Treatment of Epigallocatechin-3-Gallate Inhibits Matrix Metalloproteinases-2 and -9 via Inhibition of Activation of Mitogen-Activated Protein Kinases, c-jun and NF-κB in Human Prostate Carcinoma DU-145 Cells

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BACKGROUND. Matrix metalloproteinases (MMPs) are involved in tumor progression including the carcinoma of the prostate (CaP). Therefore, the effect of (−)-epigallocatechin-3-gallate (EGCG) was determined on the synthesis and activation of tumor invasion-specific MMP-2 and MMP-9 in human prostate carcinoma DU-145 cells.
METHODS. MMP-2 and MMP-9 were determined by zymography and Western blot analysis. Since fibroblast conditioned medium (FCM) partially mimics in vivo tumor-host microenvironment, DU145 cells were co-cultured in FCM.
RESULTS. Treatment of EGCG to DU-145 cells resulted in dose-dependent inhibition of FCM-induced pro and active both forms of MMP-2 and MMP-9 concomitant with marked inhibition of phosphorylation of ERK1/2 and p38. In identical conditions, treatment of EGCG or inhibitors of MEK or p38 to DU-145 cells inhibited FCM-induced phosphorylation of ERK1/2 and/or p38 concomitant reduction in MMP-2 and -9. EGCG also inhibited androgen-induced pro-MMP-2 expression in LNCaP cells. Further, treatment of EGCG also resulted in inhibition of activation of c-jun and NF-κB in in vitro DU-145 cells.
CONCLUSIONS. The inhibition of MMP-2 and MMP-9 in DU145 cells by EGCG is mediated via inhibition of phosphorylation of ERK1/2 and p38 pathways, and inhibition of activation of transcription factors c-jun and NF-κB. EGCG may play a role in prevention of invasive metastatic processes of both androgen-dependent and -independent prostate carcinoma. *Prostate 59: 33–42, 2004.* © 2003 Wiley-Liss, Inc.

KEY WORDS: **green tea; metalloproteinases; prostate cancer; prevention**

INTRODUCTION

Cancer of the prostate (CaP) is a major malignant disease and a leading cause of death due to cancer among males in the developed countries including United States. An estimated 220,900 new cases of prostate cancer will be diagnosed and an estimated 28,900 deaths will occur from prostate cancer in the year 2003 in USA [1]. Despite these alarming figures and consistent efforts to prevent this disease, the causes of prostate cancer development in humans still remain unclear. While prostate-confined disease is curable by surgery, metastatic prostate cancer remains essentially incurable. The invasion of prostate cancer cells through the basement membrane is one of the earliest events in the metastatic spread of CaP, and proteolytic degradation of the extracellular matrix proteins is a necessary

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step in this process [2]. Proteolytic degradation of components of the extracellular matrix such as collagens, proteoglycans, laminin, elastin, and fibronectin is considered to be a prerequisite for tumor invasion and metastasis. Matrix metalloproteinases (MMPs) are able to degrade essentially all of the protein components of the extracellular matrix [3,4]. In addition, MMPs substantially contribute to other steps in the metastatic cascade, such as angiogenesis, differentiation, proliferation, and apoptosis [3–5]. 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 [6]. Therefore, it is desirable to develop newer and effective chemopreventive agents or inhibitors of MMPs, which can inhibit the synthesis of MMPs at tumor site, and thus prevent, reverse, or slowed down the age-related development of CaP in humans.

Among various MMPs, two particular members of the MMP family, gelatinases A and B (MMP-2 and MMP-9), seem to play an important role in tumor invasion and metastasis [7]. These two type IV collagenases are the dominant MMPs released by most of the epithelial and endothelial cells [8,9]. Since these enzymes play an important role in the homeostasis of the extracellular matrix, an imbalance in their expression or activity may have important consequences in various pathologies including the development of prostate cancer [10–14]. Moreover, MMP gene expression is primarily regulated at the transcriptional (through AP-1 via mitogen activated protein kinase (MAPK) pathways) and posttranscriptional levels, and at the protein level via their activators, inhibitors, and their cell surface localization [11,12]. Hence, MMPs and their regulatory pathways have been considered promising targets for anti-cancer drugs and chemopreventive agents [11,13,14].

Chemoprevention is a means of cancer control by which the incidence can be prevented, delayed progression, or reversed by using dietary or synthetic compounds. Anti-cancer properties have been associated with the components of various dietary supplements including but not limited to polyphenols from green tea [15,16]. Green tea (*Camellia sinensis*) is widely consumed as a popular beverage worldwide [15]. Epidemiological as well as extensive experimental studies conducted in laboratory animals have detected an association between tea consumption and decreased cancer risk [15,16]. In recent years, cancer chemoprevention by biologically active dietary or non-dietary botanical supplements has generated considerable interest in view of their putative role in attenuating the risk of cancer incidence [15,16]. Against this background, green tea polyphenols are promising chemoprotective agents against several major epithelial and non-epithelial cancers [15]. The major polyphenolic components present in green tea are (−)-epicatechin, (−)-epicatechin-3-gallate, (−)-epigallocatechin, and (−)-epigallocatechin-3-gallate (EGCG) [15]. Of these components, EGCG has been shown to be the most potent anti-oxidant and major chemopreventive constituent of green tea and has been the focus of a great deal of attention in cancer prevention studies [reviewed in References 15, 16]. EGCG has been demonstrated to induce apoptosis in human prostate cancer cells LNCaP, PC-3, and DU145 [17], reduce the size of implanted tumors in athymic nude mice [18] and reduce substantially the spontaneous development of tumor burden in TRAMP mice [19]. Chemopreventive activity of EGCG has been attributed to its ability to inhibit tumor invasion [20] and angiogenesis [21], crucial steps for the growth and metastasis of solid tumors.

In the present study, we evaluated the chemopreventive effects of EGCG on MMP-2 and MMP-9 activities in vitro in androgen-independent human prostate carcinoma DU-145 cells. We determined whether the inhibition of these MMPs by EGCG treatment is mediated through the cellular signaling pathways such as by the inhibition of phosphorylation of MAPK proteins and blocking the activation and translocation of AP-1 and NF-κB. Since the clinical diagnosis of most of the CaP represent a mixture of androgen-dependent (sensitive) and androgen-independent (insensitive) cells, we were also interested to determine whether treatment of EGCG inhibits MMP expression in androgen-dependent LNCaP cells. These findings may provide a new insight how the dietary component, like green tea polyphenols, can be targeted against MMPs for the prevention of human prostate cancer.

MATERIALS AND METHODS

Reagents and Antibodies

Antibodies against MMP-2 and MMP-9, and inhibitors of MEK (PD095089) and p38 (SB203580) were purchased from Sigma Chemical Company (St. Louis, MO). Phospho-specific antibodies for ERK1/2, p38, and JNK as well as antibodies against ERK1/2, p38, and JNK proteins and phospho c-Jun were obtained from Cell Signaling Technologies (Beverly, MA), and monoclonal antibody to NF-κB was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Synthetic androgen R1881 was obtained from Perkin Elmer (Boston, MA). Reagents and chemicals for electrophoresis were purchased from Bio-Rad (Hercules, CA). PhastGel Blue R was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell Culture

Human prostate carcinoma DU-145 (androgen receptor negative), LNCaP (androgen receptor positive) cells, and human foreskin fibroblast HS-68 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The cells were maintained in monolayer culture at 37°C under an atmosphere of 5% CO_2 in Dulbeccos Modified Eagle Medium (DMEM) (Cellgro, Herndon, VA). The medium was supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin (100 U/ml), and streptomycin (100 μg/ml). Purified EGCG (99% pure) was obtained as a gift from Dr. Yukihiko Hara (Mitsui Norin Co., Shizuoka, Japan).

Preparation of Fibroblast Conditioned Medium (FCM)

FCM from human foreskin fibroblast HS-68 cells was used to induce MMP synthesis and secretion in DU-145 human prostate carcinoma cells. FCM was prepared by incubating sub-confluent HS-68 fibroblasts in 10 ml of serum free media (SFM) at 37°C containing 0.5 μg/ml bovine serum albumin for 24 hr. Thereafter, media was collected, centrifuged at 14,000*g* for 15 min at 4°C and supernatant was stored at −80°C for further use. In order to avoid batch-to-batch variation during the preparation of FCM, large volumes of FCM was prepared at a time, pooled and tested for its ability to induce MMPs by DU-145 cells.

Preparation of Nuclear Fraction

Nuclear fractions from different treatment groups of DU-145 cells were prepared as described previously [22,23]. 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 $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 2.0 μg/ml leupeptin, and 2.0 μg/ml aprotinin) in a microfuge tube. Then, 12.5 μl 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 μl of nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1.5 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM DTT, 1.0 mM PMSF, 0.1% IGEPAL CA-630, 2.0 μg/ml leupeptin, and 2.0 μg/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. Protein concentration was determined using DC Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol.

Preparation of Cell Lysates

After treatment of the cells for desired time, media was collected for zymography and the cells were washed with cold PBS and incubated in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% IGEPAL CA-630, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, 1 mg/ml leupeptin, and 1 mg/ml aprotinin) over ice for 30 min. The cells were scraped and lysates were collected in a microfuge tube and passed through a 21 G needle till the sample viscosity was reduced. The cell lysate was cleared by centrifugation at 14,000*g* for 15 min at 4°C and the supernatant (total cell lysate) was used immediately or stored at −80°C for further use for Western blotting.

Zymography

Analysis of MMP-2 and MMP-9 in DU-145 cells was performed using sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) zymography. Briefly, DU-145 cells (2×10^6 cell) were seeded in 24-well plates. Treatment with EGCG was done in 500 μl FCM for 24 hr. For the controls, cells were treated with 500 μl FCM or serum free medium (SFM) alone containing 0.5 μg/ml BSA. After incubation at 37°C for 24 hr, the medium from each well was collected, centrifuged and analyzed for MMPs by zymography. Zymography was performed using a single step staining method as described elsewhere [24]. Samples (15 μl) of media collected from each well were mixed 2:1 (v/v) with sample loading buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol [v/v], 1% SDS [w/v], and 0.01% bromophenol blue) and left to denature it for 10 min at room temperature. Samples were loaded on upper 4% stacking gel, resolved on a lower 10% separating gel co-polymerized with 0.1% gelatin prepared in a disposable gel cassette (Novex, Carlsbad, CA) and electrophoresed in a mini slab gel apparatus (Novex) at 4°C and constant 100 V. After electrophoresis, the gels were washed in 2.5% Triton X-100 on a shaker for 1 hr, changing once the solution after 30 min, to eliminate SDS completely. The gels were rinsed twice with zymogen activation buffer (50 mM Tris-HCl, pH 7.6, containing 10 mM $CaCl_2$ and 0.2 M NaCl) and were incubated for 18 hr at 37°C in the same buffer. After incubation, the gels were rinsed briefly in distilled water and were stained for 2 hr with PhastGel Blue R stain diluted 1:20 (v/v) in destain solution (1:3:6, glacial acetic acid:methanol:distilled water, v/v/v) from a stock of 0.2% prepared as described by the manufacturer.

Western Blotting

Samples of media collected from each treatment group of DU-145 cells (5–25 μl/well), cell lysates, or

nuclear fractions corresponding to 25–50 μg of protein/sample were mixed separately with sample buffer containing β-mercaptoethanol [23]. Samples were electrophoresed at 125 V on a 10% Tris-glycine acrylamide gel (Novex). Protein transfer was done by electroblotting onto nitrocellulose membrane for 2 hr at constant 25 V. The membrane was washed briefly in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) and blots were blocked for 1 hr in blocking buffer containing 5% non-fat dry milk in TBS-T. Then, the membrane was probed overnight at 4°C with corresponding primary antibody in 5% non-fat dry milk or BSA 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 hr, and the protein expression was detected by chemiluminescence using an ECL Plus detection kit (Amersham Life Sciences, Inc., Piscataway, NJ) and autoradiography with XAR-5 film (Eastman Kodak Co., Rochester, NY). To check whether treatment of EGCG to DU145 cells changes the amounts of ERK1/2, JNK, and p38, the blots were stripped and rehybridized with their respective antibodies, and thereafter followed similar protocol as detailed above. To compare the relative intensity of protein bands in Western blots from different treatment groups and in various experiments, computerized densitometry was performed using OPTIMAS 6.2 software program.

RESULTS

EGCG Treatment Inhibits FCM-Induced MMP-2 and -9 in DU-145 Cells

Our initial efforts were to generate a suitable inducing environment containing a cocktail of growth factors from normal cells, which can induce the synthesis and secretion of MMPs by DU-145 cells. This condition will partially mimic an in vivo tumor-host microenvironment. It is documented that FCM is a good chemoattractant and contains variety of growth factors that can induce MMP's by tumor cells [25]. Therefore, we selected HS-68 FCM for this purpose preferably because of its human origin and secretion of low levels of MMPs that can be detected by zymography. As shown in Figure 1A, cells treated with SFM alone did not induce active or pro forms of MMP-2 and MMP-9. Similarly, both forms (pro and active) of MMP-2 or -9 were not clearly detected in FCM alone. However, treatment of FCM to DU-145 cells highly induced the synthesis and secretion of both forms of MMP-2 and -9 into the medium. It is also evident that incubation of DU-145 cells in FCM induced more pro and active forms of MMP-2 than that of pro and active

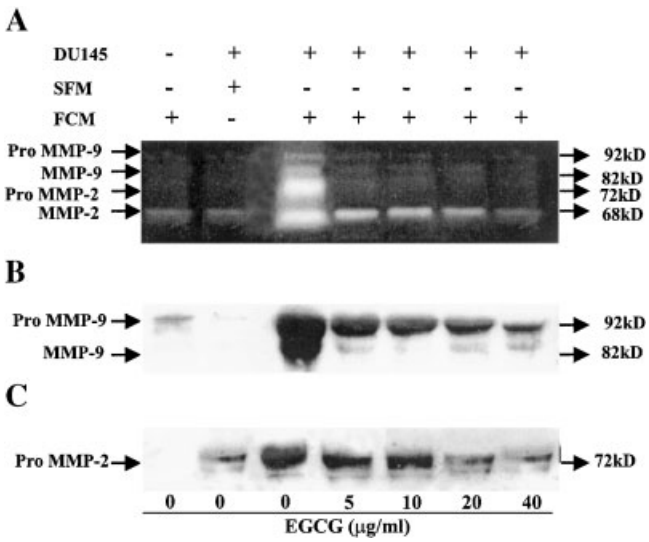

Fig. 1. Treatment of (−)-epigallocatechin-3-gallate (EGCG) inhibits FCM-induced pro and active forms of matrix metalloproteinase (MMP)-2 and MMP-9 production in human prostate carcinoma DU-145 cells. DU-145 cells (2×10^6) were cultured in SFM/FCM or FCM containing various concentrations of EGCG for 24 hr in 24-well culture plate. Thereafter, medium was collected, centrifuged, and supernatants were subjected to Zymography and Western blotting to determine extracellular secretion of MMP-2 and -9 as described in "Materials and Methods." The treatment in each lane was as marked in the figures. In case of zymography and Western blot analysis, only a representative blot from three independent experiments with identical results is shown. DU-145 cells grown in SFM demonstrate the basal level of MMPs. **A**: Treatment of EGCG to DU-145 cells dose-dependently inhibited FCM-induced extracellular secretion of pro and active forms of MMP-2 and -9, as determined by zymography. **B**: Treatment of EGCG to DU-145 cells dose-dependently inhibited FCM-induced extracellular secretion of pro and active forms of MMP-9 as determined by Western blotting. **C**: Treatment of EGCG to DU-145 cells dose-dependently inhibited FCM-induced extracellular secretion of pro-MMP-2 when determined by Western blotting. DU-145 cells grown in SFM demonstrate the basal level of MMP. FCM, fibroblast conditioned medium; SFM, serum free medium.

forms of MMP-9. However, treatment of varying concentrations of EGCG (5–40 μg/ml) to DU-145 cells inhibited the FCM-induced production of pro-MMP-2, and pro and active forms of MMP-9 as seen from zymography (Fig. 1A). Even very low dose of EGCG (5 μg/ml) was able to inhibit the synthesis of MMP-2 and -9.

The Western blot analysis of the culture media also indicated that EGCG treatment to DU-145 cells dose-dependently inhibited FCM-induced MMP-9 expression (Fig. 1, panel B). As shown in the Figure 1B, FCM alone and medium from cells cultured in SFM contained only slightly detectable levels of pro and active MMP-9 whereas treatment of FCM to DU-145 cells dramatically induced the increase in the synthesis and release of both forms of MMP-9. Treatment of

EGCG dose-dependently inhibited FCM-induced secretion of pro-MMP-9 whereas active form of MMP-9 was almost completely abrogated by EGCG treatment in the DU-145 carcinoma cells. Thus, decreased MMP-9 enzymatic activity as evident by zymography, correlated with the diminished synthesis of MMP-9 protein (shown by Western blot) upon treatment of EGCG. Similarly, pro-MMP-2 protein levels secreted into the medium were also examined. Treatment of EGCG dose-dependently (5–40 μg/ml) inhibited FCM-induced pro-MMP-2 protein in DU145 cells suggesting the inhibitory effect of EGCG on the synthesis or secretion of pro-MMP-2 as shown in Figure 1C. The effect of EGCG on FCM-induced MMP-2 production could not be detected clearly (data not shown).

EGCG Treatment Inhibits FCM-Induced Phosphorylation of ERK1/2 and p38 but not JNK in DU-145 Cells

Once we found that treatment of EGCG inhibited FCM-induced MMP synthesis in DU-145 cells, we were interested to look into the mechanism by which EGCG inhibited the synthesis of these MMPs, which play a crucial role in tumor invasion and metastasis. As evident from Figure 2, the cells grown in SFM demonstrated a detectable basal level of phosphorylation of all the MAPK proteins, and cells grown in FCM induced remarkably higher levels of phosphorylation of ERK1/2 (Fig. 2A, upper panel) and p38 (Fig. 2B, middle panel) in comparison to the cells which were not treated with FCM but treated with SFM. These observations suggested the possible role of phosphorylation of ERK1/2 and p38 in FCM induced MMP synthesis in human prostate carcinoma DU-145 cells. Under similar conditions, treatment of EGCG to DU-145 cells dose-dependently inhibited FCM-induced phosphorylation of ERK1/2 and p38, as shown in Figure 2 (A and B panels). Importantly, densitometry analysis of protein bands demonstrated that even the lowest dose of EGCG (5 μg/ml) treatment resulted in more than 50% inhibition in FCM-induced phosphorylation of ERK1/2 and p38 proteins. These observations further suggested that inhibition of FCM-induced phosphorylation of MAPK proteins could be involved in the inhibition of synthesis of MMP-2 and -9 by EGCG treatment. However, no marked effect on the phosphorylation of JNK was found by the treatment of FCM or its inhibition due to EGCG treatment (Fig. 2C). Moreover, 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 rehybridized with antibodies to these proteins (data not shown).

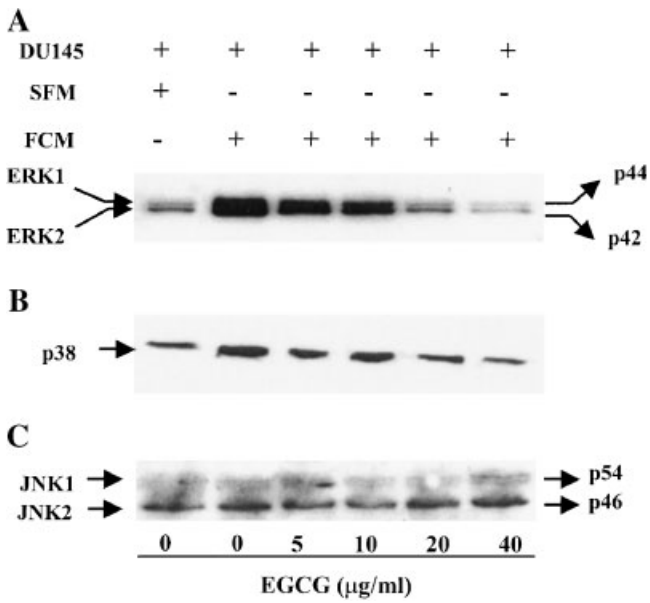

Fig. 2. Treatment of EGCG dose-dependently inhibits FCM-induced phosphorylation of MAPK proteins. DU-145 cells (2×10^6) were cultured in SFM/FCM or FCM containing various concentrations of EGCG for 24 hr in 24-well culture plate. Thereafter, cell lysates were prepared and subjected to sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) and Western blotting as detailed in "Materials and Methods." The treatment in each lane was as marked in the figure. In each case, only a representative blot from three independent experiments with identical results is shown. Details of Western blotting are described in "Materials and Methods." Treatment of EGCG to DU-145 cells dose-dependently inhibited FCM-induced phosphorylation of ERK1/2 (**A**), p38 (**B**), but did not inhibit FCM-induced phosphorylation of JNK (**C**). DU-145 cells grown in SFM demonstrate the basal level of activation of MAPK proteins.

Specific Inhibitors of ERK1/2 and p38 Inhibits FCM-Induced Secretion of MMPs in DU-145 Cells

We were also interested to determine the functional role of ERK1/2 and p38 phosphorylation in FCM-induced synthesis of MMP-2 and -9, and their inhibition by EGCG treatment in DU-145 cells. Therefore, we examined the cultured medium for the levels of MMPs in which cells were grown in presence and absence of specific inhibitors to MEK (PD095089) and p38 (SB203580) by zymography. As shown in Figure 3A, treatment of PD095089 (10–40 μM), an inhibitor of MEK (ERK1/2), dose-dependently inhibited FCM-induced phosphorylation of ERK1/2. Parallel to the inhibition in the phosphorylation of ERK1/2 after the treatment of MEK inhibitor, there was a dose-dependent inhibition in FCM-induced proteolytic activity of both pro and active forms of MMP-2 and MMP-9 (Fig. 3B) in DU-145 cells, suggesting the possible role of ERK1/2 phosphorylation in the induction of MMPs induced by FCM

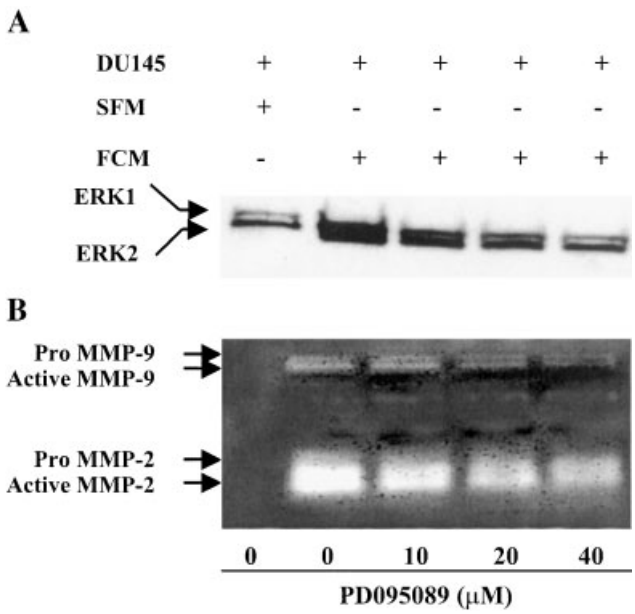

Fig. 3. Treatment of MEK inhibitor (PD095089) to DU-145 cells dose-dependently inhibited FCM-induced phosphorylation of ERK1/2 concomitantly with the inhibition of the synthesis of pro and active forms of MMP-2 and -9. The treatment in each lane is as marked in the figure. In case of zymography and immunoblotting, only a representative zymogram or blot from three independent experiments with identical results is shown. Cells were treated and subjected to zymography and immunoblotting as described in "Materials and Methods." **A**: Treatment of MEK inhibitor (PD095089) to DU-145 cells dose-dependently inhibited FCM-induced phosphorylation of ERK1/2 when determined by immunoblotting. **B**: Treatment of MEK inhibitor (PD095089) to DU-145 cells dose-dependently inhibited extracellular secretion of pro and active forms of MMP-2 and -9 when determined by zymography.

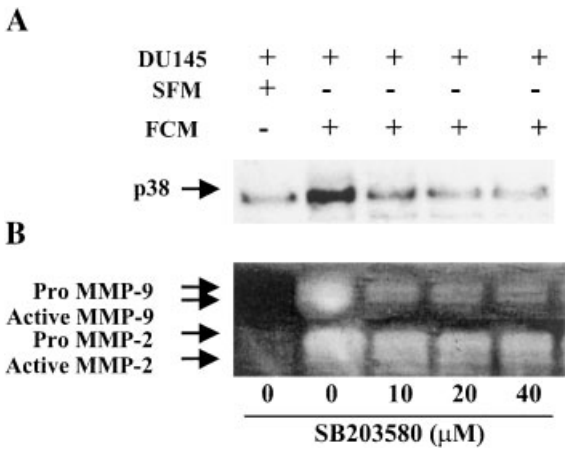

Fig. 4. Treatment of an inhibitor of p38 (SB203580) to DU-145 cells dose-dependently inhibited FCM-induced phosphorylation of p38 concomitantly with the inhibition of the synthesis of MMP-2 and -9. The treatment in each lane was as marked in the figure. In case of zymography and immunoblotting, only a representative zymogram or blot from three independent experiments with identical results is shown. Cells were treated and subjected to zymography and immunoblotting as detailed in "Materials and Methods." **A**: Treatment of p38 inhibitor (SB203580) to DU-145 cells dose-dependently inhibited FCM-induced phosphorylation of p38 when determined by immunoblotting. **B**: Treatment of p38 inhibitor (SB203580) to DU-145 cells dose-dependently inhibited extracellular secretion of pro and active forms of MMP-2 and -9 when determined by zymography. DU-145 cells grown in SFM demonstrate the basal level of MMPs.

and their inhibition by EGCG treatment. Further, treatment of p38 inhibitor (SB203580) also dose-dependently (10–40 μM) inhibited FCM-induced phosphorylation of p38 protein in DU-145 cells (Fig. 4A), concomitantly, there was a dose-dependent inhibition of pro and active forms of MMP-2 and MMP-9 was also observed (Fig. 4B). This observation also suggested the possible role of p38 MAPK pathway in the induction of MMPs induced by FCM in DU-145 cells and its inhibition by EGCG treatment. It is worth to mention that the clarity of bands of pro and active forms of MMP-2 and MMP-9 in zymograms appeared dim in Figures 3B and 4B but these bands were very clear and distinguishable in the gel.

Treatment of EGCG and Inhibitors of MAPK Inhibits FCM-Induced Intracellular Synthesis of MMPs in DU-145 Cells

To investigate the regulation of MMP synthesis by FCM and its inhibition by EGCG treatment, we also determined the cellular level of each MMP by Western blotting. As shown in the Figure 5A, there was a constitutive presence of pro-MMP-2 in cells when grown in SFM. Culturing of DU-145 cells in FCM highly increased the level of pro-MMP-2 (approximately threefold) compared to culturing with SFM alone. However, treatment of EGCG dose-dependently inhibited FCM-induced synthesis of pro-MMP-2, and further reduced below the level of constitutive pro-MMP-2 (Fig. 5A). Even a lowest dose of EGCG (2 μg/ml) treatment to DU-145 cells resulted in more than 50% inhibition of FCM-induced synthesis of pro-MMP-2 as determined by densitometric analysis. Similarly, treatment of EGCG dose-dependently inhibited the FCM-induced synthesis of both pro and active forms of MMP-9 (Fig. 5B). Further, to support the role of activation of ERK1/2 and p38 proteins in the synthesis of MMP-2 and MMP-9 in our experimental system as well as to confirm that mechanism of inhibition of MMP by EGCG is mediated through blocking the phosphorylation of ERK1/2 and p38, we used both PD095089 and SB203580 MAPK inhibitors along with other treatments. The treatment of both MAPK inhibitors to DU-145 cells resulted in inhibition of FCM-induced synthesis of MMP-2 and MMP-9 suggesting that MMP

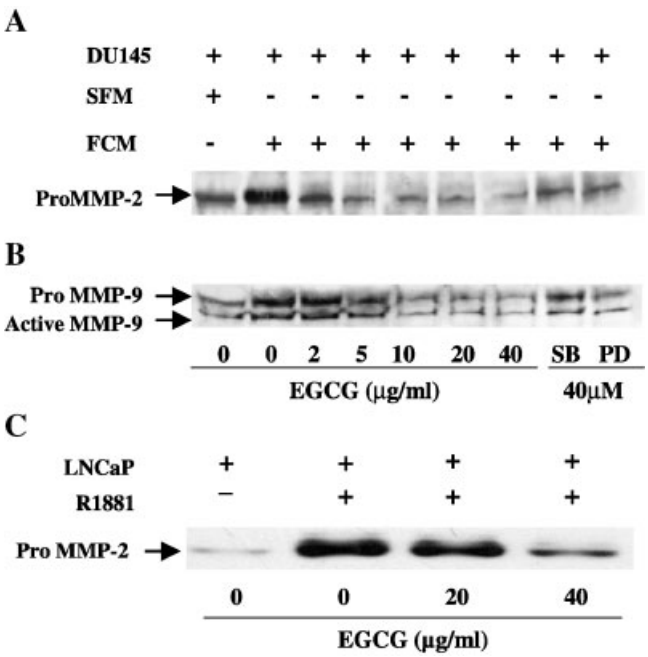

Fig. 5. Treatment of EGCG as well as inhibitors of MEK (PD095089) and p38 (SB203580) to DU-145 cells inhibits FCM-induced intracellular synthesis of pro-MMP-2 and MMP-9. The treatment in each lane was as marked in the figure. Experimental details of immunoblotting are provided in "Materials and Methods." A representative immunoblot of pro-MMP-2, and pro and active forms of MMP-9 from three independent experiments with identical results are shown. **A**: Treatment of EGCG (dose-dependently), and MEK and p38 inhibitors to DU-145 cells inhibited FCM-induced pro-MMP-2, as shown by immunoblotting. **B**: Treatment of EGCG (dose-dependently), and MEK and p38 inhibitors to DU-145 cells inhibited FCM-induced pro and active forms of MMP-9, as shown by immunoblotting. SB = SB203580, an inhibitor for p38, PD = PD095089, an inhibitor for ERK1/2. **C**: Treatment of EGCG inhibits androgen (R1881)-induced pro-MMP-2 expression in LNCaP cells. Following serum starvation for 24 hr, LNCaP cells were left untreated, or treated with R1881 alone (1.0 nM) and R1881 + EGCG (20 and 40 μg/ml) for 24 hr in serum free media. Twenty-four hours later cells were harvested and protein level of pro-MMP-2 was determined in whole cell lysate by Western blot as described in "Materials and Methods." A representative blot is shown from three independent experiments.

synthesis is activated through the phosphorylation of ERK1/2 and p38, and inhibition of MMP by EGCG is mediated through the prevention of the phosphorylation of these MAPK proteins.

Treatment of EGCG Inhibits Androgen (R1881)-Induced pro-MMP-2 Expression in Androgen Receptor Positive LNCaP Cells

In addition to look at the effect of EGCG on FCM-induced MMP-2 and -9 in androgen-independent DU145 cells, we were also interested to look at the effect of EGCG on androgen-induced pro-MMP-2 expression in androgen dependent (androgen receptor positive) LNCaP cells. The expression of pro-MMP-2 was specifically analyzed because this MMP has a crucial role in prostate cancer invasion and metastasis. Since 20 and 40 μg/ml doses of EGCG induced marked inhibition of MMP expression, in this experiment these two doses of EGCG were tested. Western blot analysis indicated that treatment of R1881 (1.0 nM) [26], a synthetic androgen, to LNCaP cells markedly induced pro-MMP-2 expression (Fig. 5C). Densitometry analysis of protein bands indicated that pretreatment of 20 and 40 μg/ml of EGCG inhibited androgen-induced pro-MMP-2 expression by 42 and 80%, respectively in LNCaP cells, as shown in Figure 5C. Pro-MMP-2 expression was assessed in the whole cell lysate 24 hr after the EGCG treatment. These data indicated that EGCG also has the ability to prevent the expression of MMP in androgen-dependent prostate cancer cells.

Treatment of EGCG to DU-145 Cells Inhibits FCM-Induced MMPs Synthesis by Blocking the Activation of NF-κB and AP-1

DNA sequence analysis has shown that *MMP* genes contain AP-1 and NF-κB binding sites and are shown to be involved in their active synthesis [27,28]. Therefore, we determined the effect of EGCG at the transcriptional level on FCM-induced MMP synthesis by analyzing the levels of phospho-specific *c-Jun*, and NF-κB transcription factors in the nuclear fractions of DU-145 cells. Cells grown in FCM induced activation of *c-Jun* and NF-κB compared to the cells grown in SFM alone

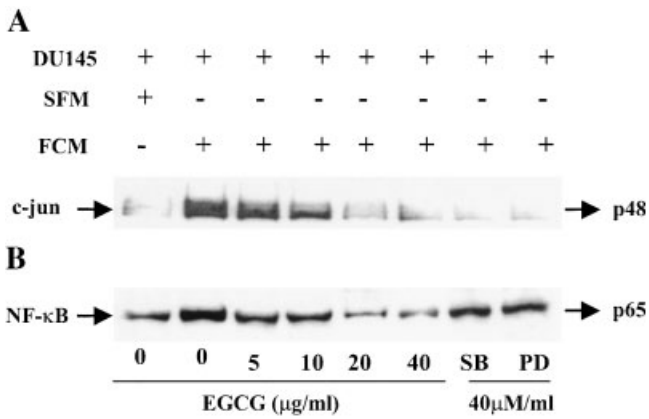

Fig. 6. Treatment of EGCG (5 – 40 μg/ml) and inhibitors of MEK (PD095089) and p38 (SB203580) to DU-145 cells inhibits FCM-induced activation of c-jun (**A**) and NF-κB (**B**). The treatment in each lane was as marked in the figure. A representative immunoblot of *c-jun* and NF-κB from three independent experiments with identical results is shown. Experimental details are provided in "Materials and Methods." DU-145 cells grown in SFM demonstrates the basal level of c-Jun and NF-κB. SB = SB203580, an inhibitor for p38, PD = PD095089, an inhibitor for ERK1/2.

(Fig. 6). Western blot analysis revealed that treatment of EGCG to DU-145 cells dose-dependently inhibited FCM-induced activation of *c-Jun* (p48) and NF-κB (p65) in nuclear fractions, as shown in Figure 6. Densitometric analysis of protein bands from different treatment groups indicated that even a low dose of EGCG (5 μg/ml) afforded a marked inhibition against FCM-induced activation of *c-Jun* and NF-κB by more than 50% when compared to FCM alone treated cells, suggested that the inhibition of MMP-2 and MMP-9 syntheses occurred at the transcription level by EGCG treatment. Further to define the role of MAPK in transcriptional regulation of MMPs, DU-145 cells were treated with respective inhibitors of p38 (SB203580) and ERK1/2 (PD095089). Treatment of PD098059 or SB203580 to DU-145 cells resulted in inhibition of FCM-induced activation of *c-jun* and NF-κB in the nuclear fractions compared to the cells grown in FCM alone. These observations suggested that the inhibition of MMP-2 and -9 by EGCG could be associated with the blocking of translocation of these transcription factors from the cytoplasm to the nucleus.

DISCUSSION

The expression of MMPs in tumors is regulated in a paracrine manner by growth factors and cytokines secreted by tumor infiltrating inflammatory cells as well as by tumor or stromal cells [7,9,10,28,29]. Fibroblast growth factors or FCM have been shown to induce MMP in a paracrine manner in several mesenchymal and cancer cell types [30–32], but not in normal epithelial cells. In vivo presence of these growth factors stimulate the induction of MMPs and thus stimulate the growth of tumors. The use of FCM in this in vitro study, therefore, partially mimics an in vivo tumor-host microenvironment. We found that treatment of EGCG to DU-145 cells dose-dependently inhibited FCM-induced increased levels of MMP-2 and MMP-9 when determined in extracellular (Fig. 1) as well as in cellular system (Fig. 5). MMP-2 and MMP-9 are thought to be key enzymes for degrading type IV collagen in the extracellular matrix and basement membrane that facilitates local invasion, metastasis of tumor cells, and angiogenesis within the various types of tumors including prostate cancer [5,7,13,14]. The inhibition of MMPs by EGCG may represent the inhibition of either activity or synthesis of MTI-MMP located in the membrane that cleaves pro-MMP-2 into its active form [33]. MMP-9 thought to be synthesized and immediately secreted in a latent form, but we found both active and pro-MMP-9 within the cells. This observation suggests that EGCG inhibited the synthesis of pro-MMPs at either transcriptional or translational level and it is also possible that the stimulators of MMP-9 may also be inhibited by EGCG as active MMP-9 level was also reduced in a dose-dependent manner.

MAPK pathway is the key regulatory mechanism for the control of growth and the induction of stress responses in cells [34]. Transcription of variety of proteins is regulated by phosphorylation by MAPK that mediate signals from cell membrane receptors triggered by growth factors, cytokines, hormones, and cell–cell and cell–matrix interactions to the nucleus [34]. Role of MAPK in the synthesis of MMPs have been studied using different cell types [34–38]. In malignant melanomas, it has been demonstrated that MMP-2 is a potential downstream target for stress-activated p38 MAPK [34]. The expression of MMP-9 has been shown to regulate by p38 inhibitors in squamous cell carcinoma [35]. In fibroblasts, the expression of MMP-13 is regulated by p38 MAPK [36]. Similarly ERK1/2 phosphorylation has been shown to mediate the induction of MMP-9 in several cell types, such as in astrocytes [37], ovarian cancer cells [38] etc. In our study, treatment of EGCG inhibited FCM-induced phosphorylation of ERK1/2 and p38 and concomitant reduction in the levels of MMP-2 and -9 (Figs. 2 and 5A,B) in DU145 cells indicating the possible mechanism of inhibition of MMPs synthesis by EGCG. This fact was further supported by the use of MEK and p38 inhibitors in our experimental system. It was reported that MAPK signaling is not only involved in the synthesis of MMPs but also involved in their activation [39]. Therefore, it is suggested that EGCG may play a role not only in inhibiting the synthesis of MMPs but also in inhibiting the conversion of pro form to its active form. Further, in vitro treatment of EGCG to androgen-dependent LNCaP cells results in inhibition of androgen-induced pro-MMP-2 expression (Fig. 5C), which demonstrate the chemopreventive efficacy of EGCG against both androgen-sensitive and androgen-insensitive prostate tumor cells. Similar to the inhibition of MMP expression by EGCG, Gupta et al. [40,41] demonstrated that treatment of EGCG causes growth inhibition, cell-cycle dysregulation and induction of apoptosis in both androgen-sensitive LNCaP and androgen-insensitive DU145 human prostate carcinoma cells. Thus, provided alternate mechanism of prevention of prostate cancer in vitro by EGCG.

MMP-9 is an inducible gene and *MMP-2* gene is constitutively expressed. *MMP-9* gene is regulated by 670 base pairs of upstream sequence, which includes motifs corresponding to AP-1, NF-κB, PEA3, and Sp1 binding sites [11]. Indeed, one or more of these binding sites have been implicated in mediating the effects of a diverse set of agents. Activation of MAPK, AP-1, and NF-κB is involved in many disease processes such as inflammation, neoplastic transformation, cancer cell invasion, metastasis, and angiogenesis [reviewed in

Reference 16]. Therefore, blocking the factors that bind to these regulatory elements seems to be an appropriate approach to the inhibition of MMP synthesis. Previously, EGCG has been shown to modulate NF-κB in cancer cells stimulated by TNF-α as well as LPS [23]. Inhibition of MAPK and AP-1 activities by EGCG has been demonstrated in JB6 cells and in the corresponding H-ras transformed cell line, and ultraviolet irradiated normal human epidermal keratinocytes [42,43]. We have also found that DU-145 cells cultured with FCM induced activation of *c-jun* and NF-κB, and the treatment of EGCG to DU-145 cells resulted in inhibition of FCM-induced activation and translocation of these factors into the nucleus (Fig. 6A,B). On the basis of these data, it appears that inhibitory effect of EGCG on the synthesis of MMPs may be associated with the inhibition of activation of MAPK and subsequently inhibition of transcription factors that activate the synthesis of MMPs. This observation is supported by the use of inhibitors of p38 and ERK1/2. Alternatively, the synthesis and/or activation of transcription factors in the AP-1 [44] and NF-κB [45] families can also be regulated by ERK-dependent signaling cascade. Thus, it is suggested that a dual inhibition at the synthesis and translocation of these transcription factors may play a role in the inhibition of synthesis of MMP-2 and MMP-9. It has also been suggested that the inhibition of gelatinases by EGCG is due to zinc chelation [46]; polyphenols do, in fact, have high complexation affinity to metal ions, and zinc is essential for enzymatic activity [47]. However, because addition of excess zinc ions with EGCG failed to restore full gelatinolytic activity during zymography, this hypothesis is not likely [47].

Thus based on the data obtained in this study, it can be suggested that a marked inhibitory effect of EGCG on MMP inhibition and thus the invasion of tumor cells may result in significant inhibition of development and metastasis of prostate cancer. However, further studies are warranted in this direction to provide more insight into the possible chemopreventive mechanism of action of EGCG in vivo CaP system and the role of EGCG in altering the host–tumor relationship in favor of the host. A positive outcome of such in vivo studies could be beneficial because green tea is widely consumed as a popular beverage worldwide, and more importantly it is non-toxic in nature.

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