Effect of a Prodrug of the Green Tea Polyphenol (-)-Epigallocatechin-3-Gallate on the Growth of Androgen-Independent Prostate Cancer In Vivo

Suk-Ching Lee, Wing-Ki Chan, and Tak-Wing Lee
Department of Anatomy, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, HKSAR, China

Wai-Har Lam
Department of Applied Biology and Chemical Technology and the Institute of Molecular Technology for Drug Discovery and Synthesis, the Hong Kong Polytechnic University, Hong Kong SAR, China

Xianghong Wang
Department of Anatomy, University of Hong Kong, Hong Kong, HKSAR, China

Tak-Hang Chan
Department of Applied Biology and Chemical Technology and the Institute of Molecular Technology for Drug Discovery and Synthesis, the Hong Kong Polytechnic University, Hong Kong SAR, China

Yong-Chuan Wong
Department of Anatomy, University of Hong Kong, Hong Kong, HKSAR, China

Epigallocatechin-3-gallate (EGCG) is the major and most potent polyphenol compound of green tea that has been shown to have anticancer effects against various types of cancers. In this study, in addition to the EGCG compound, a synthetic derivative, the per-acetate of EGCG (EGCG-P), was used to investigate the inhibitory effects on growth of androgen-independent prostate cancer in vivo. The advantage of EGCG-P is that it may act as a prodrug, leading to higher bioavailability than EGCG itself. The aim of our study was to compare the differences between EGCG and EGCG-P on their inhibitory effect on androgen-independent prostate cancer, CWR22R, xenograft model in nude mice. The mice were administrated daily with solvent dimethyl sulfoxide, EGCG, and EGCG-P separately through intraperitoneal injection for 20 days. Tumor volume and body weight of nude mice were recorded daily. Serum prostate-specific antigen (PSA) levels were also measured before and after the treatment. The effects of both EGCG and EGCG-P on tumor cell proliferation were assessed by immunohistochemical (IHC) method using antibodies against Ki-67 and proliferating cell nuclear antigen. The apoptotic effect was evaluated by IHC against B-cell non-Hodgkin lymphoma-2 and terminal deoxynucleotidyl transferase dUTP nick-end labeling assay by in situ apoptosis detection kit. Moreover, the potential suppression of angiogenesis by EGCG and EGCG-P on prostate cancer was examined by IHC against CD31. Our results revealed that treatment of EGCG and EGCG-P compounds suppressed the growth of CWR22R xenografts without causing any detectable side effects in nude mice. The suppression of growth of the tumor was correlated with the decrease of serum PSA level together with the reduction in tumor angiogenesis and an increase in apoptosis on prostate cancer cells. The results showed that treatment of EGCG and EGCG-P inhibited tumor growth and angiogenesis while promoting apoptosis of the prostate cancer cells in vivo. Our results suggest that EGCG-P may be a more stable and useful compound for increasing the therapeutic anticancer effects in androgen-independent prostate cancer.

INTRODUCTION

Prostate cancer remains one of the most common malignancies diagnosed in men and the second leading cause of male mortality in Western countries (1). Nowadays, many patients without proven metastases are receiving some form of androgen deprivation therapy (ADT), such as luteinizing hormone-releasing hormone superagonist or an antiandrogen. ADT is increasingly utilized in conjunction with radiotherapy for patients in low- and intermediate-risk categories. For patients with high-risk, localized prostate cancer who choose radiotherapy, a combination of ADT and radiation therapies has become the standard of care (2). Chemotherapy with cytotoxic agents has
been tried on metastatic prostate cancer, either alone or in combination, both in primary therapy and in relapsed cases (3). However, some studies have concluded that hormone-refractory prostate cancer had limited response to cytotoxic agents (2,3).

According to the World Cancer Report, the incidence rate of prostate cancer in the United States is 104.3 per 100,000, with similar rates in many Western countries (4). On the other hand, the corresponding incidence rate is only 1.7 in China and about 10 in Japan (5). It has been suggested that dietary or environmental factors may account for the difference. In Japan, a case-controlled study supported the hypothesis that the traditional Japanese diet, rich in soybean products and fish, may be protective against prostate cancer (6). Another case-controlled study in Southeast China found that the prostate cancer risk declined with increasing frequency, duration, and quantity of green tea consumption (7).

Green tea is an aqueous infusion of dried unfermented leaves, derived from the plant *Camellia sinensis* (Theaceae), which is a popular beverage in China and Japan. It contains many polyphenolic compounds, and the most abundant constituents are flavonols, commonly known as catechins (8). Of the several catechins present in green tea, including epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate (EGCG), EGCG (Fig. 1, Panel 1) is the major constituent present in green tea, accounting for about 50% of the total polyphenols. The biological activity of green tea has been attributed mainly to EGCG. It has been suggested that green tea has a protective effect against the development of prostate cancer (8). The inhibition of tumor progression by green tea was associated with reduced tumor cell proliferation, tumor angiogenesis, and decline in prostate-specific antigen (PSA) levels (9). EGCG treatment of lymph node carcinoma of the prostate (LNCaP) cells induced apoptotic cell death by changes in nuclear morphology and DNA fragmentation (10). Also, it has been shown to induce apoptosis by inhibiting fatty acid synthase (11). Recently, some studies have found that EGCG can inhibit cyclooxygenase-2 (COX-2) without affecting COX-1 expression at both the protein and messenger RNA levels in androgen-sensitive LNCaP and androgen-insensitive PC-3 human prostate carcinoma cells (12). It has been suggested that green tea may play a role in chemoprevention or therapy of prostate cancer in the future (13). In a study of the effect of green tea on an animal tumor model, known as transgenic adenocarcinoma of the mouse prostate (TRAMP), oral administration of green tea to mice with TRAMP showed decline of overall incidence of tumor development, decreased tumor burden, and decreased incidence of distant metastases compared with the control group (14). However, in spite of these encouraging in vitro and animal data, a phase II clinical trial of green tea in the treatment of patients with androgen independent metastatic prostate carcinoma gave disappointing results (15). It was concluded that green tea carries a limited antineoplastic activity among patients with androgen-independent prostate carcinoma (15). However, because green tea powder containing caffeine was used as the drug in the clinical trial, and there was no study on the effect of pure EGCG on an animal model of androgen-independent prostate cancer in vivo, it was not clear whether the disappointing result of the clinical trial was due to the inherent lack of activity of EGCG or the use of insufficient dose and duration of the drug or other factors. We were interested, therefore, to undertake a study of the effect of EGCG on the growth of androgen-independent prostate cancer in an animal model.

One of the disadvantages of EGCG is its poor bioavailability. The low bioavailability was thought to be partly due to the poor stability of EGCG in alkaline or neutral solutions (16). In fact, the pH values of intestine and body fluid are neutral or slightly alkaline. Green tea catechins will be unstable inside the human body, thus leading to reduced bioavailability of EGCG (17). Another factor may be due to in vivo metabolic transformation of EGCG into various metabolites (18,19). Therefore, the bioavailability of EGCG is greatly reduced in vivo (20). Recently, we demonstrated that (→)-EGCG peracetate (EGCG-P; Fig. 1, Panel 2), a synthetic derivative of EGCG by acetylation can act as a prodrug of EGCG (16). EGCG-P is converted under cellular conditions to EGCG (17), and others have since reported that EGCG-P enhanced the bioavailability of EGCG in vivo (21). Consistently, EGCG-P showed much higher potency than EGCG to inhibit proliferation and transforming activity and to induce apoptosis in human prostate, breast, leukemia, and simian virus 40-transformed cells (22). Therefore, EGCG-P has a potential to be developed into a novel anticancer drug. The aim of this study was to compare the differences between EGCG and EGCG-P on their inhibitory effects on androgen-independent prostate cancer in vivo using the CWR22R xenograft in nude mice.

**MATERIALS AND METHODS**

**CWR22R Animal Model**

CWR22R is a human prostate cancer xenograft originally derived from a prostate cancer patient. It has been passaged in nude mice for many generations. A total of 24 male nude mice of 4 to 5 wk old were used and castrated via scrotal approach 2 days before tumor inoculation. About 1 mm³ of CWR22R tumor tissue was inoculated subcutaneously into each nude mouse.
Drugs

EGCG was obtained from Sigma (St. Louis, MO). EGCG-P was synthesized from (-)-EGCG as described (17). The drugs were dissolved in dimethyl sulfoxide (DMSO) (Sigma) and stored at 4°C. The EGCG stock concentration was 20 mg/ml, and the dose used in mice was 50 mg/kg. Because the molecular weights of EGCG and EGCG-P were different, 458.4 and 794.5, respectively, the equivalent concentration of EGCG-P in DMSO was 34.7 mg/ml, and the dose used in nude mice was 86.7 mg/kg.

Treatment Strategy and Collection of Samples

Treatment of animals began 7 days after inoculation to allow time for establishment of tumors. The sizes of tumors at the beginning of the treatment experiment were also determined. We randomly divided 24 castrated mice with xenografts into 3 groups: DMSO, EGCG, and EGCG-P. Each group was injected intraperitoneally (ip) daily with DMSO solvent (2.5 groups: DMSO, EGCG, and EGCG-P. Each group was injected intraperitoneally (ip) daily with DMSO solvent (2.5 µl/g of mice body weight), EGCG (20 mg/ml in DMSO and 50 mg/kg dose of mice body weight), and EGCG-P (34.7 mg/ml in DMSO and 86.7 mg/kg dose of mice body weight), respectively, for 20 days. Body weight and tumor volumes of mice were measured daily and recorded. At the end of the experiment, mice were sacrificed by cervical dislocation. Tumors, kidneys, livers, and lungs were harvested and fixed in 4% neutral buffered formalin. Then the tissues were processed and embedded in paraffin blocks. Sections were cut 4 µm thick for histopathological evaluation.

Body weight of mice was recorded daily during the experiment. Tumor volumes were also recorded at the same time by caliper measurement and calculated by the equation tumor volume = length × (width)² × π/6 and subsequently transformed into relative values (vol), vol = Vt/V0, where V0 was the tumor volume at initiation of treatment, whereas Vt was the tumor volume at any given day during the entire treatment period.

Blood samples were drawn from the femoral vein of mice before initiation of drug administration and also at the end of the treatment. The collected blood samples were centrifuged at 800 rpm for 15 min to obtain serum and stored at −70°C. Total serum PSA measurements were made using the Enzyme Immunoassay Kit according to manufacturer’s recommendations (CanAg Diagnostics AB, Gothenburg, Sweden).

Immunohistochemistry (IHC)

IHC staining was performed on 4 µm paraffin sections. For antibodies against Ki-67, B-cell non-Hodgkin lymphoma-2 (Bcl-2), and CD31, the standard avidin-biotin complex (ABC) procedures were employed. For proliferating cell nuclear antigen (PCNA), SuperPicTure™ Polymer Detection kit (Zymed Polymer Detection System, San Francisco, CA) was used. The advantage of using the polymer system was that it could reduce the nonspecific binding due to endogenous biotin activity in the tumor section.

For the ABC method, after deparaffinization and rehydration, the slides were dipped in methanol containing 0.6% hydrogen peroxide for 20 min to block the endogenous peroxidase reaction. Antigen retrieval was carried out by treating the slides with 10 mM citrate buffer, pH 6.0, for 9 min at high to medium powers of a microwave oven. The nonspecific binding of the antibody was blocked by 10% normal horse serum (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA) in Tris-Buffered Saline (TBS). The sections were incubated with mouse antibodies against Ki-67 (clone MM1, 1:300 dilution; Novocastra Laboratories Ltd., Newcastle, United Kingdom), Bcl-2 (C-2, 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and CD31 (JC70A, 1:30 dilution; Dako Corp., Carpinteria, CA) overnight at 4°C in a humidified chamber. Negative controls were run in parallel and treated with TBS instead of a specific antibody solution and under identical conditions. After overnight incubation, the sections were incubated with a diluted biotinylated secondary antibody and then followed by ABC complex reagent. The color of sections was visualized by treatment with dianisobenzidine (DAB; Dako Corp., Produktionsvej 42, Glostrup, Denmark). The sections were counterstained with Mayer’s hematoxylin followed by dehydration, clearing, and mounting.

For the SuperPicTure polymer detection system, the sections were incubated with primary antibody against PCNA (NA03, 1:600 dilution; Calbiochem, Darmstadt, Germany), then horseradish peroxidase polymer was added. The sections were then treated with DAB and finally counterstained with hematoxylin, followed by dehydration, clearing, and mounting.

IHC Detection of Apoptotic Cells by Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick-End Labeling (TUNEL) Staining Method

IHC was performed on the formalin fixed, paraffin embedded, 4 µm sections of tumor tissues by using ApopTag Peroxidase In Situ Apoptosis Detection Kit S7100 (Chemicon International, United States and Canada) following the manufacturer’s protocols. A positive control section, which was treated with 0.5 µg/ml of deoxyribonuclease before the labeling procedures, was included. A negative control section treated with only equilibration buffer instead of TdT enzyme reagent was also included.

Analytic Methods of Experimental Results

The reactivities of Ki-67, PCNA, and TUNEL in the nuclei were quantified in 10 randomly selected fields at ×400 magnification for the 3 groups: DMSO, EGCG, and EGCG-P. The percentage of positive cells was calculated by the number of positive cells/total number of cells ×100.

For Bcl-2, which is located in cytoplasm, 10 randomly selected fields at ×400 magnification from the 3 groups were examined. The scoring grades of positive cells were based on their staining intensity from Grade 0–II. Grade 0 (negative) was defined as no or marginal staining of <5% of stained cells,
Grade I was defined as mild to moderate staining of 5–50% of stained cells, and Grade II was defined as moderate to intense staining of >50% of stained cells.

CD31 is specific for endothelial cells of blood vessels. Microvessel density was estimated by the mean number of vessels stained in 10 random fields under ×400 magnifications. All positive stained individual cells or clustering cells with or without visible lumina were counted as 1 vessel.

Measurement of Serum PSA of Nude Mice

Serum were collected by centrifugation of blood samples and stored at −70°C. Total serum PSA measurements were performed by using the Enzyme Immunometric Assay Kit (CanAg Diagnostics AB, Gothenburg, Sweden) for quantitative determination. The PSA concentrations of samples were calculated from the standard curves generated by using PSA calibrators provided in the kit. All calibrators, controls, and samples were run in duplicated sets. The PSA values of samples were calculated from the calibration curve by the value of mean absorbance of each sample.

Statistical Analysis

All statistical analysis was performed using SPSS version 11.1 software (SPSS Inc., Chicago, IL). Normal distribution was first tested using Kolmogorov–Smirnoff test. Nuclear scores were presented as mean and standard error of the mean. Nuclear score within groups was compared by either analysis of variance or Kruskal–Wallis test, whereas comparison between 2 groups was performed by either Independent T or Mann–Whitney U tests. The associations of cytoplasmic immunostaining and different histological or clinical relevant subgroups were evaluated by Pearson’s χ² test. Differences were considered significant for a P value of <0.05, and all reported P values were 2-sided. For analysis of the tumor volume and PSA levels, the Kolmogorov–Smirnoff test was applied first to test for normal distribution. The statistical significance of difference between control and treated groups, or between the EGCG and p-EGCG treated groups, was determined by Mann–Whitney test or Student’s t-test. A P value of less than 0.05 was as regarded statistically significant.

RESULTS

Effect of EGCG and EGCG-P on the Growth of Androgen-Independent Human Prostate Cancer CWR22R Xenograft

As explained earlier, 24 mice were selected and divided into 3 groups: DMSO, EGCG, and EGCG-P. Solvent (DMSO, control) and drugs (EGCG and EGCG-P) were administrated ip daily for a period of 20 days.

For the DMSO control group, the gross size of tumor was gradually increased during the period studied. For EGCG treated mice, the tumor size was also gradually increased as the control group from Days 1–14. However, from 15–20 days, the growth of tumor was stabilized in a steady state and the final tumor size was significantly (P < 0.05) smaller (36.3%) than the DMSO control group. As for EGCG-P treated mice, the tumor growth rate was much slower than the control group or the EGCG-treated group starting from Day 4, and the eventual tumor size was much smaller (62.9%) than the control (P < 0.001) at the end of experiment (i.e., 20 days), as shown in Fig. 2A. The tumor size of the EGCG-P-treated group was also significantly smaller (40.1%) than the EGCG group (P < 0.05). This suggested that EGCG-P was more effective in suppressing the growth of prostate cancer than the EGCG.
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FIG. 3. Effects of epigallocatechin-3-gallate (EGCG) and the peracetate of EGCG (EGCG-P) on the proliferations of tumor cells in CWR22R xenograft. A: Ki-67. Immunohistochemical micrographs showing immunoreactive positive cells in the control and the 2 treated groups. Note that treatment with EGCG significantly reduced ($P < 0.001$) the Ki-67 index but not when treated with EGCG-P ($P > 0.05$). There is also no difference between the 2 treated groups ($P > 0.05$). B: Proliferating cell nuclear antigen (PCNA). The results show that treatment with EGCG-P effectively reduced the PCNA index ($P < 0.05$) but not when treated with EGCG ($P > 0.05$). The difference between the treated groups is marginally significant ($P = 0.05$). All the data are presented as mean ± standard error of the measurement (SEM); $n = 10$, scale bar = SEM ×400. DMSO, dimethyl sulfoxide.

Clinically, the level of PSA is a significant indicator for the early detection, staging, and diagnosis of prostate cancer (3). In our study, serum PSA level was measured within the 3 groups of mice before and after 20 days treatment. As shown in Fig. 2B, 24 h before the treatment, PSA levels were low for all 3 groups of mice. At the end of 20 days treatment, PSA levels were increased in all 3 groups. However, the PSA levels of EGCG- and EGCG-P-treated groups were significantly lower than the control group (both $P < 0.05$). EGCG-P showed a slightly higher suppression effect on PSA levels than the EGCG group, but the difference was not statistically significant ($P > 0.05$). Thus, both EGCG and EGCG-P are effective in suppressing the growth of tumor concurrent with a decrease in serum PSA levels.

Toxicity Assessment of EGCG and EGCG-P in Nude Mice

The body weights of mice in 3 groups from Day 1 to 20 were recorded daily. The body weights of EGCG- and EGCG-P-treated mice were all maintained at a steady state and showed little changes throughout when compared to the DMSO control group (data not shown). Moreover, the effect of EGCG and EGCG-P on the vital organs, such as kidney, liver, and lung, between the control and treated groups were assessed histologically by hematoxylin and eosin staining. There were no significant morphological alterations or damages to the kidney, liver, and lung between the control and treated groups (data not shown). Therefore, under the limited conditions of dose investigated, the toxic effects of EGCG and EGCG-P to vital organs of nude mice were unremarkable, and no significant structural changes were found.

**Effect of EGCG and EGCG-P on Tumor Cell Proliferation Rate**

To evaluate the suppression effect on the cancer cell proliferation, the tumor markers of Ki-67 and anti-PCNA were studied. As shown in Fig. 3A, there were higher percentages of Ki-67 positive-stained tumor cells nuclei in the DMSO control (39%) as compared to the EGCG (19%) and EGCG-P (32%) groups. The difference between the control and EGCG-treated group was statistically significant ($P < 0.001$) but not between control and EGCG-P-treated groups ($P > 0.05$). There was also no difference between the 2 drug-treated groups ($P > 0.05$).

The results of the PCNA tumor marker are shown in Fig. 3B. The percentages of PCNA positive nuclei in DMSO control (59%) were higher than in EGCG (56%) and EGCG-P (44%) groups. The difference between the control and EGCG-P-treated group was statistically significant ($P < 0.001$) but not between control and EGCG-P-treated groups ($P > 0.05$). There was also no difference between the 2 drug-treated groups ($P > 0.05$).

The results showed some inconsistencies in suppression of tumor cell proliferation between the 2 drugs. The effect seemed to be more significant by EGCG when the Ki-67 marker was used, whereas the EGCG-P was more effective when the PCNA marker was used. The results suggested that, for both drugs, dampening the tumor cell proliferation may not be the key factor in the suppression of tumor growth.

**Effect of EGCG and EGCG-P on Apoptosis of CWR22R Xenograft**

One of the anticancer effects on tumor cells can operate through the induction of apoptosis. We used 2 markers, Bcl-2
FIG. 4. Effects of epigallocatechin-3-gallate (EGCG) and the peracetate of EGCG (EGCG-P) on the tumor cell apoptosis of CWR22R xenograft. A: B-cell non-Hodgkin lymphoma-2 (Bcl-2). Micrographs showing illustrative results of semiquantitation of Bcl-2 staining in EGCG treated, EGCG-P treated, and the dimethyl sulfoxide (DMSO) control tumors. (Staining intensity score: Grade 0 = negative to marginal staining of <5% of cells; Grade I = mild to moderate staining of 5–50% of cells; Grade II = moderate to intense staining comprising >50% of cells.) The results show a significant downregulation of Bcl-2 expression after treatment of EGCG and EGCG-P. The data are expressed as mean ± standard error of the measurement (SEM); n = 10, scale bar = SEM × 400. B: Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). Apoptotic effect was measured by immunohistochemical staining by using TUNEL assay. Panels 1, 2, and 3 are from DMSO, EGCG, and EGCG-P, respectively, whereas Panels 4 and 5 are negative and positive reaction control, respectively. The graph shows that the control and the 2 treated groups are significant (both at P < 0.001). The difference between the 2 treated groups is also significant (P < 0.05). The data are expressed as mean ± standard error of the measurement (SEM); n = 8, scale bar = SEM × 400.

and TUNEL, for this assay. Bcl-2 is a well-known marker for inhibition of normal apoptosis process, whereas TUNEL is a marker for promotion of normal apoptosis. Figure 4A shows the positive reactivities of Bcl-2 ranging from mild to intense (i.e., Grade I to II) for DMSO, EGCG, and EGCG-P groups, respectively. The percentages of Grade I and II within the 3 groups (i.e., DMSO, EGCG, and EGCG-P) were 21%, 8%, and 11%, respectively. The reduced reactivities of Bcl-2 (including both mild and intense) reflected the stronger inhibition effect on normal apoptosis process. Our results showed that the percentages of Grade I to II staining were lower in EGCG (P < 0.001) and EGCG-P (P < 0.001) treated samples. Therefore, the results indicate the inhibition of apoptosis on tumor cells were decreased under the influence of EGCG and EGCG-P compounds (thus allowing more cells to undergo apoptosis). The results also show that EGCG was able to decrease the expression of Bcl-2 more than EGCG-P (P < 0.05).

The results of the TUNEL assay is shown in Fig. 4B, which reaffirmed the results of Bcl-2 in Fig. 4A. There was significantly higher percentage of TUNEL-positive cells in the EGCG (60%) and EGCG-P (49%) groups than the DMSO control (17%) group. The results show that EGCG and EGCG-P were both able to induce a higher apoptotic rate compared to control (control vs. EGCG, P < 0.001; control vs. EGCG-P, P < 0.05, respectively). There was also a difference in apoptotic rate between the 2 treated groups, with the EGCG-treated group having a higher rate (P < 0.05). Overall, the results from both Bcl-2 and TUNEL assays suggest that EGCG and EGCG-P can induce apoptosis by downregulation of Bcl-2 and activation of TUNEL. Moreover, from the results, the induction of apoptosis by EGCG appears to be more profound than the EGCG-P group.

Alteration in Microvessel Density Under the Effect of EGCG and EGCG-P

The measurement of microvessel density in prostate cancer is a valuable prognostic marker. By IHC staining of CD31, an endothelial cell-specific antigen, we evaluated the effect of the treatment of EGCG and EGCG-P on angiogenesis of CWR22R prostate tumor xenograft. Figure 5 shows that there was significant decrease in density of CD31-positive endothelial cells (arrows) in vessels of tumor in the DMSO control (71.4 positive cells per ×400 magnification field) as compared with the EGCG (34.9 positive cells per ×400 magnification field, P < 0.001) and the EGCG-P (23.6 positive cells per ×400 magnification field, P < 0.001) groups. The difference between the 2 treated groups was, however, not significant (P > 0.05). The results indicate that EGCG and EGCG-P can exert an inhibition effect in prostate cancer angiogenesis through suppression of microvessel formation.
DISCUSSION

Using the human androgen-independent prostate tumor xenograft, CWR22R, the in vivo anticancer properties of EGCG and EGCG-P were evaluated in nude mice. The CWR22R xenograft was developed from the CWR22 tumor, which was originally derived from a primary human prostate cancer cells and was maintained in castrated athymic nude mice by serial transplantation (23). CWR22R has been widely used in molecular study on progression of prostate cancer and evaluation of drug activities in vivo. This is because CWR22R expresses androgen receptors, secretes PSA, and is able to grow and proliferate under androgen-independent conditions. Therefore, CWR22R has been considered as a valuable tool for studying prostate cancer progression to a hormone refractory state because it has all the features of clinically advanced prostate cancer (24–26).

In our study, treatment of EGCG and EGCG-P on the CWR22R xenograft showed inhibitory effects as evidenced by the decrease in tumor volumes (Fig. 2A) and serum PSA levels (Fig. 2B). The effects of EGCG and EGCG-P on the suppression of tumor cells appeared to be exerted by promoting apoptosis on one hand (Fig. 4) while inhibiting tumor angiogenesis on the other hand (Fig. 5). The effects of the 2 drugs on cell proliferation from this study were less clear (Fig. 3). Previously, in an earlier study on the TRAMP model of prostate cancer, green tea polyphenols were shown to reduce the PCNA reactivities in tumor tissues (14). Other studies have demonstrated that EGCG could exert induction of apoptosis in human prostate cancer cells through the mediation of a number of cellular regulatory proteins (10,27–29) In our study, the results of immunostaining on Bcl-2 and TUNEL (Fig. 4) showed that both compounds promoted apoptosis, in agreement with literature results (28,29).

Angiogenesis is another facet that is important in affecting tumor growth. Our results showed that both EGCG and EGCG-P significantly inhibited tumor angiogenesis using CD31 as the marker. EGCG-P appeared to have a slightly stronger inhibitory effect on angiogenesis than EGCG, but the difference was statistically insignificant. Folkman (30) suggested that tumor growth is dependent on the formation of new blood vessels, that is, angiogenesis. The oxygen and nutrients supplied by the vascular system are crucial for cell survival and function. During organogenesis, the mechanism is to ensure the coordinated growth of vessels and parenchyma. Therefore, induction of tumor angiogenesis can be caused by upregulation of the angiogenic factors or by downregulation of its inhibitors (31,32). Microvessel density, the measurement of small blood vessels quantitatively in a unit area, has been recognized as one of the prognostic factors important in solid tumors including prostate cancer (33).
Our study demonstrated that both EGCG and EGCG-P were effective in retarding microvessel formation in the CWR22R xenograft.

All these data suggest that a combination of factors account for the antitumor effect of EGCG and EGCG-P. On the whole, EGCG-P appeared to be more effective than EGCG in controlling the growth of tumors (Fig. 2A), and the difference was statistically significant ($P < 0.05$). However, the results from analysis on tumor proliferation and apoptosis rates were either not striking (Fig. 3) or statistically insignificant (Fig. 4) between the EGCG- and EGCG-P-treated tumors. Therefore, the mechanistic studies seem to be contradictory to the increased in vivo anticancer effect of EGCG-P. Although we do not have an explanation for these inconsistent results, it is possible that the increased anticancer efficiency observed in the EGCG-P-treated mice may be the result of a combination of several factors. First, although EGCG-P did not show an increased ability to suppress the labeling index of Ki-67, the percentage of PCNA positive cells was lower in the EGCG-P-treated tumors, which reached statistical significance ($P = 0.05$, Fig. 3B). Although both Ki-67 and PCNA labeling indexes have been used together to estimate cell proliferation, recently it was shown in prostate cancer patients that higher PCNA labeling index but not Ki-67 was more closely associated with poor prognosis (34). Based on this evidence, it is possible that in this study, the lower PCNA labeling index may reflect the better anticancer effect of EGCG-P in the CWR22R xenograft. Second, as discussed previously, angiogenesis is essential for tumor growth. In this study, we found that there was an over 10% decrease in microvessel density in the EGCG-P-treated tumors compared to the EGCG-treated group (23.6% vs. 34.9%; Fig. 5). Because microvessel density alone is able to predict the prognosis of prostate cancer (35), it is possible that the reduced microvessel density in the EGCG-P-treated tumors may also contribute to its anticancer effect in CWR22R xenograft. Taken together, our results suggest that the combination of an increased inhibitory effect on cell proliferation and a moderately increased negative effect on microvessel formation may lead to a better anticancer effect of EGCG-P in the CWR22R prostate cancer nude mice model.

One explanation for the improved efficacy of EGCG-P over EGCG may be due to the improved bioavailability. Previously, we had suggested that EGCG-P, the peracetate of EGCG, functioned seemingly as a prodrug form of EGCG by showing that EGCG-P was converted to EGCG using cell-free lysates (17). This suggestion was recently supported by a report that EGCG-P was rapidly converted to EGCG by HCT116 human colon cancer cells resulting in a 2.8- to 30-fold greater intracellular concentration of EGCG as compared to treatment with EGCG itself (21). Lambert et al. further showed that intragastric administration of EGCG-P to CF-1 mice resulted in higher bioavailability compared to administration of equimolar doses of EGCG (21). In a related study, we showed that EGCG-P can be converted to EGCG in human breast cancer MDA-MB-321 cells (36), leading to a higher intracellular concentration (>2.4-fold) of EGCG than those cells treated with same dose of EGCG on one hand and better in vivo inhibition of proteosome activity on the other hand. Although we have not done similar studies with the prostate cancer cells, it may be possible to suggest that EGCG-P may provide better bioavailability than an equivalent dose of EGCG. With improved bioavailability, EGCG-P might be able to exert its antitumor effect in mice more effectively and also almost immediately, whereas it took a much longer period for EGCG to show its antitumor effect (Fig. 2A). This may have clinical significance. In the phase II clinical trial of using green tea for androgen independent prostate cancer patients (15), the patients did not show improvement after a month’s intake of green tea powder, and the trial was discontinued with the conclusion that the treatment protocol was ineffective. The animal data in this study suggest that a longer duration may be required for EGCG to show its antitumor effect and that pure EGCG instead of green tea powder should be used for the treatment.

In conclusion, the results of our study show that both EGCG and EGCG-P are potential compounds that could be used as therapeutic anticancer agents for androgen-independent prostate cancer. With its better bioavailability, EGCG-P may be a better option than EGCG itself. However, more in-depth studies on EGCG-P are clearly needed before its development to become a new chemotherapeutic agent for advanced prostate and perhaps other cancers.

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