Epigallocatechin-3-gallate prevents tumor cell implantation/ growth in an experimental rat bladder tumor model

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Abstract. The aim of this study was to determine the efficacy of epigallocatechin-3-gallate (EGCG) (Polyphenon E®) in comparison with mitomycin C (MMC) to prevent tumor cell implantation/growth in an animal model of superficial bladder cancer and search for possible mechanism(s) of action. Female Fisher 344 rats were used to study the effects of EGCG and mitomycin C for the prevention of transitional cell tumor implantation (AY-27). Twenty rats served as a control, tumor implantation and saline wash only. Sixty rats were treated with EGCG (100, 200 and 400 μM) intravesically for 60 or 120 min after tumor implantation. Thirty other rats were divided equally and pretreated with 400 μM EGCG or saline for 120 min before tumor initiation. In a separate series of experiments, 30 rats were treated 2 weeks after tumor initiation with saline or EGCG (400 μM). In a different experiment 39 rats were treated with: saline (n=10) EGCG (n=9) 400 μM, MMC (n=10) 0.5 μM, MMC (n=10) 400 μM. Rats were sacrificed 3 weeks following treatment. Gross and histological analyses were performed on the bladders. EGCG and mitomycin C prevented intravesical tumor growth in a concentration- and time-dependent manner. EGCG pretreatment or treatment 2 weeks post tumor implantation did not have therapeutic effects. Molecular modeling suggests that EGCG inhibits urokinase and matrix metalloproteinase-9. EGCG prevents intravesical tumor implantation/growth with a slightly better efficacy than mitomycin C in this experimental model. The data suggest that EGCG lowers proteolytic activity and lowers probability of cancer cell implantation rather than direct cancer cell killing.

Introduction

Bladder cancer is the fourth leading cause of cancer in American men. The majority of bladder cancers present as superficial disease, restricted to the bladder mucosa or submucosal layer. Most superficial tumors (60-70%) have a tendency for recurrence after transurethral resection of a bladder tumor and 15-25% are at high risk for progression to muscle invasion. Tumor implantation at tumor resection site may account in part for the high rate of tumor recurrence (1,2). People who are at risk of recurrence will have either intravesical chemotherapy, most commonly mitomycin C or Bacillus Calmette-Guérin (BCG) (3,4). None of these agents have proved to fully prevent disease progression and have some side effects (3).

Numerous pre-clinical and clinical studies have presented convincing evidence that green tea may protect against cancer (5,6). Green tea extract contains catechins such as epigallocatechin-3-gallate (EGCG), epigallocatechin, epicatechin gallate and epicatechin and the anticarcinogenic properties of green tea are attributed to the biological activities of these polyphenol components. EGCG is the most abundant component and the most widely studied (5,7-9). EGCG has been shown to induce apoptosis, inhibit cancer cell proliferation, and inhibit invasion and metastasis of cancers (10-14). This polytrophic molecule affects many different pathways altering expression and activity of numerous proteins (15).

Matrix dissolution is an essential step during carcinogenesis where matrix metalloproteinase (MMP-9) and urokinase (uPA) are one of the most important proteases functioning during invasion and tumor spread (16,17). Expression and activity of both enzymes are inhibited by EGCG.

MMPs are a family of zinc-dependent endopeptidases which are involved in the breakdown of extracellular matrix. Among MMPs, MMP-9 (gelatinase B) has gained attention for its role in tumor invasion and metastasis, since MMP-9 has an exceptional ability to degrade type IV collagen, a major constituent of the basement membrane. Accordingly, elevated expression of MMP-9 has been associated with increased metastatic potential in many cancer types (18). Furthermore, direct inhibition of MMP-9 activity or suppression of the expression via blockage of the ERK-MAP kinase pathway results in the inhibition of tumor invasion and metastasis (18-21). It has been found that green tea polyphenol epigallocatechin-3-gallate (EGCG) inhibits tumor invasion and implantation by directly inhibiting the MMP-9 activity (11,12,18) and suppressing the ERK-MAP kinase pathway (22).

Urokinase activates plasminogen to plasmin an enzyme that degrades extracellular matrix enabling cancer cells to invade and metastasize (23,24). Similarly to MMP-9, inhibi-

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tion of uPA results in inhibition of cancer cell implantation, invasion and metastasis (8,13,23-25). EGCG inhibits expression of uPA most likely by suppression of NFκB (26,27) and urokinase activity (13-15,27). Consequently, the inhibition of MMP and uPA expression and/or activity by EGCG signifies potential novel therapeutic strategies for the treatment of cancer patients.

We evaluated EGCG as an intravesical agent for the prevention of transitional cell tumor implantation in the past. We previously found that intravesical instillation of EGCG inhibits the growth of AY-27 rat transitional cells implanted in this model (1,28). In the current study we evaluated a large number of animals treated with different doses of EGCG or mitomycin C and propose a possible mechanism of action.

Materials and methods

Cell line. AY-27 rat urothelial derived tumor cells (29) were maintained in RPMI-1640 medium with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich®), and 10% FBS (Atlanta Biologicals®, Lawrenceville, GA, USA).

Animals. All experiments using animals in this study were approved by the University of Toledo institutional animal care and use committee and performed under the guidance of the Department of Laboratory Animal Resources. Female Fischer 344 rats (Charles River®, Wilmington, MA, USA) were used in conjunction with the transplantable AY-27 rat bladder tumor cell line.

Tumor initiation. Tumor growth was initiated in the rat bladder by injuring the bladder mucosa and immediately instilling a suspension of AY-27 tumor cells obtained from stock cultures. Surgical exposure of the bladder was necessary to ensure proper placement of the cautery and visualization of the cauterization procedure under general anesthesia. A blunt tipped 24-gauge copper wire was advanced through the catheter. Six hundred volts current (60 W maximum) was used to cauterize the bladder wall, creating a small white puckered area. The bladder was catheterized with an 18-gauge Teflon i.v. catheter allowing easy access to the bladder lumen for cauterization, instillation of the tumor cells and treatment solutions. The concentration of the EGCG and MMC treatment solutions was up to 400 μM. After mucosal injury a suspension of tumor cells in RPMI media ($1x10^6$ cells/0.5 ml) was instilled into the bladder. The tumor cell suspension remained in the bladder for 30 min. Small tumors were palpable within 2-3 weeks.

Treatment solutions. Treatment solutions were prepared by diluting MMC and EGCG in a 0.9% normal saline solution. The EGCG concentration of treatment solutions were based on the total EGCG content (63%) in the PE used. EGCG and NS control solutions were adjusted to pH 5.0, pH of EGCG in the highest concentration.

Animal treatment. Phase 1 consisted of 8 groups (80 animals, 10 rats/group). Bladders of groups I and II (n=10), the control groups, were instilled with a 0.9% normal saline solution. Dwell time for group I was 60 min and 120 min for group II. Groups III (n=10, dwell time 60 min) and IV (n=10, dwell time 120 min) bladders were instilled with a 100-μM concentration of PE. Groups V (n=10, dwell time 60 min) and VI (n=10, dwell time 120 min) bladders were instilled with a 200-μM concentration of PE. Groups VII (n=10, dwell time 60 min) and VIII (n=10, dwell time 120 min) bladders were instilled with a 400-μM concentration of EGCG. All rats in phase 1 were euthanized 3 weeks after tumor initiation for direct examination of the bladder to determine the incidence of tumor growth.

Phase 2 of the *in vivo* study investigated the potential effectiveness of EGCG as a pretreatment prior to bladder injury and instillation of AY-27 tumor cells. This phase consisted of 2 groups; a control group, group IX (n=15) was pretreated with a 0.9% normal saline solution, group X (n=15) was pretreated with 400 μM of EGCG for 120 min. The bladders were instilled with 0.5 ml of treatment solution and then were gently emptied after the first 60 min and re-filled with fresh treatment solution for the remaining 60 min. Thirty minutes after the treatment time with PE or saline, the tumor initiation process, as described above in phase 1 was performed. All rats in phase 2 were euthanized 3 weeks after tumor initiation for direct examination of the bladder to determine the incidence of tumor growth.

Phase 3 of the *in vivo* study investigated the potential effectiveness of PE as a treatment after establishment of early stage bladder cancer. The bladder tumor initiation process, as described above in phase 1 was performed on 30 rats. Two weeks after tumor initiation the rats were divided into 2 groups of 15 rats each. Rats in group XI (n=15), or the control group, had their bladders instilled with saline for 120 min. The treatment group, group XII (n=15) had their bladders instilled with 400 μM of EGCG for 120 min. After the first 60 min, the bladders were emptied and refilled with control or treatment solution for the remaining 60 min.

Phase 4 consisted of four groups. Thirty minutes post bladder injury and tumor cell instillation, the tumor cell suspension was gently expelled and the bladders were instilled with 0.5-1.0 ml of the treatment solution. Group XIII (n=10), or the control group, involved the instillation of a 0.9% normal saline solution. Group XIV (n=10) involved the instillation of 400 μM EGCG. Group XV (n=9) involved the instillation of 0.5 μM of MMC and group XVI (n=10) involved the instillation of 400 μM of MMC. The dwell time for all groups was 2 h; the bladders were gently emptied after the first 60 min and re-filled with fresh treatment solution for the remaining 60 min. At the end of the treatment time the bladders were gently expressed and gently rinsed with normal saline. All rats in phase 4 were euthanized 4 weeks after treatment for direct examination of the bladder.

All animals were euthanized by CO_2 inhalation. The bladders were removed, fixed in 10% phosphate-buffered formalin for 24 h and then bisected. The bladders were examined grossly and photographed. The bladders were removed, placed in 10% phosphate-buffered formalin solution and fixed. They were hemisected with a razor, examined under x4 magnification and sent for histopathological sectioning.

Molecular modeling. Two dimensional structure of EGCG was created by AccelrysDraw v. 4.0 in 'skc' format. 2D structure was converted into three dimensional and 'pdb' format file

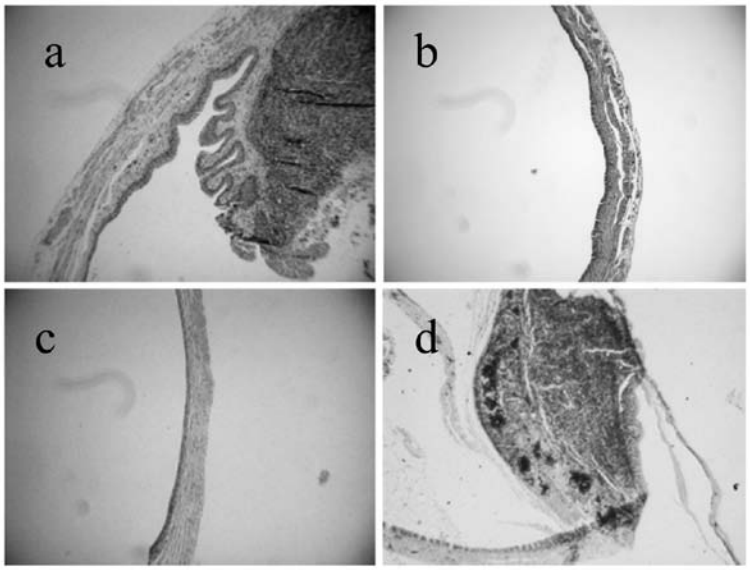

Figure 1. Typical microscopic appearance (reduced from x40) of: (a) control bladder with tumors; (b) tumor-free bladder treated with 400 μM of EGCG; (c) tumor-free bladder treated with 400 μM of MMC; (d) bladder with tumors treated with 400 μM of MMC.

by web based program (http://www.molecular-networks.com/products). Dockings of EGCG to proteins were done by VINA Autodock (30). Protein structures and DNA sequences were downloaded from http://www.rcsb.org/pdb/home/home.do as: **2ovx** - MMP-9 (31), **2vnt** - urokinase (32). Search box was set up with following parameters for: MMP-9 (center: x = 27, y = 6, z = 51; size: x = 40, y = 56, z = 40), urokinase (center: x = -37, y = 171, z = 70; size: x = 40, y = 40, z = 40). Inhibitors present in pdb structures were used to determine center of search and later removed from structure. Small molecules were kept flexible by allowing rotation around single bonds. By default, VINA Autodock analyzes eight different protein/inhibitor complexes (different conformers and the one with the lowest free energy is considered the most probable. Free energy can be converted to K_i by the below formula (30,33-36):

$$K_i = \exp(\Delta G/(R*T))$$

The final analyses of structures generated by Autodock and figures generation were done utilizing PyMOL v. 1.4. (37,38). DNA sequences were downloaded from databases (39,40).

Statistical analysis. Tumor implantation and growth between the groups was assessed by Fisher's 2-way exact test.

Results

In vivo studies. Overall similarity between human and rat uPA is 77% and differs by two amino acids in specificity pocket where uPA inhibitors bind (8,13,39) and 79% similarity in case of MMP-9 (40). Thus we expect that data collected on rats will be applicable in humans.

Tumor growth was observed in both control groups (100%) (Table I). While concentration of EGCG and time of treatment increases, tumor growth ratio decreases, statistically significant in comparison with control (groups IV-VIII, $p \leq 0.03$). In highest concentration of EGCG and longest time of treatment we observed no tumor growth at all in 10 of 10 animals ($p<0.0001$). Pretreat animals with EGCG demonstrated 100% tumor growth (phase 2, Table II). Similarly, there was no effect on tumor growth in group of animals treated with EGCG 2 weeks after establishment of early stage bladder cancer, all 15 animals had tumor growth (phase 3, Table III). In phase 4 animals were treated with EGCG and MMC (Table IV). A statistically significant difference was seen between: control and EGCG treated ($p<0.0001$), control and MMC treated groups (400 μM, $p<0.0007$; 0.5 μM $p<0.0325$).

Table I. Tumor growth in animals of control group and treated with different concentration of EGCG as a function of time.

Group	EGCG in μM	Dwell time (min)	Tumor growth ratio
I	0	60	10/10
II	0	120	10/10
III	100	60	7/10
IV	100	120	5/10
V	200	60	4/10
VI	200	120	2/10
VII	400	60	2/10
VIII	**400**	**120**	**0/10**

Table II. Tumor growth ratios in animals pretreated with EGCG.

Group	EGCG in μM	Dwell time (min)	Tumor growth ratio
IX	0	120	14/15
X	400	120	15/15

Table III. Tumor growth ratios, treated with EGCG 2 weeks post tumor initiation.

Group	EGCG in μM	Dwell time (min)	Tumor growth ratio
XI	0	120	15/15
XII	400	120	15/15

Table IV. Tumor growth ratios in animals treated with EGCG and MMC.

Group	Treatment solution	Concentration (μM)	Tumor growth ratio
XIII	NS	0	10/10
XIV	**EGCG**	**400**	**0/9**
XV	MMC	0.5	5/10
XVI	MMC	400	2/10

Molecular modeling. Fig. 2 shows that EGCG occupies space where MMP-9 inhibitor 5-(4-phenoxyphenyl)-5-(4-pyrimidin-2-ylpiperazin-1-yl)pyrimidine-2,4,6(2h,3h)-trione is present (31). Calculated affinity of EGCG is -8.1 kcal/M or 1.20 μM.

EGCG is located in the specificity pocket of urokinase superimposed over uPA inhibitor: 1-({4-chloro-1-[(diamino-methylidene)amino]isoquinolin-7-yl}sulfonyl)-d-proline (32). Calculated affinity of EGCG to uPA is -7.9 kcal/M or 1.67 μM.

Discussion

Prediction of disease outcome in superficial transitional cell carcinoma of the bladder in an individual patient is still impossible. It has been recommended that in patients the initial treatment, transurethral resection of bladder tumor (TURBT) should be followed by immediate intravesical instillation with mitomycin C to prevent a tumor cell implantation during TURBT. Drug efficacy has been clearly demonstrated in short term, but the long term effects remain a question (41,42). Therefore, there exist a need for a novel agent to be used as an adjunct to endoscopic bladder tumor resection.

Wang *et al* observed a protective effect on bladder cancer in group of green tea drinkers of Asian people, but did not proposed the biological mechanism (43). Contrary to that Wu *et al* found no relationship between drinking green tea and bladder cancer risk (44) despite numerous reports on cancer preventing effects of EGCG *in vitro* and *in vivo* (45-47). That may be related to fact that the concentrations of EGCG used in some *in vitro* and *in vivo* experiments (20-100 μM) are much higher than the plasma and tissue concentrations observed in humans after drinking a few cups of green tea (mostly <0.5 μM) (48). An additional complicating factor in assessment of effects of EGCG on bladder cancer is consumption of large amounts of fruits and vegetables by Asian population. Kamat and Lamm found that even in a high-risk group such as nuclear-bomb survivors in Japan, high consumption of vegetables and fruits is protective against bladder cancer (49).

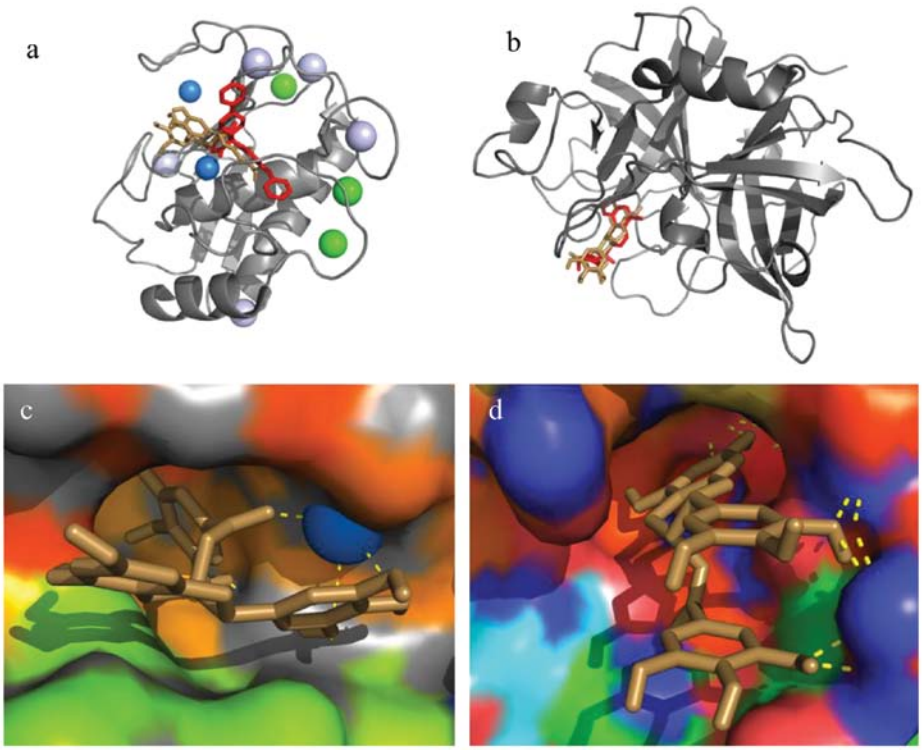

Figure 2. Ribbon model of: (a) MMP-9/inhibitrors; (b) uPA/inhibitors; surface of active site of: (c) MMP-9; (d) uPA. Inhibitors of MMP-9 and uPA are shown as sticks in red. EGCG is shown as stick model in light brown. Ions in MMP-9 are shown as speres: Zn^{2+} in blue, Ca^{2+} in light blue, Cl^{-} in green. Polar contacts are shown as a yellow doted lines.

Intravesical instillation EGCG into bladder permits choice of concentration and time of treatment and is free of these uncertainties. Kemberling *et al* (1) observed cell lethality of the AY-27 cell line at concentrations >100 μM. In the same study they found that intravesical instillation of EGCG inhibits the growth of AY-27 rat transitional cells implanted in Fisher rats. Selman and Keck studied EGCG and mitomycin C and on their *in vitro* and *in vivo* effects using AY-27 cells. They found that at 72 h after exposure to MMC there was 100% lethality at all doses >30 μM, treatment with EGCG reduces the number of AY-27 cells by ~80% at the 200- and 400-μM concentrations. *In vivo* studies using the same animal model there was 100% tumor growth in control treated animals (10 of 10), no tumor growth after 4 weeks in animals treated with EGCG (0 of 10) and no tumor growth in 8 of 10 animals treated with MMC (28). This study is continuation of our previous experiments done in the same laboratory on larger number of animals and in different concentrations of EGCG and MMC. We found the same results as previously for highest concentration of EGCG (tumor-free in 9 of 9 animals) and MMC (tumor-free in 8 of 10 animals). This raises the question why MMC superior in cell killing in the cell culture is inferior to EGCG in animal studies. Both are lethal to cancer cells but it seems that some other mechanism is more relevant in this case. One of the possibilities is that EGCG prevents cancer cell implantation into the bladder wall. As we stated in the introduction EGCG inhibits MMP-9 and uPA proteolytic enzymes critical for cell implantation. Maeda-Yamamoto *et al* reported that EGCG inhibited completely MMP-9 activity at concentration of 40 μM (50) while Demeule *et al* reported that EGCG inhibited 80% activity at concentration of 30 μM (51). Our molecular modeling calculations provides evidence that EGCG binds to the active site as a competitive inhibitor with Ki within range reported above. EGCG inhibits uPA activity in the μM level and the molecular modeling calculation also suggest competitive mechanism in the μM range (13-15). There are some reports that EGCG down regulates expression of both proteases (11,12,15,51-54). End result will be the same, lower proteolytic activity and less probable cancer cell implantation. However, this mechanism seems to be less probable since treatment was limited to two hours and these proteases are already synthetized in cancer cells.

Our data show the slightly better effects of EGCG than MMC in this experimental model. Since mitomycin C requires special precautions during handling and has side effects (55,56) we propose use of EGCG as an agent to decrease tumor cell implantation and consequent intravesical cancer growth in a bladder. EGCG is a potential novel therapeutic strategy for its use as an adjunct to endoscopic bladder tumor resection.

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