Induction of apoptosis in human bladder cancer cells by green tea catechins

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ABSTRACT

Cell culture and animal studies have demonstrated strong chemopreventative effects of green tea and its associated polyphenols in multiple cancers, though the exact mechanisms of action are not well understood. This *in vitro* study examined the antiproliferative/pro-apoptotic potential of green tea extract (GTE), polyphenon-60 (PP-60), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin-3-gallate (EGCG) in both normal and malignant human bladder cells. Cell growth (proliferation/apoptosis) was measured in UROtsa (normal), SW780 (tumorigenic; low-grade), and TCCSUP (tumorigenic; high-grade) human bladder urothelial cells by cell proliferation (XTT) assay after treatment with 0–80 μg/mL of GTE, PP-60, ECG and EGCG for 72 h. Molecular signaling pathways of catechin-induced apoptosis were analyzed using Human signal transduction RT^2 Profiler PCR array (SuperArray). Compared to control-treated cells, treatment with catechin agents significantly suppressed cell growth in a dose-dependent fashion ($P < 0.01$), with strongest effects evoked by ECG and EGCG in UROtsa cells, ECG in low-grade RT4 and SW780 cells, and PP-60 and EGCG in high-grade TCCSUP and T24 cells. Microarray analysis indicated distinct differences in mRNA gene expression regarding growth signaling pathway activation induced by EGCG in normal/tumorigenic human bladder cell lines, providing a rationale for the putative therapeutic usage of green tea polyphenols against bladder disease.

Bladder cancer (BlCa) remains one of the leading causes of death among men and women in the United States. According to statistics of the American Cancer Society (1), BlCa is the fourth and eighth most common cancer in men and women, respectively. In the United States alone last year, nearly 68,810 new cases of BlCa were to be diagnosed, with 14,100 resulting deaths. The incidence of BlCa increases with age, occurring most commonly in people between 50 and 70 years of age (1). Overall,

men are nearly 3–4 times more likely to develop BlCa than women. Other risk factors for BlCa include smoking, industrial chemical exposure, race, family history and chronic bladder inflammation.

Green tea, extracted from *Camellia sinensis*, is a widely consumed beverage throughout the world second only to water (7). Green tea contains multiple catechin components, though (−)-epigallocatechin-3-gallate (EGCG; Fig. 1) is the primary catechin accounting for 50–80% of catechins in a brewed cup (reviewed in 9). ECG [(−)-epicatechin-3-gallate] (Fig. 1) is the second most concentrated catechin component of green tea, and is associated with its anti-inflammatory/anti-oxidant activities (16). Other major catechins found in green tea (Fig. 1) include (−)-epicatechin (EC) and (−)-epigallocatechin (EGC). Because of its low cost, low cytotoxicity and widespread availability, green tea has enormous potential as a chemopreventative agent for a variety of human diseases. Indeed, the inhibitory effects of

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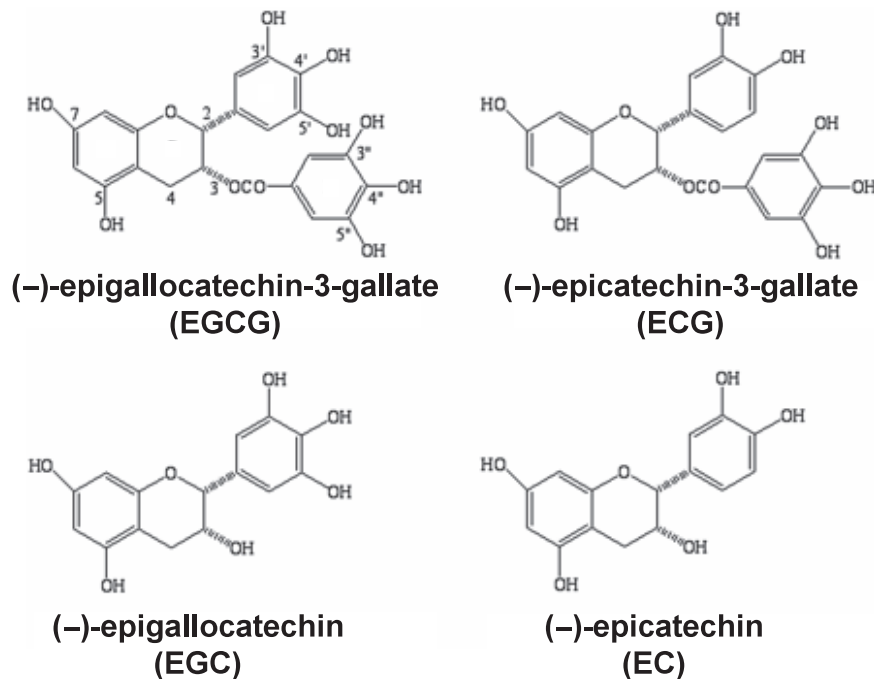
Fig. 1 Chemical structures of (–)-epigallocatechin-3-gallate (EGCG), (–)-epicatechin-3-gallate (ECG), (–)-epigallocatechin (EGC) and (–)-epicatechin (EC) [Revised from 22]. EGCG and ECG have strong antioxidant activities and are believed to afford protection against a variety of cancer types.

green tea and its associated components on the growth of various cancers, including BlCa (14, 17, 18), have been reported (4, 5, 8, 11, 15, 19, 20, 23–26). However, their therapeutic use in the prevention/treatment of BlCa has not been fully evaluated in both experimental and clinical studies.

Due to its promising anticancer properties, the purpose of the current study was to investigate the potential anti-proliferative effects (and associated general mechanisms of action) of green tea extract (GTE), as well as polyphenon-60 (PP-60; 60% pure catechins) and two major GTE catechin components (ECG and EGCG) on the proliferation of both normal and malignant (low-grade/high-grade) human bladder uroepithelial cells *in vitro*. Our data demonstrate that some of these catechin agents can differentially down-regulate the growth of human bladder urothelial cells *in vitro* via modulation of multiple signal transduction pathways, suggesting clinical relevance regarding dietary prevention and treatment for bladder disease.

MATERIALS AND METHODS

Chemicals. Green tea extract (GTE), obtained from General Nutrition Corporation (USA), was a mixture of multiple catechin compounds (14% polyphe-

nols). Polyphenon-60 (PP-60), ECG and EGCG were purchased from Sigma Chemical Co. (USA). Stock solutions of GTE (1 mg/mL), PP-60 (1 mg/mL), ECG (0.5 mg/mL) and EGCG (1 mg/mL) were prepared in Earle's buffered salt solution (Gibco, USA) under sterile conditions, and stored at 4°C protected from light.

Cell culture. The human bladder transitional cell carcinoma (TCC) cell line TCCSUP was cultured in Minimum Essential Media with phenol red, supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin (PS). Human SW780 TCC-derived cells (ATCC, USA) were cultured in Leibovitz's L-15 Media without phenol red, supplemented with 10% (v/v) FBS. Human BlCa T24 and RT4 cells (ATCC) were cultured in McCoy's (Modified) 5A Media with phenol red, supplemented with 10% (v/v) FBS and 1% (v/v) PS. Normal human urothelium UROtsa cells were kindly provided by Dr. Scott Garrett (University of North Dakota, USA) and cultured in Dulbecco's Modified Eagle Media without phenol red, supplemented with 5% (v/v) FBS, 1% (v/v) PS and 1.5 mM L-glutamine. Cell lines were maintained at 37°C in a humidified 5% CO_2 incubator.

Cell proliferation (XTT) assay. Cell proliferation was measured using the Sigma *In Vitro* Toxicology Assay Kit (XTT) according to manufacturer's instructions. The XTT-based system is a reliable index to measure mitochondrial activity and thus cell viability/function. Briefly, cells were seeded in triplicate in 24-well plates at a density of 50,000–75,000 cells/well, and grown for 24 h at 37°C. After reaching 25–35% confluence, cells were treated in the presence or absence of increasing concentrations of GTE, PP-60, ECG and EGCG (0, 10, 20, 40, 60 and 80 µg/mL) for 72 h. Following addition of 200 µL XTT reagent diluted in serum-free media (68 h after initiation of incubation), plates were covered and incubated at 37°C for 4 h. Supernatants were removed and added to 96-well plates in triplicate, and the absorbance measured at 450 nm. Reduction in cell proliferation was expressed as a percentage of total activity/cell number compared to non-treated control cells. Control cells were considered to be 100% viable.

Apoptosis (Annexin V) assay. Apoptosis was probed using the Annexin V detection kit (Molecular Probes, Inc., USA) per manufacturer's instructions. Briefly, UROtsa (50,000 cells/chamber), TCCSUP (50,000 cells/chamber) and RT4 (75,000 cells/chamber) cells were seeded in duplicate in 8-chambered Nunc Permanox plastic slides, grown for 24 h at 37°C, then treated in the presence or absence of 40 µg/mL PP-60, ECG or EGCG for either 24 h, 48 h or 72 h at 37°C. As a positive control, cells were exposed to either 200 mM (48 h, 72 h) or 500 mM (24 h) ethanol. After treatment, cells were washed ($2 \times$) with cold phosphate-buffered saline (PBS) and incubated with 20 µL Annexin V-Alexa 488 conjugate diluted in buffer (pH 7.4) for 30 min at 37°C. Finally, cells were washed with buffer, coverslipped and imaged using a bright field/fluorescence microscope and digital camera. Fluorescence, expressed as relative fluorescence units (RFU) per cell, was measured by the area under the curve method following correction for background.

Real-time quantitative PCR (QPCR)/Microarray analysis. UROtsa (1×10^6 cells/plate), RT4 (2×10^6 cells/plate) and TCCSUP (1×10^6 cells/plate) cell lines were seeded in 100 mm plates, grown for 24 h at 37°C, then treated in the presence or absence of 40 µg/mL ECG (RT4) or EGCG (UROtsa, TCCSUP) for 24 h at 37°C. After treatment, medium was removed and cells were washed with PBS before being trypsinized. Sample RNA from UROtsa, RT4 and TCCSUP bladder cells was purified using RNeasy kits and quantified by spectrophotometry.

Real-time PCR (QPCR) reactions for critical signaling pathway genes were analyzed in total RNA using the Human signal transduction RT^2 Profiler PCR array (SuperArray Biosciences Corporation, USA) according to the manufacturer's protocol. Briefly, cDNA was prepared from 1 µg total RNA using a RT^2 PCR array first strand kit (SuperArray). QPCR reactions were conducted in a 25 µL mixture, which included 12.5 µL of $2 \times RT^2$ QPCR master mix, 11.5 µL of nuclease-free H_2O, and 1 µL of template cDNA. QPCR amplification was conducted with an initial 10-min step at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Real-time quantitations were conducted using the Mx3000P QPCR detection system (Stratagene, USA).

The Human signal transduction RT^2 Profiler PCR array profiles 84 key genes representative of 18 distinct signal transduction pathways including: Mitogenic, Wnt, Hedgehog, TGF-β, Survival, p53, Stress, NFκB, NFAT, CREB, Jak-Stat, Estrogen, Androgen, Calcium and Protein Kinase C, Phospholipase C, Insulin, LDL and Retinoic Acid. Data were analyzed via SuperArray PCR Array Data Analysis software using the comparative threshold method with normalization of the raw data to several housekeeping genes including β-2-microglobulin, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a, GAPDH and β-actin. For each bladder cell line, results were expressed as the ratio of gene expression in ECG- or EGCG-treated cells relative to the gene expression in untreated cells.

Data analysis. Statistical significance was evaluated by one-way analysis of variance (ANOVA) and Bonferroni *t* post-test using Prism (statistical software) from GraphPad. A level of $P < 0.01$ was considered significant. Values are reported as mean ± standard error of measure (SEM).

RESULTS

Green tea extract/catechins modulate growth of normal/tumorigenic human bladder cells
To ascertain the effects of GTE, PP-60, ECG and EGCG (0–80 µg/mL) on the viability of normal/malignant human bladder cells after 72 h, we performed XTT assays to examine agent dose dependency. This concentration range of green tea/catechins (0–80 µg/mL) was selected based upon those concentrations previously reported in the literature (4, 8, 14, 17,

**Table 1** *Summary of human bladder cell viability studies: Effects of catechin agents*

Bladder Cell Line	Relative EC_{50} in μg/mL			
	GTE	PP-60	ECG	EGCG
Normal:				
UROtsa	58	38	18	20
Tumorigenic, Low-Grade:				
RT4	> 80	> 80	47	> 80
SW780	> 80	> 80	35	> 80
Tumorigenic, High-Grade:				
T24	> 80	41	59	38
TCCSUP	> 80	32	54	33

Relative effective concentrations (EC_{50}) are indicated for each agent.

18, 20), and catechin concentrations potentially attainable *in vivo* (12). A comparative summary of viability effects (EC_{50}) induced by GTE, PP-60, ECG and EGCG is shown in Table 1. We observed that PP-60 ($EC_{50} = 38$ μg/mL), ECG ($EC_{50} = 18$ μg/mL) and EGCG ($EC_{50} = 20$ μg/mL) significantly inhibited the growth of normal UROtsa bladder urothelial cells in a dose-dependent manner ($P < 0.001$) when compared to control-treated cells (Fig. 2). GTE also significantly down-regulated UROtsa cell growth dose-dependently ($P < 0.001$), though the overall effect was less pronounced ($EC_{50} = 58$ μg/mL). In low-grade RT4 (Fig. 3A) and SW780 (Fig. 3B) BlCa cells, ECG ($EC_{50} = 47$ μg/mL and 35 μg/mL, respectively) significantly inhibited cell growth in a dose-dependent fashion ($P < 0.001$) relative to the growth of non-treated control cells. In contrast, GTE and PP-60 demonstrated limited, though significant ($P < 0.001$), anti-proliferative effects on cell growth, while EGCG exhibited significant ($P < 0.001$) effects, predominantly at higher doses (40–80 μg/mL). Growth of high-grade TCCSUP (Fig. 4A) and T24 (Fig. 4B) BlCa cells was significantly inhibited in a dose-dependent manner ($P < 0.001$) by treatment with PP-60 ($EC_{50} = 32$ μg/mL and 41 μg/mL, respectively), ECG ($EC_{50} = 54$ μg/mL and 59 μg/mL, respectively) and EGCG ($EC_{50} = 33$ μg/mL and 38 μg/mL, respectively). In comparison to controls, GTE elicited modest, significant ($P < 0.001$) anti-proliferative effects in TCCSUP and T24 BlCa cells (Fig. 4).

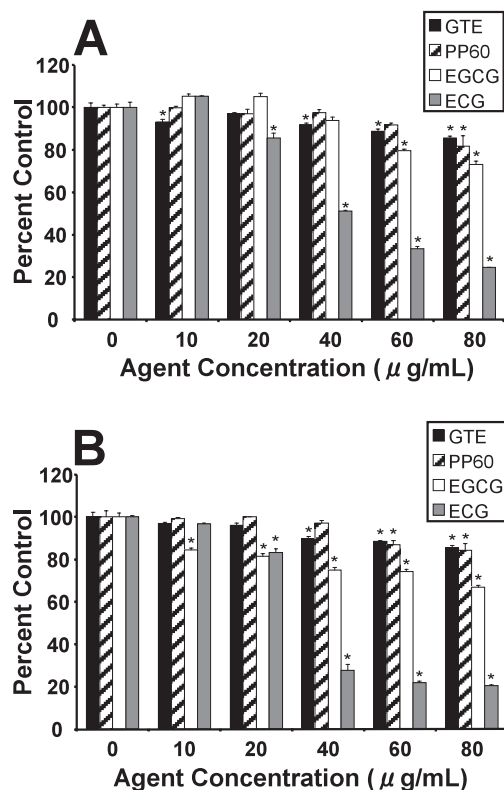*Green tea extract/catechins suppress human bladder cell growth via apoptosis*
We next determined whether catechin-mediated loss of normal (UROtsa) and malignant (RT4, TCCSUP) human bladder cell viability is mediated through apoptosis. Because a majority of UROtsa, RT4 and TCCSUP cells were not viable and were detached
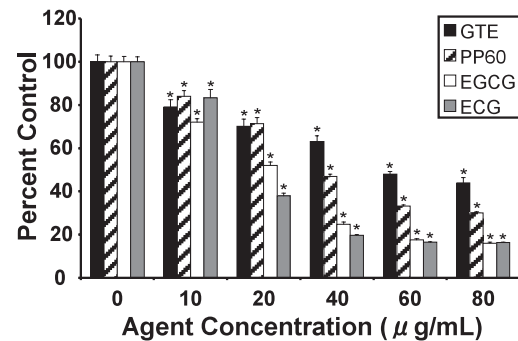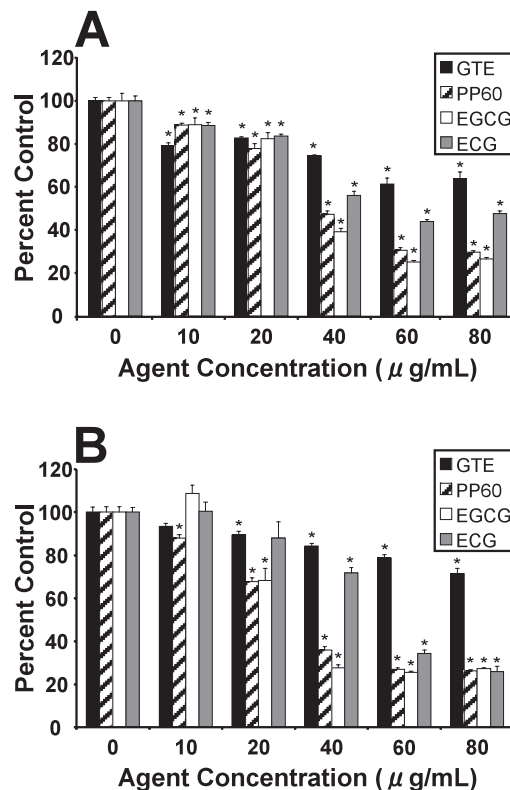

Fig. 2 Effects of green tea extract (GTE) and catechins (PP-60, ECG, EGCG) on normal human bladder UROtsa cell proliferation. Data (means ± SEM) are representative of three independent experiments. *Significant difference at $P < 0.01$ versus control.

from the culture plates following 72 h catechin treatment (XTT proliferation data not shown), 72 h exposure was not assessed for apoptotic studies. Consequently, doses of 40 μg/mL PP-60, ECG and EGCG with exposure times of 24 h and 48 h were selected based upon XTT proliferation results (Figs. 2–4). Also, GTE was not utilized in these studies due to modest anti-proliferative effects (Figs. 2–4). Apoptosis was confirmed by fluorescent staining with Annexin V (10) and analysis of cellular characteristics of apoptosis (*i.e.*, cell shrinkage, membrane blebbing).

Figure 5 shows representative fluorescent images (24 h and 48 h post-treatment) of Annexin V staining (cell death marker) in treated and control-treated UROtsa (Fig. 5A, normal) and TCCSUP (Fig. 5B, tumorigenic; high-grade) human bladder cells. In all cell lines, quantitative fluorescence measurement (RFU/cell) revealed increased Annexin V staining in PP-60-, ECG- and EGCG-treated bladder cells compared to non-treated control cells. Specifically, relative to untreated cells, catechin-treated cells av-

Fig. 3 Effects of green tea extract (GTE) and catechins (PP-60, ECG, EGCG) on low-grade human bladder cancer RT4 (A) and SW780 (B) cell proliferation. Data (means ± SEM) are representative of three independent experiments. *Significant difference at $P < 0.01$ versus control.

Fig. 4 Effects of green tea extract (GTE) and catechins (PP-60, ECG, EGCG) on high-grade human bladder cancer TCCSUP (A) and T24 (B) cell proliferation. Data (means ± SEM) are representative of three independent experiments. *Significant difference at $P < 0.01$ versus control.

eraged approximately 2-fold greater RFU/cell in UROtsa cells (1500 RFU/cell vs 700 RFU/cell) and in RT4 cells (tumorigenic; low-grade) (2400 RFU/cell vs 1200 RFU/cell, data not shown), and 1.2-fold greater RFU/cell in TCCSUP cells (1500 RFU/cell vs 1300 RFU/cell). In most cases, particularly with RT4 (data not shown) and TCCSUP (Fig. 5B) BlCa cells, phase-contrast imaging also revealed decreased cell density (approximately 3-fold) at 24 h and 48 h post-treatment compared to non-treated control cells, confirming both the XTT proliferation data (Figs. 2–4) and the pro-apoptotic effects of these catechin agents.

Microarray analysis: Green tea extract/catechins activate differential signaling pathways in human bladder cells
To identify the molecular signaling pathways of EGCG-induced apoptosis, we analyzed changes in gene expression profiles of normal UROtsa and high-grade TCCSUP BlCa cells following exposure to 40 μg/mL EGCG for 24 h using the Human sig-

nal transduction RT^2 Profiler PCR array (SuperArray, USA). A dose of 40 μg/mL EGCG with an exposure time of 24 h was selected based upon XTT proliferation (Figs. 2 and 4) and apoptosis (Fig. 5) results. Furthermore, because EGCG is: 1) the most prevalent polyphenol in green tea, 2) thought to be responsible for most of the anti-inflammatory/cancer-preventative properties of green tea, and 3) the most studied and characterized of the green tea catechins, this agent was utilized for gene expression analysis.

Major gene expression profile changes for UROtsa and TCCSUP cell lines following treatment with EGCG are shown in Table 2. Exposure of both cell lines to EGCG led to a down-regulation of key regulatory genes involved in cell growth/survival. For example, gene expression of *Tmepai* (androgen growth signaling) was down-regulated nearly 2-fold in UROtsa cells and approximately 4-fold in TCCSUP cells. Also, *Wnt2* gene expression, involved in hedgehog signaling and implicated in the development of some cancers, was down-regulated almost 2-fold in UROtsa cells and approximately 3-fold in

24 h   **48 h**
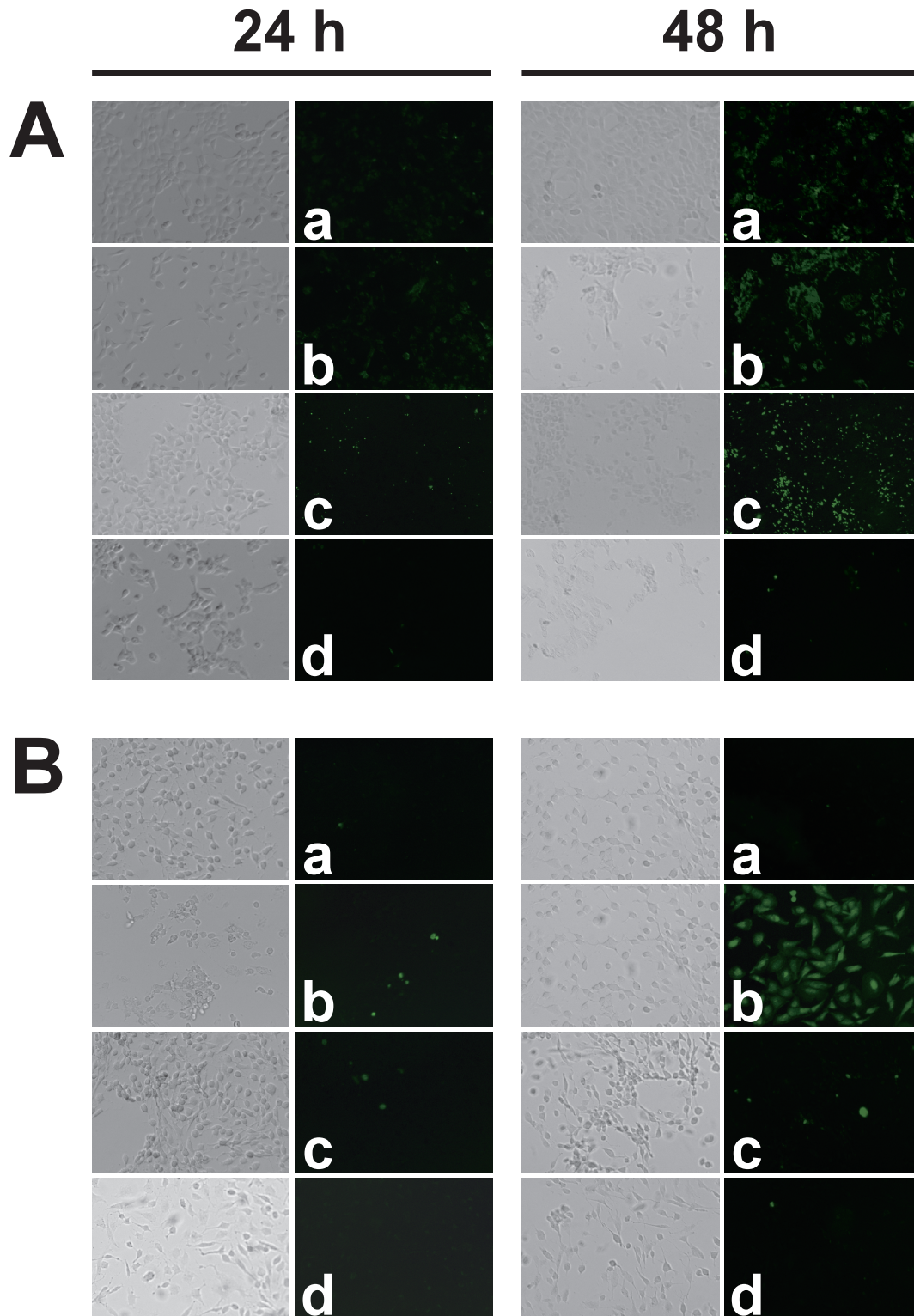
Fig. 5 Green tea extract (GTE)/catechin induction of apoptosis in UROtsa (A) and TCCSUP (B) human bladder cells. Cells were treated with 40 µg/mL agent for either 24 h or 48 h and analyzed for apoptosis using Annexin V. Annexin V-labeled apoptotic cells (green fluorescence) within the same microscopic field were viewed and photographed by bright field or by fluorescence (FITC filter). For quantification, fluorescence was expressed as relative fluorescence units (RFU) per cell. (a) Negative control; (b) Positive (ethanol) control; (c) EGCG treatment; (d) PP60 treatment. Representative images (10X magnification) are from three independent experiments.

Table 2 *Effects of EGCG on gene expression in normal UROtsa and tumorigenic TCCSUP bladder cells*

Gene Name	Fold Change		Pathway/Gene Function
	UROtsa	TCCSUP	
Similar Expression:			
Tmepai	1.8-Fold Down	4.3-Fold Down	Androgen/Cell Growth
Wnt2	2-Fold Down	3.2-Fold Down	Hedgehog/Cell Growth
Differential Expression:			
Ccl20	2.1-Fold Down	> 10-Fold Up	NFkB/Inflammation
Il-8	3.7-Fold Down	> 10-Fold Up	NFkB/Inflammation
Pecam1	4.8-Fold Up	Unchanged	NFkB/Cell Adhesion
Wisp1	4.8-Fold Up	3-Fold Down	Wnt/Cell Survival

Gene expression profiles were analyzed in untreated and treated (40 μg/mL EGCG for 24 h) UROtsa and TCC-SUP bladder cells using the Human signal transduction RT^2 Profiler PCR array (SuperArray, USA). Fold changes in the expression of select genes (and associated functions/signaling pathways) in EGCG-treated cells relative to the expression in untreated cells are indicated.

TCCSUP cells. However, significant differences in signaling pathway activation were noted between normal UROtsa cells and high-grade TCCSUP BlCa cells. For instance, gene expression of *Ccl20* and *Il-8*, both involved in nuclear factor kappa B (NFκB)-mediated signaling, were down-regulated (approximately 3-fold) in UROtsa cells, but markedly up-regulated (> 10-fold) in TCCSUP BlCa cells (Table 2). In addition, *Pecam1*, whose expression is involved in cell adhesion, was up-regulated nearly 5-fold in UROtsa cells, but remained unchanged in TCCSUP BlCa cells. Furthermore, gene expression of *Wisp1*, a key regulator of WNT cell survival signaling, was up-regulated (roughly 5-fold) in UROtsa cells, though down-regulated (approximately 3-fold) in TCCSUP BlCa cells. These findings indicate that EGCG differentially affects signaling pathway activation, and thus overall cell survival, of normal UROtsa cells and high-grade TCCSUP BlCa cells.

DISCUSSION

In this study, we evaluated the anti-proliferative effects of GTE, PP-60, ECG and EGCG in both normal and malignant human bladder cell lines. Our *in vitro* results demonstrate that PP-60, ECG and EGCG induce growth arrest/apoptosis in both low- and high-grade BlCa cell lines. These findings are consistent with recent observations in T24 BlCa cells (14) and in cell lines from cancers of the pancreas (11), prostate (4, 11, 15), breast (20, 26) and colon (8), among others (reviewed in 9). However, to our knowledge, this is the first report demonstrating differential pro-apoptotic effects of catechin compounds in multiple human bladder cell lines (both normal and tumorigenic) that are mediated by

the differential activation of signaling pathways.

To date, very little is known about the growth inhibitory activities of green tea/catechins on human bladder carcinoma cells. Additionally, even less is known about the mechanism(s) of action regarding growth inhibition/apoptosis evoked by these polyphenolic agents. Qin *et al*. (2007) recently observed that EGCG induces apoptosis in human BlCa T24 cells via the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, involving Bcl-2 family proteins and activation of caspase-3 and Poly (ADP-ribose) polymerase. Although other studies have demonstrated that EGCG, as well as ECG, inhibits the proliferation and viability of multiple human cancers (4, 8, 9, 15, 19, 20, 26), Qin and others (2007) were the first to link a mechanism of action for EGCG-induced apoptosis in human BlCa cells. Regarding cell migration/invasion, Rieger-Christ and others (2007) demonstrated that EGCG treatment of bladder carcinoma cells causes inactivation of Akt signaling, concomitant with reduced *in vitro* migration.

Our *in vitro* results demonstrate that EGCG can inhibit the growth of both normal and tumorigenic human bladder cells through the induction of apoptosis. However, we found that programmed cell death results in part from the differential activation of distinct signaling pathways between normal UROtsa and high-grade BlCa TCCSUP cells evoked by this green tea catechin component (Table 2). For example, it is well known that WNT pathway activation is a key for diverse cellular processes such as cell proliferation and differentiation. Indeed, the fact that 40 μg/mL EGCG treatment caused nearly 3-fold down-regulation of *Wisp1* gene expression in TCC-SUP BlCa cells is consistent with both the role of WNT in cell growth/survival and aberrant *Wisp1*

gene expression in carcinogenesis (2, 21). Conversely, *Wisp1* gene expression was up-regulated approximately 5-fold in normal UROtsa following EGCG treatment.

In addition, the transcriptional activity of the nuclear factor NFκB is known to be an essential player in inflammation, cell proliferation and apoptosis via gene regulation. We found that *Ccl20* and *Il-8* gene expression, both associated with NFκB-mediated inflammation, were highly up-regulated (> 10-fold) in TCCSUP BlCa cells, but down-regulated (nearly 3-fold) in normal UROtsa cells. Gene expression of another NFκB signaling member, *Pecam1*, whose function is involved in cell adhesion, remained unchanged following 40 μg/mL EGCG treatment in TCCSUP BlCa cells, but was up-regulated nearly 5-fold in normal UROtsa cells.

Based upon previous studies (3, 13), EGCG appears to have strong anti-proliferative actions against various human cancer cell types, unlike their respective normal counterparts. However, in our studies, growth of normal UROtsa bladder cells were adversely affected by EGCG treatment (EC_{50} = 33 μg/mL). It should be noted that this concentration is nearly 30 × greater than the estimated bioavailability of EGCG in human plasma (12). However, it is clear from our *in vitro* studies that normal and tumorigenic human bladder cells are differentially sensitive to green tea and its catechin components (see Table 1), with associated differential gene expression (see Table 2). In addition, our findings indicate numerous gene expression differences between low-grade RT4 and high-grade TCCSUP BlCa cells following EGCG treatment (data not shown). Whether these differential effects are mediated by general antioxidant properties of these compounds and/or via EGCG-mediated specific gene regulation remains undetermined. As suggested by Chen *et al*. (1998), target sites of EGCG in cancer cells may be different from those sites in normal cells. Thus, it is plausible that intracellular target sites in normal/tumorigenic bladder cells, in comparison to other human tissues, are cell- and tissue-specific.

Although we have identified potentially a few signaling proteins/pathways, the differential apoptotic effects of EGCG on UROtsa and TCCSUP bladder cells likely reflect, in part, the pleiotropic effects of WNT and NFκB cascade signaling. Future studies (*i.e.,* Northern blot analysis and real-time quantitative PCR) are planned to validate the gene expression profile changes observed with the RT^2 Profiler PCR array. In addition, we will examine dose-/time-dependent effects of both ECG and EGCG in low-/high-grade BlCa cell lines (including primary culture cells) for direct comparison of gene expression profiles in attempt to further elucidate underlying apoptotic signaling mechanisms.

In summary, we have demonstrated that EGCG stimulates the activation of distinct cell fate signaling pathways in normal UROtsa and high-grade BlCa TCCSUP cells. Given the scarcity of mechanistic-based studies in bladder, these novel findings may provide relevant information for clinical therapeutic trials involving green tea. Indeed, because the bladder provides a convenient closed system, bladder installation of elevated catechin doses (40 μg/mL or greater) can be achieved as a means of putative catechin-based therapy for bladder disease. We recently reported (6) that green tea polyphenols can protect against H_2O_2-mediated oxidative stress/damage and bladder cell death, suggesting clinical relevance regarding bladder inflammation/disease. Further investigations, however, involving specific gene regulation are needed to determine the importance of growth modulation by green tea and its catechin components.

REFERENCES

1. American Cancer Society: *Cancer Facts & Figures 2007*. Atlanta: American Cancer Society.
2. Cervello M, Giannitrapani L, Labbozzetta M, Notarbartolo M, D'Alessandro N, Lampiasi N, Azzolina A and Montalto G (2004) Expression of WISPs and of their novel alternative variants in human hepatocellular carcinoma cells. *Ann NY Acad Sci* **1028**, 432–439.
3. Chen ZP, Schell JB, Ho CT and Chen KY (1998) Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts. *Cancer Lett* **129**, 173–179.
4. Chung LY, Cheung TC, Kong SK, Fung KP, Choy YM, Chan ZY and Kwok TT (2001) Induction of apoptosis by green tea catechins in human prostate cancer DU145 cells. *Life Sci* **68**, 1207–1214.
5. Clark J and You M (2006) Chemoprevention of lung cancer by tea. *Mol Nutr Food Res* **50**, 144–151.
6. Coyle CH, Philips BJ, Morrisroe SN, Chancellor MB and Yoshimura N (2008) Antioxidant effects of green tea and its polyphenols on bladder cells. *Life Sci* **83**, 12–18.
7. Dreosti IE (1996) Bioactive ingredients: antioxidants and polyphenols in tea. *Nutr Rev* **54**, S51–S58.
8. Hwang JT, Ha J, Park IJ, Lee SK, Baik HW, Kim YM and Park OJ (2007) Apoptotic effect of EGCG in HT-29 colon cancer cells via AMPK signal pathway. *Cancer Lett* **247**,

115–121.

9. Khan N, Afaq F, Saleem M, Ahmad N and Mukhtar H (2006) Targeting multiple signaling pathways by green tea polyphenol (−)-epigallocatechin-3-gallate. *Cancer Res* **66**, 2500–2505.

10. Koopman G, Reutelingsperger CPM, GAM Kuijten, Keehnen RMJ, Pals ST and van Oers MHJ (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* **84**, 1415–1420.

11. Lyn-Cook BD, Rogers T, Yan Y, Blann EB, Kadlubar FF and Hammons GJ (1999) Chemopreventive effects of tea extracts and various components on human pancreatic and prostate tumor cells *in vitro*. *Nutr Cancer* **35**, 80–86.

12. Nakagawa K and Miyazawa T (1997) Chemiluminescence-high-performance liquid chromatographic determination of tea catechin, (−)-epigallocatechin 3-gallate, at picomole levels in rat and human plasma. *Anal Biochem* **248**, 41–49.

13. Nihal M, Ahmad N, Mukhtar H and Wood GS (2005) Antiproliferative and proapoptotic effects of (−)-epigallocatechin-3-gallate on human melanoma: possible implications for the chemoprevention of melanoma. *Int J Cancer* **114**, 513–521.

14. Qin J, Xie LP, Zheng XY, Wang YB, Bai Y, Shen HF, Li LC and Dahiya R (2007) A component of green tea, (−)-epigallocatechin-3-gallate, promotes apoptosis in T24 human bladder cancer cells via modulation of the PI3K/Akt pathway and Bcl-2 family proteins. *Biochem Biophys Res Commun* **354**, 852–857.

15. Ravindranath MH, Saravanan TS, Monteclaro CC, Presser N, Ye X, Selvan SR and Brosman S (2006) Epicatechins purified from green tea (*Camellia sinensis*) differentially suppress growth of gender-dependent human cancer cell lines. *Evid Based Complement Alternat Med* **3**, 237–247.

16. Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM and Pridham JB (1995) The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radic Res* **22**, 375–383.

17. Rieger-Christ KM, Hanley R, Lodowsky C, Bernier T, Vemulapalli P, Roth M, Kim J, Yee AS, Le SM, Marie PJ, Libertino JA and Summerhayes IC (2007) The green tea compound, (−)-epigallocatechin-3-gallate downregulates N-cadherin and suppresses migration of bladder carcinoma cells. *J Cell Biochem* **102**, 377–388.

18. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A and Rath M (2006) Antitumor effect of ascorbic acid, lysine, proline, arginine, and green tea extract on bladder cancer cell line T-24. *Int J Urol* **13**, 415–419.

19. Shammas MA, Neri P, Koley H, Batchu RB, Bertheau RC, Munshi V, Prabhala R, Fulciniti M, Tai YT, Treon SP, Goyal RK, Anderson KC and Munshi NC (2006) Specific killing of multiple myeloma cells by (−)-epigallocatechin-3-gallate extracted from green tea: biologic activity and therapeutic implications. *Blood* **108**, 2804–2810.

20. Thangapazham RL, Singh AK, Sharma A, Warren J, Gaddipati JP and Maheshwari RK (2007) Green tea polyphenols and its constituent epigallocatechin gallate inhibits proliferation of human breast cancer cells in vitro and in vivo. *Cancer Lett* **245**, 232–241.

21. Tian C, Zhou ZG, Meng WJ, Sun XF, Yu YY, Li L, Luo HZ, Yang L, Zhou B and Gu J (2007) Overexpression of connective tissue growth factor WISP-1 in Chinese primary rectal cancer patients. *World J Gastroenterol* **13**, 3878–3882.

22. Waltner-Law ME, Wang XL, Law BK, Hall RK, Nawano M, and Granner DK (2002) Epigallocatechin gallate, a constituent of green tea, represses hepatic glucose production. *J Biol Chem* **277**, 34933–34940.

23. Wang ZY, Hong JY, Huang MT, Reuhl KR, Conney AH and Yang CS (1992) Inhibition of *N*-nitrosodiethylamine- and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced tumorigenesis in A/J mice by green tea and black tea. *Cancer Res* **52**, 1943–1947.

24. Wang ZY, Huang MT, Ho CT, Chang R, Ma W, Ferraro T, Reuhl KR, Yang CS and Conney AH (1992) Inhibitory effect of green tea on the growth of established skin papillomas in mice. *Cancer Res* **52**, 6657–6665.

25. Xiao GS, Jin YS, Lu QY, Zhang ZF, Belldegrum A, Figlin R, Pantuck A, Yen Y, Li F and Rao JY (2006) Annexin-I as a potential target for green tea extract induced actin remodeling. *Int J Cancer* **120**, 111–120.

26. Zhao X, Tian H, Ma X and Li L (2006) Epigallocatechin gallate, the main ingredient of green tea induces apoptosis in breast cancer cells. *Front Biosci* **11**, 2428–2433.