

Proanthocyanidins from grape seeds inhibit expression of matrix metalloproteinases in human prostate carcinoma cells, which is associated with the inhibition of activation of MAPK and NFκB

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Prostate cancer (PCA) is the second most frequently diagnosed and leading cause of cancer-related deaths in men in the USA. The recognition that matrix metalloproteinases (MMPs) facilitate tumor cell invasion and metastasis of PCA has led to the development of MMP inhibitors as cancer therapeutic agents. As part of our efforts to develop newer and effective chemopreventive agents for PCA, we evaluated the effect of proanthocyanidins from grape seeds (GSP) on metastasis-specific MMP-2 and -9 in human prostate carcinoma DU145 cells by employing western blot and gelatinolytic zymography. Treatment of GSP dose-dependently inhibited cell proliferation (15–100% by 5–80 μg/ml of GSP), viability (30–80% by 20–80 μg/ml of GSP) and fibroblast conditioned medium (FCM)-induced expression of MMP-2 and -9 in DU145 cells. Since the signaling cascade of mitogen-activated protein kinases (MAPK) have been shown to regulate the expression of MMPs in tumor cells, we found that the treatment of DU145 cells with GSP (20–80 μg/ml) resulted in marked inhibition of FCM-induced phosphorylation of extracellular signal regulated kinase (ERK)1/2 and p38 but had little effect on c-Jun N-terminal kinase under similar experimental conditions. GSP treatment (20–80 μg/ml) to DU145 cells also dose-dependently inhibited FCM-induced activation of NFκB concomitantly with inhibition of MMP-2 and -9 expression in the same system. Additionally, the treatment of inhibitors of MEK (PD98059) and p38 (SB203580) to DU145 cells resulted in the reduction of FCM-induced phosphorylation of ERK1/2 and p38 concomitantly marked reduction in MMP-2 and -9 expressions. In further studies, treatment of androgen-sensitive LNCaP cells with a synthetic androgen R1881, resulted in an increase of MMP-2 and -9, which were completely abrogated in the presence of GSP (20–60 μg/ml). These data suggest that inhibition of metastasis-specific MMPs in tumor cells by GSP is associated with the inhibition of activation of MAPK and NFκB pathways, and thus provides the molecular basis for the development of GSP as a novel chemopreventive agent for both androgen-sensitive and -insensitive prostate cancer therapies.

Abbreviations: ERK, extracellular signal regulated kinase; FCM, fibroblast conditioned medium; GSP, proanthocyanidins from grape seeds; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NFκB, nuclear factor κB; PCA, prostate cancer; SFM, serum-free medium.

Introduction

The incidence of prostate cancer (PCA) is increasing steadily in almost all countries, yet we know little about the real causes of this disease (1). PCA is the most common neoplasm and the second leading cause of cancer-related deaths among US men (2–4). According to recent statistics, about 220 900 new cases of prostate cancer will be diagnosed and an estimated 28 900 deaths will occur in the USA during the year 2003 (1,2). Androgen ablation via surgical castration or anti-androgen therapy is the first-line therapy in metastatic prostate cancer (5). However, following such therapy, the PCA almost invariably progress to more aggressive androgen-refractory prostate cancer rendering androgen ablation therapy ineffective (6,7). One of the earliest events in the metastatic spread of prostate cancer is the invasion through the basement membrane and proteolytic degradation of the extracellular matrix proteins such as collagens, laminin, elastin and fibronectin, etc., that also make up the basement membrane (8). Thus, prevention of tumor invasion could be considered as a promising target and appropriate strategy to prevent prostate cancer. The matrix metalloproteinase (MMP) family is comprised of secreted and membrane-associated zinc-dependent endopeptidases that can selectively degrade extracellular matrix proteins and non-matrix proteins (9,10) and therefore play a central role in all the processes of tumor invasion, angiogenesis and metastasis (11–14). Thus, MMPs are important regulators of tumor growth, both at the primary site and in distant metastases. Given the clear implications of MMPs in many human cancers, MMPs remain important targets for cancer therapy (12). Therefore, it is desirable to develop newer and effective chemopreventive agents or inhibitors of MMPs, which can inhibit the synthesis of MMPs at the tumor site, and thus prevent, reverse or slow down the age-related development of PCA in humans.

Among various MMPs, two particular members of the MMP family, gelatinase A (MMP-2) and gelatinase B (MMP-9) seem to play an important role in prostate cancer invasion and metastasis (15). Importantly, it has been shown that MMP-2 is secreted by the human prostate gland, both *in vivo* and *in vitro*, and higher expression levels of MMP-2 are associated with increasing Gleason score, tumor metastasis and aggressive behavior of prostate cancer (16–18). MMP gene expression is primarily regulated at the transcriptional (through AP-1 via mitogen activated protein kinase pathways) and post-transcriptional levels, and at the protein level via their activators, inhibitors and their cell surface localization (18–20). Hence, MMPs and their regulatory pathways have been considered promising targets for anticancer drugs and chemopreventive agents (18–21). Moreover, the expression of MMP in tumors is regulated in a paracrine manner by growth factors secreted by tumor infiltrating inflammatory cells as well as by tumor or stromal cells (22–24). Fibroblast growth factors have been shown to mediate transcriptional

regulation of the MMP gene, such as matrilysin (MMP-7) in human prostate carcinoma cells but not in normal prostate epithelial cells (24).

Considerable interest among the human population is developing with regard to the use of dietary botanical supplements for the prevention of age-related diseases like PCA. Among the dietary supplements with a broad range of biological activities, the antioxidant class of compounds has gained considerable interest. One among them is the proanthocyanidins from grape seeds (GSP). Grape seeds (*Vitis vinifera*) are an excellent source of proanthocyanidins in which the most abundant classes of flavonoids are the flavan-3-ols. Some of the compounds in the flavan-3-ol class are also referred to as catechins, which comprise the monomeric compounds, (+)-catechin and (–)-epicatechin, as well as epicatechin 3-*O*-gallate (25,26). However, the major proportions of flavan-3-ols are found in oligomeric and polymeric forms (Table I). Studies indicate that the higher polymers (such as trimers and oligomers) are linked mainly to the beneficial effects of grape seed polyphenols (27,28). The chemopreventive effects of GSP have been associated with their high antioxidant activity (reviewed in ref. 29). GSP has also been shown to have a wide variety of biological activities such as antimutagenicity, inhibition of low-density lipoprotein oxidation, anti-inflammatory, antiviral, antihypertensive, etc. (29). Cytotoxic effects of GSP have been shown in a variety of cancer cell lines (30) with respect to the induction of apoptosis in human prostate cancer cells without affecting the growth and viability of the normal cells (31,32). Recently, it has been shown that grape seed extract inhibits epidermal growth factor-induced and constitutively active mitogen-activated protein kinase (MAPK) signaling in DU145 cells, which may have a possible role in anti-proliferation and apoptosis of cancer cells (33). In the present study, we demonstrate the effects of proanthocyanidins from grape seeds on cell proliferation, viability and most importantly on invasion-specific MMP expression and their regulatory mechanism in androgen-insensitive human prostate carcinoma DU145 cells by employing gelatinolytic zymography and western blot analysis. The treatment of GSP resulted in inhibition of cellular proliferation and the expression of MMP-2 and -9 in DU145 cells, as well as synthetic androgen R1881-induced MMP-2 and -9 expressions in androgen-sensitive human prostate carcinoma LNCaP cells. We also demonstrate that inhibition

of MMP expression by GSP is associated with the inhibition of activation of proteins of MAPK family, such as extracellular signal regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK) and p38, and inhibition of activation of nuclear factor κ B (NF κ B) in human prostate carcinoma DU145 cells. Thus, defining a possible mechanism of inhibition of MMPs associated with the invasion and metastasis of PCA.

Materials and methods

Reagent and antibodies

Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin, fetal bovine serum (FBS) and trypsin-EDTA, were purchased from Cellgro (Herdon, VA). Acrylamide and the protein assay kit were obtained from Bio-Rad (Hercules, CA). Polyclonal anti-MMP-2 and anti-MMP-9, MEK inhibitor PD095089 and p38 inhibitor SB203580 were purchased from Sigma Chemical (St Louis, MO). Antibodies against ERK1/2, JNK and p38 and for their phospho-specific counterparts were obtained from Cell Signaling Technology (Beverly, MA), and antibodies to NF κ B were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). R1881 was obtained from Perkin Elmer (Boston, MA). An enhanced chemiluminescence (ECL) reagent kit, PhastGel Blue R and protein molecular weight markers were purchased from Amersham Pharmacia Biotech (Piscataway, IL). Purified GSP was obtained from Kikkoman, Japan, for this study. Chemical composition of GSP was analyzed by Kikkoman and given in Table I. All other chemicals used in this study were purchased from authentic sources and of highest grade and purity.

Cell cultures and cell lines

Human prostate carcinoma DU145 and LNCaP cells, and human foreskin fibroblast (HS 68) cells were purchased from the American Type Culture Collection (Rockville, MD). These cells were routinely maintained in a regular culture medium, i.e. phenol red-containing DMEM supplemented with 10% FBS, 4 mM glutamine, and penicillin (100 U/ml) and streptomycin (100 μ g/ml) in an incubator at 37°C in a humidified atmosphere containing 5% CO₂.

Preparation of fibroblast conditioned medium (FCM)

To prepare FCM, sub-confluent HS-68 cells in 75 cm³ culture flask were incubated at 37°C in a humidified atmosphere containing 5% CO₂ in 10 ml of serum-free medium (SFM) containing 0.5 μ g/ml bovine serum albumin for 24 h as described earlier (34). After 24 h, the medium was collected, centrifuged and the supernatant was used as a FCM either immediately or stored at –80°C for further use. To determine the chemopreventive effects of GSP on the synthesis of MMPs, DU145 cells (2×10^6) were cultured in 500 μ l of FCM or SFM containing different concentrations of GSP for 24 h in 24-well culture plates. Media was collected to perform both gelatinolytic zymography and western blotting and simultaneously cells were used to prepare the cell lysates to detect the expressions of MMP, and nuclear fractions to detect the activation of NF κ B.

Clonogenic assay/colony-forming potential

The anti-proliferative effect of GSP on DU145 cells was measured by a colony-formation assay. Approximately 500 cells were seeded into 35 mm Petri plates in triplicate for each group and allowed to adhere for 24 h. Thereafter cell culture medium was changed and cells were treated with or without GSP containing medium (5–80 μ g/ml). The cells were allowed to incubate at 37°C in the incubator undisturbed for 14 days. During this period each individual surviving cell would proliferate and form colonies. On day 15, the colonies were then washed with cold phosphate buffer saline, fixed with 70% ethanol and stained with 0.02% aqueous trypan blue solution. The colonies, which had 50 cells or more per colony were counted. Data were represented as number of colonies formed per 500 cells plated with and without the treatment of GSP, which expressed the colony-forming potential of the DU145 cells in different treatment conditions.

MTT assay

Standard procedure was adopted to determine cell proliferation and viability of prostate cancer cells with or without the treatment of GSP (35). Briefly, cells were plated in 96-well culture plates (1×10^4 cells/well) in eight replicates and kept overnight in an incubator. Thereafter, cells were treated with the required concentration of GSP. Control wells (non-GSP) with tumor cells were maintained in culture medium without the treatment of GSP. Wells containing culture medium without cells were used as blanks. The plates were incubated at 37°C in an incubator for 24, 48 and 72 h. Upon completion of the incubation, stock MTT dye solution (50 μ l, 5 mg/ml in media) was added to each well. After 2 h incubation, dimethylsulfoxide (100 μ l) was added to solubilize the

Table I. Chemical composition of grape seed extract used in this study

Components	Percent
Total proanthocyanidins	89.3
Dimers ^a	6.6
Trimers ^b	5.0
Tetramers	2.9
Oligomers	74.8
Total monomeric flavanols	6.6
(+)-Catechin	2.5
(–)-Epicatechin	2.2
(–)-Epigallocatechin	1.4
(–)-Epigallocatechin-3-gallate	0.5
Moisture	2.2
Protein	1.1
Ash	0.8

^aDimers containing procyanidin B1, procyanidin B2, procyanidin B3, procyanidin B4 and procyanidin B5.

^bTrimers containing procyanidin B5-3'-gallate and procyanidin C1.

MTT formazan. The optical density (OD) of each well was then measured on a microplate spectrophotometer at a wavelength of 540 nm.

Gelatinolytic zymography

Gelatinolytic zymography was used to detect the expression of MMP in the supernatant obtained from the media with or without the treatment of GSP as described previously (36). Briefly, the collected media (15 μ l) after treatment was loaded on to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel copolymerized with 0.1% gelatin and subjected to electrophoresis at 100 V for 1.5 h. In order to remove SDS, the gel was washed twice with 2.5% Triton X-100 solution for 30 min each, rinsed with incubation buffer (0.05 M Tris-HCl buffer pH 8.0, 5 mM CaCl₂, 5 μ M ZnCl₂), and incubated at 37°C overnight. The gel was stained with PhastGel Blue R at room temperature for 2 h as described previously (36). Gelatinases in the media were detected as unstained gelatin degraded zones on the gel.

Preparation of cell lysates and nuclear fraction, and western blot analysis

The cell lysates were prepared as described previously (37). Briefly, the cells were washed in PBS, incubated on ice with ice-cold lysis buffer containing protease inhibitors [Tris-HCl, 50 mM (pH 7.4) 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF, and aprotinin and leupeptin 1 μ g/ml each] for 30 min. Cells were scraped, passed through a 22 G needle several times to break the aggregation of the cells and centrifuged at 14 000 r.p.m. for 15 min at 4°C and the supernatants were collected for western blot analysis. Supernatants were used either immediately or stored at -80°C. Nuclear fractions were also prepared as described previously (38). Briefly, the cells were washed twice with ice-cold PBS followed by incubation on ice for 15 min with 0.4 ml of ice-cold lysis buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2.0 μ g/ml leupeptin and 2.0 μ g/ml aprotinin] in a microfuge tube. Then, 12.5 μ l of 10% IGEPAL CA-630 was added and mixed. The suspension was vortexed and centrifuged at 14 000 g for 1 min at 4°C. The pellets were incubated on ice for 30 min with 25 μ l of nuclear extraction buffer [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1.5 mM MgCl₂, 1.0 mM EDTA, 1.0 mM dithiothreitol, 1.0 mM PMSF, 0.1% IGEPAL CA-630, 2.0 μ g/ml leupeptin and 2.0 μ g/ml aprotinin]. The resulting homogenates were centrifuged at 4°C at 14 000 g for 5 min. The supernatants were collected and used immediately or stored at -80°C until use.

Samples of culture media (10–25 μ l/well), cell lysates or nuclear fractions (25–60 μ g of proteins) were resolved on Tris-glycine gel (10%). Protein transfer was done by electroblotting onto nitrocellulose membrane and thereafter membranes were blocked for 1 h in blocking buffer containing 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBS-T), pH 7.6. The membranes were probed overnight at 4°C with primary antibody in 5% non-fat dry milk or bovine serum albumin (3%) solution prepared in TBS-T. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody in TBS-T containing 5% non-fat dry milk and incubated for 1 h. Antigen detection was achieved by incubating the membrane for 1 min at room temperature with 0.125 ml/cm² of enhanced chemiluminescence reagents (Amersham, Piscataway, NJ) and performed autoradiography with XAR-5 film (Eastman Kodak Co., Rochester, NY). To check whether treatment of GSP to DU145 cells affects the total amounts of ERK1/2, JNK and p38, the blots were stripped and re-hybridized with their respective antibodies, and thereafter followed a similar protocol as detailed above. The relative intensity of protein bands in western blots and zymograms from different treatment groups and in various experiments were measured using computerized densitometry software program OPTIMAS 6.2.

Quantitative estimation of NF κ B by ELISA

Quantitative analysis of NF κ B/p65 was performed using ELISA. For this purpose, we employed commercially available Trans-AM kit (Active Motif, Carlsbad, CA) using the manufacturer's protocol. For this assay, the nuclear extract of DU145 cells was prepared using the Nuclear Extraction Kit (Active Motif) according to the manufacturer's protocol. This Trans-AM detection ELISA kit is 10-fold more sensitive than the electromobility shift assay (EMSA). The assay was done in triplicate and absorbance read at 450 nm with reference taken at 650 nm. Results are expressed in terms of OD of different treatment samples.

Results

Treatment of GSP inhibits cellular proliferation and viability of DU145 cells

To assess the cytotoxic, anti-proliferative or anticarcinogenic effect of GSP on DU145 cells, dose-response curves were

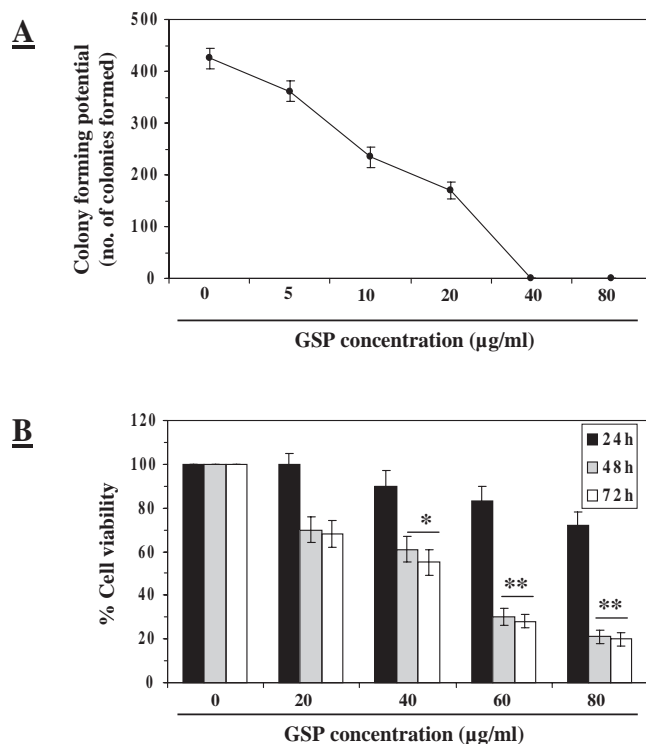


Fig. 1. Treatment of GSP to DU145 cells dose-dependently inhibited cells proliferation. (A) Treatment of GSP (5–80 μ g/ml) inhibits the proliferation potential of human prostate carcinoma DU145 cells in a dose-dependent manner. Proliferation of cells was assayed by the clonogenic assay as described in the Materials and methods section. The colony formation by DU145 cells with and without the treatment of GSP is represented in terms of total number of colonies formed at the end of the 15-day period protocol. Treatment of DU145 cells with GSP dose-dependently inhibited colony-formation capacity of the cells. The experiment was repeated three times and each point indicates the mean \pm SD of the number of colonies formed. (B) Treatment of GSP (20–80 μ g/ml) dose-dependently inhibited the cell viability of DU145 cells. Cells were plated in 96-well plates at a cell concentration of 10 000/well and treated with and without different concentrations of GSP and incubated for 24, 48 and 72 h and analyzed the viability of the cells by MTT assay as described in the Materials and methods section. The data are presented as % cell viability in terms of control (non-GSP treatment) at different time points from three independent experiments. Statistical significance, * P < 0.05; ** P < 0.001.

generated using data from an anchorage-dependent colony-forming assay and MTT assay. The colony-forming assay is based on the fact that a single isolated cell in medium proliferates independently and ultimately from an individual colony. Thus, it measures the ability of the cells to maintain their reproductive capacity under various treatment conditions. We found that GSP treatment results in dose-dependent cytotoxic effects on the human prostate carcinoma DU145 cells and significantly inhibited the colony-forming potential of the cells (Figure 1A). The doses of GSP treatment at 40 and 80 μ g/ml have shown almost complete cessation in the colony-forming potential or proliferation ability with no colonies being observed at the end of the experimental time period of 15 days.

Treatment of GSP to human prostate carcinoma DU145 cells significantly inhibited cell viability of the DU145 cells in both a time and dose-dependent manner when evaluated by MTT assay, as shown in Figure 1B. A dose of 60 μ g/ml of GSP resulted in 70–75% (P < 0.001) inhibition in cell viability at 48–72 h, respectively, compared with non-GSP-treated

(vehicle, DMSO treated) cells (Figure 1B). Inhibition at 24 h after its treatment was not found significant. Further, higher inhibition in cell viability by GSP was observed at the dose of 80 $\mu\text{g/ml}$ ($P < 0.001$) after its treatment at 48 and 72 h. The 20 and 40 mg/ml doses of GSP induced lesser inhibitory effect on cell viability at 48 and 72 h compared with that of 60 and 80 mg/ml as is evident from Figure 1B. However, it was observed that treatment of GSP to DU145 cells for 24 h did not induce significant inhibitory effect on the cell viability.

GSP inhibits FCM-induced secretion of MMP-2 and -9 in DU-145 cells

Since treatment of GSP to DU145 cells inhibited cell proliferation and cell viability, we extended our study to examine the mechanism of inhibition of tumor cell growth/viability with an emphasis on the inhibition of MMP expression in *in vitro* prostate cancer cells. This emphasis was based on the fact that enhanced expressions of MMP facilitate tumor cell invasion and metastasis and thus increase the mortality among the prostate cancer patients. Our efforts were concentrated on the use of a suitable inducing microenvironment containing a cocktail of growth factors from normal cells, which can induce the synthesis and secretion of MMP by DU145 cells in the *in vitro* system. This *in vitro* system partially mimics an *in vivo* tumor–host microenvironment where tumor cells are exposed to higher concentrations of growth factors that induce MMP synthesis (22–24). Previously, we have shown that treatment of DU145 cells with FCM induced the expressions of gelatinase-A (MMP-2) and gelatinase-B (MMP-9) (34); therefore, we again used FCM in this model to induce the expression of MMP, which play a major role in invasion and metastasis of prostate cancer cells. Incubation of DU145 cells with SFM for 24 h did not induce the extracellular secretion of MMP-2 or -9 as shown by gelatinolytic zymography (Figure 2A). However, substantial amounts of activated MMP-2 and -9 were secreted in the medium when cells were treated with FCM for 24 h. As shown in Figure 2A, treatment of GSP at the dose of 60 mg/ml and higher (60–125 $\mu\text{g/ml}$) to DU145 cells markedly inhibited FCM-induced expression of MMP-2 and -9. Densitometric analysis of the bands indicated that treatment of GSP to DU145 cells inhibited FCM-induced expression of MMP-2 and -9 by 4–26% at the doses of 20 and 40 $\mu\text{g/ml}$ whereas at the doses of 60–125 $\mu\text{g/ml}$ the inhibition of FCM-induced expression of extracellular MMP-2 and -9 was 26–83 and 63–93%, respectively (Figure 2A). It is worth mentioning that the bands of MMP-2 and -9 in zymograph were very clear and distinct when looked in the gel itself as compared with the scanned image and made into the final figures. As we concentrated our attention on the activated forms of MMP, the expression or inhibition of pro-MMP was not included in these figures and discussion as well. The dose-dependent inhibitory effect of GSP (20–125 $\mu\text{g/ml}$) on FCM-induced secretion of MMP-2 and -9 was further evident at the protein level when determined by western blot analysis using the same culture medium (Figure 2B). Densitometric analysis of the bands in western blot indicated that treatment of GSP to DU145 cells dose-dependently inhibited MMP-2 secretion by 30–90% whereas MMP-9 secretion was inhibited by 47–68%, as shown in Figure 2B. The bands of MMP-2 and -9 were detected by comparison with standard protein markers and positive controls of MMP.

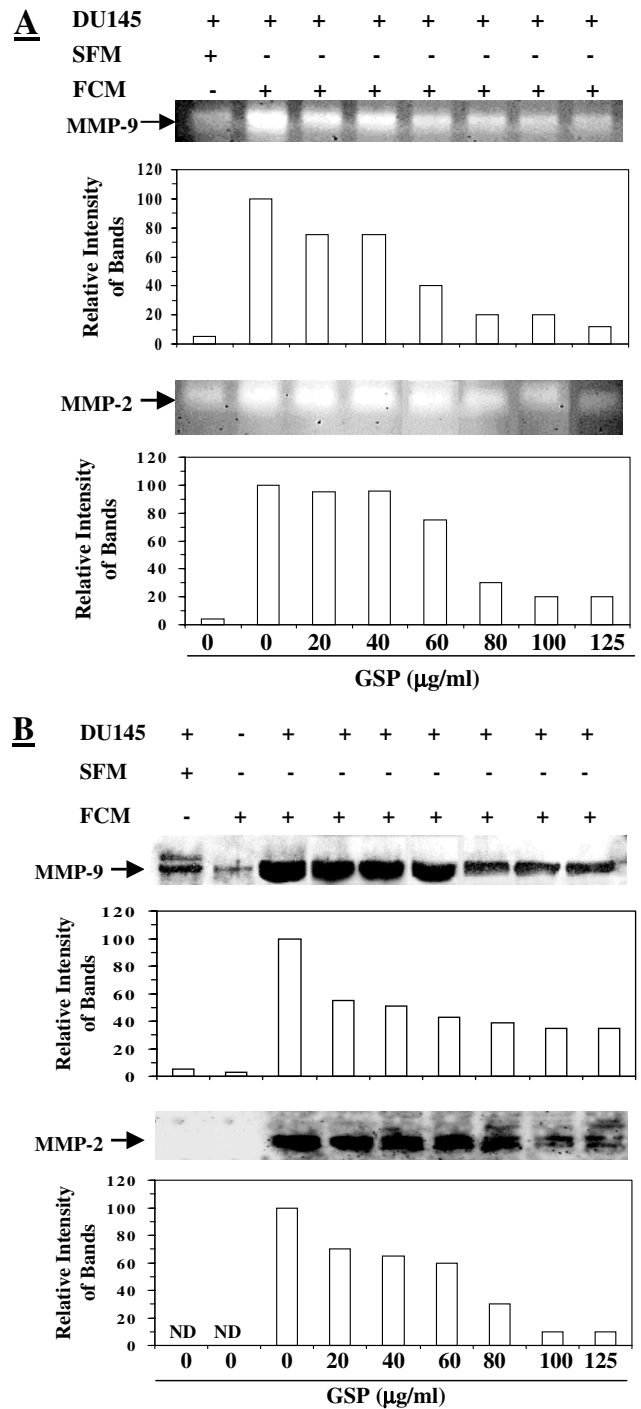


Fig. 2. (A) Treatment of GSP to human prostate carcinoma DU145 cells inhibits FCM-induced extracellular secretion of MMP-2 and -9 when determined by gelatinolytic zymography. The 2×10^6 cells were cultured in SFM/FCM or FCM containing various concentrations of GSP for 24 h. The medium was collected and subjected to zymography as described in the Materials and methods section. To analyze the percent inhibition of FCM-induced expression of MMP-9 and -2 by GSP, the relative intensity of bands was measured as shown below the zymograph. (B) Treatment of GSP to human prostate carcinoma DU145 cells inhibits FCM-induced extracellular secretion of MMP-2 and -9 when determined by western blot analysis. The same culture medium was used to analyze MMP-2 and -9 by western blotting, which was used in gelatinolytic zymography (A), as detailed in the Materials and methods section. In each case, only representative blots or gels from three independent experiments with similar results are shown. To analyze the percent inhibition of FCM-induced expression of MMP-2 and -9 by GSP, the relative intensity of bands was measured as shown below the western blot.

GSP inhibits FCM-induced phosphorylation of MAPK proteins

As we have shown that treatment of GSP to DU145 cells inhibited FCM-induced expression or extracellular secretion of MMP-2 and -9 in the media, we were interested to look at the mechanism of inhibition of MMP secretion by GSP. It is documented that activation of MAPK proteins, such as ERK1/2, p38 and JNK, regulate the synthesis of MMP (8). Therefore, we conducted an experiment to define the inhibitory mechanism of MMP by GSP treatment in DU145 cells. Further, as we observed that the doses of GSP ranging from 20 to 80 $\mu\text{g/ml}$ significantly inhibited the proliferation, cell viability and FCM-induced extracellular secretion of MMP-2 and -9, we used only these doses in further mechanistic studies. Our study demonstrated that cells grown in SFM showed a basal level of ERK1/2, p38 and JNK 1/2 whereas treatment of FCM enhanced the phosphorylation of ERK1/2, p38 and JNK1/2 proteins as shown in Figure 3. Under identical conditions, treatment of GSP (20–80 $\mu\text{g/ml}$) to human prostate carcinoma DU145 cells dose-dependently inhibited FCM-induced phosphorylation of ERK1/2 (58–94%/42–95%) and p38 (28–100%) after 24 h of treatment. As is evident in Figure 3, GSP dose-dependently inhibited FCM-induced phosphorylation of ERK1/2 (Figure 3A) and p38 (Figure 3B) MAPK proteins whereas the levels of JNK1/2 (Figure 3C) were increased (32–57%/6–19%) by GSP treatment at the doses of 20 and 40 $\mu\text{g/ml}$. However, higher doses of GSP (60 and 80 $\mu\text{g/ml}$) treatment resulted in inhibition of FCM-induced phosphorylation of JNK1 (100%) and JNK2 (56%). Additionally, no significant change in total amount of ERK1/2, p38 and JNK proteins among any of these treatment groups were observed when the blots were stripped and re-hybridized with antibodies to these proteins and following the identical western blot conditions, as shown in Figure 3A–C.

In order to confirm the functional role of activated ERK1/2 and p38 in the induction of MMPs by FCM and its inhibition by GSP in DU-145 cells, we examined the culture medium in which cells were grown in the presence and absence of specific inhibitors to MEK (PD098059) and p38 (SB203580) for the levels of MMPs by gelatinolytic zymography. The effect of MEK inhibitor PD095089 on the phosphorylation of ERK1/2 in DU145 cells treated with and without FCM is demonstrated in Figure 4A. Treatment with PD095089 (10–40 μM) inhibited FCM-induced phosphorylation of ERK1/2 in a dose-dependent manner. Parallel to the changes in the phosphorylation of ERK1/2 there was a dose-dependent inhibition of both MMP-2 and -9 secretion by PD095089 in DU145 cells suggesting the possible role of ERK1/2 phosphorylation in the induction of MMP induced by FCM and its inhibition by GSP. Similar to the MEK inhibitor, treatment of DU-145 cells with different concentrations of p38 inhibitor, SB203580 (10–40 μM), markedly decreased FCM-induced phosphorylation of p38. Concomitantly, there was a dose-dependent inhibition of MMP-9 and -2 as shown by zymography (Figure 4B). This observation demonstrated the involvement of the p38 MAPK pathway in the induction of MMPs induced by FCM in DU-145 cells and its inhibition by GSP treatment.

GSP inhibits synthetic androgen R1881-stimulated MMP-2 and -9 expressions in androgen-sensitive human prostate cancer LNCaP cells

After looking into the effect of GSP in androgen-insensitive human prostate cancer DU145 cells, we determined the effect

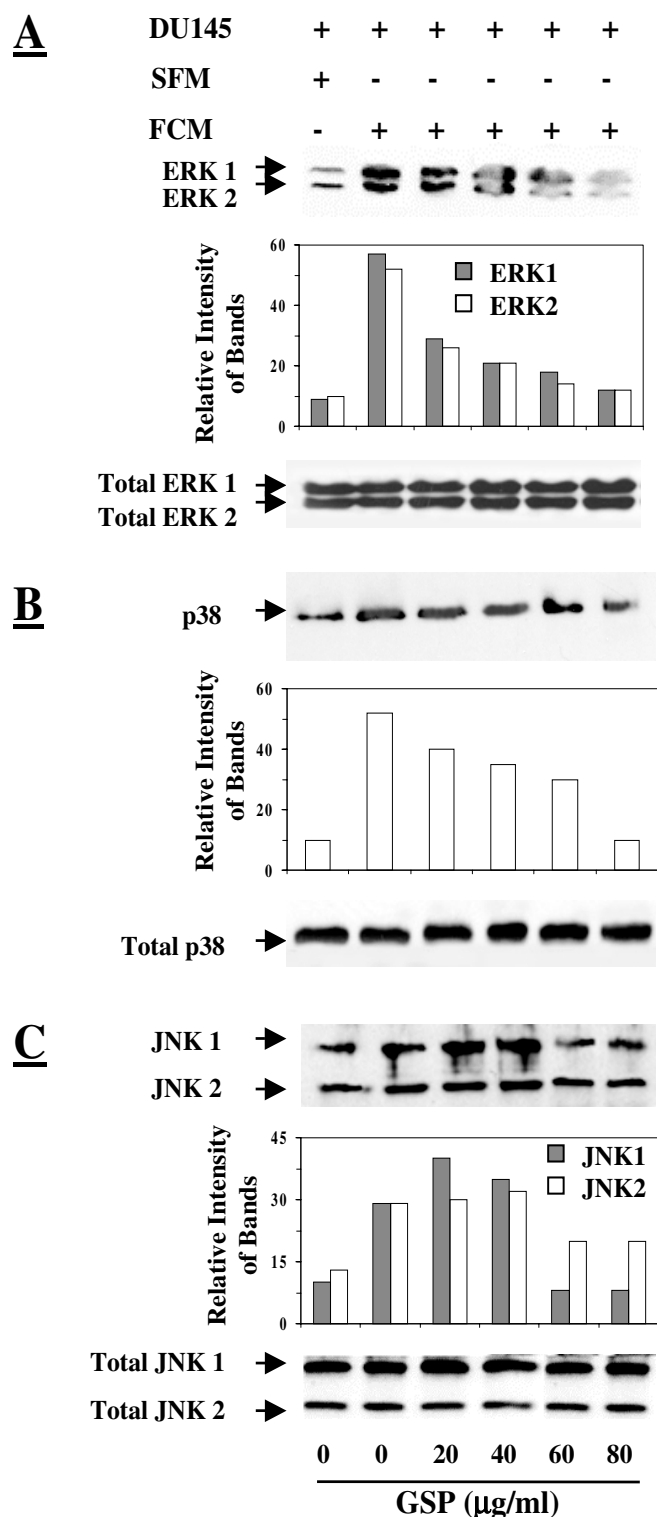


Fig. 3. Treatment of GSP to DU145 cells inhibits FCM-induced phosphorylation of MAPK proteins. 2×10^6 cells were cultured in SFM/FCM or FCM containing various concentrations of GSP for 24 h under identical conditions. Cell lysates were prepared and western blotting was performed as detailed in the Materials and methods section. The treatment in each lane is as marked in the figure. In each case, only a representative blot from three independent experiments with similar results is presented. Immunoblot analysis of phospho-specific ERK1/2 (A), p38 (B) and JNK (C) are shown in the figure. Just below the phospho-specific ERK1/2, p38 and JNK1/2, the densitometric analyses of respective western blot bands are given. Total amounts of ERK1/2, p38 and JNK1/2 proteins in each sample are also shown under identical experimental conditions.

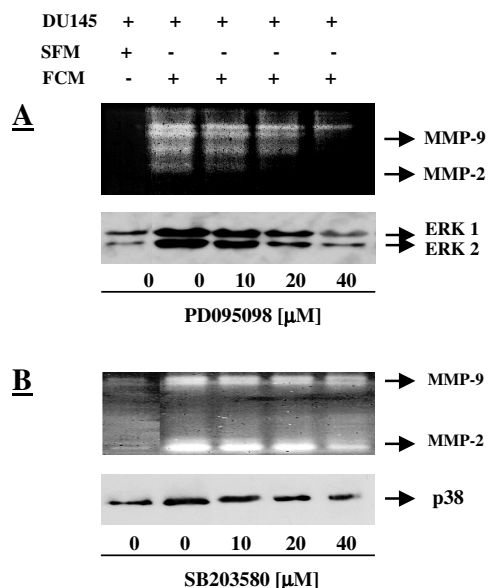


Fig. 4. Treatments of MEK and p38 inhibitors to DU145 cells inhibited the phosphorylation of FCM-induced ERK1/2 and p38 concomitantly with the inhibition of MMP-2 and -9. Phospho-specific ERK1/2 and p38 were analyzed by western blot analysis and MMP-2 and -9 were determined by using gelatinolytic zymography as detailed in the Materials and methods section. (A) Treatment of PD095098, an inhibitor of ERK1/2, inhibits the phosphorylation of ERK1/2 and concomitantly inhibits the synthesis of MMP-2 and -9 in FCM-stimulated DU-145 cells. The treatment in each lane is as marked in the figure. A representative blot from three independent experiments with identical results is shown. (B) Treatment of SB203580, an inhibitor of p38, inhibits FCM-induced phosphorylation of p38 and concomitantly inhibits the synthesis of MMP-2 and -9 in DU-145 cells. The treatment in each lane is as marked in the figure. A representative blot from three independent experiments with identical results is shown.

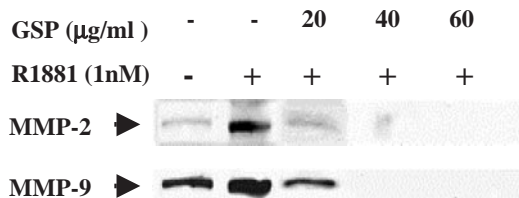


Fig. 5. Treatment of GSP to androgen-sensitive human prostate cancer cells, LNCaP, markedly inhibits synthetic androgen R1881 stimulated expressions of MMP-2 and -9. Following serum starvation for 24 h, LNCaP cells were left untreated or treated with R1881 (1 nM) with or without different doses of GSP (20, 40 and 60 mg/ml) for 24 h in serum-free media. Twenty-four hours later, cells were harvested and cell lysates were prepared to determine MMP-2 and -9 using western blot analysis, as detailed in the Materials and methods section. Data represent two independent experiments.

of GSP on synthetic androgen R1881-stimulated MMP-2 and -9 in androgen-sensitive human prostate cancer LNCaP cells. The LNCaP cell line is a commonly used *in vitro* model with well-characterized features of androgen responsiveness for prostate cancer research (39). Following serum starvation for 24 h, the cells were treated with synthetic androgen R1881 (1 nM) in serum-free conditions (40) with or without GSP treatment for another 24 h. Thereafter, cells were harvested and the cellular content of MMP-2 and -9 in whole-cell lysates was determined by western blot. As is evident in Figure 5, treatment of R1881 stimulated the synthesis of both MMP-2 and -9, and treatment of GSP even at the minimum dose of 20 μg/ml

almost completely inhibited R1881-stimulated MMP-2 and -9 expressions (Figure 5). The inhibition of R1881-stimulated MMP-2 and -9 expressions by GSP in LNCaP cells indicated the chemopreventive efficacy of GSP against androgen-sensitive human prostate cancer cells.

GSP inhibits FCM-induced activation of NFκB, which leads to the inhibition of MMP-9 and -2 expression

DNA sequence analysis has shown that MMP genes contain NFκB binding sites and are shown to be involved in their active synthesis (8). Therefore, we investigated the regulation of MMP synthesis by GSP induced by FCM at the transcription level by determining the activation of the NFκB transcription factor in DU145 cells. Our study demonstrated the presence of low levels of activated NFκB in cells grown in SFM alone (Figure 6A). However, there was a significant increase in the activation of NFκB in the nucleus when cells were grown in FCM. When DU145 cells were induced by FCM and treated with different concentrations of GSP, a dose-dependent inhibition in NFκB activation was observed, which was determined by western blot analysis. Densitometric analysis of bands revealed the dose-dependent inhibition of activation of NFκB, which varies from 30 to 100% by GSP treatment (20–80 μg/ml) compared with that of FCM-alone treated cells (Figure 6A). A similar dose-dependent inhibitory effect of GSP was obtained when the quantitative analysis of NFκB/p65 activation in nuclear fraction was performed using Trans-AM ELISA kit (Active Motif) following the manufacturer's protocol, as shown in Figure 6B. Treatment of GSP to DU145 cells resulted in the dose-dependent inhibition of FCM-induced activation of NFκB by 20, 54 ($P < 0.01$), 83 and 91% ($P < 0.001$), respectively, at the doses of 20, 40, 60 and 80 μg/ml at 24 h after its treatment. These data suggested the possibility that inhibition of activation of NFκB by GSP in DU145 cells play a role in inhibition of the expression of MMP. To confirm this fact, we determined further the levels of MMP-2 and -9 in whole cell lysates from each treatment group of this experiment following western blot analysis. As shown in Figure 6, treatment of GSP dose-dependently inhibited FCM-induced expression of MMP-9 (Figure 6C) and -2 (Figure 6D). Densitometric analysis of the bands indicated that this inhibition was in the range of 57–93 and 73–100%, respectively, for MMP-9 and -2. These observations further support the fact that inhibition of FCM-induced activation of NFκB by GSP resulted in a reduction in the transcription of MMP-2 and -9.

Discussion

Invasion of extracellular matrix and basement membrane barriers by tumor cells is a critical step in tumor invasion and metastasis. To facilitate invasion, tumor cells use complex, specific and multi-step mechanisms that allow cells to attach, initiate proteolysis of matrix proteins and migrate through the pathway made by a series of multiple enzymatic cleavages (18,19,41). The synthesis, secretion, activation and use of proteases to degrade the components of basement membranes and the extracellular matrix are, therefore, believed to be indispensable aspects of an invasive phenotype. The expression of MMPs can be induced by growth factors that function through a cascade of phosphorylation of proteins of the MAPK family (24,34). This in turn activates many transcription factors in the cytoplasm and active forms translocate into the nucleus and bind to specific sequences in the

promoter regions of MMP genes to activate their transcription (18,19). Therefore, these sequential cellular pathways offer a therapeutic opportunity to prevent invasion, metastasis and progression and for the treatment of advanced cancer.

In search of new dietary botanicals that can inhibit tumor cell growth and MMP synthesis and thus invasion of tumor cells, we found that *in vitro* treatment of GSP inhibited tumor cell viability (Figure 1), and FCM-induced extracellular secretion of MMP-2 as well as MMP-9 into the medium (Figure 2). Proanthocyanidins constitute ~89% of whole GSP used in this study, which has a high molecular weight in comparison with their monomers; therefore, we believe that the high molecular weight fraction of GSP is involved in the inhibition of MMP expression induced by FCM. There are several studies, using

different cell types, indicating that MAPK like ERK1/2, p38 and JNK/SAPK seem to play a central role in regulating the expression of MMPs (8). Thus, inhibition of the MAPK pathway might have the potential to prevent invasion, metastasis and angiogenesis for a wide range of tumors. In the present study, we found that GSP treatment inhibited FCM-induced phosphorylation of ERK1/2, p38 and comparatively less inhibition was found in JNK1/2 activation at higher doses of GSP (60 and 80 $\mu\text{g/ml}$, Figure 3) and concurrent reduction in the levels of MMP-2 and -9 (Figures 2A and B and 6) indicating a possible mechanism of inhibition of MMPs synthesis by GSP. With respect to ERK1/2 and JNK, Tyagi *et al.* (33) reported that the treatment of grape seed extract increases the phosphorylation of ERK1/2 and JNK1/2 in DU145 cells. This variation may be due to the fact that: (i) Tyagi *et al.* used whole grape seed extract while in the present study a purified fraction of proanthocyanidins was used. The presence of other components in whole grape seed extract may have a role in increased phosphorylation of ERK1/2 and JNK1/2; (ii) the *in vitro* system, which we used, is different. In our system, the tumor cells were induced by FCM and thereafter the effect of GSP was determined on the phosphorylation of MAPK proteins. This was not the case with the reported study (33). The involvement of MAPK pathways was further supported by the use of MEK and p38 inhibitors in our experimental model. The treatment of inhibitors of MEK and p38 to DU145 cells inhibited the phosphorylation of proteins of the MAPK family and simultaneous inhibition of MMPs synthesis or secretion (Figure 4). ERK1/2 and p38 pathways are independent pathways however; the use of both inhibitors MEK and p38 separately inhibited FCM-induced MMP synthesis. The exact reason for this fact is unclear but it appears that each pathway plays a major role in regulation of MMP synthesis.

Androgens such as R1881 produce most cellular responses through their cognate nuclear receptor, androgen receptor. On binding to the hormone, the androgen receptor forms a homodimer. The dimerized protein then interacts with specific DNA sequences directly through an androgen-responsive element (42). The result is an alteration in protein synthesis and the

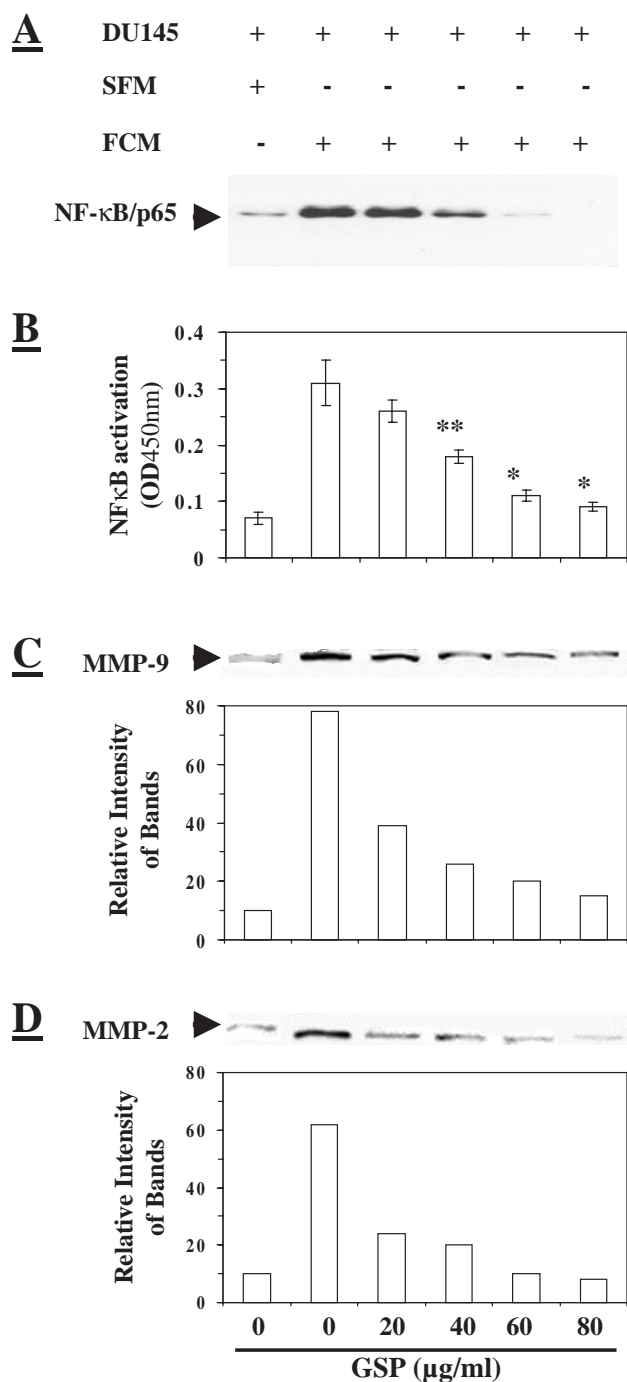


Fig. 6. Treatment of GSP dose-dependently inhibits FCM-induced activation of NFκB/p65 and expression of MMP-9 and -2 in human prostate carcinoma DU145 cells. Following serum starvation for 24 h, DU145 cells were treated with either SFM or FCM with or without GSP (20, 40, 60 and 80 $\mu\text{g/ml}$). Twenty-four hours later, cells were harvested and whole cell lysates and nuclear fractions were prepared for western blot analysis and ELISA, as detailed in the Materials and methods section. (A) Dose-dependent inhibition of FCM-induced activation of NFκB in nuclear fraction by GSP treatment. A representative immunoblot from three independent experiments with identical results is shown. The treatment in each lane is as marked on the figure. (B) Quantitative analysis of activation of NFκB/p65 by ELISA. Nuclear fraction was prepared following the vendor's protocol for the determination of NFκB by ELISA. The data are expressed as mean \pm SD from three independent experiments in terms of OD at 450 nm for NFκB. Statistical significance, ** $P < 0.01$; * $P < 0.001$. (C) Treatment of GSP to DU145 cells dose-dependently inhibited FCM-induced expression of MMP-9 in whole cell lysates as determined by the western blot analysis and detailed in the Materials and methods section. Densitometric analysis of bands was performed to analyze the levels of MMP-9 in each treatment group and shown below the bands. (D) Treatment of GSP to DU145 cells dose-dependently inhibited FCM-induced expression of MMP-2 in whole cell lysates as determined by the western blot analysis and detailed in the Materials and methods section. Densitometric analysis of bands was performed to analyze the levels of MMP-2 in each treatment group and shown below the bands. Cellular treatments in each panel were identical as detailed on the top of (A), and at the bottom of (D).

generation of a cellular response. In this study, we demonstrated that R1881-stimulated expressions of MMP-2 and -9 were abolished by the treatment of GSP in androgen-responsive human prostate cancer LNCaP cells (Figure 5) that may block androgen receptor-mediated gene transcription similar to other inhibitors (43). From these observations, it appears that proanthocyanidins from grape seeds have the ability to inhibit the expressions of metastasis-specific MMP in human prostate cancer cells having both androgen-sensitive (like LNCaP) and androgen-insensitive (like DU145) properties.

MMP-9 is an inducible gene and the MMP-2 gene is constitutively expressed. The MMP-9 gene is regulated by AP-1, NF κ B, PEA3 and Sp1 binding proteins (8). Indeed, one or more of these binding sites have been implicated in mediating the effects of a diverse set of agents. Increased expression of MMP-2, the promoter regions of which do not contain conserved *cis* elements, has also been observed in malignancies, indicating overlapping mechanisms in the regulation of the expression of these genes (8). Elevated MAPK and NF κ B activities are involved in many disease processes such as inflammation, neoplastic transformation, cancer cell invasion, metastasis and angiogenesis. Therefore, blocking the factors that bind to these regulatory elements seems to be an appropriate approach to the inhibition of MMP synthesis. In the present study, the incubation of DU145 cells with FCM increased the activation of NF κ B/p65 in the nucleus, and treatment of GSP blocked the activation as well as translocation of NF κ B into the nucleus (Figure 6A and B). We have shown that the use of inhibitors of p38 and ERK1/2 inhibited the activation of NF κ B as well as the expression of MMP-2 and -9 (34). This observation suggests that the reduction in secretion of MMP-2 and -9 by GSP may be associated with the inhibition of phosphorylation of proteins of MAPK family and activation of NF κ B. This observation is supported by the use of inhibitors of p38 and ERK1/2. The observation that the inhibition of NF κ B may lead to the inhibition of MMP synthesis, was further supported by the fact that treatment of GSP to DU145 cells dose-dependently inhibited FCM-induced expression of MMP-2 and -9 concomitantly with the inhibition of NF κ B activation under identical conditions (Figure 6C and D), thus confirming our observation that inhibition of NF κ B by GSP treatment results in a reduction in the expression of MMP-2 and -9. Moreover, this observation is also supported when the treatment of GSP to DU145 cells inhibited FCM-induced extracellular secretion of MMP-2 and -9 (Figure 2). We have shown that *in vitro* treatment of (–)-epigallocatechin-3-gallate from green tea to DU145 cells inhibited the activation of NF κ B concomitantly with the reduction in the transcription of MMP-2 and -9 (34), and this further supports our observation that inhibition of NF κ B by GSP results in a reduction of MMP synthesis.

Recent studies on the role of MMP in the multi-stage tumor development have shown that in addition to their role in the degradation of extracellular matrix and basement membrane proteins, MMP also have a specific role in survival, proliferation and growth of the tumor cells. It was demonstrated that MMP-mediated ectodomain shedding and release from matrix binding of many growth factors was essential for the survival and proliferation of tumor cells (6,7,20). Therefore, the anti-carcinogenic effect of GSP on prostate cancer cells may be explained, at least in part, by its ability to inhibit the synthesis of MMPs or inhibit the activators of MMPs that subsequently block the release of active growth factors necessary for

optimum growth of tumor cells. However, further studies are required to provide more insight into the possible chemopreventive mechanism of action of the GSP *in vivo* PCA model and the role of GSP in altering the host–tumor relationship in favor of the host. A positive outcome of such *in vivo* studies could also be useful because a considerable interest in consumption of dietary botanical supplements among humans is increasing to prevent age-associated diseases and importantly GSP is non-toxic, *in vitro* (31,32) and in *in vivo* animal system (44).

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