Oral Grape Seed Extract Inhibits Prostate Tumor Growth and Progression in TRAMP Mice

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Abstract

Prostate cancer chemoprevention is an alternative and potential strategy to control this malignancy. Herein, we evaluated the chemopreventive efficacy of grape seed extract (GSE) against prostate cancer in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice where animals were fed with GSE by oral gavage at 200 mg/kg body weight dose during 4 to 28 weeks of age. Our results showed a significant reduction (46%, $P < 0.01$) in the weight of genitourinary tract organs in the GSE-fed mice. The GSE-fed group of mice had a higher incidence of prostatic intraepithelial neoplasia but showed strong reduction in the incidence of adenocarcinoma compared with mice in control group. Prostate tissue from the GSE group showed ~50% ($P < 0.001$) decrease in proliferating cell nuclear antigen (PCNA)–positive cells and 64% ($P < 0.01$) reduction in total PCNA protein level compared with the control group; however, GSE increased apoptotic cells by 8-fold. Furthermore, GSE strongly decreased the protein levels of cyclin B1, cyclin A, and cyclin E by 84% ($P < 0.05$), 96% ($P < 0.05$), and 89% ($P < 0.001$), respectively. The protein expression of cyclin-dependent kinases 2 and 6 and Cdc2 was also decreased by more than 90% ($P < 0.05$) in the prostate from the GSE-fed group. Together, for the first time, we identified that oral GSE inhibits prostate cancer growth and progression in TRAMP mice, which could be mediated via a strong suppression of cell cycle progression and cell proliferation and an increase in apoptosis. [Cancer Res 2007;67(12):5976–82]

Introduction

Prostate cancer is the most common malignancy in elderly American men and second only to lung cancer in deaths (1). According to the American Cancer Society, there would have been an estimated 234,460 new prostate cancer cases and 27,350 associated deaths in 2006 in the United States alone. Lifestyle and dietary habits have been identified as major risk factors in prostate cancer growth and progression (2, 3). Epidemiologic data indicate that vegetables and fruits with chemopreventive agents could have protective effect against cancer (4). Management of cancer by chemoprevention may not necessarily eliminate the lesions; however, it is expected to delay the neoplastic progression and that would certainly improve the morbidity and survival time in prostate cancer patients (5, 6). In the last few years, considerable progress has been made in this direction, which has led to the identification of novel cancer chemopreventive agents and their mode of action (7, 8). One such agent is grape seed extract (GSE), which has shown promising chemopreventive and anticancer effects in various cancer cells and animal tumor models (9–12).

GSE is a complex mixture of polyphenols containing dimers, trimers, and other oligomers (procyanidins) of catechin and epicatechin and their gallate derivatives together known as the proanthocyanidins (13–15). GSE is marketed as a dietary supplement in the United States, owing to several health benefits mainly attributed to its antioxidant property (16–18). In addition, studies conducted in our laboratory have shown that GSE inhibits *in vitro* growth of hormone-refractory advanced human prostate carcinoma DU145 cells by induction of apoptosis via caspase activation (19), inhibition of constitutive as well as tumor necrosis factor-α–induced nuclear factor-κB activation, and inhibition of epidermal growth factor–induced or constitutive activation of mitogenic signaling (20). Our studies have also shown that GSE inhibits *in vivo* growth of DU145 xenograft in nude mice via an inhibition of cell proliferation and an induction of apoptosis (21). Additionally, we have also shown that GSE induces anoikis and apoptosis in androgen-dependent human prostate carcinoma LNCaP cells in culture (22). In the present study, for the first time, we evaluated the chemopreventive efficacy of oral GSE against prostate cancer growth and progression in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model.

The TRAMP model has been developed in the inbred C57BL/6 strain of mice using the minimal rat probasin promoter to drive the expression of SV40 early genes (T/t; Tag) specifically in the prostatic epithelium (23, 24). The transgene is hormonally regulated, expressed at sexual maturity, and induces spontaneous neoplastic epithelial transformation (25). The SV40 large T antigen abrogates p53 and retinoblastoma (Rb) function and thus acts as an oncoprotein. As a consequence, TRAMP males develop spontaneous progressive stages of prostatic disease with time from early lesions of prostatic intraepithelial neoplasia (PIN) of the dorsolateral prostate to the late-stage metastatic adenocarcinoma and thus mimic the progressive forms of human prostatic carcinoma (26–29). Therefore, our present finding of antitumor efficacy of GSE and associated mechanisms in TRAMP model could have potential clinical significance.

Materials and Methods

GSE. Standardized commercial preparation of GSE, constituting of 89.3% (w/w) procyanidins, 6.6% of monomeric flavonols, 2.24% of moisture content, 1.06% of protein, and 0.8% of ash, was a kind gift from the Kikkoman Corp. (Noda City, Japan), and its chemical constituents are well characterized and defined in recent studies (22).

Note: K. Raina and R.P. Singh contributed equally to this work and share first authorship.

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Animals, treatment, and necropsy. Heterozygous TRAMP females developed on a pure C57BL/6 background were cross-bred with non-transgenic C57BL/6 breeder males and maintained in the Laboratory Animal Care facility at the University of Colorado Health Sciences Center, Denver, CO. Mouse-tail DNA was isolated from the litter and subjected to PCR-based screening assay as described previously by Greenberg et al. (25). The routinely obtained 4-week-old TRAMP male mice were randomly distributed into control and treatment groups (n = 20 mice per group) and gavaged with sterile saline (control group) or GSE (200 mg/kg body weight) in sterile saline, 5 days/wk for a period of 24 weeks. Animal care and treatments were in accordance with institutional guidelines and approved protocol. During the study, animals were permitted free access to AIN-76A diet and drinking water. Body weights were recorded weekly, and the animals were monitored daily for their general health.

At 28 weeks of the age, animals were sacrificed by ketamine injection (containing heparin). Each mouse was weighed, and the lower genitourinary tract, including bladder, seminal vesicles, and prostate, was removed *en bloc*. The genitourinary tract wet weight was recorded, and the dorsolateral prostate was microdissected whenever possible (when a tumor obscured the boundaries of the lobes, it was taken as such) and divided into two portions. One portion of the dorsolateral prostate was snap-frozen and stored at −80°C. The other portion was fixed overnight in 10% (v/v) phosphate-buffered formalin and then transferred into 70% ethanol before standard tissue processing. The fixed tissues were dehydrated in ascending grades of ethanol, cleared in toluene, and embedded in paraffin wax. Sections (5 μm) were cut with microtome and mounted on superfrost slides (Fisher Scientific) coated with 0.01% poly-L-lysine (Sigma-Aldrich). Tissues were processed and stained with H&E for routine histopathologic evaluation. At the time of necropsy, the animals were also examined for gross pathology, and any evidence of edema, abnormal organ size, or appearance in non-target organs was also noted.

Immunohistochemical analysis of proliferating cell nuclear antigen expression. The paraffin-embedded sections (5-μm thick) were deparaffinized and stained using antibody against proliferating cell nuclear antigen (PCNA) followed by 3,3′-diaminobenzidine staining, as previously described (21). The primary antibody used was mouse monoclonal anti-PCNA antibody IgG2a (1:250; DAKO), and the biotinylated secondary antibody used was rabbit anti-mouse antibody IgG (1:200 in 10% normal rabbit serum; DAKO). The proliferating cells were quantified by counting the PCNA-positive cells and the total number of cells at 10 randomly selected fields at ×400 magnification. The proliferation index (per ×400 microscopic

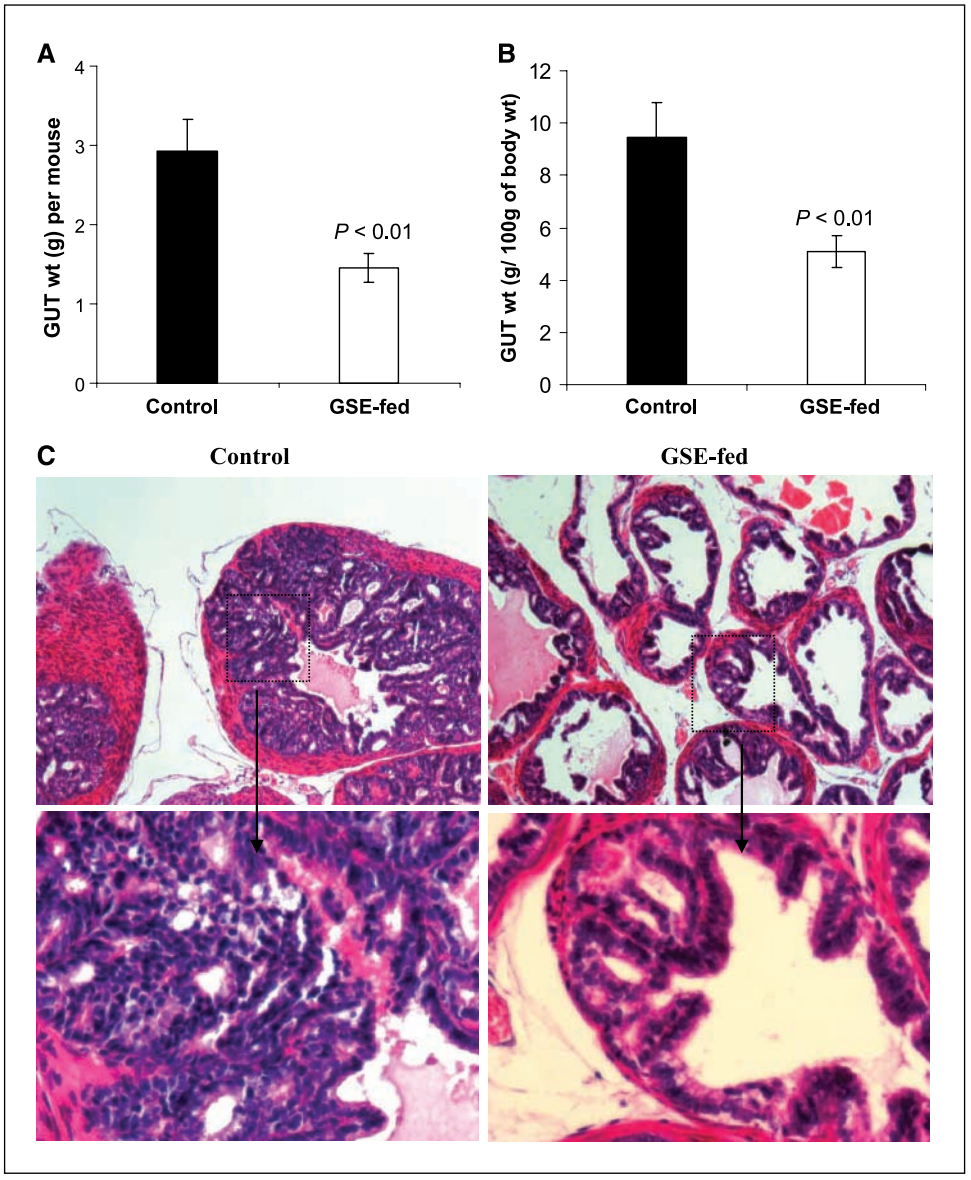

Figure 1. Inhibitory effect of oral GSE on prostate tumorigenesis in TRAMP mice. *A* and *B,* at the time of necropsy, after 24 wks of GSE feeding (200 mg/kg, 5 d/wk) starting from 4th week of age, each mouse was weighed, and the lower genitourinary tract (*GUT*), including the bladder, seminal vesicles, and prostate, was removed *en bloc* and weighed. $P < 0.01$ (Student's t test). *Columns*, mean (n = 20 mice per group); *bars,* SE. *C,* H&E staining of the dorsolateral prostate of control and GSE-fed mice sacrificed after 28 wks of age. *Top left,* magnification (×100) of a section through the dorsolateral prostate of the control mice showing increased epithelial stratification that almost fills the lumen of the ducts, enlarged diameter of the glands, distorted and bulging duct profiles, stromal thickening, and invasion. *Bottom left,* magnification (×400) of the bracketed section of the top left image. *Top right,* magnification (×100) of a section through the dorsolateral prostate of the GSE-fed mice showing duct profiles with infoldings and luminal space, intact basement membrane, and focal lesions of proliferating epithelial cells. *Bottom right,* magnification (×400) of the bracketed section of the top right image.

field) was determined as number of PCNA-positive cells × 100 / total number of cells.

***In situ* apoptosis detection by terminal deoxynucleotidyl transferase–mediated nick-end labeling staining.** The paraffin-embedded 5-μm-thick sections were used to identify apoptotic cells by terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) staining using Dead End Colorometric TUNEL System (Promega Corp.). The apoptosis was evaluated by counting the positive cells (brown-stained cells) as well as the total number of cells at 10 randomly selected fields at ×400 magnification. The apoptotic index (per ×400 microscopic field) was calculated as number of apoptotic cells × 100 / total number of cells.

Immunoblot analysis of tissue lysates. The dorsolateral prostate samples dissected out from control and GSE-fed groups of mice were homogenized in non-denaturing lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.3 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L sodium orthovanadate, 0.5% NP40, 5 units/mL aprotinin]. Protein concentration in lysates was determined using Bio-Rad detergent-compatible protein assay kit (Bio-Rad Laboratories) by the Lowry method. For immunoblot analyses, 50 to 80 μg of protein per lysate was denatured in 2× SDS-PAGE sample buffer and subjected to SDS-PAGE on 12% or 16% Tris-glycine gel as needed. The separated proteins were transferred onto nitrocellulose membrane followed by blocking with 5% nonfat milk powder (w/v) in TBS (10 mmol/L Tris, 100 mmol/L NaCl, 0.1% Tween 20) overnight at 4°C. Membranes were probed with different primary antibodies, including anti-cyclin A (sc-751), anti-cyclin B1 (sc-245), anti-cyclin D1 (sc-718), anti-cyclin D3 (sc-182), anti–cyclin-dependent kinase 2 (Cdk2; sc-163), anti-Cdk4 (sc-749), anti-Cdk6 (sc-177), anti-Cdc2 (sc-54; Santa Cruz Biotechnology); anti-cyclin E (Ab-1), anti-Kip1/p27 (Neomarkers); and anti-Cip1/p21 (Upstate). The membranes were then incubated with specific peroxidase-conjugated secondary antibody, anti-rabbit IgG (Cell Signaling Technology), or anti-mouse IgG secondary antibody (Amersham Corp.) followed by detection using enhanced chemiluminescence kit (Amersham). To confirm equal protein loading per lane, the membranes were stripped and reprobed with mouse monoclonal anti-β-actin primary antibody (Sigma).

Statistical and microscopic analyses. All statistical analyses were carried out with Sigma Stat software version 2.03 (Jandel Scientific). $P < 0.05$ was considered significant. χ^2 analysis was used to compare the incidence of PIN and adenocarcinoma in control and GSE-fed groups. For other data, the difference between control and GSE-fed groups was calculated by Student's t test. Densitometric analysis of the immunoblots (adjusted with β-actin as the loading control) was done by Scion Image program (NIH, Bethesda, MD). All the microscopic histopathologic and immunohistochemical analyses were done by Zeiss Axioscope 2 microscope (Carl Zeiss, Inc.), and the photomicrographs were captured by Carl Zeiss AxioCam MrC5 camera with the Axiovision Rel 4.5 software.

Results

GSE feeding reduces the weight of genitourinary tract in TRAMP mice. Oral gavage feeding of GSE at 200 mg/kg body weight, 5 days/wk from 4 to 28 weeks of age, did not show any considerable change in diet consumption during 24 weeks of treatment (data not shown). In addition, there was no considerable difference in body weight (adjusted with genitourinary tract weight) between control and GSE-fed mice (data not shown); however, at necropsy, reduced size of genitourinary tract organs was observed compared with those of the control group. At the time of necropsy, all animals were examined for gross and microscopic pathology, where necropsy did not show any evidence of edema, abnormal organ size, or appearance in non-target organs. There was a significant difference between the genitourinary tract weight of control and GSE-fed group. The genitourinary tract weight of the GSE-fed group was 52% ($P < 0.01$) lesser than that of the control group (Fig. 1*A*). When the genitourinary tract weight was normalized to body weight (Fig. 1*B*), the difference in weight followed the same trend; the GSE-fed group of mice showed 46% ($P < 0.01$) lesser genitourinary tract weight compared with control mice. This finding clearly indicates the inhibitory effect of GSE on abnormal growth of the prostate in TRAMP mice, which was also examined by histopathologic analysis.

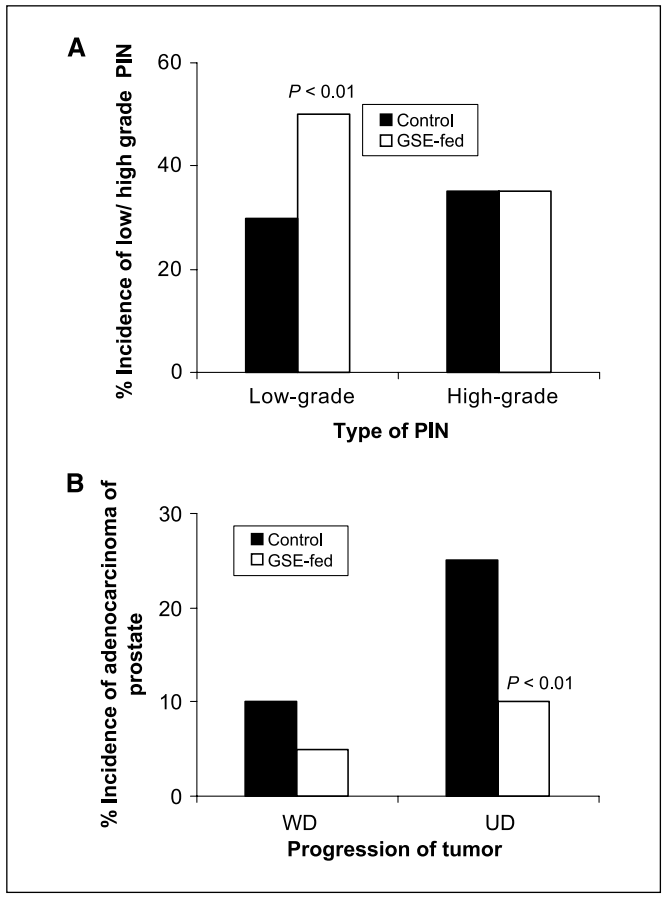

Figure 2. Oral GSE inhibits neoplastic progression of prostate in TRAMP mice. The prostate glands from the study detailed in Fig. 1 were histopathologically analyzed for the different stages of the neoplastic progression of the dorsolateral prostate. *A,* effect of GSE feeding on the incidence of LGPIN and HGPIN lesions in TRAMP mice. *B,* effect of GSE on the incidence of adenocarcinoma of prostate in TRAMP mice. $P < 0.01$ (χ^2 test). *WD,* well-differentiated tumors; *UN,* undifferentiated (both moderately and poorly differentiated) tumors.

GSE feeding reduces the incidence of adenocarcinoma in TRAMP mice. A detailed histopathologic analysis of the neoplastic progression of the dorsolateral prostate in both control and GSE-fed groups was done. H&E-stained sections (Fig. 1*C*) were microscopically examined and classified (30) as (*a*) low-grade PIN (LGPIN) having foci with two or more layers of atypical cells with elongated hyper chromatic nuclei and intact gland profiles; (*b*) high-grade PIN (HGPIN) having increased epithelial stratification, foci of atypical cells fill or almost fill the lumen of the ducts, enlarged diameter of the glands, distorted duct profiles, increase in nuclear pleomorphism, hyper chromatic nuclei, and cribriform structures; (*c*) well-differentiated adenocarcinoma showing invasion of basement membrane, loss of intraductal spaces, and increased quantity of small glands; (*d*) moderately differentiated adenocarcinoma showing total loss of intraductal spaces and relatively solid growth; and (*e*) poorly differentiated adenocarcinoma showing sheets of poorly differentiated cells with remnants of

trapped glands. As shown in Fig. 2*A*, there was a difference in PIN incidences between GSE-fed and control groups. In most animals, the tumor progression was arrested at LGPIN stage in the GSE-fed group compared with that in the control group (LGPIN incidence 50% versus 30%, respectively; $P < 0.01$); however, no difference was noted in the incidence of HGPIN between the groups. The control group exhibited features characteristic of microinvasive carcinoma with penetration of PIN involved glands into the surrounding stroma to form small nests of cells. As shown in Fig. 2*B*, there was a 50% reduction in the incidence of well-differentiated tumors in the GSE-fed group together with a 60% reduction ($P < 0.01$) in the incidence of undifferentiated (both moderately and poorly differentiated) tumors (Fig. 2*B*). These results suggest that GSE inhibits tumor progression in the neoplastic stage, thereby reducing the incidence of adenocarcinoma, which is also supported by an accumulation of prostatic tissue at LGPIN stage in GSE-fed TRAMP compared with control mice.

GSE feeding reduces proliferation index in dorsolateral prostate of TRAMP mice. To assess the *in vivo* effect of GSE feeding on the proliferation index in dorsolateral prostate, the tissue samples were analyzed by PCNA immunostaining. Qualitative microscopic examination of PCNA-stained sections showed a substantial decrease in PCNA-positive cells in the prostate tissue samples from GSE-fed mice compared with vehicle controls (Fig. 3*A* and *B*). The quantification of PCNA staining showed $33 \pm 1.9\%$ PCNA-positive cells in GSE-fed group compared with $65 \pm 2.6\%$ PCNA-positive cells in controls (Fig. 3*C*), which clearly shows that GSE feeding decreased proliferating cells by $\sim 50\%$ ($P < 0.001$) compared with control group. The negative controls, in which PBS was used instead of PCNA, did not show any considerable positive staining (data not shown). These results were further confirmed by immunoblot analysis of the tissue lysates for the PCNA protein, which showed lower levels in GSE-fed group (Fig. 3*D*). The densitometric analysis of the bands (adjusted with β-actin as the loading control) exhibited a 64% ($P < 0.01$) decrease in PCNA protein expression in GSE-prostate tissue samples compared with controls (Fig. 3*D*). These results suggest the *in vivo* antiproliferative effect of oral GSE on dorsolateral prostate in TRAMP mice.

GSE increases apoptosis in dorsolateral prostate of TRAMP mice. *In vivo* apoptotic response of GSE feeding on prostate tumorigenesis in TRAMP mice was investigated by TUNEL staining. Microscopic examination of the tissue sections showed an increased number of TUNEL-positive cells in GSE-fed group (Fig. 4*A* and *B*). The number of TUNEL-positive apoptotic cells in the GSE-fed group was $22 \pm 1.2\%$ compared with $3 \pm 0.2\%$ in the control group, accounting for $\sim$8-fold ($P < 0.001$) increase in apoptotic cells (Fig. 4*C*). This finding suggests that in addition to antiproliferative effect, proapoptotic effect could be another potential mechanism underlying inhibitory effect of GSE in prostate tumorigenesis in TRAMP mice.

GSE modulates cell cycle regulators in inhibition of prostate tumor progression in TRAMP mice. The GSE-caused decrease in proliferation index in the mouse prostate prompted us to investigate its effect on cell cycle regulatory molecules. Immunoblot analysis of the prostate tissue lysates showed that GSE downregulates the expression of mitotic cyclins A, B, and E (Fig. 5). A summary of the densitometric analysis of the data (adjusted with β-actin as the loading control) is shown in Fig. 5. In the GSE-fed group, the expression of cyclin B1 and cyclin A was decreased by 84% and 96%, respectively ($P < 0.05$, for both; Fig. 5). Similarly, cyclin E protein level was also decreased by 89% ($P < 0.001$) in the GSE-fed group (Fig. 5). There was, however, no change in the protein levels of cyclin D1 and D3 in both the groups; in fact, there was only a faint expression of the D type cyclins in both the groups (data not shown).

Because cyclins act as the regulatory subunits that mediate the activation of the Cdks, we also examined the expression levels of

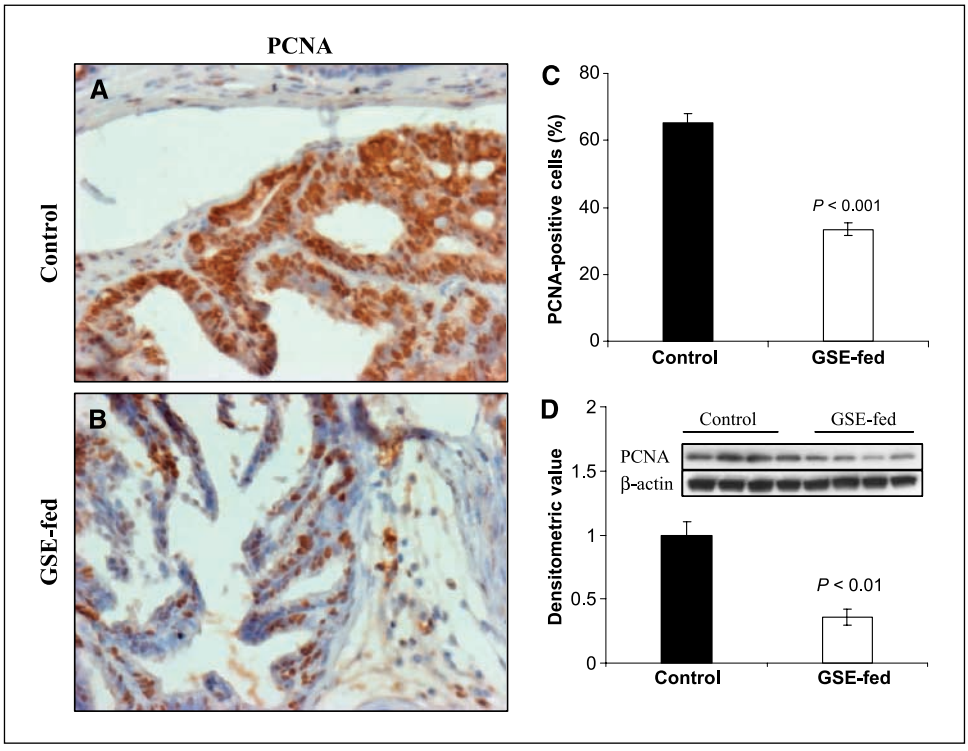

Figure 3. *In vivo* antiproliferative effect of GSE feeding on dorsolateral prostate in TRAMP mice. *A* and *B,* immunohistochemical staining for PCNA (×400 magnification) in prostate was based on 3,3′-diaminobenzidine staining as detailed in Materials and Methods. *C,* quantification of PCNA-positive cells for determination of proliferation index. *Columns,* mean of 20 samples in each group; *bars,* SE. $P < 0.001$ (Student's *t* test). *D,* four prostate tissue samples from individual mice were randomly selected from each group for PCNA immunoblotting. Reactive protein bands were visualized by enhanced chemiluminescence detection system, and membrane was stripped and probed with β-actin as loading control. Densitometric analysis of the PCNA band intensity adjusted with β-actin. *Columns,* mean of the four bands from individual mouse prostate in each group; *bars,* SE. $P < 0.01$ (Student's *t* test).

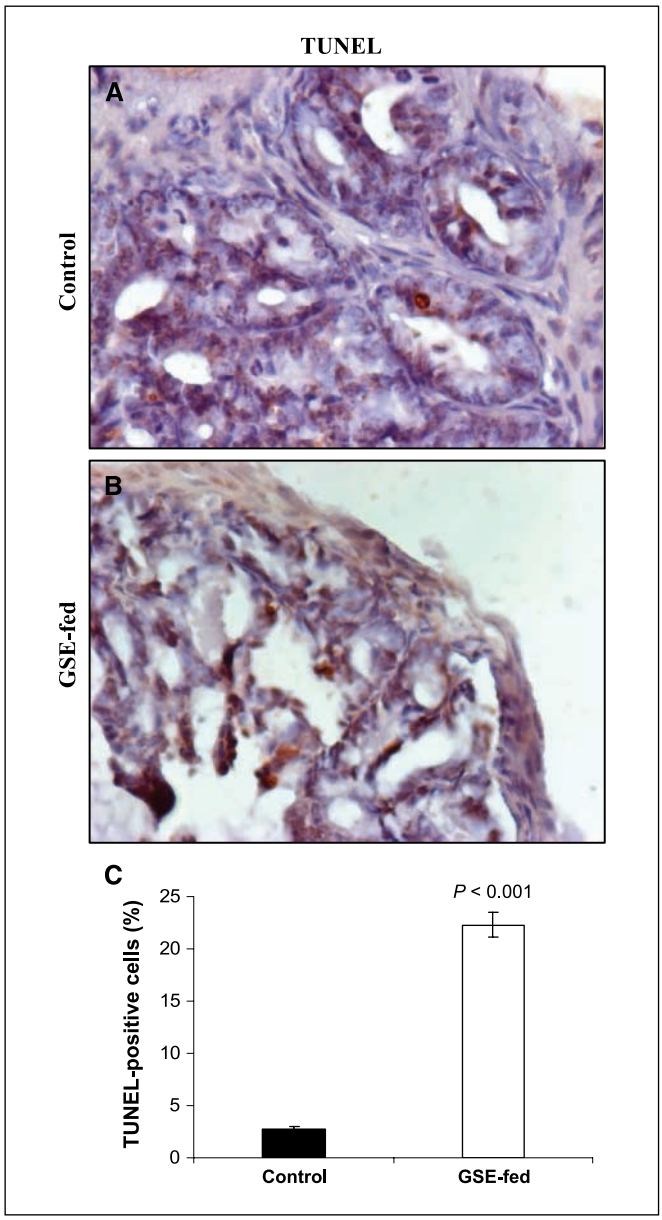

Figure 4. *In vivo* proapoptotic effect of GSE feeding on dorsolateral prostate in TRAMP mice. *A* and *B,* apoptosis was analyzed by TUNEL staining in prostate tissues as detailed in Materials and Methods. Brown-colored TUNEL-positive cells depicted at ×400 magnifications. *C,* apoptotic index was calculated as the number of positive cells × 100 / total number of cells counted under ×400 magnification in 10 randomly selected areas in each sample (n = 20). *Columns,* mean; *bars,* SE. $P < 0.001$ (Student's t test).

different Cdk molecules in the prostate tissue lysates. GSE feeding strongly decreased the protein levels of Cdk2, Cdk6, and Cdc2 in the TRAMP mice (Fig. 5). We did not observe any significant difference in the expression of Cdk4 in control versus GSE-fed groups, which can be attributed to a large degree of variability (>50%) in the expression levels of Cdk4 in the individual prostate samples within the control group (Fig. 5). The expression levels of Cdk2, Cdk6, and Cdc2 (Fig. 5) were decreased by 96%, 99%, and 94%, respectively (for each, $P < 0.05$). However, we did not observe any significant difference in the expression levels of Cdk inhibitors Cip1/p21 and Kip1/p27 between both the groups (Fig. 5). Together, these results indicate that GSE strongly decreases the expression of cyclins and Cdks regulating G_1-S and G_2-M checkpoints in cell cycle progression, which could potentially inhibit prostate tumor progression in TRAMP mice.

Discussion

The novel finding in the present study is that oral consumption of GSE, a commonly used dietary supplement, inhibits prostate tumor progression in TRAMP, a mouse model in which the course of prostate tumorigenesis closely mimics the human form of this malignancy. This antitumor efficacy of GSE was associated with its antiproliferative and proapoptotic effect in prostate tumor tissue. Furthermore, for the first time, we also identified that GSE strongly decreases the *in vivo* expression of cyclins and Cdks, which are known to regulate the checkpoints during cell cycle progression. Therefore, we anticipate that cyclins and Cdks could constitute potential molecular targets in the inhibition of prostate tumor progression by GSE.

Although most organs regress with age, prostate grows leading to prostatic hypertrophy in elderly men (31–33). A percentage of these could develop carcinoma of the prostate, which has become the biggest killer of elderly men due to cancers in United States (1). However, the onset is gradual, and most men fail to realize that anything is wrong with their prostate; therefore, many of them battle on and accept the progressive reduction in their quality of life as simply a part of aging until other complications eventually supervene. The socioeconomic implications of this disease are considerable, and with the trend towards increasing life span, the statistics will increase in the future (34). Recently, there have been considerable activities directed toward the identification of dietary components for both prevention and intervention of cancer, including prostate cancer (7, 8). This is supported by the present study, wherein GSE showed strong preventive efficacy against prostate tumorigenesis in TRAMP mice. Oral GSE also showed a significant reduction in the weight of genitourinary tract organs including prostate.

The TRAMP model is an autochthonous transgenic mouse model for prostate cancer in which the minimal rat probasin promoter drives the expression of SV40 early genes (T/t; Tag), specifically in the prostatic epithelium. The transgene is hormonally and developmentally regulated and induces spontaneous neoplastic epithelial transformation in the prostate of the transgenic mice (23–25, 27, 28). In humans, prostate cancer progression is a multistage process involving the onset as a small carcinoma of low histologic grade progressing slowly to metastatic lesions of higher grade. Prostate cancer in the TRAMP model mimics this human type of prostate cancer progression events in a stochastic fashion (26, 29). In the present study, GSE-fed mice showed a higher incidence of PIN with a concomitant reduction in the incidence of adenocarcinoma. This suggests that GSE feeding for 24 weeks starting from the 4th week of age causes suppression of the tumor progression in the neoplastic stage, thereby reducing the incidence of adenocarcinoma. Both immunohistochemical and immunoblot analyses for PCNA revealed that GSE feeding significantly decreases cell proliferation in the dorsolateral prostate, which was also accompanied by an increase in apoptotic cell death. Furthermore, it was anticipated that antiproliferative effect of GSE against prostate tumor progression might involve cell cycle regulatory mechanisms.

An aberrant regulation of cell cycle has been an underlying cause of the uncontrolled cell proliferation and survival leading to the

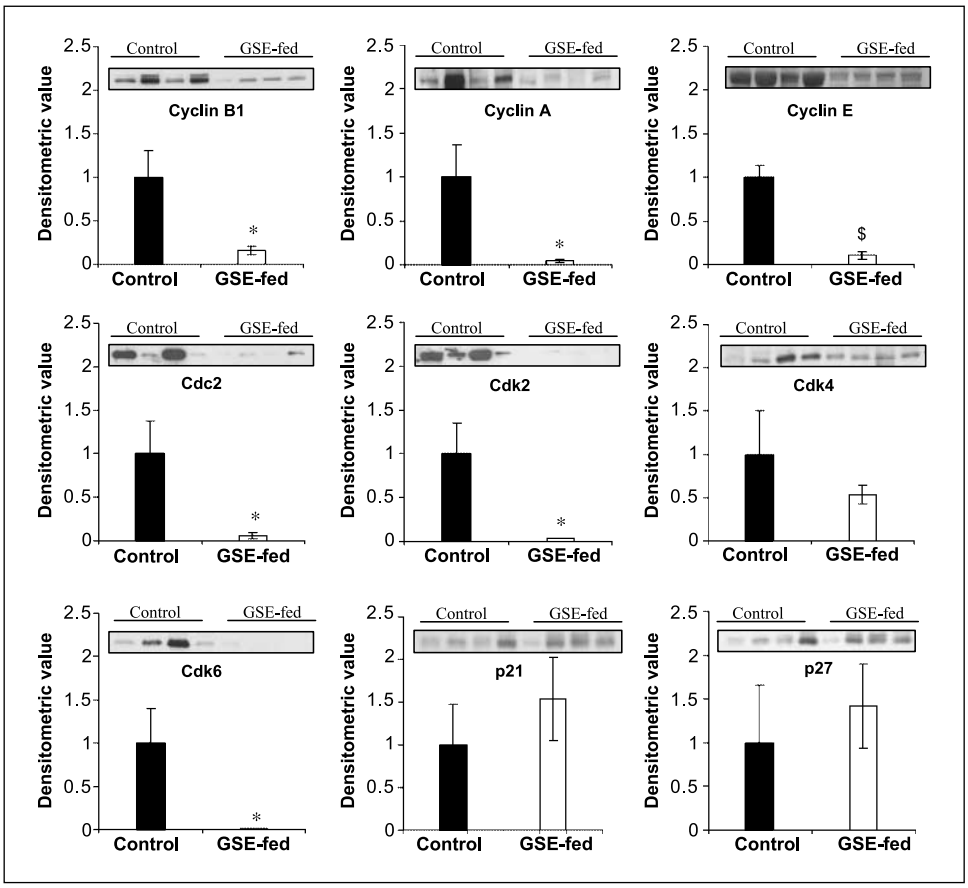

Figure 5. GSE feeding alters the expression levels of cell cycle regulatory molecules in the prostate of TRAMP mice. Four prostate tissue samples from individual mice were randomly selected from each group for immunoblot analyses detailed in Materials and Methods. Reactive protein bands for the expression of cyclin B1, cyclin A, cyclin E, Cdc2, Cdk2, Cdk4, Cdk6, Cip1/p21, and Kip1/p27 were visualized by enhanced chemiluminescence detection system, and membrane was stripped and probed with β-actin as loading control. Densitometric analysis of band intensity for each protein was adjusted with β-actin (blots not shown). *Columns,* mean of the four bands from individual mouse prostate in each group; *bars,* SE. $, $P < 0.001$; *, $P < 0.05$ (Student's *t* test).

neoplastic transformation and cancer progression (35). Cell cycle progression is regulated by the activity of Cdks in association with their regulatory subunits, cyclins, and Cdk inhibitors (36). Activation of different Cdks is specific to the distinct phases of the cell cycle; for example, the association of Cdk4/Cdk6 with D-type cyclins regulates the early and mid-G_1 phase progression, whereas Cdk2-cyclin E for late-G_1 and G_1-S transition, Cdk2-cyclin A for S phase progression, and Cdc2 (or Cdk1)-cyclin B1 for G_2-M phase transition (37). The changing patterns of cyclins and Cdks have been characterized during the progression of prostate cancer in the TRAMP model (38), wherein an up-regulation of mitotic cyclins, including cyclin A, B, and E, and a concomitant decrease in cyclin D1 have been observed during the progression of cancer. However, the expression levels of cyclin D3, although they increase compared with normal prostate, do not vary much during the disease progression (38). In the present study, GSE down-regulated the expression of cyclins E, A, and B1 as well as Cdk2, Cdk6, and Cdc2 in the prostate of TRAMP mice. A slight increase, although not significant, in the protein expression of Cdk inhibitors Cip1/p21 and Kip1/p27 was also observed by the GSE treatment. Therefore, it is more likely that GSE could inhibit aberrant cell cycle progression through late G_1 and G_1-S transition as well as G_2-M transition in the prostate of TRAMP mouse. This cell cycle inhibitory effect of GSE was complemented by its strong inhibitory effect on cell proliferation as observed by PCNA analysis. Additionally, it could be also possible that chronically arrested cells in cell cycle can undergo apoptosis as observed by the GSE treatment.

The present *in vivo* findings are supported by our cell culture studies where a polyphenolic fraction isolated from grape seeds is found to decrease both Cdks and cyclins protein levels together with inducing cell cycle arrest and apoptotic death of human prostate carcinoma DU145 cells (39). Furthermore, gallic acid, recently isolated and identified by us as an active agent in GSE (14), has also shown its cell cycle arrest and apoptotic effects in DU145 cells via a decrease in Cdks and cyclins protein levels as well as an inactivating phosphorylation of cdc25A/cdc25C-cdc2 via ataxia telangiectasia mutated kinase (ATM)/checkpoint kinase-2 (Chk2) activation (40). Our other studies with GSE or GSE fractions have also shown strong apoptotic effects in DU145 cells (19, 39–41), involving dissipation of mitochondrial membrane potential, cytochrome *c* release, and caspase activation as well as ATM-p53 activation in LNCaP cells (22). Many of these *in vitro* findings are yet to be investigated *in vivo* for the anti–prostate cancer effects of GSE.

Studies in the TRAMP model with other chemopreventive agents, such as green tea (also rich in catechins/epicatechins and their gallate esters), have shown inhibition of progression of prostate cancer via modulation of insulin-like growth factor receptor signaling pathway and apoptosis induction (42). Genistein, the primary isoflavone from soy, has been shown to reduce the incidence of poorly differentiated tumors in TRAMP mice by down-regulation of specific sex steroid receptor and growth factor signaling pathways (43, 44). Whereas more studies are needed in future to assess whether the anti–prostate cancer efficacy of GSE also involves similar molecular changes as observed with green tea and genistein, to our knowledge, this is the first study in the TRAMP model with strong prostate cancer chemopreventive efficacy of GSE involving the modulation of cell cycle regulatory molecules.

In summary, GSE feeding inhibits prostate tumor growth as well as progression in TRAMP mice without any adverse health effects. At molecular level, GSE decreased the expression of cyclins and Cdks, thereby arresting cell cycle progression that was accompanied by the decreased cell proliferation and an enhanced apoptosis. Therefore, it could be suggested that cyclins and Cdks are potential *in vivo* molecular targets for GSE efficacy in arresting the tumor grade at an earlier stage during prostate tumor progression in the TRAMP mice. Findings in the present study together with our earlier findings in human prostate tumor xenograft in athymic nude mice (21) suggest strong prostate cancer chemopreventive efficacy of GSE with scientific rationale and advocate for its potential clinical trial in human prostate cancer patients.

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