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The Green Tea Compound, (−)-Epigallocatechin-3-gallate Downregulates N-Cadherin and Suppresses Migration of Bladder Carcinoma Cells

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Abstract Green tea has been reported as potential dietary protection against numerous cancers and has been shown to have activity in bladder tumor inhibition in different animal models. The goal of this study was to examine the effects of (−)-epigallocatechin gallate (EGCG—the major phytochemical in green tea) on growth inhibition and behavior of human bladder carcinoma cells and to identify the altered signaling pathway(s) underlying the response to EGCG exposure. EGCG inhibited the in vitro growth of invasive bladder carcinoma cells with an IC_{50} range of 70–87 μM. At a concentration of 20 μM, EGCG decreased the migratory potential of bladder carcinoma cells with concomitant activation of p42/44 MAPK and STAT3 and inactivation of Akt. Using biochemical inhibitors of MAPK/ERK, and siRNA to knockdown STAT3 and Akt, inhibition of migration was recorded associated with Akt but not MAPK/ERK or STAT3 signaling in bladder cells. In addition, EGCG downregulated N-cadherin in a dose-dependent manner where reduction in N-cadherin expression paralleled declining migratory potential. Continuous feeding of EGCG to mice prior to and during the establishment of bladder carcinoma xenografts in vivo revealed >50% reduction in mean final tumor volume ($P \leq 0.05$) with no detectable toxicity. EGCG inhibited bladder carcinoma cell growth and suppressed the in vitro migration capacity of cells via downregulation of N-cadherin and inactivation of Akt signaling. Continuous administration of EGCG to mice revealed significant inhibition of tumor growth in vivo indicating a possible preventative role for green tea in bladder cancer. J. Cell. Biochem. 102: 377–388, 2007.

Key words: N-cadherin; EGCG; bladder cancer; Akt; migration

Green tea has been reported as potential dietary protection against numerous cancers and has been shown to have activity in bladder tumor inhibition in different animal models [Kamat and Lamm, 2002; Kemberling et al., 2003; Sato and Matsushima, 2003; Chen et al., 2004]. Epidemiological studies have suggested that the low incidence of some cancers in Asian countries is linked to the regular drinking of green tea [Galanis et al., 1997; Imai et al., 1997; Nakachi et al., 1998]. Indeed in areas of green

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tea production significantly lower death rates are reported for gastrointestinal cancers [Gao et al., 1994; Inoue et al., 1998; Ji et al., 1999; Zhang et al., 2002]. However, one epidemiological study from Taiwan [Lu et al., 1999] and one from Japan [Wakai et al., 2004] suggests that green tea is associated with an increased risk of bladder cancer. In the United States superficial bladder tumors account for 75–80% of neoplasms, while the remaining 20–25% are invasive or metastatic at the time of presentation. Over 70% of patients affected with superficial tumors will have one or more recurrences after initial treatment, and about one-third of those patients will progress, presenting at a later date with a more aggressive lesion. In bladder oncology, invasive, locally advanced and metastatic cancer, remain therapeutic challenges where cystectomy, systemic chemotherapy, and radiation treatment comprise the spectrum of interventions.

Green tea contains several polyphenolic compounds where (−)-epigallocatechin gallate (EGCG) is the major constituent displaying the most potency in the inhibition of cell growth. EGCG has also been shown to affect multiple signaling pathways in different models including inhibition of growth factor receptor phosphorylation [Adhami et al., 2004; Hou et al., 2005; Shimizu et al., 2005], the induction of apoptosis [Hibasami et al., 1996; Baliga et al., 2005; Park et al., 2006; Zhao et al., 2006], inhibition of DNA methyltransferase [Fang et al., 2003], and cell cycle G_0–G_1 phase arrest [Ahmad et al., 2000; Gupta et al., 2000; Chen et al., 2004]. In a rat bladder cancer model green tea has been shown to inhibit bladder tumor growth and the growth of cells by downregulation of the cyclin D1, cyclin-dependent kinase 4/6 and the retinoblastoma signaling pathway involved in cell-cycle progression [Chen et al., 2004]. Such events can change cell behaviors including migration, invasion, and metastasis associated with late-stage cancer. Alternative molecules involved in bladder cell invasion include the cadherin family whereby loss or reduced expression of the invasive suppressor gene E-cadherin is a common event in late-stage disease. We have shown that loss of E-cadherin expression in bladder carcinoma cells may be accompanied by novel expression of N-cadherin, the latter acting as an invasive promoter gene. Concomitant with this cadherin switching event in bladder is activation of Akt, a signaling event intimately involved in the acquisition of the invasive phenotype [Rieger-Christ et al., 2004]. For these reasons we have identified these molecules as prime targets for new therapeutic approaches and in this study investigate the expression and activation status of each in bladder carcinoma cells exposed to EGCG.

MATERIALS AND METHODS

Cell Culture and Proliferation Assay

The human bladder carcinoma cell lines J82, UM-UC-3, EJ, KK47, T24, and TCCSUP were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal bovine serum and penicillin/streptomycin. Cells (293T) were maintained in 10% fetal bovine serum and penicillin/streptomycin. EGCG was purchased from Sigma (St. Louis, MO).

The effect of each concentration of EGCG on the viability of bladder carcinoma cells was determined by clonogenic assays. Cells were seeded at a density of 1×10^3 cells per 60 mm dish and allowed to attach. After 48 h, cells were incubated with fresh medium containing an appropriate concentration of the EGCG (0–100 μM). After 2 days of treatment the media was removed, cells were washed with phosphate buffered saline (PBS) and maintained for an additional 7–10 days in standard culture media. Cells were then washed three times with PBS, fixed for 20 min in 3.7% formaldehyde, and stained with crystal violet (0.2%). Following a 15-min incubation with crystal violet, cells were rinsed in water and dried at room temperature. Surviving colonies with >50 cells were counted without optical aid. All experiments were repeated at least three times.

Antibodies and Inhibitors

The following antibodies were used in this study: N-cadherin, clone 13A9 (kindly provided by Dr. M. Wheelock, Toledo, OH); γ-catenin, β-catenin, and $p120^{ctn}$ (Transduction Laboratories, Lexington, KY); c-myc (Oncogene Research Products, Boston, MA); p42/44 MAPK, phospho-p42/44 MAPK, Akt and phospho-Akt (Ser 473), STAT3, and pSTAT3 727 (Cell Signaling Technology, Beverly, MA); E-cadherin (Zymed, San Francisco, CA). The biochemical inhibitors were

purchased from Calbiochem (San Diego, CA). Cells were pre-treated with p42/44 MAPK/ERK (PD98059-40 μM) or PI3 kinase (LY294002-20 μM) inhibitors for 1 h prior to inclusion in assays.

SiRNA Knockdown

Pre-designed siRNA for Akt (SignalSilence Akt siRNA, Cell Signaling Technology Danvers, MA), STAT3 (SMARTpool siRNA, Dharmacon, Lafayette, CO), and scrambled controls were purchased. Cells were plated (1.2×10^5/well) in a 12-well plate 24 h prior to transfection. For knockdown of Akt, transfection of the RNA oligonucleotide and a scrambled control sequence was performed using transfection reagent provided in the siRNA kit (Cell Signaling) according to the manufacturer's instructions. For knockdown of STAT3, transfection of the RNA oligonucleotide and a scrambled control sequence was performed using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. All experiments were repeated on three separate occasions.

Western Blot Analysis

Subconfluent dishes of cells were washed in PBS followed by lysis in hot sample buffer ($2 \times$ ESB – 0.08 M Tris, pH 6.8; 0.07 M SDS, 10% glycerol, 0.001% bromophenol blue, and 1 mM $CaCl_2$) and sheared through a 26-gauge needle. Lysates were then assayed for protein concentration using the BSA method (Pierce, Rockford, IL). After determination of protein content, β-mercaptoethanol (1%) was added to each sample. Samples were boiled for 5 min and loaded in each lane of a 7.5% or 12.5% polyacrylamide gel. Electrophoretically separated proteins were transferred overnight onto nitrocellulose. Membranes were blocked in 10% milk in TBS with 0.05% Tween (TBST) and placed on primary antibody overnight at 4°C. Blots were washed in TBST, three times for 15 min each, followed by incubation with secondary antibody for 60 min at room temperature. Blots were then washed as described above and developed with an ECL kit (Amersham, Arlington Heights, IL). UM-UC-3 and J82 cells were incubated in the presence or absence of 25 μM cycloheximide (in DMSO) for 4 h, followed by exposure to 20 μM EGCG for an additional 18 h. Cells were lysed and lysates were loaded onto gels as described above. All experiments were repeated on three separate occasions.

Reporter Assays

Cell line 293T was transfected with pGL3-basic (control) or the N-cadherin promoter [Le Mée et al., 2005] linked to the firefly luciferase gene. Twenty-four hours post-transfection different concentrations of EGCG were added (see text). A 60 mm dish was lysed 48 h post-transfection in 150 μl Promega Lysis Buffer (Promega, Madison, WI). After scraping the cells and subjecting them to two freeze-thaw cycles, lysates were rocked for 30 min at 4°C and then spun for 30 min at 14,000 rpm. Luciferase activity was measured using the Luciferase Reporter Assay System (Promega) according to the manufacturer's protocols. Transfection efficiency was normalized using transfected RSV-β-galactosidase. β-galactosidase activity was quantitated using the colorimetric substrate *o*–nitrophenyl-β–D-galactopyranoside (Sigma, St Louis, MI). All experiments were repeated a minimum of three times.

Migration and Invasion Assays

In vitro migration and invasion assays were carried out using modified Boyden chambers consisting of Transwell (8 μm pore size; Corning Costar Corp., Cambridge, MA) membrane filter inserts in 24-well tissue culture plates. For invasion assays the upper surfaces of the membranes were coated with Matrigel (Collaborative Biomedical Products, Bedford, MA) and placed into 24-well tissue culture plates containing 600 μl of NIH/3T3 conditioned media (experimental) or plain DMEM (control). No matrigel was used for migration assays where the chemoattractant used was fibronectin (10 μg/ml). In both assays, cells (1×10^5) were added to each Transwell chamber and allowed to migrate/invade toward the underside of the membrane for 16 h at 37°C. Cells that pass through the membrane were fixed in methanol, stained with crystal violet, and counted under a light microscope. In additional assays, the cells were pre-incubated for 1 h (PD98059-40 μM and LY294002-20 μM) before the cells were added to the Matrigel-coated wells. All assays were performed in triplicate and repeated three times. Statistical analysis was performed by a Student's *t*-test, where $P < 0.05$ was considered significant.

Establishment of Bladder Cancer Xenografts and Treatment with EGCG

The human bladder carcinoma cell line UM-UC-3 has been shown to be tumorigenic in this model and was considered representative of a late stage bladder lesion. Mice were fed EGCG (0.05% w/v) in the drinking water 7 days prior to inoculation of cells. Cells were harvested and resuspended in PBS. Six-week-old male BALB/c nude (nu/nu) mice were injected subcutaneously with inoculums of 1×10^6 UM-UC-3 cells per site. Mice were kept under barrier conditions and maintained on a 12-h day/night light cycle with food and water ad libitum. Mice were randomly assigned to two groups of ten animals, the experimental group receiving EGCG in water and the control group water alone. Mice were weighed weekly and animal behavior, food intake, and excrement were monitored throughout the course of the experiment to assess the toxicity of the treatment. Tumors were measured at the same time using calipers and tumor volume was calculated using the following formula: tumor volume = length $\times$ width $\times$ height $\times \pi/6$. The study period was determined by the size of the tumors in the control group. The in vivo data were presented as mean tumor volume plus standard error of the mean. *P*-values at the end of the experiment were established using the Student's *t*-test.

RESULTS

Green Tea Polyphenol EGCG on In Vitro Cell Growth

Figure 1A shows that in a clonogenic assay EGCG caused a concentration-dependent growth inhibition in a panel of invasive bladder carcinoma cells. Fifty percent growth inhibition (IC_{50}) was recorded in a range of 70–87 μM EGCG. Trypan blue exclusion on floating and attached cells revealed a >99% loss of viability in the former, with only 60% of attached cells retaining the ability to exclude the dye.

Effect of EGCG on Bladder Xenograft Growth

To establish whether EGCG had anti-tumor activity in a human bladder cancer model, mice were fed EGCG in the drinking water (0.05%) 7 days prior to subcutaneous inoculation of UM-UC-3 cells. Animals were provided with fresh EGCG every 2 days throughout the course of the study. Administration of EGCG resulted in 53% reduction in tumor volume compared to vehicle-treated control animals (Fig. 1B). The toxicity of EGCG was assessed by mouse survival along with careful monitoring of body weight, behavior, and food intake. In both experimental and control groups mice gained between 4 and 5 g in body weight relative to their weight at the start of the experiment.

EGCG Suppresses Migration and Invasive Potential

Screening of bladder cell lines in in vitro migration assays revealed significant inhibition in all cases when assays were performed in the presence of EGCG. Concentration-dependent inhibition of cell migration was demonstrated in bladder carcinoma cells and observed at concentrations as low as 5 μM EGCG (Fig. 1C). The bladder carcinoma cell lines within the panel revealed identical, reproducible results in migration and invasion assays hence, in subsequent experiments we have used a representative cell line (UM-UC-3) along with migration potential as the indicator of EGCG action. All subsequent experiments in this study were performed at an EGCG concentration of 20 μM.

Signaling Pathways Altered by EGCG

Multiple signaling pathways have been identified as altered by the action of EGCG in different cell types. Figure 2A shows the activation status of two such pathways in the UM-UC-3 bladder carcinoma cell line following 48-h exposure to increasing concentrations of EGCG. Activation of p42/44 MAPK, as assessed by levels of phosphorylated p42/44 MAPK, was first observed at an EGCG concentration of 10 μM and was sustained through to the highest concentration (50 μM) while inactivation of Akt was recorded over the same concentration range (Fig. 2A).

In order to assess the timing of the alterations in various signaling pathways we treated UM-UC-3 cells with 20 μM for periods of time up to 24 h and performed Western blot analysis. Twenty-four hour exposure of bladder carcinoma cells to EGCG revealed activation of p42/44 MAP kinase. However, tracking the temporal sequence of events revealed a biphasic activation of p42/44 MAP kinase. The first activation phase occurred within 30 min and was sustained until the 2-h time point. Beginning after 4 h exposure of cells to EGCG

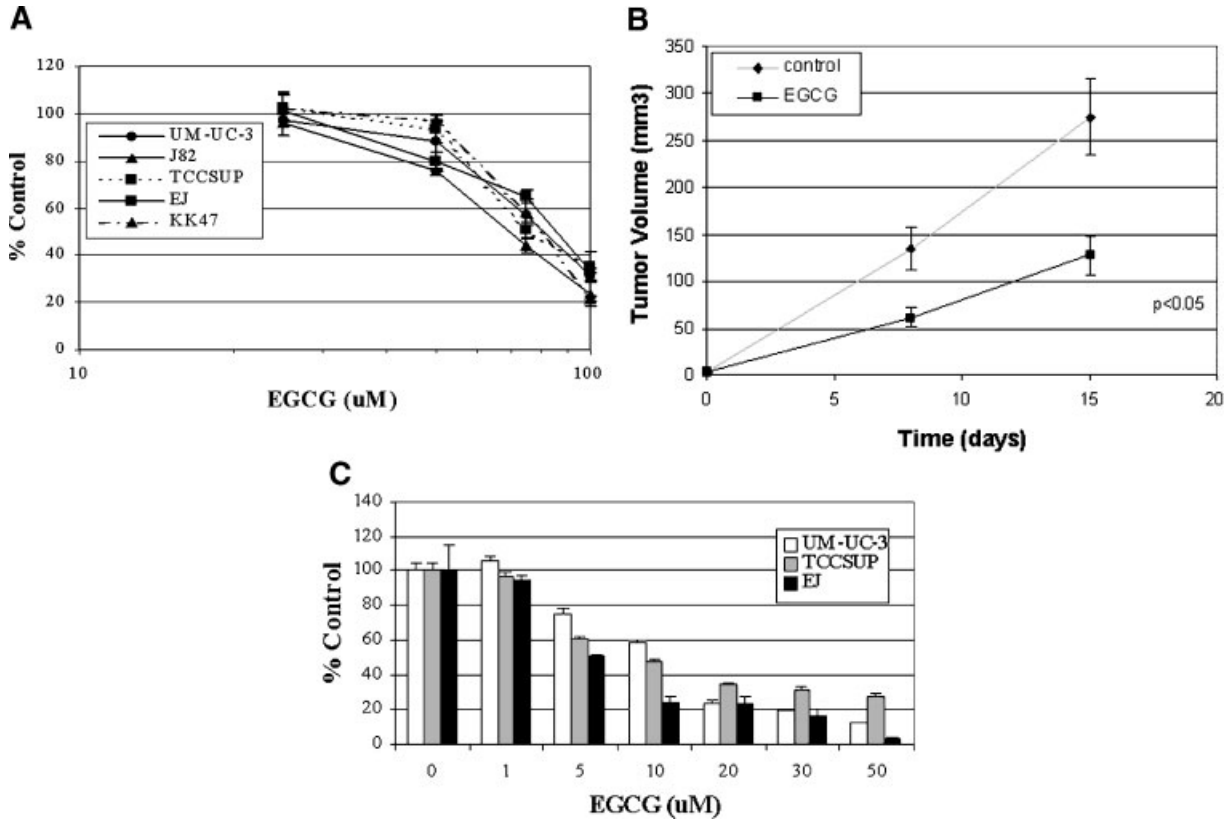

Fig. 1. **A**: Results from clonogenic assays with a panel of invasive bladder carcinoma cells following exposure to different concentrations of EGCG. Results are expressed as percentage of the colonies surviving in control plates (±SEM). Colonies were fixed and stained 7–10 days following exposure to EGCG with colonies >50 cells counted and recorded. The data shown are from three experiments performed in triplicate. **B**: Tumor volume in mice fed EGCG or water alone. Mice were fed EGCG (0.05% w/v) in the drinking water (n = 10), or water alone in the control group (n = 10), 7 days prior to subcutaneous inoculation of UM-UC-3 cells (day 0) and throughout the duration of the study. At the end of the study period (day 15), tumor volumes were significantly lower ($P < 0.05$) in EGCG fed mice compared to control mice. No adverse effect of EGCG was recorded in the experimental group where mice gained between 4 and 5 g in body weight relative to their weight at the start of the experiment. **C**: Concentration-dependent inhibition of in vitro migration in invasive bladder cell lines exposed to EGCG. Numbers are expressed as percent control where migration after treatment with DMSO was considered 100%. The data shown are the mean (+SEM) of one experiment performed in triplicate.

treatment, phospho-p42/44 MAPK levels decreased over time and returned close to baseline levels by 12 h. p42/44 MAPK then became re-activated by 18 h and continued to increase through the 24-h exposure period (Fig. 2B).

STAT3 is a signaling target of MAP kinase and its activation is implicated in numerous different models of neoplastic progression. Assessment of the activation status of STAT3 following the addition of EGCG to bladder carcinoma cells was performed in Western blot analysis using antibodies that specifically recognize the serine (727) STAT3 where the phosphorylation status is indicative of activation. Following the addition of EGCG (20 μM) to bladder cells, STAT3 activation was recorded after 30 min and remained activated throughout the 24-h time course of continuous exposure to EGCG (Fig. 2B).

An alternative signaling pathway implicated in the migrational and invasive behavior involves Akt kinase. In contrast to STAT3, inactivation of Akt in bladder carcinoma cells was recorded within 30 min of exposure to EGCG with maintenance of the deactivated state throughout the 24-h EGCG exposure period (Fig. 2B).

Investigation of Signaling Events Linked to EGCG Reduced Migration

MAP kinase is one pathway that has also been implicated in establishing the invasion status of carcinoma cells [Reviewed in Reddy et al.,

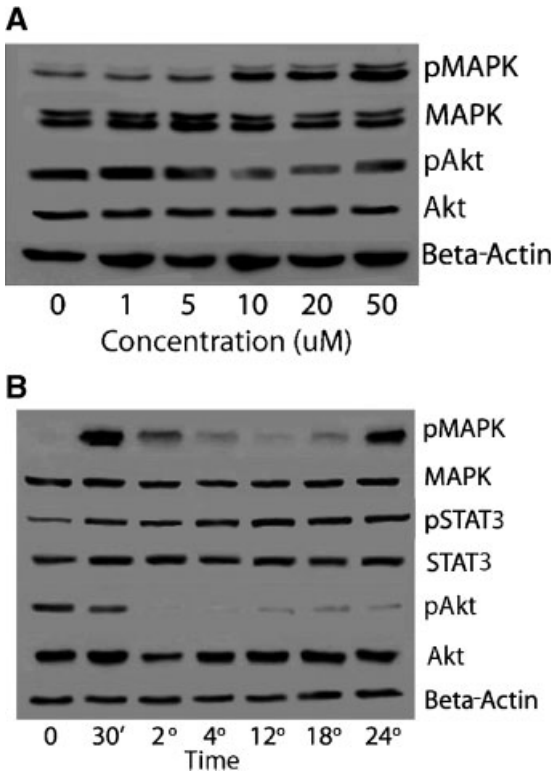

Fig. 2. **A**,**B**: Activation status of different signaling events after 48-h exposure of UM-UC-3 bladder carcinoma cells to increasing concentrations of EGCG (A) and the temporal sequence of events (B) following exposure of UM-UC-3 cells to 20 μM EGCG. Total cell lysates from EGCG treated or control cells were protein standardized prior to loading on gels and probed in Western blot analysis using specific antibodies. An antibody to β-actin served as a loading control.

2003]. To determine whether MAP kinase activation was essential for the inhibition of migration by EGCG, we performed migration assays in the presence of the MAP kinase/ERK inhibitor PD98059. Figure 3 shows inhibition of migration by EGCG in the presence and absence of PD98059 with no inhibition observed when the biochemical inhibitor was used alone. Confirmation of inhibition of phospho-p42/44 MAP kinase by PD98059 was determined in Western blot analysis (data not shown).

Since activation of STAT3 has been shown to be essential in tumorigenesis in different models [Bromberg and Darnell, 2000; Turkson and Jove, 2000; Silva, 2004] we used siRNA to knockdown STAT3 expression in UM-UC-3 cells. Confirmation of STAT3 knockdown was performed in Western blot analysis (Fig. 4A) where the same pool of cells was used in parallel migration assays in the presence and absence of EGCG. No difference in migration was observed between control and STAT3 knockdown UM-UC-3 cells (Fig. 4B).

Inhibition of migration of bladder carcinoma cells was recorded when a biochemical inhibitor of PI3 kinase was used in migration assays with UM-UC-3 cells (Fig. 3). Combining of EGCG with the PI3 inhibitor in migration assays revealed no significant difference between the percentage inhibition of migration recorded compared with either agent alone (Fig. 3). To assess the role of Akt in the EGCG-dependent decrease in migration we used siRNA to knockdown Akt. In the absence of EGCG, we recorded a 30% inhibition of migration as compared to control. When EGCG and Akt siRNA were used in combination the migratory potential was reduced to 86% of control (Fig. 4C). Western blot analysis confirmed reduced levels of pAkt in knockdowns with and without EGCG treatment (Fig. 4D).

EGCG Modulates Cadherin/Catenin Expression

Within the panel of bladder carcinoma cells used in this study all cell lines express N-cadherin in the absence of E-cadherin. This cadherin expression profile is indicative of late-stage lesions displaying an invasive phenotype. Because we have shown that novel expression of N-cadherin in bladder carcinoma cells promotes migration and invasion, we investigated the expression status of N-cadherin following exposure to EGCG. Reduced expression of N-cadherin was found to be concentration dependent where downregulation of this cadherin family member was first recorded at a concentration of 5 μM EGCG (Fig. 5A). Probing the same cell lysates with antibodies to β-, γ-, and $p120^{ctn}$ revealed concomitant reduction of β- and γ-catenin with no change in the $p120^{ctn}$ expression status. Probing cell lysates for E-cadherin expression revealed no reactivation of expression of this cadherin family member by EGCG (data not shown). Analysis of the timing of downregulation of N-cadherin revealed significant loss of N-cadherin protein expression between 12 and 24 h exposure to 20 μM EGCG (Fig. 5B). Inactivation of Akt paralleled the timing of downregulation of N-cadherin in this experiment. Downregulation of N-cadherin was observed in each of our bladder cell lines expressing N-cadherin in the absence of E-cadherin at a concentration of 20 μM EGCG (Fig. 5C).

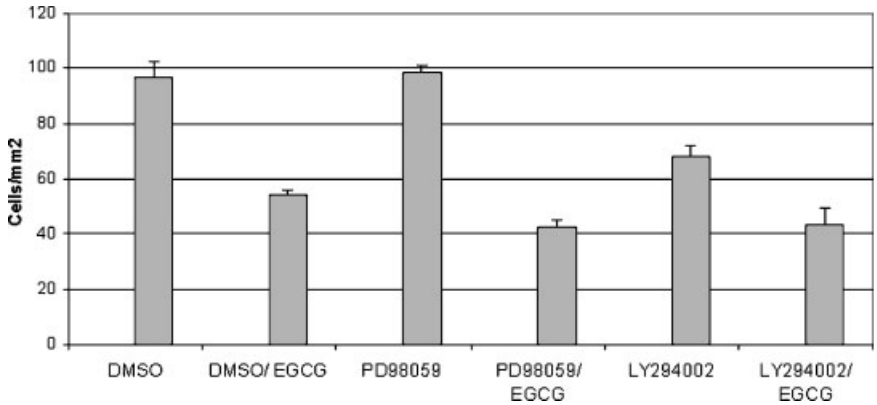

Fig. 3. The effects of various biochemical inhibitors on the migration potential of UM-UC-3 cells in the presence or absence of EGCG. UM-UC-3 cells were treated with DMSO as the control, or inhibitors of MAPK/ERK (PD98059), or PI3 kinase (LY294002). Migration assays were run with each inhibitor in the presence or absence of 20 μM EGCG. Numbers were expressed as cells/mm^2. The data shown are the mean (+SEM) of one experiment performed in triplicate.

EGCG Does Not Reduce N-cadherin Promoter Activity

To test the hypothesis that the decrease in N-cadherin protein was due to the ability of EGCG to alter N-cadherin promoter activity, reporter studies were performed. The baseline luciferase level of control promoter activity detected in 293T cells remained unaltered when these transfected cells were exposed to increasing concentrations of EGCG. When the N-cadherin promoter construct was introduced into 293T cells, a dramatic increase in luciferase activity was recorded as has previously been reported [Le Mee et al., 2003]. When these transfectants were exposed to increasing concentrations of EGCG the level of promoter activity did not change (data not shown).

EGCG Acts on N-cadherin at the Post-Transcriptional Level

Since EGCG did not affect N-cadherin promoter activity, we analyzed its effects at the post-transcriptional level. UM-UC-3 and J82 cells were treated with the protein synthesis inhibitor, cycloheximide followed by exposure to 20 μM EGCG and the levels of N-cadherin expression were assessed. Analysis of N-cadherin expression is shown in Figure 6, where in the absence of cycloheximide N-cadherin levels were reduced in the presence of EGCG. As expected, cells treated with cycloheximide alone showed reduced levels of N-cadherin in the absence of newly synthesized protein. In the presence of cycloheximide and EGCG both UM-UC-3 and J82 cell lines showed further reduction in N-cadherin expression. In UM-UC-3 cells, the level of N-cadherin was undetectable with this combination whereas low levels were detected in J82 cells with the same treatment.

DISCUSSION

In this study we show that EGCG suppresses at least two features of late-stage bladder carcinoma cells, proliferation and migration. In the presence of EGCG, in vitro cell growth and migration were reduced in a concentration-dependent manner in the bladder cell lines tested. In addition, mice fed EGCG in the drinking water 7 days prior to subcutaneous inoculation of bladder carcinoma cells presented with a significantly reduced tumor burden as compared to mice receiving water alone. Together these results suggest that EGCG may have use as a therapeutic agent for bladder cancer.

Both activation [Facchini et al., 2003; Siddiqui et al., 2004; Kim et al., 2005] and inactivation of MAPK [Dong et al., 1997; Wang and Bachrach, 2002; Kim et al., 2004; Sah et al., 2004; Shimizu et al., 2005] have been reported in cells in response to various concentrations of EGCG. In the bladder carcinoma cell lines EGCG activated p42/44 MAPK and STAT3 and inactivated Akt with a different temporal sequence of events. In evaluating the underlying mechanism of EGCG's ability to reduce the migratory potential of bladder carcinoma cells we utilized biochemical inhibitors of

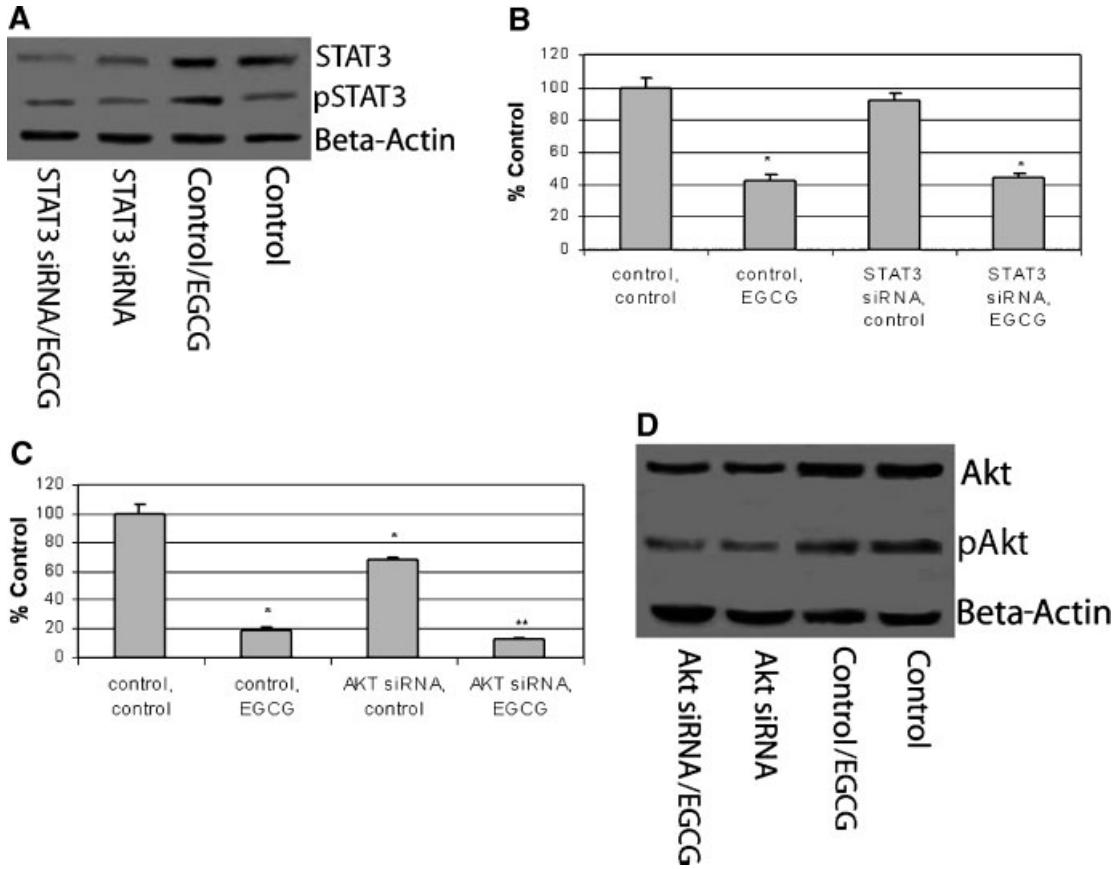

Fig. 4. **A**,**B**: siRNA directed against STAT3 downregulates protein expression but does not reduce in vitro migration. UM-UC-3 cells were transfected with siRNA directed against STAT3 or control transfected with a scrambled siRNA. Twenty-four hours post-transfection, cells were maintained in the presence (20 μM) or absence of EGCG. Cells were lysed 48-hrs post-transfection and Western blots were probed with anti-STAT3 or anti-β-actin antibodies (A) or used in migration assays (B). The cells transfected with siRNA directed against STAT3 show downregulation of the protein but did not display a statistically significant decrease in migration capacity compared to the controls. Numbers were expressed as percent control where scrambled control transfectants were considered 100%. * Signifies a statistically significant difference ($P < 0.05$) compared to control cells without EGCG treatment. The data shown are from one experiment performed in triplicate. **C**,**D**: siRNA directed against Akt downregulates protein expression and reduces in vitro migration. UM-UC-3 cells were transfected with siRNA directed against Akt or control transfected with a scrambled siRNA. Twenty-four hours post-transfection, cells were maintained in the presence (20 μM) or absence of EGCG. Forty-eight hours post-transfection cells were used in a migration assay (C) or analyzed in Western blots (D) probed with anti-Akt or anti-β-actin antibody. The cells transfected with siRNA directed against Akt show downregulation of the protein and a statistically significant decrease in migration capacity compared to the controls. Numbers were expressed as percent control where scrambled control transfectants were considered 100%. * Represents a statistically significant difference ($P < 0.05$) compared to control cells without EGCG treatment. ** Represents a statistically significant difference ($P < 0.05$) as compared to scrambled control transfects treated with EGCG. The data shown are from one experiment performed in triplicate.

several signal transduction pathways. These studies suggest that alterations in MAPK/ERK and STAT pathways are not responsible for the reduction of migratory potential observed in the presence of EGCG since inhibiting these pathways did not alter the migratory potential of the bladder cells. A role for the Akt pathway however was demonstrated when siRNA to Akt reduced in vitro migration and in combination with EGCG showed a significant further increase in the inhibition of migration. From this we conclude that although activated Akt contributes to the migratory potential of bladder carcinoma cells additional pathways involved in migration are affected by the action of EGCG.

There have been a few reports on the alteration of cadherins and catenins by EGCG [Dashwood et al., 2002; Tang et al., 2003; Dashwood et al., 2005; Ju et al., 2005; Park et al., 2006; Peng et al., 2006]. In the bladder carcinoma panel, cell lines characterized as established

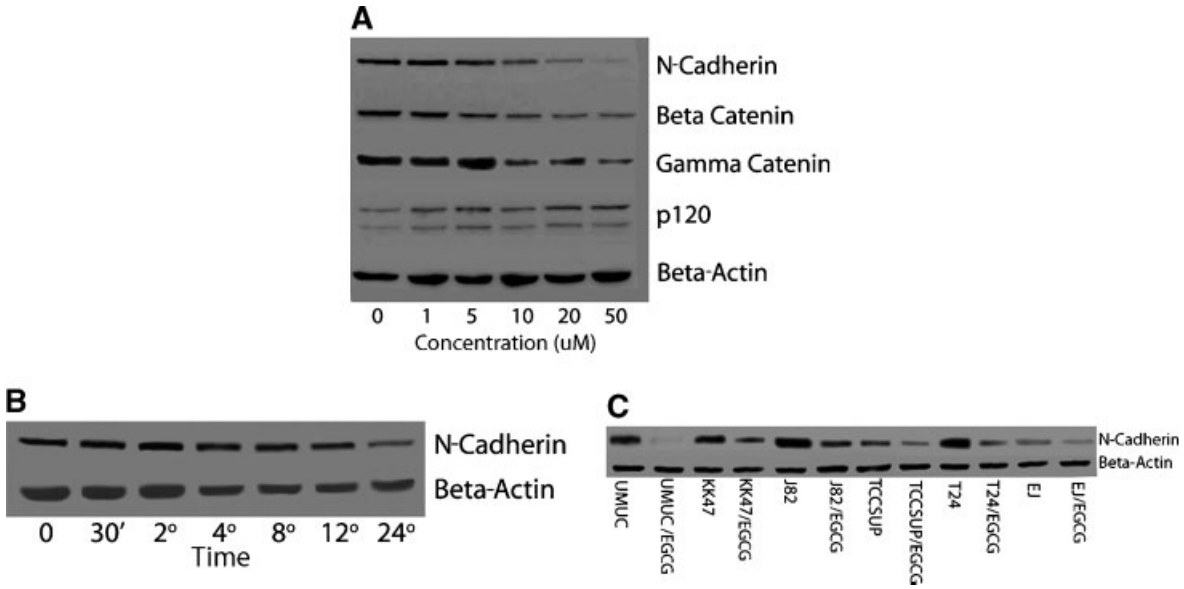

Fig. 5. **A**,**B**: Expression level of different elements of the cadherin complex after exposure of UM-UC-3 bladder carcinoma cells to increasing concentrations of EGCG (A) and the temporal sequence of events (B) following exposure of UM-UC-3 cells to 20 μM EGCG. Total cell lysates from EGCG treated or control cells were protein standardized prior to loading on gels and probed in Western blot analysis using specific antibodies. An antibody to β-actin served as a loading control. **C**: Western blot analysis of N-cadherin expression in the panel of invasive bladder carcinoma cell lines exposed for 24 h to 20 μM EGCG. Total cell lysates from EGCG treated or control cells were protein standardized prior to loading on gels and probed in Western blot analysis using specific antibodies. An antibody to β-actin served as a loading control.

from late-stage tumors were represented. The cadherin/catenin expression profile in such cell lines showed a loss of E-cadherin expression accompanied by novel expression of N-cadherin and reduced expression of γ-catenin [Canes et al., 2005]. Several groups have demonstrated upregulation of E-cadherin after treatment with EGCG and indeed, EGCG has been reported to inhibit DNA methyltransferase [Fang et al., 2003] resulting in the re-expression of silenced genes in cancer cell lines. However, a recent study by Stresemann et al. [2006] showed that EGCG did not inhibit DNA methylation and suggested that the cellular effects observed might be attributed to indirect effects of the compound, such as oxidative stress. In addition, there is one report of modulation of the phosphorylation of VE-cadherin by EGCG resulting in inhibition of VEGF-induced angiogenesis [Tang et al., 2003]. In this study we show that EGCG treatment reduced the expression levels of N-cadherin, β-, and γ-catenin in bladder carcinoma cells without restoration of E-cadherin expression in the panel of cell lines. The reduction in expression of the cadherin and catenins was not due to cell death since the 20 μM concentration used throughout this study was below the IC_{50} for all cell lines. Also, the expression level of another member of the cadherin/catenin complex, $p120^{ctn}$, remained unaltered in the presence of the same concentration of EGCG. Although EGCG can directly bind to DNA and RNA our results showed that the reduction in N-cadherin protein after EGCG treatment is not due to EGCG binding to the N-cadherin promoter as no alteration in promoter activity

Fig. 6. Post-transcriptional levels of N-cadherin. Total cell lysates from J82 and UM-UC-3 cells in the presence and absence of 25 μM cycloheximide for 4 h followed by 20 μM EGCG for 18 h were protein standardized prior to loading on gels and probed in Western blot analysis using specific antibodies. An antibody to β-actin served as a loading control.

was observed with the addition of EGCG. These results are similar to another study where we observed an increase in HBP1 protein after EGCG treatment that was due to an increase in HBP1 mRNA stability rather than increased promoter activity [Kim et al., 2006]. In this study we have shown that EGCG treatment of bladder carcinoma cell lines leads to reduced levels of N-cadherin even in the presence of cycloheximide, suggesting that EGCG is acting at a post-transcriptional level.

We and others have demonstrated that novel expression of N-cadherin plays a primary role in the migration and invasion of cancer cells [Nieman et al., 1999; Hazan et al., 2000; Rieger-Christ et al., 2004]. In this study we report that EGCG downregulates the expression of N-cadherin resulting in a reduction in the migratory capacity of bladder carcinoma cells. The signaling pathway by which N-cadherin affects migration is reportedly different depending on the cell type studied. In bladder carcinoma cells we have shown that Akt is one pathway by which N-cadherin modulates migration. Here we also show that EGCG reduced bladder cell migration in part via inactivation of Akt and downregulation of N-cadherin.

Multiple signaling pathways have been identified as altered by the action of EGCG, however, changes were often recorded at higher concentrations of EGCG than was used in this study. An ongoing concern with the use of green tea as a therapeutic agent is whether sufficient concentrations of EGCG can accumulate in vivo to trigger the cellular events reported in in vitro models. In the bladder, intravesical installation of EGCG presents the opportunity to closely control the concentration administered in vivo. Kuzuhara et al. [2006] recently reported that EGCG accumulates in cells and that both DNA and RNA molecules act as biological reservoirs for EGCG. Thus by consumption of green tea throughout the day, the concentration of EGCG used in this study is likely attainable in vivo. One recent publication has reported that oral administration of green tea catechins to men with high-grade prostate intraepithelial neoplasia resulted in a reduction in tumor incidence after 1 year [Bettuzzi et al., 2006]. This occurred in the absence of significant side effects or adverse events with an indication that such treatment can ease the symptoms of benign prostate hyperplasia. Such clinical studies support the use of green tea in a therapeutic setting. In this study we have shown inhibition of migration and the suppression of tumorigenicity by EGCG in bladder carcinoma cell lines representative of late-stage cancer. Future efforts will be directed at using EGCG in combination with inhibitors or small molecules to target multiple signaling events underlying the acquisition of the invasive phenotype. In addition, we will extend these studies to assess the therapeutic efficacy of green tea catechins in superficial bladder disease.

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