B, top). However, treatment of T24/83-GRP78 cells with EGCG and etoposide increased the percentage of apoptotic cells to 14.1% (Fig. 5B, bottom). These results indicated that, compared with T24/83-pcDNA cells, T24/83-GRP78 cells were more resistant to etoposide. However, treatment with EGCG in combination with etoposide seemed, at least partially, to overcome the resistance of T24/83-GRP78 cells to etoposide-induced apoptosis (2).

To verify that EGCG can facilitate etoposide-induced apoptosis in other malignant cancer cell lines, we used human breast adenocarcinoma MDA-MB-231 and T-47D cells to study the effect of EGCG on the activation of caspase-7. Results indicated that etoposide could induce caspase-7 activation in either MDA-MB-231 (Fig. 5C, left) or T-47D cells (Fig. 5C, right), but 10 μmol/L EGCG alone had no effect on the activation of caspase-7. However, treatment of cells with both etoposide and EGCG induced a dose-dependent increase in the activation of caspase-7. These results suggested that EGCG could enhance etoposide-induced caspase-7 activation, which strongly indicated that EGCG might prevent the antiapoptotic effects of GRP78. To further test this idea, we next examined the effect of EGCG on etoposide-induced apoptosis using flow cytometry analysis. EGCG treatment of etoposide-treated MDA-MB-231 cells increased the etoposide-induced apoptosis from 17.7% to 22.0% and 28.6%, respectively (Fig. 5D, top). For T-47D cells, the percentage of apoptosis increased from 8.8% to 13.5% and 15.2%, respectively (Fig. 5D, bottom). These results are consistent with the idea that EGCG prevents the formation of the inhibitory complex comprising endogenous GRP78 and caspase-7, especially following treatment with etoposide. Overall, these results strongly indicated that EGCG could prevent the protective effect of GRP78, in which it suppresses the caspase-mediated cell death pathways in drug-treated cells, contributing to the development of drug resistance.

**EGCG sensitizes human breast cancer cells to etoposide.** Etoposide has been shown to induce apoptosis in a variety of tumor cell lines (43, 44). It is an important cancer chemotherapeutic agent with clinical activity against a broad range of human malignancies (45). To evaluate the effect of EGCG in etoposide-inhibited colony formation, we carried out soft agar clonogenic assays using human breast cancer cell lines MDA-MB-231 and T-47D (Fig. 6A and B). Results indicated that treatment of human breast cancer cells with etoposide and EGCG together significantly reduced colony number as well as colony size compared with cells treated with either etoposide or EGCG alone (Fig. 6A and B), which had little effect on colony number or size. Taken together, these data indicate that EGCG can assist in preventing the drug resistance of MDA-MB-231 and T-47D breast cancer cells against etoposide.

**Discussion**

EGCG is the major polyphenol component of green tea and the most active single anticancer factor found in tea (27). Several reports have shown that tea components target specific cell signaling pathways, which are responsible for regulating cell proliferation and cell transformation (21, 46). Although several mechanisms explaining the anticancer activities of EGCG have been reported in cell culture studies, the mechanism of EGCG action is not yet fully understood.

In the present study, using affinity chromatography, two-dimensional electrophoresis, and MALDI-TOF analysis, we are the first to show that EGCG binds with the molecular chaperone GRP78, which is one of the major regulatory proteins in the ER. GRP78 normally functions to facilitate the proper folding or maintenance of proteins in a folded competent state and also prevents protein folding intermediates from aggregating (36, 47). GRP78 is also involved in the translocation of newly synthesized precursors across the ER membrane (48). We have provided clear evidence that EGCG directly binds with GRP78, which was subsequently confirmed by both in vitro and in vivo binding assays.
The association of EGCG with GRP78 was found to be markedly ATP dependent, and our observations support the idea that the GRP78-binding region for EGCG and ATP is the same. This suggested that EGCG specifically inhibits GRP78 ATPase activity, which is essential for the chaperone function of the HSP70 family (38, 48, 49). In most HSP70 proteins, ATP hydrolysis is the rate-limiting step in the ATPase cycle that consists of alterations between the ATP state of low affinity and...

Figure 5. Effect of EGCG on etoposide-induced apoptosis in cancer cells. A, effect of EGCG on etoposide-induced activation of caspase-7 in GRP78-overexpressing cells. T24/83-pcDNA and T24/83-GRP78-transfected cells were either treated or not treated with 10 μmol/L EGCG in serum-free medium for 3 hours and then treated or not treated with 20 μmol/L etoposide for an additional 5 hours. Then, 24 hours after drug treatment, total protein lysates were prepared. The protein samples (50 μg) were subjected to SDS-PAGE and followed by detection with immunoblotting using antibodies against GRP78, caspase-7, or β-actin. Caspase-7 (35 kDa) and the cleaved caspase-7 (20 kDa) proteins. B, top and bottom, fluorescence-activated cell sorting (FACS) analysis of apoptotic cells after the addition of etoposide (Etop; 20 μmol/L) in the presence or absence of EGCG (10 μmol/L). At 24 hours following drug treatment, the T24/83-pcDNA (top) or T24/83-GRP78 (bottom) cells were labeled with Annexin V-FITC and propidium iodide. The distribution pattern of live and apoptotic cells was determined by FACS analysis. Bottom left quadrants, viable cells are those displaying low Annexin or no Annexin and propidium iodide staining; bottom right quadrants, early-stage apoptotic cells are represented by high Annexin and low propidium iodide staining; top right quadrants, late-stage apoptotic cells are represented by high Annexin and high propidium iodide staining; top left quadrants, necrosis is represented by cells with high propidium iodide and low Annexin staining. The percentage of apoptosis in EGCG-treated cells compared with untreated cells is representative of at least three independent experiments. Columns, mean of three independent experiments; bars, SD. Asterisk, significant enhancement of etoposide-induced apoptosis by EGCG. *, P < 0.05; **, P < 0.005; ***, P < 0.0001. C, effect of EGCG on etoposide-induced caspase-7 activation in MDA-MB-231 (left) or T-47D (right) breast cancer cells. MDA-MB-231 and T-47D cells were either treated or not treated with 10 μmol/L EGCG in serum-free medium for 3 hours and then treated or not treated with 20 μmol/L (MDA-MB-231 cells) or 40 μmol/L (T-47D cells) etoposide for 48 hours. The analysis of proteins (50 μg) was as for (A). D, flow cytometry analysis of apoptosis of MDA-MB-231 cells (top) or T-47D cells (bottom) was done as for (B). The percentage of apoptosis is representative of at least three independent experiments. Columns, mean; bars, SD. Asterisk, significant enhancement of etoposide-induced apoptosis by EGCG. *, P < 0.05; ***, P < 0.0001.
fast exchange rates for substrates and the ADP state of high affinity and low exchange rates for substrates (38). The GRP78 protein exists in multiple forms, including ATP-mediated monomeric, dimeric, and oligomeric forms (39, 40, 50). The GRP78 monomeric species reflect maximal activity, whereas the various dimeric or oligomeric species are less active or inactive. Our in vitro and in vivo results showed that EGCG could convert GRP78 from the active monomeric to the inactive dimeric or oligomeric form. In addition to EGCG, the ECG analogue is also capable of inhibiting ATPase activity and inducing the conversion of the GRP78 protein from the monomeric to the oligomeric form. These data strongly indicated that the gallate group might be critical for binding with GRP78 and inhibiting GRP78 ATPase activity.

Based on these results, we hypothesized that EGCG might be involved in the regulation of GRP78 function in cells. Heat shock proteins have a cytoprotective role and inhibit the apoptotic response. Previous studies by other investigators have shown that GRP78 and a related protein, GRP94, can protect cells against cell death (2, 6, 42). Whereas the mechanisms explaining the antiapoptotic effect of GRP78 are still under investigation, recent studies have revealed that GRP78 can inhibit ER-induced apoptosis through the direct binding and subsequent inhibition of caspase-7 activation (2, 6). GRP78 may be an ideal drug target because of its function as a chaperone for key molecules involved in apoptosis. Our results showed that EGCG prevents the formation of the complex between GRP78 and caspase-7, therefore suppressing the antiapoptotic effect of GRP78.

The protective effect of GRP78 against apoptosis caused by ER disturbance induced by pharmacologic agents has been established (2, 3). Herein, we examined the effect of EGCG on etoposide-induced apoptosis in GRP78-overexpressing T24/83 bladder carcinoma cells and MDA-MB-231 and T-47D breast cancer cells. Results indicated that EGCG sensitized these cells to etoposide-induced caspase-7 activation and apoptosis. A tumor cell may escape from caspase-mediated apoptosis by overexpressing GRP78, and increased GRP78 expression has been observed in many human cancer cell types (7, 51). For drug resistance of cancer cells involving induction of GRP78, we examined the preventive effect of EGCG on clonogenesis of the breast cancer cells in soft agar and results indicated that EGCG sensitized breast cancer cells to etoposide. The human breast cancer cells treated with etoposide and EGCG together

![Figure 6. Effect of EGCG on phenotype expression (colony formation) of human breast cancer cells.](image-url)
had a significant reduction in their ability to express their transformed phenotype. These studies imply that GRP78 is a general contributor to tumor growth and drug resistance in human cancer and therefore is a potential drug target. Based on our results, we propose a new mechanism of EGCG in cancer prevention and chemotherapy that occurs through its suppression of the antiapoptotic function of GRP78. From a therapeutic standpoint, suppression of GRP78 functions in cancer cells by EGCG could represent a novel approach to cancer chemotherapy.

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