Inhibition of Bcl-X<sub>L</sub> Phosphorylation by Tea Polyphenols or Epigallocatechin-3-Gallate Is Associated with Prostate Cancer Cell Apoptosis

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ABSTRACT

Prostate cancer cells demonstrate slow growth kinetics and chemoresistance. Tea polyphenols have been shown to exert prostate cancer-preventative effects. Here we report that growth-arrested prostate cancer cells expressed high levels of a hyperphosphorylated Bcl-X<sub>L</sub> in mitochondria. Treatment with tea polyphenols or the major tea component epigallocatechin-3-gallate blocked expression of the hyper-, but not hypophosphorylated Bcl-X<sub>L</sub> in mitochondria, accompanied by cytochrome c release, caspase activation, and apoptosis. Studies using specific inhibitors suggest that tea inhibits p38 mitogen-activated protein kinase and the proteasome activities, leading to inhibition of Bcl-X<sub>L</sub> phosphorylation and induction of prostate cancer cell death.

Epidemiological and animal studies have demonstrated the cancer preventative properties of green tea polyphenols (GTP) (Liao et al., 1995; Fujiki, 1999; Gupta et al., 1999; Yang, 1999). Four major green tea components are (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epicatechin (EC), all of which are also present in black and other teas. Among the tea polyphenols, EGCG has been the most extensively investigated because of its relative abundance and strong cancer preventative properties (Fujiki, 1999; Yang, 1999). Tea polyphenols have been found to affect numerous cancer-related proteins, including mitogen-activated protein kinase (Chung et al., 2001), matrix metalloproteinase (Demeule et al., 2000), the androgen receptor (Ren et al., 2000), EGF receptor (Yang et al., 1999), activator protein 1 (Chung et al., 1999), and nuclear factor-κB (Lin and Lin, 1997). Most recently, we have found that tea polyphenols containing ester bonds, such as EGCG or ECG, potently inhibit the proteasomal chymotrypsin-like, but not trypsin-like, activity in vitro and in vivo at concentrations similar to those found in the serum of green tea drinkers. In contrast, tea polyphenols without ester bonds, such as EGC or EC, are not proteasome inhibitors (Nam et al., 2001). Regardless of all the above findings, the detailed molecular mechanisms responsible for tea-mediated cancer prevention are still not established.

Under in vivo conditions, many human tumor cells contain an unduplicated DNA content, indicating growth arrest in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle (Cross et al., 1989; Pardee, 1989). Solid tumor cells are also often exposed to hypoxia and low-nutrient environment in vivo (Harrington et al., 1994; Dang and Semenza, 1999). Those nonproliferating tumor cells are resistant to many types of current anticancer drugs that are primarily effective against rapid dividing cancer cells (Kessel, 1994; Tomida and Tsuruo 1999; Smith et al., 2000). Indeed, human prostate cancer (PCa) cells demonstrate very slow growth kinetics and are resistant to current cancer therapies (Tang and Porter 1997; Ripple and Wilding, 2000). This work was supported in part by research grants from the National Cancer Institute, National Institutes of Health, the United States Army Medical Research and Material Command, and H. Lee Moffitt Cancer Center and Research Institute (to Q.P.D.) as well as by the Flow Cytometry Core Facility at Moffitt Cancer Center and Research Institute.

ABBREVIATIONS: GTP, green tea polyphenols; EGCG, (−)-epigallocatechin-3-gallate; EGC, (−)-epigallocatechin; ECG, (−)-epicatechin-3-gallate; EC, (−)-epicatechin; PCa, prostate cancer; PD169316, 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)1H-1,2,4-triazole; PD98059, 2′-amino-3′-methoxyflavone; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase; COX, cyclo-oxygenase.
1999). Thus, novel drugs need to be identified to either eradicate slow-growing/nonproliferating PCa cells or sensitize them to current chemotherapy. Understanding the molecular mechanism for the chemo-resistance of PCa cells should help us to achieve this goal.

Activation of the cellular apoptotic program is a current strategy for the treatment of human cancer. It has been demonstrated that radiation and standard chemotherapeutic drugs kill some tumor cells through induction of apoptosis (Fisher, 1994). Upon apoptosis stimulation, several key events occur in mitochondria, including the release of cytochrome c (Green and Reed, 1998; Gross et al., 1999). The mitochondrial cytochrome c release can be inhibited by expression of an antiapoptotic Bcl-2 family member (i.e., Bcl-2 or Bcl-X<sub>L</sub>) and induced by expression of a proapoptotic member of Bcl-2 family, [i.e., Bax or Bid (Green and Reed, 1998; Gross et al., 1999)].

Here we report that growth-arrested human PCa cells express high levels of a hyperphosphorylated Bcl-X<sub>L</sub> in mitochondria. Treatment with GTP or EGCG completely blocked the hyperphosphorylated, but not hypophosphorylated, Bcl-X<sub>L</sub> expression, associated with cytochrome c release, caspase activation, and apoptosis induction. Further studies using specific pharmacological inhibitors demonstrate that tea may target both p38 MAP kinase- and the proteasome-mediated pathways, which are required for Bcl-X<sub>L</sub> phosphorylation and PCa cell survival. Our study suggests that down-regulation of phosphorylated Bcl-X<sub>L</sub> in mitochondria is at least one of the molecular mechanisms responsible for tea-mediated cancer-preventative function.

Materials and Methods

Materials. Highly purified tea polyphenols (EGCG (>95%), ECG (>98%), EGC (>98%), and EC (>98%)) and green tea polyphenols (Polyphenon 100) were purchased from Sigma (St. Louis, MO) and used directly without further purification. Lambda (λ) protein phosphatase was obtained from New England BioLabs (Beverly, MA). The selective inhibitors to p38 MAP kinase (PD169316) MAP kinase kinase/MEK (PD98059), and phosphatidylinositol 3-kinase (Wortmannin) as well as the fluorogenic peptide substrate N-acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin (for the caspase-9 activity) were purchased from Calbiochem (San Diego, CA). The specific proteasome inhibitor lactacystin was from Biomol (Plymouth Meeting, PA). Polyclonal antibodies to a sequence of amino acids 201 to 216 of human Bcl-X<sub>L</sub> (Ab-1; the carboxyl or C-terminal antibody) was from Oncogene Research Products (Cambridge, MA); to the amino terminus of human Bcl-X<sub>L</sub> (M-125), to Bax (N20) and to actin (C-11) were from Santa Cruz Biotechnology (Santa Cruz, CA); to human poly(ADP-Ribose) polymerase (PARP) was from Roche Applied Science (Indianapolis, IN). Monoclonal antibodies to the Bcl-X<sub>L</sub> N terminus and to cytochrome c were from BD PharMingen (San Diego, CA); to Bcl-2 from DAKO Co. (Glostrup, Denmark); to cytochrome oxidase unit II (COX) from Molecular Probes (Eugene, OR).

Cell Culture and Treatment. Human PCa cell lines LNCaP and PC-3 were grown in RPMI 1640 and Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Cell cultures were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO<sub>2</sub>. To induce G<sub>1</sub> arrest, 80 to 90% confluent cells were incubated in serum-free medium for 72 h. The growth-arrested cells were then treated with GTP, a purified tea polyphenol or a pharmacological inhibitor, as described in legends of figures.

Whole Cell Extract, Subcellular Fractionation, and Western Blot Assay. A whole-cell extract was prepared as described previously (An and Dou, 1996). Both cytosolic and mitochondrial fractions were isolated at 4°C using a previous protocol (Gao and Dou, 2000). Western blot assay with the enhanced chemiluminescence system was performed as we described previously (An and Dou, 1996; Gao and Dou, 2000). For densitometric analysis, intensities of interested protein bands detected in Western blotting were scanned, and ratios of these proteins to the loading control protein (such as actin or p48) were calculated (Nam et al., 2001).

In Vitro Phosphatase Treatment. After 72 h of serum starvation, prostate cancer cells were harvested, washed with PBS, and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol). In vitro phosphatase treatment was performed according to a protocol provided by the manufacturer (New England Biolabs). Briefly, a protein extract aliquot (40 μg) was incubated with either λ protein phosphatase (400 units) or the control buffer at 30°C for 4 h in a phosphatase reaction buffer containing 2 mM MnCl<sub>2</sub>. After incubation, protein samples were analyzed by Western blot assay.

Flow Cytometry and Cell-Free Caspase Activity Assay. Cell cycle analysis based on DNA content was performed as we described previously (Nam et al., 2001). The cell-cycle distribution is shown as the percentage of cells containing G<sub>1</sub>, S, G<sub>2</sub>, and M DNA judged by propidium iodide staining. The apoptotic population (Ap) is determined as the percentage of cells with sub-G<sub>1</sub> DNA content. To measure caspase-9 activity, a protein extract (20 μg) was incubated for 2 h at 37°C with 20 μM of a fluorogenic peptide substrate, N-acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin, in a 96-well plate. After incubation, the hydrolyzed AFC groups were measured by a Wallac Victor1240 Multilabel counter (Turku, Finland) with 405/535 nm filters.

Results

Increased Expression of a Hyperphosphorylated Form of Bcl-X<sub>L</sub> in G<sub>1</sub> Prostate Cancer Cells. When human PCa LNCaP cells were serum-starved for 72 h, their G<sub>1</sub>/G<sub>0</sub> population was increased by ~30% (Fig. 1A). We determined changes in Bcl-X<sub>L</sub> protein levels during serum starvation process. A specific polyclonal antibody to the C terminus of human Bcl-X<sub>L</sub> protein detected doublet bands with a molecular mass of 34 to 36 kDa (Fig. 1B, a), which was later found to be a hyperphosphorylated form of Bcl-X<sub>L</sub> (named as Bcl-X<sub>L</sub>-hyper; see Fig. 1C). The levels of the Bcl-X<sub>L</sub>-hyper were low in growing LNCaP cells, but increased by 6-fold after 24-h serum starvation and by 10- to 11-fold after 48 or 72 h (Fig. 1B, a), as determined by densitometric analysis. The same Bcl-X<sub>L</sub> C-terminal antibody also detected doublet band(s) of ~48 kDa with unknown nature, whose expression was relatively unchanged in LNCaP cells during serum starvation and therefore used as a loading control (Fig. 1B, a; see also Fig. 1C, a).

A specific polyclonal antibody to the N terminus of human Bcl-X<sub>L</sub> protein did not detect the Bcl-X<sub>L</sub>-hyper in LNCaP cells; instead, it detected another Bcl-X<sub>L</sub> band with a molecular mass of 31 kDa, a hypophosphorylated form of Bcl-X<sub>L</sub> (named as Bcl-X<sub>L</sub>-hypo; see Fig. 1C, b). In contrast to Bcl-X<sub>L</sub>-hyper, levels of Bcl-X<sub>L</sub>-hypo were relatively unchanged during serum starvation (Fig. 1B, b). Constitutive levels of Bcl-X<sub>L</sub>-hypo were also observed when a monoclonal Bcl-X<sub>L</sub> N-terminal antibody was used (data not shown). When a mixture of both C- and N-terminal polyclonal antibodies was applied in Western blotting, increased levels of Bcl-X<sub>L</sub>-hyper, but not Bcl-X<sub>L</sub>-hypo, were again detected in growth-arrested LNCaP cells (Fig. 1B, b). In this experiment, constitutive.
levels of actin protein were used as a control (Fig. 1B, c). When PCa PC-3 cells were serum-starved, Bcl-XL-hyper expression was again increased, whereas Bcl-XL-hypo levels were unchanged (data not shown).

The Bcl-XL-hyper Is a Phosphorylated Form of Bel-XL. Bcl-XL protein is phosphorylated in vivo, which leads to a gel mobility shift (Poruchynsky et al., 1998; Fan et al., 2000). We hypothesized that the Bcl-XL-hyper observed under our experimental conditions is a phosphorylated form of Bel-XL. To test this hypothesis, PC-3 and LNCaP cells were serum-starved and then used for protein extraction. Aliquots of the protein extracts were treated with either λ protein phosphatase or the control buffer, followed by measurement of levels of Bcl-XL-hyper and Bcl-XL-hypo in Western blot assay. The phosphatase treatment significantly decreased the expression of Bcl-XL-hyper band, as detected by the C-terminal antibody, which was associated with appearance of a new band of ~28 kDa, which should be the unphosphorylated Bcl-XL (named as Bcl-XL-unphos; Fig. 1C, a). The increased intensity of the Bcl-XL-unphos band was probably due to a strong interaction of this form of Bcl-XL with the antibody. Associated with decreased levels of Bcl-XL-hyper, the levels of Bcl-XL-hypo were slightly increased after the phosphatase treatment (Fig. 1C, b), indicating a conversion of Bcl-XL-hyper to Bcl-XL-hypo by dephosphorylation. The Bcl-XL N-terminal antibody also detected the appearance of a similar Bcl-XL-unphos band (Fig. 1C, b). When a mixture of both antibodies was used, decreased Bcl-XL-hyper expression and slightly increased Bcl-XL-hypo levels, as well as the new Bcl-XL-unphos band, were again detected (Fig. 1C, c). In this experiment, levels of the LNCaP-specific p48 protein remained unaffected and served as a control (Fig. 1C, a and c).

Down Regulation of Bcl-XL-hyper, but not Bcl-XL-hypo, by Green Tea Polyphenols in the Mitochondria of G1 Prostate Tumor Cells. It has been suggested that Bcl-XL plays a key role in survival and chemo-resistance of PCa cells (Green and Reed, 1998; Gross et al., 1999) and that green tea has chemopreventative effects on prostate cancer (Liao et al., 1995; Fujiki, 1999; Gupta et al., 1999; Yang, 1999). We hypothesized that green tea-mediated cancer preventative function is related to inhibition of Bcl-XL. If so, treatment of prostate tumor cells with GTP should be able to decrease Bcl-XL protein expression. Indeed, when serum-starved LNCaP cells were treated with GTP for 3 h, expression of Bcl-XL-hyper was decreased to an undetectable level, whereas the p48 levels were unaffected (Fig. 2A, a, lanes 3 versus 1). The decreased Bcl-XL-hyper expression was caused by effects of GTP, because when the same LNCaP cells were treated with the vehicle H2O, the Bcl-XL-hyper levels were not decreased (Fig. 2A, a, lanes 2 versus 1). In contrast to the dramatic reduction of Bcl-XL-hyper expression, levels of Bcl-XL-hypo were only slightly decreased in the LNCaP cells treated with GTP (Fig. 2A, b). Furthermore, the GTP treatment had no inhibitory effect on expression of Bax protein (Fig. 2A, c). Down-regulation of Bcl-XL-hyper expression was found to be GTP concentration-dependent: the lowest GTP concentration needed in LNCaP cells was between 10 and 25 μg/ml (Fig. 2B).

Treatment of serum-starved PC-3 cells with GTP, but not the vehicle, also inhibited expression of Bcl-XL-hyper, but not Bcl-XL-hypo (Fig. 2C, a versus b). In this experiment, expression of Bcl-2 protein was only slightly inhibited by GTP (Fig. 2C, c). To determine whether GTP was able to down-regulate Bcl-XL-hyper expression in other tumor or transformed cells, human breast cancer MCF-7 (data not shown) and simian virus-transformed human fibroblast VA-13 cells (Fig. 2D) were serum-starved and then treated with GTP. Again, this treatment completely inhibited expression of Bcl-XL-hyper, but not Bax (Fig. 2D). Taken together, it seems that GTP is able to selectively inhibit expression of Bcl-XL-hyper in prostate and other tumor or transformed cells.

We next determined where Bcl-XL-hyper was localized and where GTP exerted the inhibitory effect. After serum-starved LNCaP cells were treated with the vehicle H2O, most of the Bcl-XL-hyper doublets were found in the mitochondrial fraction, whereas a small portion of the top (but not the bottom)
band of the Bcl-X<sub>L</sub> hyper doublets was in the cytosol (Fig. 2E, a, lanes 1 and 3). Similar levels of Bcl-X<sub>L</sub> hypo were found in both mitochondrial and cytosolic fractions, and more of the LNCaP-specific p48 band was observed in the cytosolic fraction (Fig. 2E, a, lanes 1 and 3). Treatment with GTP completely inhibited expression of the Bcl-X<sub>L</sub> hyper doublets in mitochondria and also decreased the top band of Bcl-X<sub>L</sub> hyper doublets in the cytosol (Fig. 2E, a, lanes 2 versus 1 and 4 versus 3). Similar to the result using a whole-cell extract (Fig. 2A), GTP had no inhibitory effects on the levels of either Bcl-X<sub>L</sub> hyper or the p48 in both mitochondrial and cytosolic fractions (Fig. 2E, a). As controls, the mitochondria-specific COX protein was detected only in the mitochondrial fraction (Fig. 2E, b), and more actin protein was observed in the cytosol than in the mitochondrial fraction (Fig. 2E, c). GTP treatment did not affect levels of either COX or actin in the isolated cellular fractions (Fig. 2E, b and c). Therefore, it seems that GTP selectively inhibits expression of Bcl-X<sub>L</sub> hyper in the mitochondria of prostate cancer LNCaP cells.

**Down-Regulation of Bcl-X<sub>L</sub> hyper by GTP Is Associated with Prostate Cancer Cell Apoptosis.** We then tested whether decreased level of mitochondrial Bcl-X<sub>L</sub> hyper by GTP treatment in PCa cells was associated with cytochrome c release, caspase activation, and apoptosis. Treatment of serum-starved LNCaP cells with GTP for 3 h induced cytochrome c release from the mitochondria to the cytosol (Fig. 3A). In addition, after down-regulation of Bcl-X<sub>L</sub> hyper (Figs. 2A and 3B) and cytochrome c release (Fig. 3A) at 3 h, caspase-9 was activated by GTP, as measured by cell-free activity assay (Fig. 3B). The activity of caspase-9 was increased by ~2-fold at 6 h and by ~7-fold at 12 h (Fig. 3B). Furthermore, the apoptosis-specific cleavage fragment p85 of PARP was first detected after 6 h of GTP treatment and its levels increased significantly at 12 h (Fig. 3C). Associated with that, the pre-G<sub>1</sub> apoptotic population was increased by ~10% at 6 h and by ~35% at 12 h (Fig. 3D). All the apoptotic events, including cytochrome c release, caspase-9 activation, PARP cleavage, and pre-G<sub>1</sub> population increase were not observed in the vehicle-treated LNCaP cells (Fig. 3, A-D). The apoptosis-specific PARP cleavage was also observed in GTP- but not vehicle-treated PC-3 cells (Fig. 2C, e, lane 3), further demonstrating that induction of PCa cell death by GTP is tightly associated with down-regulation of Bcl-X<sub>L</sub> hyper expression (compare Figs. 3 and 2).

**EGCG among Tea Polyphenols Has the Greatest Potency to Down-Regulate Bcl-X<sub>L</sub> hyper Expression and Induce Prostate Cancer Cell Apoptosis.** To determine which component(s) of GTP is responsible for their ability to down-regulate Bcl-X<sub>L</sub> hyper expression, we first compared effects of purified EGCG and EGC. Treatment of growth-arrested LNCaP or PC-3 cells with EGCG at 50 μM for 3 h completely blocked Bcl-X<sub>L</sub> hyper expression, which mimics the effect of GTP (in Figs. 2, A and C, a, and 4, A and B). In contrast, EGC had no effects under the same experimental conditions (Fig. 2, A and C, and Fig. 4, A and B). EGCG at 5, 10, 25, and 50 μM inhibited 15, 85, 93, and 100% of Bcl-X<sub>L</sub> hyper expression, respectively (as determined by densitometric analysis), in serum-starved LNCaP cells, indicating a concentration-dependent effect (Fig. 4C). Both EGCG and EGC had little or no effect on expression of Bcl-X<sub>L</sub> hypo (Fig. 2, A and C, b). In addition, neither EGCG nor EGC affect levels of Bax or Bcl-2, compared with the vehicle (Fig. 2, A and C, c, lanes 4 and 5). Importantly, apoptosis-specific PARP cleavage was induced by only EGCG but not EGC in LNCaP, PC-3, and DU145 cells (Fig. 2C, e, and data not shown). When LNCaP and PC-3 cells were treated with 50

![Fig. 2](image-url) Down-regulation of Bcl-X<sub>L</sub> hyper expression by GTP or EGCG in G<sub>i</sub> PCa cells. LNCaP(A) or PC-3(C) cells were serum-starved for 72 h (0 h), followed by a 3-h treatment with the solvent (H<sub>2</sub>O), 50 μg/ml GTP, 50 μg/ml EGCG, or 50 μg/ml EGC. B, 72-h serum-starved LNCaP cells were treated with increasing concentrations of GTP (5, 10, 25, and 50 μg/ml) for 12 h. D, 72-h serum-starved VA-13 cells were treated with 50 μg/ml GTP for 3 or 24 h. E, 72-h serum-starved LNCaP cells were treated with either the solvent H<sub>2</sub>O or 50 μg/ml GTP for 3 h, followed by isolation of cytosolic and mitochondrial fractions. Samples prepared in each experiment were used in Western blot assay using each indicated antibody (a mixture of both Bcl-X<sub>L</sub> C- and N-terminal antibodies were used in E, a). Molecular masses of Bcl-2, Bax, PARP, and COX are 26, 21, 116, and 26 kDa, respectively. The apoptosis-specific PARP cleavage fragment p85 is also indicated in C. Similar results were obtained in at least three independent experiments.
A 55% inhibition of Bcl-XL-hyper expression was observed (Fig. 4, A and B, lanes 4 versus 1). In contrast, EC, similar to EGC, was inactive (Fig. 4, A and B, lane 5). Taken together, these results demonstrate that EGC is the major green tea polyphenol that is responsible for down-regulating Bcl-XL-hyper expression and inducing prostate cancer cell apoptosis.

It has been shown that c-Jun NH₂-terminal protein kinase pathway plays a role in Bcl-XL phosphorylation in vivo (Fan et al., 2000) and that EGC is able to directly inhibit Erk activity in vitro (Chung et al., 2001). To determine whether EGC targets a Bcl-XL kinase pathway in PCa cells, we examined effects of different kinase inhibitors on Bcl-XL-hyper expression. PD169316, a specific inhibitor of p38 MAP kinase (Kummer et al., 1997), was found to inhibit Bcl-XL-hyper expression in a concentration-dependent manner: 55% at 10 μM and nearly 100% at 25 μM, with potency similar to that of EGC (Fig. 4D, lanes 4 and 5 versus 1; compare also to Fig. 4C). In contrast, the specific MEK inhibitor PD98059 and the phosphatidylinositol 3-kinase inhibitor Wortmannin had no or very little inhibitory effects (Fig. 4D, lanes 2, 3, 6). These data indicate that p38 MAP kinase is involved in Bcl-XL phosphorylation in PCa cells.

We have also reported that EGC and ECG, but not EGC and EC, potently and specifically inhibit the chymotrypsin-like activity of the proteasome in vitro and in vivo (Nam et al., 2001). To determine whether inhibition of the proteasome activity is required for down-regulation of Bcl-XL-hyper by tea polyphenols (Fig. 4, A and B), we tested effects of the specific proteasome inhibitor lactacystin. Lactacystin potently inhibited Bcl-XL-hyper expression by 80% at 10 μM and by 100% at 25 μM (Fig. 4D, lanes 7–9), whose potency was comparable with those of EGC and PD169316 (Fig. 4D, D versus C). It seems that the proteasome-mediated pathway also regulates Bcl-XL phosphorylation in vivo in prostate cancer cells.

**Discussion**

Our current study has reported that treatment with GTP or EGC can down-regulate expression of Bcl-XL-hyper, but not Bcl-XL-hypo, protein in PCa cell mitochondria, associated with cytochrome c release and apoptosis induction. This novel mechanism may contribute to the previously demonstrated cancer-preventative properties of green tea (Liao et al., 1995; Fujiki 1999; Gupta et al., 1999; Yang, 1999).

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**Fig. 3.** Induction of PCa cell apoptosis by GTP is associated with inhibition of Bcl-XL-hyper expression. LNCaP cells, after serum starvation for 72 h (0 h), were treated with either 50 μg/ml GTP or the control vehicle (H₂O) for 3 (A), 6, or 12 h (B-D). A, cytosolic fraction was prepared and immunoblotted for levels of cytochrome c (molecular mass, 17 kDa) and actin. B and C, whole cell extracts were used to determine levels of Bcl-XL-hyper (with the C-terminal antibody) or PARP cleavage as well as cell-free caspase-9 activity. D, flow cytometry analysis. Ap, the apoptotic cell population with <G₁ DNA content. Control, 12-h H₂O treatment gave similar results. Similar results were obtained in three independent experiments.

**Fig. 4.** Inhibition of Bcl-XL-hyper expression by EGC, ECG, specific p38 kinase and proteasome inhibitors. LNCaP (A) and PC-3 cells (B) were serum-starved for 72 h, followed by treatment with either the solvent H₂O or purified EGC, EEC, ECG, or EC at 50 μM for 3 h. C, 72-h serum-starved LNCaP cells were treated for 12 h with either solvent H₂O or EGC at indicated concentrations. D, 72-h serum-starved LNCaP cells were treated for 3 h with either control solvent dimethyl sulfoxide (C) or pharmacological inhibitors, Wortmannin (Wort), PD169316 (PD16), PD98059 (PD98), or lactacystin (Lact), at indicated concentrations. Samples prepared in each experiment were used in Western blot assay using specific antibody to the Bcl-XL C terminus or actin. Similar results were obtained in two to four independent experiments.
The following arguments support that the Bcl-X<sub>L</sub> band, we named Bcl-X<sub>L</sub>-hyper, is a phosphorylated form. First, several groups have shown that Bcl-X<sub>L</sub> protein is phosphorylated in vivo which leads to a mobility shift (Poruchynsky et al., 1998; Fan et al., 2000). Second, EGCG has been found to directly inhibit activities of several kinases, including IκB kinase (Yang et al., 2001), p70 S6 kinase (Nomura et al., 2001), and Erk (Chung et al., 2001) under cell-free conditions. Third, the Bcl-X<sub>L</sub>-hyper band seems to be selectively recognized by a specific polyclonal antibody to the C terminus of human Bcl-X<sub>L</sub> protein (Fig. 1C). Only the Bcl-X<sub>L</sub>-hyper band was detected in PC-3 cell extracts by the antibody (Fig. 1C, a, lane 1), although another p48 band was also detected in LNCaP cell extracts (Fig. 1C, a, lane 3). Fourth, the mobility of the Bcl-X<sub>L</sub>-hyper band is slower than those of the Bcl-X<sub>L</sub>-hypo and Bcl-X<sub>L</sub>-unphos (Fig. 1C). Finally, in vitro phosphatase treatment significantly decreased the level of Bcl-X<sub>L</sub>-hyper, associated with appearance of a new band with faster mobility that should be unphosphorylated form of Bcl-X<sub>L</sub> (Fig. 1C, a). The phosphatase treatment did not affect levels of p48 expression (Fig. 1C), demonstrating specificity on phosphorylated proteins. We plan to look further into the involved molecular mechanism by developing an in vitro Bcl-X<sub>L</sub> phosphorylation assay.

Our results also indicated that phosphorylation of Bcl-X<sub>L</sub> is associated with G<sub>i</sub> arrest (Fig. 1). We have found increased levels of Bcl-X<sub>L</sub>-hyper during serum starvation. This starvation arrested 82% of LNCaP cells in G<sub>i</sub> phase of the cell cycle (Fig. 1A). We hypothesized that the increased Bcl-X<sub>L</sub>-hyper in G<sub>i</sub> phase contributes to resistance of PCa cells to apoptosis induction. Indeed, some studies have shown that under serum-deprived condition Bcl-X<sub>L</sub> expression is increased, protecting cells from apoptosis (Zhang et al., 2000; Takehara et al., 2001). In addition, in vivo many human tumor cells (including prostate cancer) contain high percentages of G0/G1 DNA content (Cross et al., 1989; Pardee 1989) and are hypoxic and low-nutrient (Harrington et al., 1994; Dang and Semenza, 1999). Many tumor cells also overexpress the antiapoptotic proteins Bcl-X<sub>L</sub>, Bcl-2, and are resistant to chemotherapy and radiotherapy (Green and Reed, 1998; Gross et al., 1999). It should be noted that although serum deprivation is commonly used to synchronize cell lines in the G<sub>i</sub>/G<sub>1</sub> phase of the cell cycle, there are other differences between serum-starved conditions and in vivo tumor microenvironments.

Another important finding in the present study is the tight association between inhibition of Bcl-X<sub>L</sub> phosphorylation by GTP and EGCG and induction of PCa cell apoptosis. Previous animal and human epidemiological studies have suggested that the polyphenols present in green tea have protective effects against a variety of cancers including prostate cancer (Liao et al., 1995; Fujiki, 1999; Gupta et al., 1999; Yang, 1999). Different molecular mechanisms have been suggested for tea polyphenols’ anticancer activity but none of them have been shown to be directly responsible for the cancer-preventive properties of tea (see Introduction). We hypothesized that GTP and EGCG might inhibit Bcl-X<sub>L</sub> phosphorylation and consequently induce PCa cell apoptosis, which contributes to green tea-mediated cancer preventative function. Indeed, we observed that GTP and EGCG selectively downregulated the expression of Bcl-X<sub>L</sub>-hyper, but not Bcl-X<sub>L</sub>-hypo in preparations of PCa cell extracts and mitochondria (Fig. 2, A, C, and E). This reduction in Bcl-X<sub>L</sub>-hyper expression by GTP or EGCG was time- and concentration-dependent (Figs. 2–4) and found in prostate cancer (Figs. 2–4), breast cancer (data not shown) and simian virus 40-transformed cells (Fig. 2D). Furthermore, GTP or EGCG had little effect on expression of Bcl-2 and Bax proteins (Fig. 2), indicating selectivity to Bcl-X<sub>L</sub> in the phosphorylated form. Importantly, reduction of the mitochondrial Bcl-X<sub>L</sub>-hyper by GTP (Fig. 2E) was associated with induction of cytosolic cytochrome<sub>c</sub> release, caspase-9 activation, PARP cleavage, and apoptosis (Fig. 3). Our results are consistent with other studies that showed that the mitochondrial Bcl-X<sub>L</sub> prevents apoptosis via inhibition of cytochrome<sub>c</sub> release (Green and Reed, 1998; Gross et al., 1999).

The direct target of green tea and EGCG that regulates Bcl-X<sub>L</sub> phosphorylation is currently unknown. It is possible that tea and EGCG can directly inhibit the Bcl-X<sub>L</sub> kinase activity in vivo, resulting in decreased level of the Bcl-X<sub>L</sub>-hyper. Consistent with this argument, it has been shown that the c-Jun NH<sub>2</sub>-terminal protein kinase pathway plays a role in Bcl-X<sub>L</sub>-hypo activity in vivo (Fan et al., 2000) and that EGCG is able to directly inhibit Erk activity in vitro (Chung et al., 2001). Our results also showed that the specific p38 MAP kinase inhibitor PD169316 could inhibit expression of the Bcl-X<sub>L</sub>-hyper in a concentration-dependent manner (Fig. 4D). In contrast, the MEK and phosphatidylinositol 3-kinase inhibitors had no or very little inhibitory effects (Fig. 4D). These data at least suggest that p38 kinase is involved in the Bcl-X<sub>L</sub> phosphorylation pathway.

We also found that EGCG and ECG, both of which contain an ester bond, inhibited expression of Bcl-X<sub>L</sub>-hyper, but EGC and EC without ester bond had no effect (Fig. 4, A and B). We have reported that EGCG and ECG, but not EGC and EC, potently and specifically inhibit the chymotrypsin-like activity of the proteasome in vitro and in vivo (Nam et al., 2001). These results suggest that the proteasome may regulate the Bcl-X<sub>L</sub> phosphorylation pathway in PCa cells. Indeed, lactacystin, a specific proteasome inhibitor, also inhibited expression of Bcl-X<sub>L</sub>-hyper in a concentration-dependent manner (Fig. 4D). Identification of the Bcl-X<sub>L</sub> kinase, establishment of an in vitro Bcl-X<sub>L</sub> phosphorylation assay, and use of synthetic EGCG analogs (Smith et al., 2002) will help to uncover the target of tea and EGCG.

Although the present studies focused on the level of Bcl-X<sub>L</sub> phosphorylation, it should be noted that Bcl-X<sub>L</sub> transcription can be up-regulated by signal transducer and activator of transcription 3 (Stat 3) or Stat 5, which are regulated by various kinase pathways (Catlett-Falcone et al., 1999; Horita et al., 2000; Sevilla et al., 2001). EGCG might also be able to inhibit Stat 3-mediated Bcl-X<sub>L</sub> transcription (Masuda et al., 2001). However, the novel aspect of our investigation is the demonstration of the inhibition of Bcl-X<sub>L</sub> phosphorylation by tea polyphenols and EGCG in prostate cancer cells. These studies have implied that inhibition of Bcl-X<sub>L</sub> phosphorylation in mitochondria may contribute to the prostate cancer preventative properties of tea polyphenols.

Our future studies will focus on characterization of the Bcl-X<sub>L</sub>-hyper phosphorylation sites, how Bcl-X<sub>L</sub>-hyper inhibits the mitochondrial cytochrome<sub>c</sub> release, how p38 MAP kinase and the proteasome regulate Bcl-X<sub>L</sub> phosphorylation, and the detailed molecular mechanisms for EGCG-mediated inhibition of p38 kinase and the proteasome. Synthetic ana-
logs of natural polyphenols (Smith et al., 2002) should help to identify the tea target(s) regulating Bcl-Xₐ phosphorylation and create more potent and specific compounds for the prevention and treatment of human prostate and other cancers. Many of the current chemotherapeutic drugs were originally developed from natural products. We believe that the research presented here is the initial step for further developing such novel cancer-preventative agents.

References


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