

## GRAPE SEED EXTRACT INHIBITS ADVANCED HUMAN PROSTATE TUMOR GROWTH AND ANGIOGENESIS AND UPREGULATES INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3

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Dietary intake of many fruits and vegetables has been shown to be associated with reduced risk of cancer. We investigated the *in vivo* efficacy of grape seed extract (GSE, patented as Traconol) against prostate cancer (PCA) and associated molecular events. Athymic nude mice were implanted with hormone-refractory human prostate carcinoma DU145 cells and fed with 100 and 200 mg/kg/day (5 days/week) doses of GSE for 7 weeks. At the end of experiment, tumors were immunohistochemically analyzed for cell proliferation, apoptosis and angiogenesis. Our data show that GSE feeding strongly inhibited tumor growth that accounted for 59–73% ( $p < 0.001$ ) inhibition in tumor volume and 37–47% ( $p < 0.05$ ) decrease in tumor weight at the end of the experiment. It did not show any significant change in body weight gain profile and diet consumption. Immunohistochemical analysis of tumors showed that GSE decreases proliferation index by 51–66% ( $p < 0.001$ ) and increases apoptotic index by 3–4-fold ( $p < 0.001$ ). CD31 staining for endothelial cells, showed decrease in intratumoral microvasculature in GSE-fed group of mice. Control tumors showed  $64.0 \pm 1.6$  CD31 positive cells/400 $\times$  field compared to  $23.2 \pm 0.9$  and  $15.7 \pm 0.08$  ( $p < 0.001$ ) CD31 positive cells in 100 and 200 mg/kg doses of GSE-treated tumors, respectively. GSE strongly inhibited (47–70%,  $p < 0.05$ ) vascular endothelial growth factor (VEGF) secretion in conditioned medium by DU145 cells. Recently, the circulating level of insulin-like growth factor binding protein (IGFBP)-3 is shown to inversely related with PCA risk, growth and prognosis. Consistent with this, we observed 6–7-fold ( $p < 0.001$ ) increase in tumor-secreted levels of IGFBP-3 after GSE feeding. In other immunohistochemical studies, compared to controls, tumor xenografts from GSE-fed groups of mice showed a moderate decrease in VEGF but an increase in IGFBP-3 levels. These findings suggest that GSE possesses *in vivo* anticancer efficacy against hormone-refractory human PCA, which is associated with its antiproliferative, proapoptotic and antiangiogenic activities together with upregulation of IGFBP-3.

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**Key words:** prostate cancer; cell proliferation; apoptosis; angiogenesis; grape seed extract

Resistance (acquired or inducible) of tumors to chemotherapy or radiotherapy is a common clinical problem.<sup>1</sup> Increased concentrations of cytotoxic drugs and higher doses of radiation fail to improve the pharmacotherapeutic response in resistant cancer cells, such as prostate carcinoma DU145 cells.<sup>2</sup> At present, the major therapy for the localized prostate cancer (PCA) is androgen deprivation/suppression, which induces apoptosis of androgen-dependent PCA cells resulting in tumor regression and improved prognosis.<sup>3</sup> Within a few years, however, tumor re-growth occurs that is largely due to progression of initially androgen-dependent PCA cells to an androgen-independence.<sup>2</sup> The androgen-independent PCA cells are resistant to apoptosis that limits the success of cytotoxic chemotherapy or radiotherapy, and these PCA cells continue to grow vigorously and metastasize to other tissues.<sup>2,4</sup> It is also accepted widely that cancer cells shift the balance between proangiogenic and antiangiogenic molecules toward angiogenesis facilitating tumor growth and metastasis.<sup>5</sup> Therefore identification of new agents that inhibit growth and induce apoptosis in PCA cells, as well as suppress tumor angiogenesis could have immediate significance in PCA control.

Several epidemiological studies followed by animal data or *vice versa* suggest that consumption of fresh fruits and yellow-green vegetables reduces the human cancer incidence and mortality.<sup>6–8</sup> Grapes (*Vitis vinifera*) are one of the most widely consumed fruits in the world and are rich in polyphenols of which about 60–70% exist in grape seeds as dimers, trimers and other oligomers of flavan-3-ols and known commonly as procyanidins.<sup>9,10</sup> Commercial preparations of grape seed polyphenols are marketed in the United States as ‘Grape Seed Extract’ (hereafter referred as GSE) with 95% standardized procyanidins as dietary supplement due to its several health benefits.

Recent studies have shown that procyanidins possess antioxidant, antiinflammatory, antifungal, antibacterial, antiarthritic and antiallergic activities, and prevent heart diseases and skin aging.<sup>11–13</sup> Wine, a source of procyanidins, has been reported to have many beneficial health effects.<sup>14</sup> There are some reports that GSE may have cancer chemopreventive properties against breast, lung, skin and gastro-intestinal cancers.<sup>15–18</sup> GSE is shown to enhance growth and viability of normal human gastric mucosal and murine macrophage cells.<sup>16</sup> We reported recently that GSE inhibits growth and induces apoptosis in human prostate carcinoma cells in culture.<sup>19</sup> We examined the *in vivo* efficacy of GSE against advanced human prostate tumor xenograft growth in athymic nude mice model. The studies carried out include efficacy on tumor growth followed by immunohistochemical analysis of tumors for the extensively used prognostic biomarkers in pre-clinical cancer models, namely PCNA, apoptosis and intratumoral microvasculature.

It is now well established that angiogenesis is obligatory for the growth and progression of solid tumors beyond the size limit (~2 mm diameter) imposed by simple diffusion for the nutrient supply.<sup>5,20</sup> VEGF is an important angiogenic factor, the overexpression of which has been linked to angiogenesis and more aggressive behavior of growing tumors.<sup>21,22</sup> Recent epidemiological as well as laboratory studies have demonstrated that higher circulating

**Abbreviations:** ELISA, enzyme-linked immunosorbent assay; IGFBP-3, insulin-like growth factor-binding protein-3; PCA, prostate cancer; PCNA, proliferation cell nuclear antigen; PECAM (CD31), platelet endothelial cell adhesion molecule; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; VEGF, vascular endothelial cell growth factor.

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IGF-1 level or lower IGFBP-3 level are strongly and positively correlated with increased risk of PCA, and could be an excellent determinant of PCA progression in humans.<sup>23–25</sup> Therefore, anti-angiogenic potential of GSE was also evaluated by assessing VEGF secretion from DU145 cells. Further we estimated the tumor-secreted levels of IGFBP-3, for its possible role in the suppression of PCA growth. In the present study we report that GSE inhibits human prostate carcinoma DU145 tumor xenograft growth in athymic nude mice without any apparent toxicity via decrease in proliferation, an increase in apoptotic, and inhibition of tumor angiogenesis. GSE also downregulates VEGF and upregulates IGFBP-3 in tumor cells, suggesting VEGF and IGFBP-3 as possible molecular targets in its antitumor efficacy.

## MATERIAL AND METHODS

### Cell lines and animals

DU145 human prostate carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI-1640 with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO<sub>2</sub> atmosphere. Athymic male nu/nu mice were purchased from National Cancer Institute (Frederick, MD) and housed in nude mice care facility at University of Colorado Health Sciences Center. Animal care was in accordance with current regulations and standards of National Institute of Health (Bethesda, MD) and our institutional guidelines for animal care.

### GSE preparation and treatment

GSE (item I.D. BLKG46, Bin # 05-A-2), obtained from Traco Labs Inc. (Champaign, IL), is patented as "Traconol" (patent 4,698,360). The GSE product analysis sheet provided by Traco Labs Inc shows procyanidolic value as 95 minimums. Although the chemical composition of GSE has not been specified completely, the main constituents are monomers (catechin and epicatechin) and other condensed flavonoids of various chain lengths; epicatechin and oligomeric procyanidins (also known as proanthocyanidins) are predominant in GSE. In animal studies, GSE was fed by oral gavage as a suspension in the saline. The rationale for the 5 days/week GSE treatment to mice was based on our observation in the pilot studies of GSE feeding, showing no apparent toxicity to the animals. For the cell culture study, GSE was dissolved in DMSO and used at the doses based on our earlier studies with GSE in PCA cells.<sup>15,19,36,37</sup>

### In vivo tumor xenograft study

To establish DU145 tumors in mice, DU145 cells were grown in culture, then detached by trypsinization, washed and resuspended in serum-free RPMI1640 medium. Six-week-old athymic nu/nu male mice were injected with  $2 \times 10^6$  DU145 cells mixed with matrigel, in the right flank of each mouse to initiate tumor growth. Mice were randomly divided into 3 groups each having 10 mice. After 24 hr, mice in control group were fed with 0.2 ml saline/day by oral gavage and second and third groups with 100 and 200 mg/kg/day doses of GSE in 0.2 ml of saline 5 days a week, respectively, for 7 weeks. Body weight and diet consumption were recorded twice weekly throughout the study. After xenografts started growing, their sizes were measured twice weekly. The tumor volume was calculated by the formula  $0.5236 L_1(L_2)^2$ , where  $L_1$  is the long axis and  $L_2$  is the short axis of the tumor.<sup>7</sup> At the end of experiment, tumors were excised, and weighed and stored at -80°C until further analysis.

### Immunohistochemical detection of PCNA in tumors

Tumor samples were fixed in 10% buffered formalin for 12 hr and processed conventionally. The paraffin-embedded tumor sections (5 µm thick) were heat immobilized, and deparaffinized using xylene and rehydrated in a graded series of ethanol with a final wash in distilled water. Antigen retrieval was done with 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by immersing the sections in 3.0 % H<sub>2</sub>O<sub>2</sub> in methanol

(v/v). The sections were then incubated with mouse monoclonal anti-PCNA antibody IgG2a (Dako, Carpinteria, CA), 1:400 in PBS for 1 hr at 37°C in humidity chamber. Negative controls were treated only with PBS under identical conditions. The sections were then incubated with biotinylated rabbit anti-mouse antibody IgG (1:200 in 10% normal rabbit serum) followed by conjugated horseradish peroxidase streptavidin (Dako). The sections were then incubated with 3,3'-diaminobenzidine (Sigma, St. Louis, MO) working solution for 10 min at room temperature and counterstained with diluted Harris hematoxylin (Sigma). Finally, sections were viewed and photographed under inverted Nikon TE-300 microscope equipped with a Princeton Instrument Micromax camera. Images are acquired with Image Pro-plus software (Media Cybernetics, Silver Spring, MD). Proliferating cells were quantified by counting the PCNA-positive cells and the total number of cells at 10 arbitrarily selected fields at 400× magnification in a blinded manner. The proliferation index (per 400× microscope field) was determined as number of PCNA-positive cells × 100/total number of cells.

### In situ apoptosis detection by TUNEL staining

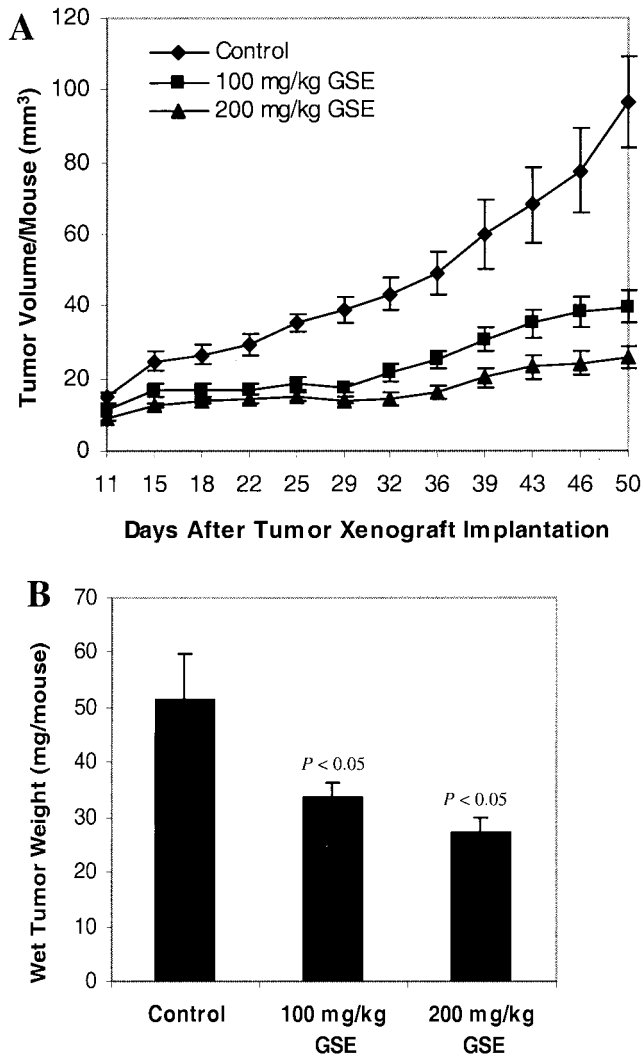
The formalin-fixed and paraffin-embedded 5 µm thick sections of all tumor samples (those used for PCNA staining) were studied by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) staining by Tumor TACS *In Situ* Apoptosis Detection Kit (R&D Systems, Inc. Minneapolis, MN) as published recently.<sup>26</sup> The apoptosis was evaluated by counting the positive cells (brown-stained) as well as the total number of cells at 10 arbitrarily selected fields at 400× magnification in a blinded manner. The apoptotic index (per 400× microscope field) was calculated as number of apoptotic cells × 100/total number of cells.

### Immunohistochemical analysis of tumors for CD31 expression

Staining procedure for CD31 (an endothelial cell specific antigen also known as PECAM-1) was similar to that of PCNA staining using specific antibodies. Sections from paraffin-embedded tumors were incubated overnight with goat anti-mouse CD31 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in PBS containing 10% rabbit serum. Then sections were incubated with biotinylated rabbit anti-goat secondary antibody (Santa Cruz Biotechnology) followed by streptavidin-conjugated horseradish peroxidase (Dako). Antigen-antibody complexes were visualized by incubation with 3,3'-diaminobenzidine substrate and counterstained with diluted Harris hematoxylin. CD31 stained (brown) cells were quantified in 10 random microscopic (400×) fields per tumor by an independent observer.

### Quantification of VEGF secreted by DU145 cells

Quantikine human VEGF immunoassay kit from R&D Systems, Inc. (Minneapolis, MN) was used to determine the VEGF concentration secreted from DU145 cells in conditioned medium, following manufacturer's instructions. Briefly, DU145 cells were grown in serum-supplemented medium to a sub-confluent stage. The cells were then washed twice with serum-free medium and treated with DMSO or GSE (40 and 80 µg/ml) in 1% serum containing medium (1% serum was recommended for the stability of VEGF in culture medium) for 12 hr. At the end of treatments, conditioned medium was harvested and analyzed for VEGF concentration. Cells were also collected, and protein concentration was determined to express VEGF concentration as pg/100 µg of protein in each treatment. In the ELISA assay, the final optical density of the developed color was determined using a microplate reader set to 450 nm with correction wavelength at 540 nm. VEGF concentration was extrapolated from the standard curve generated using recombinant human VEGF in the assay. We observed that known concentrations of recombinant human VEGF added in culture

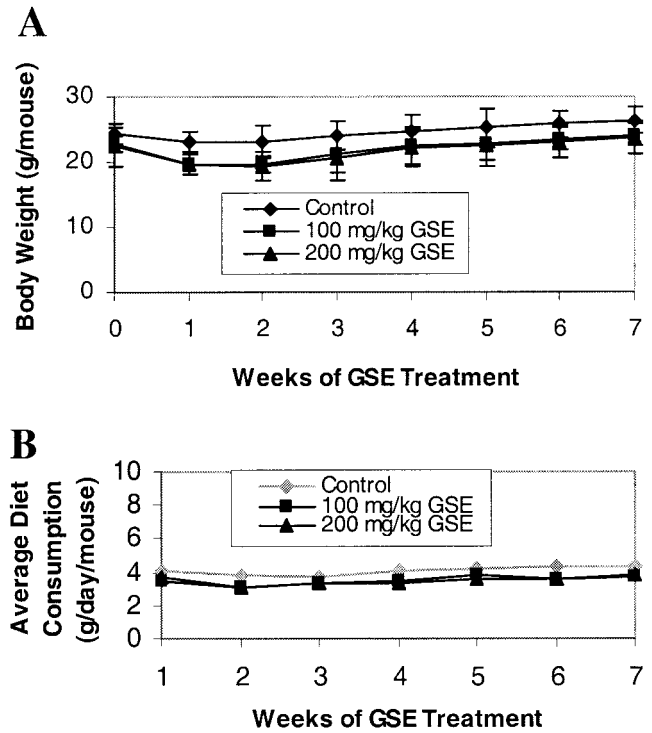


**FIGURE 1**—Effect of GSE feeding on DU145 tumor xenograft growth, and body weight gain and diet consumption profiles in athymic male nude mice. Approximately 2,000,000 DU145 cells were s.c. injected in the right flank of each mouse to initiate ectopic prostate tumor growth. GSE (100 and 200 mg/kg/day) feeding started after 24 hr of tumor cells injection and continued for 7 weeks and tumor sizes were measured twice a week. (a) Once tumor xenografts started growing, their sizes were measured twice weekly in two dimensions, throughout the study. The tumor volume was calculated by the formula  $0.5236 L_1(L_2)^2$ , where  $L_1$  is the long axis and  $L_2$  is the short axis of the tumor. Tumor volume (mm<sup>3</sup>)/mouse plotted as a function of time (week). (b) Tumor weight (mg)/mouse at the end of study. All these data are represented as mean  $\pm$  SE of 7–9 samples from individual mouse in each group. Scale bars = SE.

medium yielded linear standard curve similar to that generated in assay diluent, and the efficiency of detection in our assay was 96%.

#### Enzyme-linked immunosorbent assay for human IGFBP-3

At the termination of the experiment, blood was collected intracardiacally from mice in heparinized tubes, and plasma was separated for IGFBP-3 analysis. Quantikine human IGFBP-3 immunoassay kit from R&D Systems, Inc. (Minneapolis, MN) was used to determine the IGFBP-3 concentration secreted from DU145 tumors in mouse plasma following manufacturer's instructions. The principle of the assay was based on quantitative sandwich enzyme immunoassay employing pre-coated monoclonal antibody specific for IGFBP-3 onto a microplate for solid-phase



**FIGURE 2**—Effect of GSE feeding on body weight gain and diet consumption profiles in athymic male nude mice. Body weight and diet consumption were recorded twice a week during 7 weeks of the study as detailed in Material and Methods. (a) Body weight (g)/mouse and (b) average diet consumption/mouse/day are plotted as function of week. Body weight data are represented as mean  $\pm$  SE of 7–9 mice in each group. Scale bars = SE.

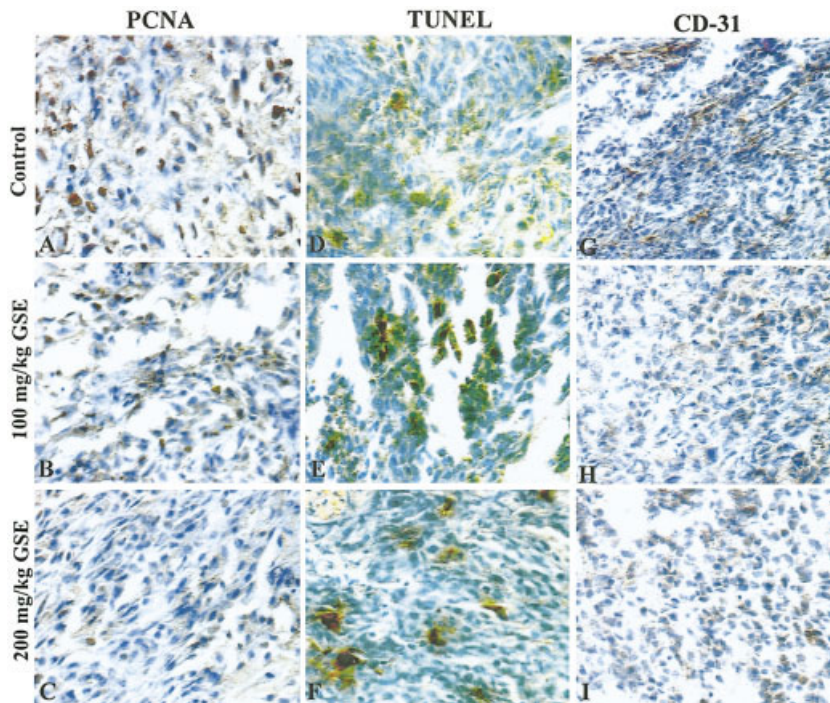
ELISA. Briefly, 50  $\mu$ l plasma was used in the assay and finally the optical density of the developed color was determined using a microplate reader set to 450 nm with correction wavelength at 540 nm as described earlier.<sup>27</sup> IGFBP-3 concentration was extrapolated from the standard curve generated using recombinant human IGFBP-3 in the assay. We observed that known concentrations of recombinant human IGFBP-3 added in mouse plasma of control mice yield linear standard curve similar to that generated in assay diluent, but efficiency of detection was only 40%. Final IGFBP-3 concentrations reported in results, therefore, are extrapolated accordingly.

#### Immunohistochemical analysis for VEGF and IGFBP-3 expression in tumors

Immunostaining for VEGF and IGFBP-3 in tumor sections was similar to that of PCNA staining using specific primary antibodies. Briefly, tumor sections were incubated overnight with human reactive rabbit anti-VEGF and goat anti-IGFBP-3 primary antibodies (Santa Cruz Biotechnology) followed by incubation with appropriate biotinylated secondary antibodies and horse-radish peroxidase-conjugated streptavidin. Antigen-antibody complexes were visualized by peroxidase reaction with DAB substrate and counterstained with hematoxylin. Sections were viewed and analyzed by microscope under low (100 $\times$ ) as well as high (400 $\times$ ) magnifications by an independent observer.

#### Statistical analysis

The statistical significance of difference between control and GSE-treated groups was determined by one way analysis of variance followed by Tukey-test for multiple comparisons. Student's two-tailed *t*-test was employed, as needed, and *p*-value was considered significant at *p* < 0.05.



**FIGURE 3**—*In vivo* antiproliferative, apoptotic and antiangiogenic effects of GSE in human prostate tumor xenograft in nude mice. (a–c) At the end of the study detailed in Figure 1, tumors were excised and processed for immunohistochemical staining for proliferation cell nuclear antigen (PCNA); (d–f) for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL); and (g–i) endothelial cell specific antigen CD31 as detailed in Material and Methods. Representative pictures were taken at 400× magnification of microscopic field from each group.

## RESULTS

### *Inhibition of prostate tumor xenograft growth by GSE feeding in nude mice*

To study the effect of GSE feeding on prostate tumor development, we used *in vivo* human prostate tumor xenograft in nude mice model. In saline-treated control, progressive tumor growth was observed during the 7 weeks of study period, and tumor cell masses become visible and measurable at Day 11, after DU145 xenograft implantation. Compared to control, GSE treatment (started 24 hr after xenograft implantation) to nude mice by oral gavage at 100 and 200 mg/kg/day doses, 5 days a week for 7 weeks resulted in a strong inhibition of prostate DU145 tumor xenograft growth throughout the study (Fig. 1a). As shown in Figure 1a, at the end of study, GSE (100 and 200 mg/kg) treatment resulted in 59 and 73% inhibition ( $p < 0.001$ ) of tumor growth, respectively, as calculated by the measurement of tumor volumes. Consistent with the decrease in tumor volumes, GSE also decreased the wet weight of tumors (37–47%,  $p < 0.05$ ) as compared to saline treated control (Fig. 1b).

Body weight-gain and diet consumption profiles are considered as some of the widely used parameters in assessment of gross toxicity or untoward effects of a test compound in cancer chemoprevention/therapy studies. Accordingly, we assessed whether GSE feeding causes any adverse health effect on mice during the study period, by monitoring body weight and diet consumption twice a week. In GSE-treated mice, body weight gain and diet consumption profiles were almost comparable to control group of mice (Fig. 2a,b). Although a slight quantitative difference (not statistically significant) remained throughout the experiment between control and treatment groups, these profiles did not decrease with the treatment time, and therefore, diet consumption per gram body weight of mouse remained similar in all the groups.

### *In vivo antiproliferative and apoptotic efficacy of GSE in prostate tumor xenografts*

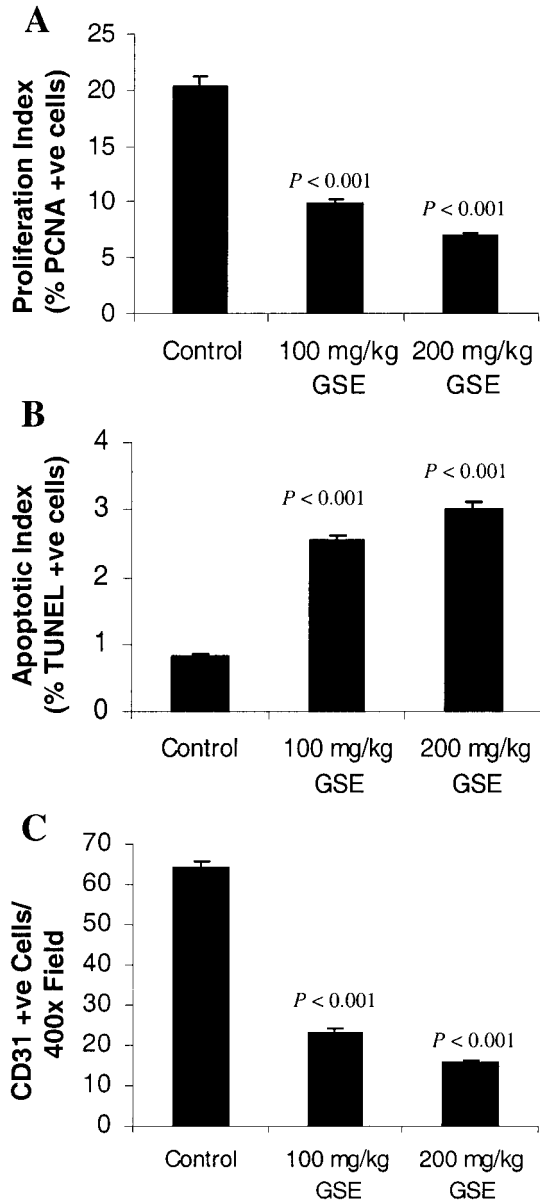
To assess the *in vivo* effect of GSE feeding to mice on its antiproliferative and apoptotic responses toward the inhibition of tumor xenograft growth in mice, the tumor samples from the study detailed above, were analyzed by PCNA and TUNEL staining. Qualitative microscopic examination of PCNA-stained tumor sec-

tions showed substantial decrease in PCNA-positive cells in GSE-fed groups as compared to that of control group (Fig. 3a–c). The quantification of PCNA immunohistochemical staining of tumors showed that GSE (100 and 200 mg/kg) feeding to nude mice results in 51 and 66% ( $p < 0.001$ ) decrease in proliferation index, respectively, as compared to saline treated control (Fig. 4a). The negative control, in which only blocking buffer was used instead of PCNA monoclonal antibody, did not show any considerable positive staining (data not shown).

Microscopic examination of TUNEL-stained tumor sections showed that compared to control, GSE increases TdT-positive (brown-stained) cells (Fig. 3d–f). The quantitative evaluation of apoptosis showed that GSE (100 and 200 mg/kg) increases the apoptotic index by 3.3- and 4.2-fold ( $p < 0.001$ ) in prostate tumor xenografts, respectively, over that of control (Fig. 4b). The positive control in which TACS-nuclease was used to generate DNA fragments with free 3'-OH end, showed positive staining in all the nuclei whereas in negative control, in which labeling buffer was used instead of TdT, did not show any considerable positive staining (data not shown).

### *GSE inhibits tumor microvessel density*

Neovascularity is an essential requirement in the progression of many cancers from latent and localized focal carcinomas to invasive carcinomas. Tumor microvessel is regarded as an important prognostic marker, and an independent predictor of pathologic stages and of malignant potential of PCA.<sup>28–30</sup> To examine whether strong inhibition of prostate tumor growth by GSE is accompanied by its *in vivo* antiangiogenic effect, we investigated intratumoral microvessel density by immunohistochemical analysis of endothelial cell specific marker CD31. The microscopic examination of tumors after immunohistochemical staining showed numerous cells positive for the expression of CD31 in control group of tumors but only sporadic positive cells in the tumors derived from the GSE-treated mice (Fig. 3g–i). Control tumors showed  $64.04 \pm 1.57$  CD31 positive cells/400× field compared to  $23.22 \pm 0.86$  ( $p < 0.001$ ) and  $15.72 \pm 0.79$  ( $p < 0.001$ ) CD31 positive cells in 100 and 200 mg/kg doses of GSE-treated tumors, respectively (Fig. 4c). The reduction in number of endothelial cells in GSE-treated prostate tumors, showed a novel



**FIGURE 4** – Quantification of antiproliferative, apoptotic and antiangiogenic effects of tumor xenografts. (a) Quantification of PCNA-positive cells for proliferation index; and (b) TUNEL-positive cells for apoptotic index in tumor samples. Both indices were calculated by number of positive cells  $\times$  100/total number of cells counted under 400 $\times$  magnification in 10 randomly selected areas in each tumor sample. The data shown are mean  $\pm$  SE of 7–9 samples from individual mouse in each group. (c) Quantification of CD31-positive cells for the assessment of intratumoral microvasculature, CD31 positive (brown) cells were counted under 400 $\times$  magnification of microscopic field in 10 randomly selected areas in each tumor sample. The data shown are mean  $\pm$  SE of 5 samples from individual mouse in each group. Scale bars = SE.

property of GSE that is the inhibition of ‘tumor angiogenesis’ that could have contributed to the inhibition of prostate tumor growth in nude mice.

#### GSE decreases DU145 cell-secreted VEGF level in conditioned medium

It is well established that tumor cells secrete proangiogenic factors for tumor angiogenesis, which is required for their growth

**TABLE I** – EFFECT OF GSE ON VEGF SECRETION IN CONDITIONED MEDIUM OF DU145 CELLS<sup>1</sup>

Treatment time	GSE dose ( $\mu$ g/ml)	VEGF content (pg/100 $\mu$ g protein)	%
12 h	0	1,217.56 $\pm$ 48.44	100
	40	646.39 $\pm$ 4.44 <sup>2</sup>	53
	80	361.24 $\pm$ 57.47 <sup>2</sup>	30

<sup>1</sup>Values are mean  $\pm$  SE of duplicate samples. Data were analyzed by one-way ANOVA followed by Tukey-test. <sup>2</sup> $p < 0.05$  as compared to control.

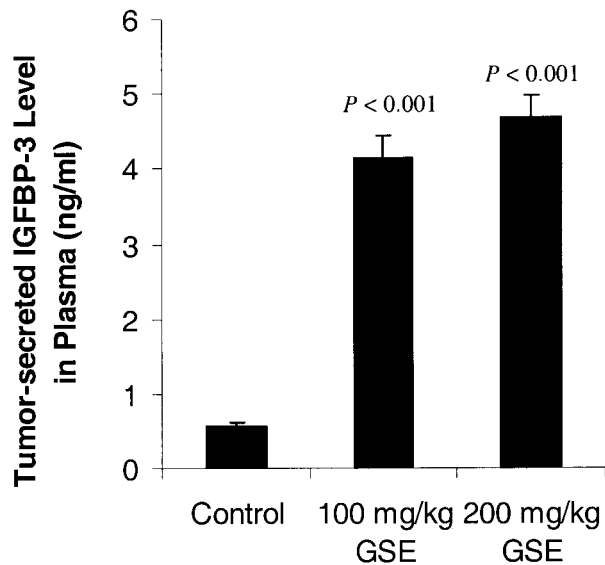
beyond a certain microscopic size.<sup>5</sup> VEGF is regarded as one of the most important angiogenic factors, which has shown to be secreted by various cancer cells including DU145 cells. Accordingly, we also investigated whether GSE has any effect on VEGF secretion from DU145 cells. It is important to mention that first we did this assay in mouse plasma samples from the xenograft experiment but could not get enough detectable levels of tumor-secreted levels of VEGF. This might be because tumors were very small and did not secrete VEGF levels in the detectable range of the kit. Next, we used cell culture system to check the effect of GSE on VEGF secretion in DU145. Treatment of semi-confluent DU145 cells with 40 and 80  $\mu$ g/ml doses of GSE for 12 hr resulted in 646.39  $\pm$  4.44 and 361.24  $\pm$  57.47 pg VEGF/100  $\mu$ g of protein compared to control showing 1,217.56  $\pm$  48.44 pg VEGF/100  $\mu$ g protein, which accounted for 47 and 70 % ( $p < 0.05$ ) inhibition, respectively (Table I). The inhibitory effect of GSE on VEGF secretion by DU145 cells also supports the *in vivo* suppression of tumor angiogenesis by GSE in tumor xenograft study.

#### GSE increases tumor-secreted IGFBP-3 level in mouse plasma

Human IGFBP-3 level in mice plasma secreted by DU145 tumor xenograft after GSE treatment was determined at the end of the experiment. A standard curve was made using recombinant human IGFBP-3 in the assay to extrapolate the plasma level of IGFBP-3 (data not shown). Plasma samples from each treatment groups were assayed for tumor secreted IGFBP-3 as described in Material and Methods, which resulted in higher OD<sub>450–540</sub> in GSE-treated samples as compared to control samples (data not shown). When these optical densities were extrapolated on standard curve, oral feeding of GSE at 100 and 200 mg/kg/day doses for 7 weeks resulted in 4.15  $\pm$  0.30 and 4.68  $\pm$  0.31 ng IGFBP-3/ml plasma as compared to 0.65  $\pm$  0.07 (ng/ml) in control group, respectively (Fig. 5). This increase in IGFBP-3 level accounted for 6.4- and 7.2-fold ( $p < 0.001$ ) increase over that of control value, respectively. This finding provides the first evidence for an *in vivo* association between IGFBP-3 upregulation and inhibition of prostate tumor xenograft growth by GSE.

#### GSE moderately decreases VEGF and increases IGFBP-3 protein expression in tumors

To assess the *in vivo* effect of GSE feeding on VEGF and IGFBP-3 protein levels in DU145 prostate tumor xenograft, paraffin-embedded tumor sections were analyzed by immunohistochemical staining for VEGF and IGFBP-3 using specific antibodies. Microscopic examination of VEGF-stained tumor sections showed slight decrease in the intensity of VEGF-positive cytoplasmic staining in GSE-fed groups of tumors as compared to control group of tumors (Fig. 6a–c). Immunohistochemical analysis for IGFBP-3 staining showed an increase in IGFBP-3-positive cytoplasmic staining in GSE-fed groups of tumors as compared to control group of tumors (Fig. 6d–f). The increased expression of IGFBP-3 in tumors by GSE showed almost similar trend to that of secreted levels of IGFBP-3 in plasma. These results were evident in about 70% of the tumor samples examined in the study. These observations support the antiangiogenic potential of GSE in prostate tumors as well as VEGF and IGFBP-3 as possible molecular targets of its anticancer efficacy.



**FIGURE 5** – Effect of oral feeding of GSE on tumor-secreted plasma level of IGFBP-3 in nude mice. At the termination of the study detailed in Figure 1, plasma concentration of human IGFBP-3 was estimated using quantikine ELISA, as mentioned in Material and Methods. A standard curve was established with known concentrations of IGFBP-3 at logarithmic scale. IGFBP-3 concentrations (ng/ml) in plasma samples were calculated by four-parameter logistic (4-PL) curve-fit generated standard curve. Values are represented as mean  $\pm$  SE of 6–8 samples (ng/ml plasma) from individual mouse in each treatment group. Scale bars = SE.

#### DISCUSSION

The results of our present study demonstrate that GSE feeding inhibits androgen-independent advanced human prostate carcinoma DU145 tumor growth in athymic nude mice without any apparent untoward toxicity. This inhibition of tumor growth is accompanied by a decrease in proliferation index, an increase in apoptotic index, and a strong inhibition of tumor angiogenesis. In DU145 cell culture, GSE strongly decreases the secreted level of the potent angiogenic factor VEGF. GSE also increases IGFBP-3 secretion from tumor cells, as one of the possible mechanisms of inhibition of tumor cell growth and survival. The results from immunostaining of tumors for VEGF and IGFBP-3 further support their roles in GSE-caused inhibition of prostate tumor xenograft growth. These results suggest the further investigation of GSE efficacy in other PCA models such as orthotopic PCA xenograft as well as those where PCA is induced chemically or by transgene. A positive outcome in such studies will support the notion of GSE efficacy in different pre-clinical PCA models, and its possible usefulness in PCA patients.

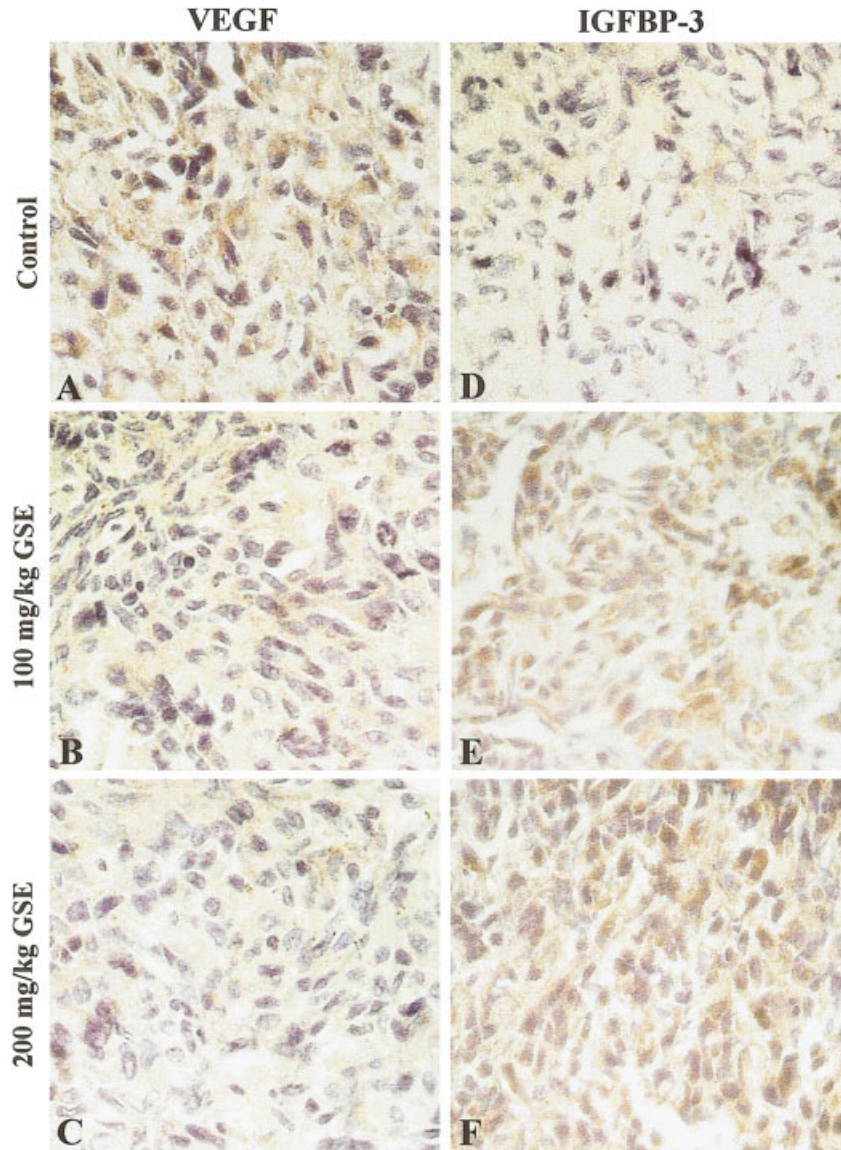
Tumorigenesis is a multistep process that requires acquisition of certain oncogenic properties such as uncontrolled cell proliferation, inhibition of apoptosis and induction of angiogenesis. The autocrine and paracrine growth factor-receptor interactions have been reported to be the causal factors for the advanced and androgen-independent PCA stage.<sup>31,32</sup> Multiple constitutively active mitogenic, cell survival and antiapoptotic pathways in PCA lead to the argument that inhibiting one pathway is possibly not enough to achieve PCA growth control, and also explain the ineffectiveness of apoptosis-inducing androgen suppression/ablation therapy in PCA patients.<sup>31–35</sup> Accordingly, it could be argued that agents that exert diversified inhibitory effects on both mitogenic and cell survival signaling pathways and induce apoptotic death in prostate carcinoma cells could be effective in controlling growth and development of PCA.

Consistent with above notion, the findings of our present study together with those reported recently clearly suggest that GSE exerts its anticancer efficacy against prostate tumor growth in nude mice by diverse mechanisms of action.<sup>19</sup> The strong inhibition of tumor growth by GSE, as evidenced by a decrease in tumor volume and proliferation index, may be attributed to the inhibition of constitutively active mitogenic and cell survival signaling in PCA cells.<sup>9,36</sup> An increased apoptotic index in the tumors from GSE-fed mice also supports its inhibitory effect on cell survival signaling or an induction of pro-apoptotic cascade by GSE. Several mechanistic studies completed by us recently support the effect of GSE on mitogenic, cell survival and both anti- and pro-apoptotic pathways in DU145 cells. For example, we have shown that GSE inhibits ERK1/2 activation and induces apoptotic death of DU145 cells in culture.<sup>19</sup> More recently, we observed that in DU145 cells, GSE impairs EGFR signaling, directly inhibits ERK1/2 kinase activity and constitutively active Elk1 and AP1 as its anti-proliferative effect, and activates JNK as its apoptotic response.<sup>36</sup> The apoptotic efficacy of GSE was further supported by our another recent mechanistic study where GSE induced mitochondrial membrane potential damage, cytochrome *c* release, caspases activation and poly (ADP-ribose) polymerase cleavage in DU145 cells leading to their apoptotic death.<sup>37</sup>

Human tumors can remain dormant for years owing to a balance between cell proliferation and apoptosis.<sup>5,38</sup> Therefore, systemic concentration of angiogenic inhibitors exceeding that of stimulators could inhibit tumor growth as well as metastasis to distal organs.<sup>38</sup> Based on this hypothesis and outcomes of preclinical studies, anti-angiogenic therapy is suggested as a most promising approach to cancer control.<sup>5,20,39</sup> Another advantage of this therapy could be that endothelial cells are generally non-transformed cells and are less prone to acquire drug resistance.<sup>40</sup> It has been suggested that the use of quantitative angiogenesis assay in future PCA clinical trials may be helpful in the early detection of the disease and monitoring the efficacy of the agents under test.<sup>41,42</sup> Consistent to these reports, GSE strongly inhibited neovascularization of prostate tumor xenograft in nude mice as analyzed by endothelial cell specific CD31 immunohistochemical staining.

Several types of solid tumors of epithelial origin have been shown to overexpress and secrete VEGF, however, stromal or endothelial cells may also express VEGF in hypoxic microenvironment of tumors.<sup>43</sup> Antiangiogenic intervention by VEGF antibodies or by blocking its receptor signaling has been shown to inhibit tumor growth.<sup>44–47</sup> Several phytochemicals have been shown to downregulate VEGF in imparting their antiangiogenic potential.<sup>48</sup> We also observed that GSE strongly inhibits the secreted level of VEGF in DU145 cell culture. The GSE-caused inhibition of VEGF secretion by prostate epithelial cancer cells might be one of the important mechanisms in controlling angiogenic switch and leading to an inhibition of overall growth and progression of PCA. This possibility was confirmed by the immunohistochemical analysis of tumors for VEGF expression. As expected, GSE decreased cytoplasmic immunoreactivity for VEGF in tumor sections. The etiology of PCA also shows an age-related increase in the prevalence of latent carcinoma of prostate. Therefore, antiangiogenic potential of GSE could be explored as one of its effects in lowering the risk of and preventing the growth and metastasis of PCA.

The importance of IGF-1 signaling and IGFbps in deregulated cellular growth has also been established in prostate carcinoma cells and transgenic mice.<sup>49</sup> It has been suggested that mitogenic as well as cell survival signaling via IGF-1/IGF-1R pathway is constitutively activated in human PCA cells and, in part, responsible for the growth and metastatic potential of PCA.<sup>25,33</sup> Anti-tumor efficacy of some natural compounds have been shown to be associated with the upregulated levels of IGFBP-3 in animal tumor models of prostate cancer.<sup>27,50</sup> Consistent to these reports, we observed that *in vivo* PCA xenograft inhibitory efficacy of GSE is associated with an induction of IGFBP-3 level in tumor xenograft



**FIGURE 6** – Effects of GSE feeding on VEGF and IGFBP-3 expression in human prostate tumor xenograft in athymic nude mice. At the end of the study detailed in Figure 1, tumor samples were immunohistochemically analyzed for (a–c) VEGF and (d–f) IGFBP-3 protein expression as detailed in Material and Methods. *Upper panel*, control; *middle panel*, 100 mg/kg dose of GSE; *lower panel*, 200 mg/kg dose of GSE. Representative pictures from each treatment group are shown at 400 $\times$  magnification.

as well as its increased secretion in plasma, suggesting that the inhibition of IGF-1R signaling might be one of the major *in vivo* mechanisms of GSE-caused PCA xenograft growth inhibition in nude mice. To support this conclusion further studies are needed to define the effect of GSE on IGF-1-IGF-1R-PI3K signaling.

Collectively, present findings provide *in vivo* evidence for antiproliferative, apoptotic and antiangiogenic effects of GSE, and their correlation with inhibition of advanced human prostate tumor xenograft growth in athymic nude mice. Upregulation of IGFBP-3 by GSE could be one of the possible *in vivo* mechanisms leading to inhibition of PCA cell growth and survival. GSE-induced decrease in VEGF secretion from PCA cells might also play a role in

inhibition of tumor angiogenesis. Based on these findings, further studies are needed to explore the preventive/therapeutic efficacy of GSE with mechanistic details in other PCA pre-clinical models for its possible implication in humans.

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