A Comparative Study of the Inhibiting Effects of Mitomycin C and Polyphenolic Catechins on Tumor Cell Implantation/Growth in a Rat Bladder Tumor Model

Steven H. Selman* and Rick W. Keck

From the University of Toledo Medical Center, Toledo, Ohio

Abbreviations and Acronyms

EGCG = (−)-epigallocatechin-3-gallate

FBS = fetal bovine serum

MMC = mitomycin C

PBS = phosphate buffered saline

PE = Polyphenon E

RPMI = Roswell Park Memorial Institute

Submitted for publication September 27, 2010.

Study received University of Toledo institutional animal care and use committee approval.

Supported by the Stranahan Endowment for Oncological Research, Geiger Foundation and Polyphenon E International, Inc.

* Correspondence: Department of Urology, University of Toledo Medical Center, Toledo, Ohio 43614 (e-mail: Steven.Selman@utoledo.edu).

Purpose: Mitomycin C (Novaplus®) is often instilled intravesically in the postoperative period to prevent tumor cell implantation/regrowth after transurethral tumor resection. In an earlier study EGCG prevented tumor cell implantation/growth in an experimental bladder tumor model simulating clinical transurethral bladder resection. We compared the efficacy of EGCG (Polyphenon E®) to that of mitomycin C to prevent tumor cell implantation/growth in this model.

Materials and Methods: Mitomycin C and EGCG were studied for their in vitro and in vivo effects. The AY-27 rat urothelial tumor cell line was used for in vitro and in vivo studies. In vitro cell viability studies included trypan blue exclusion, MTT proliferation assay and clonal growth assay. Fischer 344 female rats were used for intravesical tumor implantation/growth assay using an electrocautery injury model. Tumor growth in vivo was assessed in controls treated with phosphate buffered saline and in bladders treated with mitomycin C or EGCG by standard histological techniques using hematoxylin and eosin 4 weeks after injury.

Results: Mitomycin C and EGCG showed cytotoxicity on all in vitro assays. They were equivalent for preventing intravesical tumor growth.

Conclusions: EGCG prevents intravesical tumor growth with efficacy equivalent to that of mitomycin C in this experimental model.

Key Words: urinary bladder, urinary bladder neoplasms, epigallocatechin gallate, mitomycin, polyphenols

Low grade Ta bladder tumors recur at a rate of 50% to 70% and progress in approximately 5% of cases while high grade T1 lesions recur in more than 80% and progress in 50% within 3 years.[1] Tumor implantation at endoscopic resection may in part be responsible for bladder tumor recurrence.[2] Current practice guidelines for endoscopic bladder tumor resection recommend the instillation of chemotherapeutic agents in the perioperative period to decrease the risk of tumor recurrence.[3]

MMC is used most often in this clinical setting. Introducing MMC directly into the bladder is not without risk, including pain, bleeding, bladder calcification and even death. Also, MMC handling requires special precautions. Less toxic agents would be inherently more appealing for clinical application.[1]

EGCG as an intravesical instillation agent to prevent tumor growth was reported in an experimental model.[4] EGCG, a catechin polyphenol found in green tea, has no known toxicity and

0022-5347/11/1862-0702/0
THE JOURNAL OF UROLOGY®
© 2011 by American Urological Association Education and Research, Inc.

Vol. 186, 702-706, August 2011
Printed in U.S.A.
DOI:10.1016/j.juro.2011.03.125

requires no special precautions in handling or disposal. We compared the efficacy of EGCG (PE) to that of MMC to prevent rat AY-27 urothelial tumor cell implantation/growth after intravesical injury.

MATERIALS AND METHODS

In Vitro Studies

Cell line. AY-27 rat urothelial derived tumor cells[5] were maintained in RPMI 1640 medium with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich®), and 10% FBS (Atlanta Biologicals, Lawrenceville, Georgia).

Pharmaceuticals. Solutions of increasing molarity were prepared by diluting MMC in RPMI 1640 medium. PE was obtained as a mixture of catechins, including epicatechin, epicatechin gallate, epigallocatechin, gallocatechin gallate and EGCG. The total EGCG content in this mixture was determined to be 63% and treatment solution concentrations were based on this percent. For in vitro studies stock solutions were prepared by dissolving PE in RPMI. PE solutions were adjusted to pH 5.0.

Trypan blue viability assay. Cell culture tubes containing 1×10^6 cells were prepared from stock cultures of the AY-27 tumor cell line maintained at our laboratory. Increasing concentrations of EGCG (0, 22, 43, 86, 173 and 345 μM) or MMC (0, 0.03, 0.3, 3.0, 30, 300 and 3,000 μM) diluted in 0.5 ml RPMI medium were added to each tube and incubated at 37C for 120 minutes. Upon the completion of incubation the treatment solutions were replaced with 1.0 ml complete RPMI medium plus 10% FBS plus Pen/Strep (Invitrogen™) and placed in an incubator at 37C. Before trypan blue exclusion the tubes were gently mixed and 0.1 ml cell suspension was added to 0.4 ml 0.4% trypan blue. Cell count and viability were determined using a hemocytometer. Percent viability was determined immediately after incubation with MMC or EGCG, and again at 1 and 2 hours.

MTT cell proliferation assay. A stock culture of AY-27 tumor cells was trypsinized and 5×10^4 cells in 50 μl were dispensed per well of 96-well tissue culture plates. The plates were placed in an incubator at 37C for 24 hours to allow cell attachment. The cells were treated with increasing concentrations of MMC (0, 0.03, 0.3, 3.0, 30, 300 and 3,000 μM) or EGCG (0, 12.5, 25, 50, 100, 200 and 400 μM) diluted in RPMI medium. Treatment volume was 100 μl per well. Experiments were run in triplicate. After 2-hour incubation the treatment solution was removed, each well was rinsed with RPMI and 100 μl fresh RPMI medium plus 10% FBS plus Pen/Strep was added per well. The plates were placed in an incubator at 37C for 72 hours. After 72 hours 15 μl MTT Dye Solution (Promega®) was added to each well. The plates were placed in the incubator for 4 hours to allow cellular conversion of tetrazolium salt to a formazan product. At the end of 4 hours of incubation 100 μl Solubilization Solution/Stop Mix (Promega) were added to each well. The plates were placed in the incubator overnight to allow the formazan crystals to completely solubilize. Absorbance at 570 nm was measured and recorded using a 96-well plate reader.

Clonal assay. A stock culture of AY-27 tumor cells was trypsinized and 100 cells were added to each well of 6-well tissue culture plates. The plates were placed in an incubator at 37C for 24 hours to allow cell attachment. Medium was removed and the plates were treated with 3 ml MMC (0, 0.03, 0.3, 3, 30, 300 and 3,000 μM) or EGCG (0, 22, 43, 86, 173 and 345 μM) per well with incubation for 2 hours. Each concentration was performed in triplicate. After 2-hour incubation the treatment solution was removed, each well was rinsed with RPMI and 3 ml fresh RPMI medium plus 10% FBS plus Pen/Strep was added to each well. The plates were placed in an incubator at 37C for 4 days. After 4 days the cells were fixed and stained with 0.4% Giemsa stain. Under 4× magnification the number of colonies and the number of cells per colony in 5 random microscopic fields per well were counted and averaged in triplicate.

In Vivo Studies

Animal/tumor model. All experiments using animals in this study were approved by the University of Toledo institutional animal care and use committee, and done under the guidance of the Department of Laboratory Animal Resources. Female Fischer 344 rats (Charles River, Wilmington, Massachusetts) were used in conjunction with the transplantable AY-27 rat bladder tumor cell line maintained at our laboratory.

Tumor initiation. Animals anesthetized by isoflurane gas inhalation underwent surgery to expose the bladder via a small midline incision of the lower abdomen to ensure proper cautery placement and visualization of the cauterization procedure. A sterile 22 gauge polytetrafluoroethylene intravenous catheter was advanced transurethrally to achieve bladder access for cauterization, and instillation of tumor cells and treatment solutions. A blunt tipped 24 gauge copper wire was advanced through the catheter. Six mV current were used to cauterize the bladder wall, creating a small white puckered area. The skin and muscular layers of the abdominal wall were closed in 2 layers with nylon suture. After bladder injury a suspension of 1×10^6 tumor cells in 0.5 ml RPMI medium was instilled through the catheter. The catheter was capped and tumor cells were allowed to dwell in the bladder for 30 minutes.[4]

Treatment solutions. Treatment solutions were prepared by diluting MMC and EGCG in PBS. The EGCG concentration of treatment solutions were based on the total EGCG content (63%) in the PE used. EGCG and PBS control solutions were adjusted to pH 5.0. The concentration of the EGCG and MMC treatment solutions was 400 μM. The 400 μM dose of MMC was 40 times the lowest dose (10 μM) of MMC needed in vitro to cause 100% cell death. Higher concentrations of MMC closer to the values used clinically (1 mg/ml) were not used since higher concentrations cause extensive bladder mucosal and submucosal injury.

Intravesical treatment. At 30 minutes after tumor cell instillation the bladders were gently emptied by uncapping the urethral catheter. A total of 30 animals were divided into 3 groups of 10 each, including control group 1—PBS, group 2—EGCG and group 3—MMC. Emptied bladders were instilled with 0.5 to 1.0 ml treatment solu-

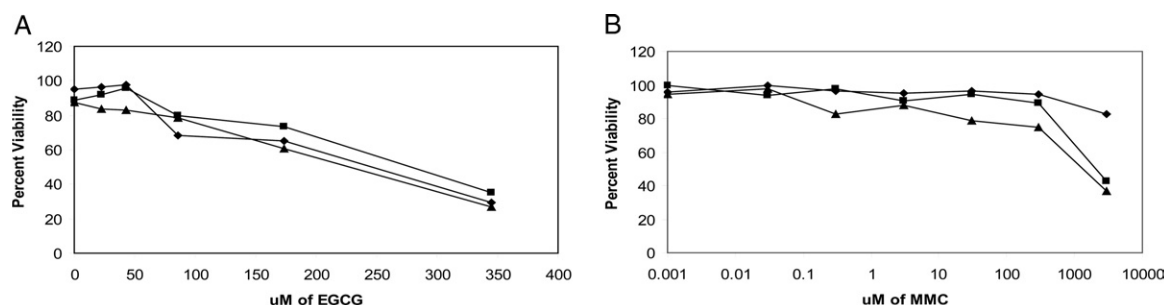

Figure 1. Trypan blue assay shows AY-27 cell percent viability immediately (diamonds), and 1 (squares) and 2 (triangles) hours after initial 120-minute incubation with various EGCG (*A*) or MMC (*B*) concentrations. *uM*, μM.

tion for 120 minutes, gently emptied after the first 60 minutes and refilled with fresh treatment solution for the remaining 60 minutes. At treatment end the bladders were emptied and gently rinsed with normal saline, and the catheters were removed.

All rats were sacrificed 4 weeks after treatment by CO_2 inhalation. The bladders were filled with 10% buffered formalin, ligated at the bladder neck, removed and placed in 10% phosphate buffered formalin for 24 hours. After fixation the bladders were bivalved longitudinally, photographed and examined grossly under a dissecting microscope at 10× to 30× magnification for evidence of tumor growth. Any tumor growth or suspicious areas were noted and marked with India ink. The specimens were submitted for histological sectioning and staining with hematoxylin and eosin, and evaluated in blinded fashion for evidence of tumor growth.

Statistics

Tumor implantation and growth between the groups was assessed by Fisher's 2-way exact test. Post hoc determination of significance was determined by Bonferroni analysis.

RESULTS

In Vitro Studies

Trypan blue assay. After 2-hour incubation with EGCG there was an increase in cell death as the concentration of EGCG increased (fig. 1, *A*). There was little change in percent viability after the 2-hour observation. There was minimal cell death immediately after the 2-hour incubation with MMC (fig. 1, *B*). Only after 1 and 2 hours after incubation with MMC was there a decrease in cell viability at the higher concentrations (300 and 3,000 μM).

MTT cell proliferation assay. At 72 hours after exposure to MMC there was 100% lethality at all doses greater than 30 μM (fig. 2). At 72 hours of treatment with EGCG the number of AY27 cells decreased by about 80% at the 200 and 400 μM levels.

Clonal assay. Four days after EGCG treatment there was a decrease in colony density and colony size compared to those of the control at all concentrations (fig. 3, *A*). At 44 μM the number of colonies decreased to about 20% while the number at higher concentrations decreased to about 6%. Colony size decreased to 3% to 5% of control size in all treatment groups. In MMC treated groups colony size also decreased with no survival at 3.0 μM or above (fig. 3, *B*). At 0.3 μM MMC there was about 55% survival with colonies about 35% the size of control colonies.

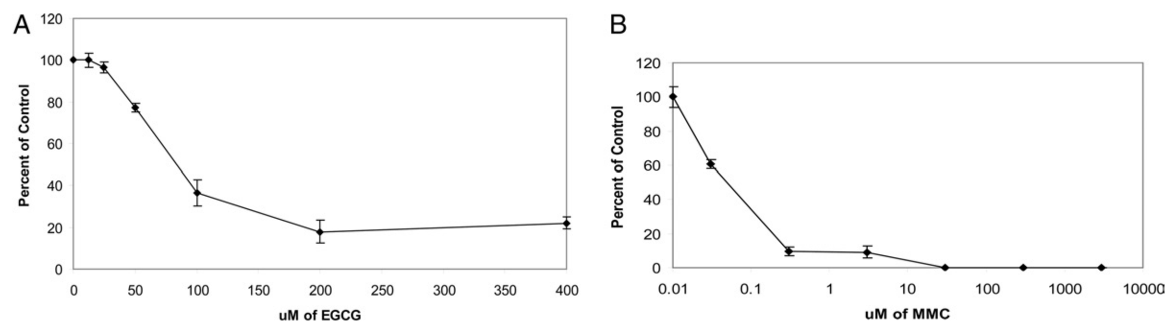

Figure 2. MTT cell proliferation assay was run in triplicate. AY-27 cells were cultured in 96-well plates and incubated with various EGCG (*A*) or MMC (*B*) concentrations for 2 hours. MTT assay was done 72 hours after incubation. Percent of control was calculated for each concentration and plotted as mean ± SE. *uM*, μM.

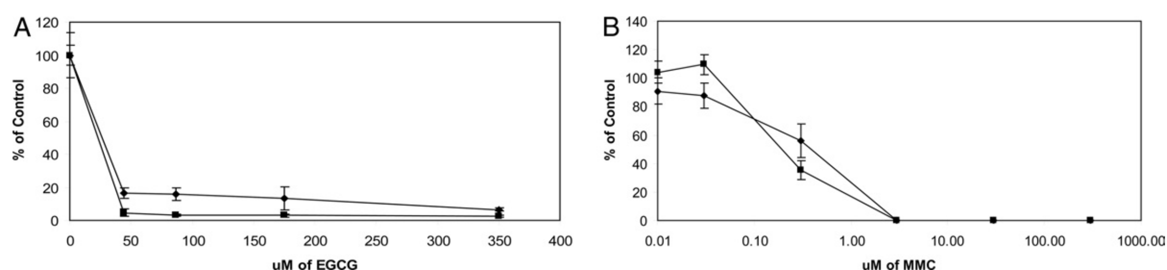

Figure 3. Clonal assay of cultured AY-27 cells treated with various EGCG (*A*) or MMC (*B*) concentrations for 2 hours and incubated for 4 days. Average numbers of colonies and cells per colony per EGCG or MMC concentration were compared to control. Percent of control was determined and plotted as mean ± SE. Diamonds represent colony density. Squares represent colony size. *uM*, μM.

In Vivo Studies

There was 100% tumor growth in control treated animals (10 of 10). There was no tumor growth after 4 weeks in animals treated with PE (0 of 10) and decreased tumor growth in animals treated with MMC (2 of 10). There was a statistically significant difference between the PBS treated group, and the groups treated with EGCG ($p < 0.001$) and MMC ($p < 0.001$). However, there was no significant difference between the EGCG and MMC groups.

Figure 4 shows representative examples of the gross appearance of the bladders and corresponding histology. Figure 4, *A* shows an example of the gross appearance of a rat bladder with obvious tumor growth. Figure 4, *B* shows the uniformity of the bladder wall thickness of a tumor-free bladder.

DISCUSSION

The putative active polyphenol in green tea, EGCG, has been investigated in a number of cell culture system for its growth inhibiting and cytotoxic properties. Recent reports were directed toward its potential use for bladder cancer. Sato reported the inhibition of bladder tumors induced by N-butyl-N-(4-hydroxybutyl)-nitrosamine in rats fed green tea.[6] Kemberling et al noted that EGCG caused apoptosis in the AY-27 cell line and prevented intravesical tumor growth in a bladder injury model.[4] Chen et al observed growth inhibition of NBT-II bladder tumor cells in a dose and time dependent manner with G0/G1 arrest in cells cultured with EGCG.[7] EGCG also down-regulated protein expression of cyclin D1, cyclin-dependent kinase 4/6 and phosphorylated retinoblastoma protein. Using T24 human bladder cancer cells Qin et al found that EGCG inhibited phosphatidylinositol 3′-kinase/Akt activation, leading to the modulation of Bcl-2 family of proteins and leading to apoptosis.[8] Philips et al studied the effects of catechins, including EGCG, on normal urothelium, and low and high grade urothelial cancer.[9] Apoptosis was induced and there was differential gene expression in normal and tumorigenic cell lines. Finally, Sagara et al reported that green tea polyphenols inhibited growth and invasion in mice with established bladder cancer through angiogenesis regulation.[10]

Current practice guidelines encourage intravesical agents in the perioperative period to decrease the rate of bladder tumor recurrence after endoscopic resection.[3] Mitomycin has been widely used in this setting. Mitomycin is not without side effects and it requires special precautions during handling.[11,12] We have been investigating EGCG as an agent to decrease tumor intravesical growth in a bladder tumor model. Our data show the equivalency of EGCG and MMC in this experimental model.

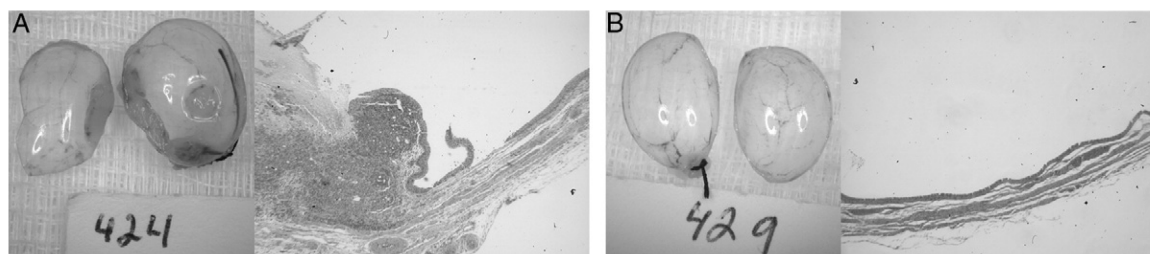

Figure 4. Typical gross and microscopic appearance of PBS control bladder with tumors (*A*) and tumor-free bladder with 400 μM EGCG (*B*). H & E, reduced from ×40.

CONCLUSIONS

EGCG has no apparent toxicity. It is water soluble and does not appear to damage normal rat urothelium. It appears that EGCG is a promising agent to pursue for its use as an adjunct to endoscopic bladder tumor resection.

REFERENCES

1. Jones JS and Campbell SC: Non-muscle-invasive bladder cancer (Ta, T1 and CIS). In: Campbell-Walsh Urology, 9th ed. Edited by AJ Wein, LR Kavoussi, AC Novick et al. Philadelphia: Saunders Elsevier 2007; vol 3, pp 2447–2467.
2. Weldon T and Soloway MS: Susceptibility of urothelium to neoplastic cellular implantation. Urology 1975; **5:** 824.
3. Guideline for the Management of Nonmuscle Invasive Bladder Cancer: (Stages Ta,T1, and Tis): Update (2007). Available at http://www.auanet.org/content/guidelines-and-quality-care/clinical-guidelines.cfm. Accessed September 13, 2010.
4. Kemberling JK, Hampton JA, Keck RW et al: Inhibition of bladder tumor growth by the green tea derivative epigallocatechin-3-gallate. J Urol 2003; **170:** 773.
5. Xiao Z, McCallum TJ, Brown KM et al: Characterization of a novel transplantable orthotopic rat bladder transitional cell tumor model. Br J Cancer 1999; **81:** 638.
6. Sato D: Inhibition of urinary bladder tumors induced by N-butyl-N-(-4-hydroxybutyl) nitrosamine (BBN) in rats by green tea. Int J Urol 1999; **6:** 93.
7. Chen JJ, Ye ZQ and Koo MWL: Growth inhibition and cell cycle arrest effects of epigallocatechin gallate in the NBT-II bladder tumor cell line. BJU Int 2004; **93:** 1082.
8. Qin J, Xie LP, Zheng XY et al: A component of green tea, (−)-epigalllocatechin-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 Comm 2007; **354:** 852.
9. Philips BJ, Coyle CH, Morrisroe SN et al: Induction of apoptosis in human bladder cancer cells by green tea catechins. Biomed Res 2009; **30:** 207.
10. Sagara Y, Miryata Y, Nomata K et al: Green tea polyphenol suppresses tumor invasion and angiogenesis in N-butyl-(-4-hydroxybutyl) nitrosamine-induced bladder cancer. Cancer Epidemiol 2010; **34:** 350.
11. Nieuwenhuijzen JA, Bex A and Horenblas S: Unusual complication after immediate postoperative intravesical mitomycin C instillation. Eur Urol 2003; **43:** 711.
12. Racioppi M, Porreca A, Foschi N et al: Bladder perforation: a potential risk of early endovesical chemotherapy with mitomycin C. Urol Int 2005; **75:** 373.