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Journal of Nutritional Biochemistry 22 (2011) 1074–1083

Journal of Nutritional Biochemistry

Green tea (−)-epigallocatechin-3-gallate inhibits HGF-induced progression in oral cavity cancer through suppression of HGF/c-Met☆

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Received 11 November 2009; received in revised form 4 September 2010; accepted 9 September 2010

Abstract

Hepatocyte growth factor (HGF) and c-Met have recently attracted a great deal of attention as prognostic indicators of patient outcome, and they are important in the control of tumor growth and invasion. Epigallocatechin-3-gallate (EGCG) has been shown to modulate multiple signal pathways in a manner that controls the unwanted proliferation and invasion of cells, thereby imparting cancer chemopreventive and therapeutic effects. In this study, we investigated the effects of EGCG in inhibiting HGF-induced tumor growth and invasion of oral cancer *in vitro* and *in vivo*. We examined the effects of EGCG on HGF-induced cell proliferation, migration, invasion, induction of apoptosis and modulation of HGF/c-Met signaling pathway in the KB oral cancer cell line. We investigated the antitumor effect and inhibition of c-Met expression by EGCG in a syngeneic mouse model (C3H/HeJ mice, SCC VII/SF cell line). HGF promoted cell proliferation, migration, invasion and induction of MMP (matrix metalloproteinase)-2 and MMP-9 in KB cells. EGCG significantly inhibited HGF-induced phosphorylation of Met and cell growth, invasion and expression of MMP-2 and MMP-9. EGCG blocked HGF-induced phosphorylation of c-Met and that of the downstream kinases AKT and ERK, and inhibition of p-AKT and p-ERK by EGCG was associated with marked increases in the phosphorylation of p38, JNK, cleaved caspase-3 and poly-ADP-ribose polymerase. In C3H/HeJ syngeneic mice, as an *in vivo* model, tumor growth was suppressed and apoptosis was increased by EGCG. Our results suggest that EGCG may be a potential therapeutic agent to inhibit HGF-induced tumor growth and invasion in oral cancer.

Keywords: HGF; c-Met; EGCG; Tumor invasion; Oral cancer; Head and neck cancer

1. Introduction

Oral cavity cancer is one of the most common head and neck cancers [1]. The World Health Organization reported oral cavity cancer as having one of the highest mortality ratios of all malignancies because of extensive local invasion and distant metastasis even at initial diagnosis [1]. Because most advanced metastasized cancers are incurable, an effort to prolong or block the process of carcinogenesis through chemoprevention has become an important and feasible strategy for cancer control and management.

The neoplastic transformation of epithelial cells and the malignant behavior of carcinoma cells are influenced by their interactions with neighboring stromal components, including fibroblasts, blood vessels, inflammatory cells and the extracellular matrix [2,3]. Meanwhile, cancer cells themselves alter their adjacent stroma to form a supportive microenvironment by producing various growth factors and cytokines [2]. An understanding of substances that mediate mutual interactions between epithelial cells and surrounding stromal cells may provide new insights into tumor biology and possible therapeutics. Hepatocyte growth factor (HGF) is known as a stromal cell-derived mediator in tumor–stroma interactions, particularly based on its close involvement in cancer invasion and metastasis. Recent work had revealed that the c-Met protein, which is the receptor for HGF, is a strong prognostic indicator of patient outcome and survival and is important in the control of cancer invasion in various types of cancer [4–6]. A direct role for c-Met in the metastatic behavior of human tumors has been proposed [7,8]. The survival rate of patients with high Met expression was found to be significantly lower than that of patients with normal expression patterns [9,10]. Overexpression of wild-type Met or HGF leads to the constitutive activation of several downstream signaling pathways, contributing to invasion and metastasis [11]. These observations suggest the importance of gaining better insight into

☆ Drs. Yoon Woo Koh, Eun Chang Choi, Sung Un Kang, Hye Sook Hwang, Mi Hye Lee, JungHee Pyun, RaeHee Park, YoungDon Lee, and Chul-Ho Kim have no conflicts of interest or financial ties to disclose.

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doi:10.1016/j.jnutbio.2010.09.005

the role of Met in tumorigenesis and progression and the potential of targeting the HGF/Met pathway in therapeutic strategies.

Tea [*Camellia sinensis* (*Theaceae*)] is second only to water as the most popular beverage consumed worldwide [12]. Epidemiological and preclinical studies have demonstrated that polyphenols derived from green tea have profound chemopreventive and antitumor effects [12–14]. The cancer-preventive activity of tea constituents has been demonstrated in many *in vitro* and *in vivo* animal models, including cancers of the skin, lung, oral cavity, esophagus, stomach, liver, pancreas, small intestine, colon, bladder, prostate and mammary gland [15].

The biological activity of green tea is due to different catechins, and (−)-epigallocatechin-3-gallate (EGCG) has been identified as the principal antioxidant, contributing about 30% of the total antioxidant capacity of green tea [16]. The major activities of EGCG include inhibition of numerous signaling pathways and protein kinases (AP-1, p44/p42 MAPK, Erk1/2, EGF-R, PDGF-R, FGF-R), inhibition of cell proliferation, induction of apoptosis, modulation of cell cycle regulation, interference with receptor binding and suppression of invasiveness and angiogenesis [17,18]. Recently, some studies showed that EGCG could inhibit the invasion and migration of human oral cancer cells through multiple mechanisms, possibly by the decreased production of MMP (matrix metalloproteinase)-2, MMP-9 and uPA or the demethylation effect on MMP inhibitors, such as RECK [19,20]. Also, EGCG has been reported to inhibit ligand-induced c-Met phosphorylation and potentially block invasive cancer growth [4,21]. Therefore, EGCG might be a useful agent to study as an adjunct to other anticancer agents.

To our knowledge, no reported study has described the effects of EGCG on HGF-induced tumor progression in oral cancer *in vitro* or *in vivo*. Thus, in the present study, we demonstrated the effects of EGCG on HGF-induced proliferation and invasion, inhibition of EGCG on the HGF/c-Met signaling pathway and antitumor effects of EGCG in a syngeneic mouse model (C3H/HeJ mice, SCC VII/SF cell line) [22,23]. The current study shows that EGCG may be a potential therapeutic agent for the inhibition of tumor growth and invasion in oral cancer, through suppression of the HGF/c-Met signaling pathway.

2. Materials and methods

2.1. Cell lines

Established human head and neck cancer cell lines KB (oral cancer cell line), FaDu (hypopharyngeal cancer cell line) and SNU-899/SNU-1086 (laryngeal cancer cell line) and murine cell line SCC VII/SF (squamous cell carcinoma cell line) were obtained from American Type Culture Collection (Manassas, VA, USA) and Korean Cell Line Bank (Seoul, Korea). The cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and penicillin–streptomycin at 100 U/ml (GIBCO, Paisley, PA, USA) at 37°C in a humidified atmosphere with 5% CO_2/95% air.

2.2. Animals and housing

Twenty 6-week-old female C3H/HeJ syngeneic mice from Samtaco (Osan, Korea) weighing around 20 g were used. After transportation, the animals were maintained in the central animal laboratory for at least 1 week. The animals were housed in independent ventilation cages and were allowed free access to water and food. The temperature was maintained at 21°C±1°C, and the lights were turned on from 8:00 a.m. to 8:00 p.m. This study was approved by the Committee for Ethics in Animal Experiments of the Ajou University School of Medicine.

2.3. Cell viability assay

To determine cell viability, we exposed various human head and neck cancer cells (FaDu, KB, SNU-899 and SNU-1066) and SCC VII/SF to various concentrations of whole green tea, EGCG, epicatechin gallate (ECG), epigallocatechin (EGC) and epicatechin (EC) (Sigma Chemical, St. Louis, MO, USA). Briefly, cells were plated in 96-well tissue culture plates at 2000 cells/well in a final volume of 100 μl of medium and were allowed to attach for 2 days. The cells were then treated once with varying doses of green tea extracts for 16 h. After completion of the treatment, the cells were incubated with 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma Chemical) for 4 h at 37°C. MTT is reduced to a colored water-insoluble formazan salt only by metabolically active cells. Isopropanol with 0.4 N HCl (1 ml) was added, and the optical density of each culture well was measured using a microplate reader (Bio-Tek, Winooski, VT, USA) at 560 nm. MTT assays were repeated at least three times in triplicate.

2.4. Wound healing assay

KB cells were seeded in 6-well plate at about 1×10^5 cells/well and were grown for 48 h in serum-free medium plus 0.1% bovine serum albumin. The monolayer was artificially injured by scratching across the plate with a sterile pipette tip (about 1.3 mm in width). The wells were washed twice to remove detached cells or cell debris. Serum-free media with increasing concentrations of EGCG (0, 10, 50 μM) in the presence and in the absence of HGF (30 ng/ml) were added, and cells were incubated. Wound healing (images of the scratched areas under each condition) was documented by photography at 12, 24, 36 and 48 h. All wound healing assays were performed on at least three occasions in duplicate.

2.5. Invasion assay

Transwell chambers (Costar, Corning, Corning, NY) were used to assay the invasion level of cells. Initially, type I collagen (6 mg per filter) dissolved in 100 μl of Dulbecco's modified Eagle's medium was poured into the upper part of the polyethylene filter (pore size, 8 μm), and coating was allowed to proceed overnight in a laminar flow hood. Fetal bovine serum medium (500 μl; 0.5%) was placed in the lower part of the well, and the well was filled with HGF (0, 10, 30 ng/ml) in the presence or absence of increasing concentrations of EGCG (0, 10, 50 μM). After preprocessing with mitomycin C (8 μg/ml for 30 min) to prevent cell proliferation, 10^5 cells (in 100 μl of growth medium) were added to the top of the filter in the upper well. The chamber was then cultivated in 5% CO_2 at 37°C for 48 h. The filter in the upper well was removed. Finally, the attached cells in the lower section were stained with hematoxylin and counted under a light microscope. All invasion assays were performed on at least three occasions in duplicate.

2.6. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay

Apoptosis in KB cells was determined by the TUNEL method using an *in situ* cell detection kit (Roche Molecular Biochemicals, Mannheim, Germany). KB cells were glass-coverslipped in 24-well culture dishes containing growth medium. After monolayers achieved 60%–70% confluence, the cells were exposed to medium supplemented with EGCG or EGCG plus HGF for 24 h. Thereafter, the cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde. The cells were then incubated with 50 μl of TUNEL reaction mixture (terminal deoxynucleotidyl transferase and fluorescein-dUTP) at 37°C for 60 min in a humid atmosphere. The cells were stained with Hoechst 33258 (5 μg/ml) for 5 min. Stained cells were observed under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from KB cells homogenized in TRIzol reagent (Gibco, Grand Island, NY, USA). An Omniscript Reverse Transcriptase Kit (Qiagen, Hilden, Germany) was used for reverse transcription of the RNA. Total RNA (2 μg) was mixed with 20 μl of the mixture: 2.0 μl of 10× RT buffer, 2.0 μl of dNTPs (5 mM each), 2.0 μl of oligo-(dT) primer (10 μM), 1.0 μl of RNase inhibitor (10 U/μl), 2 U of Omniscript reverse transcriptase and RNase-free water. Reverse transcription was carried out, and cDNA was synthesized. The synthesized cDNA was added to a mixture of 1 U of Taq DNA polymerase (Roche Molecular Biochemicals) and specific primers and amplified using an MJ Research Minicycler (Bio-Rad Laboratories, Waltham, MA, USA). The following HGF-specific and c-Met-specific primers were used: 5′-ACATCGTCACTTCTGGC-3′ for HGF-F, 5′-ATCCATCCTATGTTT GTTCG-3′ for HGF-R, 5′-AGTAGCCTGATTGTGCATTT-3′ for c-Met-F and 5′-TCTTTCATGATGCCCTC-3′ for c-Met-R. PCR was performed under the following conditions: denaturation for 3 min at 96°C followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, with extension for 5 min at 72°C. PCR products were separated by electrophoresis in 1.5% agarose gels and were detected under ethidium bromide staining and ultraviolet light (Bio-Rad Laboratories).

2.8. Western blot analysis

Total proteins were extracted using a ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, La Jolla, CA, USA) following the manufacturer's instructions. Protein concentrations were measured using the BCA assay (Pierce, Rockford, IL, USA). Proteins were separated by electrophoresis on 12% and 10% SDS polyacrylamide gels. An equal amount of protein (10 μg) was loaded in each lane. After electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membrane was blocked in Tris-buffered saline Tween-20 containing 5% nonfat milk for 1 h, followed by an overnight incubation at 4°C with primary antibodies. The antibodies used in this study were as follows: Anti-phospho-Met, anti-AKT, anti-phospho-AKT, anti-ERK and anti-phospho-ERK were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-HGF, anti-Met, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-JNK, anti-phospho-JNK and anti-caspase-3

(1:1000) came from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-poly-ADP-ribose polymerase (PARP) was from Roche Diagnostics (Indianapolis, IN, USA). Data were normalized relative to protein levels of β-actin, which was probed by polyclonal rabbit antibody (1:1000; Cell Signaling Technology). After washing the membrane extensively (10 min per wash), it was incubated with horseradish peroxidase-conjugated secondary antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. Protein bands on the blots were visualized using ECL Plus Western Blotting Detection Reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA). All Western blot analyses were repeated on these experiments.

2.9. Analysis of the antitumor effects of EGCG in a syngeneic mouse model (C3H/HeJ mice, SCC VII/SF cell line)

For an *in vivo* study, we used a syngeneic mouse model (C3H/HeJ mice, SCC VII/SF cell line) that has been introduced as an *in vivo* immunocompetent murine model for head and neck cancer [22,23]. In total, 5×10^5 SCC VII/SF cells were inoculated subcutaneously into the flank of syngeneic C3H/HeJ mice (weighing about 20 g) under pathogen-free conditions and then randomly divided into four equal groups (five mice per group). Treatment was started the day after cell implantation with either intraperitoneal injection of EGCG (25, 50 or 75 mg/kg) daily (treatment groups) or diluent control (D.W.) alone (control group). The tumor size in two perpendicular diameters was measured with calipers every second day. The tumor volume was calculated by the formula, $V=(\pi/6)\times(L\times W^2)$, where L is the large diameter and W is the smaller diameter) [24]. Animals were euthanized, and tumors were collected and weighed 22 days after implantation. Mice were killed by cervical dislocation, and tumors were excised and divided in two parts. For each sample, final tumor size and weight were calculated. Half of the tissue was snap-frozen in liquid nitrogen and used for protein extraction. The other half was fixed overnight in neutral buffered formalin and processed by routine methods. Western blotting of c-Met and HGF was performed on tumor specimens and normal soft tissue samples of C3H/HeJ syngeneic mice.

2.10. Immunohistochemistry

Caspase-3 immunohistochemistry and proliferating cell nuclear antigen (PCNA) immunohistochemistry were performed on paraffin-embedded mice SCC VII/SF tumor sections collected on polylysine-coated slides. After paraffin removal in xylene, the sections were rehydrated and incubated with 0.3% hydrogen peroxide for 10 min to eliminate endogenous peroxidase activity. Briefly, the specimens were incubated with anti-active caspase-3 (Cell Signaling Technology) and PCNA (Santa Cruz Biotechnology) mouse primary antibodies, diluted 1:400 in blocking solution, overnight at 4°C. The sections were thoroughly rinsed in PBS and incubated for 2 h at room temperature with a streptavidin–biotin–peroxidase complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). Immunolabeling was revealed after three washes in PBS using 2,3′-diaminobenzidine as a substrate, diluted 1:10 in buffer, according to the manufacturer's instructions (Roche Molecular Biochemicals). Staining was completed by incubation with 3,3′-diaminobenzidine substrate–chromogen, which results in a brown-colored precipitate at the antigen site. Measurements of active caspase-3- or PCNA-positive cells were performed on 10–15 images per slide, captured by an independent observer who was blinded to the experiment, and normalized to the total cell count by DAPI staining.

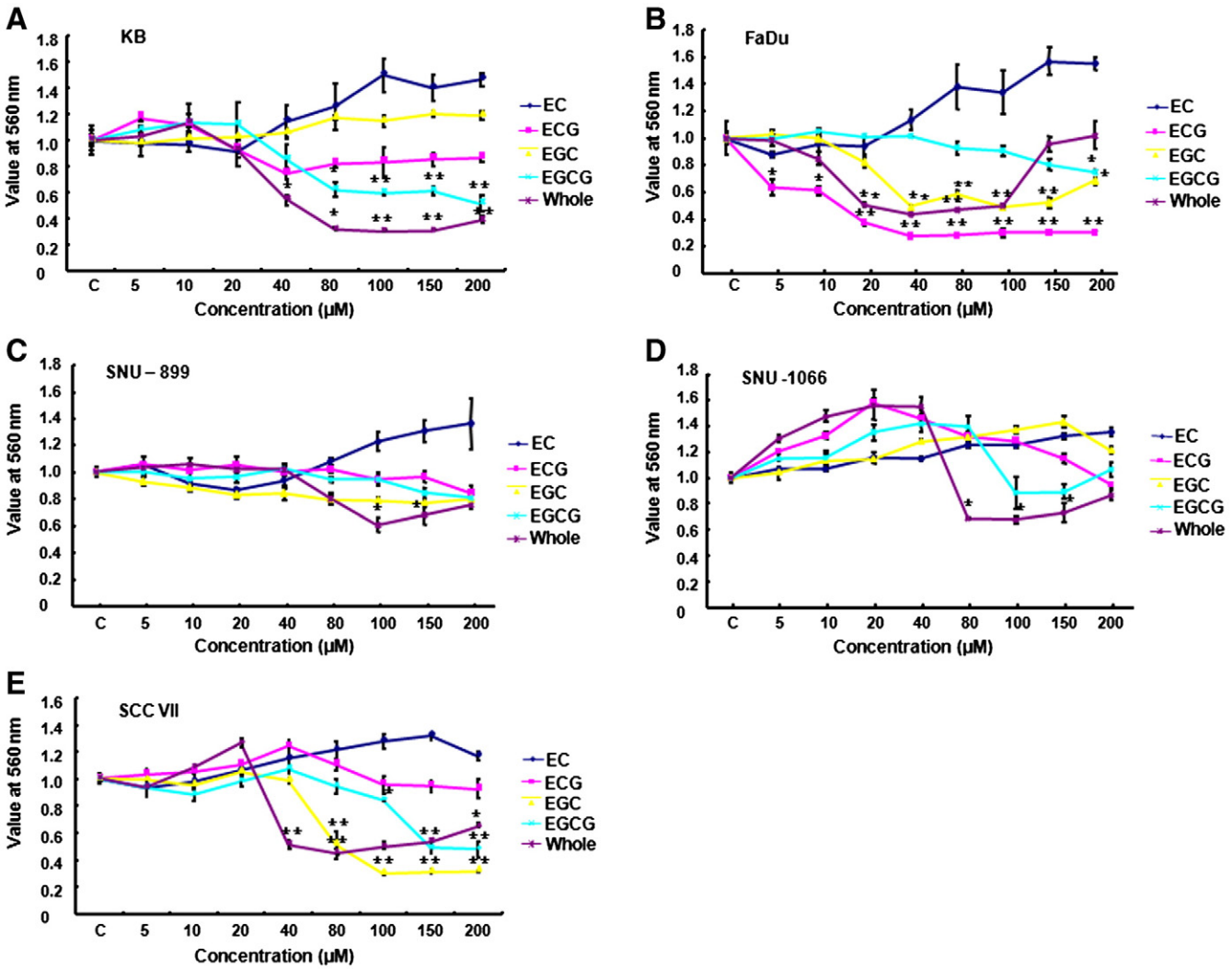

Fig. 1. Effect of EGCG on HGF-induced proliferation in the various head and neck cancer cell lines. Cells were plated in 96-well tissue culture plates at 2000 cells/well in a final volume of 100 μl of medium and were allowed to attach for 2 days. The cells were then treated once with varying doses of green tea extracts and observed after 16 h. Cell viability was estimated by the MTT assay. (A) KB (human oral cancer cell). (B) FaDu (human hypopharyngeal cancer cell). (C) SNU-899 (human laryngeal cancer cell). (D) SNU-1066 (human laryngeal cancer cell). (E) SCC VII/SF (murine squamous cell carcinoma cell line). Values represent the mean±S.D. from three or four independent experiments. $^{*}P<.05$ and $^{**}P<.01$, compared with the no-treatment control group.

2.11. Statistical analyses

Student's *t* test and one-way ANOVA were used for statistical analyses of the data. All statistical analyses were conducted using the SPSS software (version 10.0, SPSS, Chicago, IL, USA). *P* values <.05 were deemed to indicate statistical significance ($^{*}P<.05$, $^{**}P<.01$).

3. Results

3.1. Effect of EGCG on HGF-induced proliferation of head and neck cancer cell lines

We examined the effect of increasing concentrations of various green tea extracts (whole green tea, EGCG, EGC, ECG and EC) on the growth of the various head and neck cancer cells (FaDu, KB, SNU-899 and SNU-1086) and SCC VII/SF using the MTT assay. Differential sensitivities among the four head and neck cancer cells (Fig. 1) for 24 h of exposure to whole green tea, EGCG, EGC, ECG and EC were noted. Treatment with various green tea extracts (0–200 μM) inhibited the growth of the various head and neck cancer cells in a concentration-dependent manner. Extensive inhibition of cell growth was observed in groups receiving high concentrations (>100 μM) of EGCG or whole green tea. In KB cells, effective inhibition of cell growth was observed in the EGCG group (Fig. 1A). Additionally, among the four cancer cells, the most significant inhibition of cell growth was noted in the EGCG treatment of FaDu cells (Fig. 1B). However, EGCG could not effectively inhibit cell proliferation in SNU-899 and SNU-1066, human laryngeal cancer cell lines (Fig. 1C and D). In the case of SCC VII/SF, whole green tea and EGC significantly inhibited cell proliferation at relatively low doses and EGCG effectively suppressed cell proliferation at high doses (Fig. 1E).

3.2. Effect of EGCG on HGF-induced migration and invasion of KB cells

A wound healing assay was performed, as described above, and cells were pretreated with increasing concentrations of EGCG (0, 10,

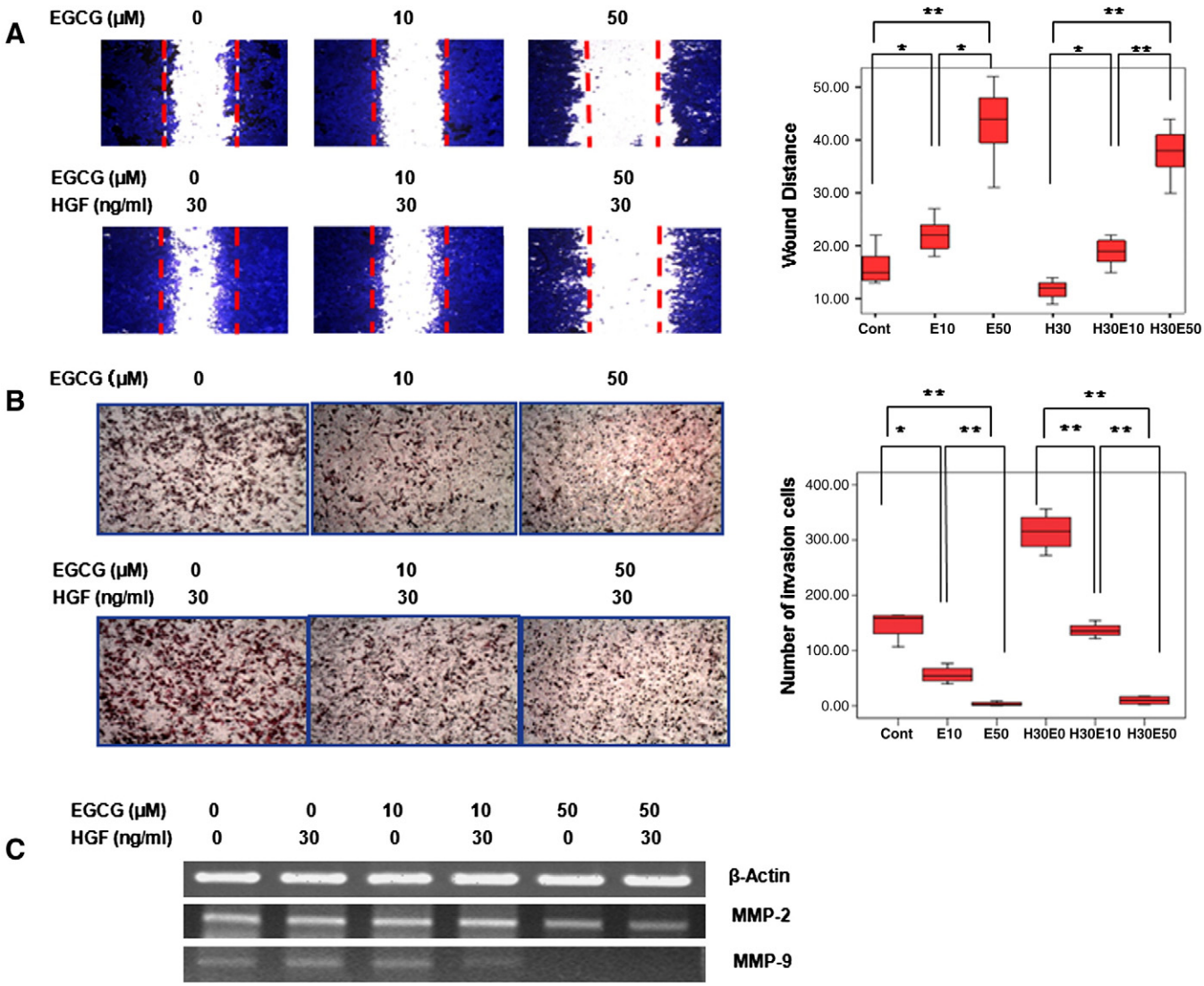

Fig. 2. Effect of EGCG on HGF-induced migration, invasion of KB cells and the expression of MMP-2 and MMP-9 in KB cells. (A) Wound healing assay. KB cells were plated in a 6-well plate and grown to confluence in serum-containing media. The cells were starved of growth factors for 48 h. The monolayer was scratched with a pipette tip and washed with PBS. KB cells were treated with EGCG (0, 10, 50 μM) and with EGCG (0, 10, 50 μM) plus HGF (30 ng/ml) to evaluate the effect of EGCG on HGF-induced migratory and proliferative activities. (B) Invasion assay. Transwell chambers were used to examine the level of cell invasiveness. KB cells were seeded on the upper membrane in EGCG (0, 10, 50 μM) in the presence and in the absence of HGF (30 ng/ml). After 48 h of incubation, plugged cells in 8-μm pores or cells attached to the undersurface of the membrane were counted, and the cells attached in the lower section were stained with hematoxylin and counted under a light microscope. (C) Effect of EGCG on the expression of HGF-induced MMP-2 and MMP-9 mRNAs in KB cells. $^{*}P<.05$ and $^{**}P<.01$, compared with the no-treatment control group.

50 μM) in the presence or absence of HGF (30 ng/ml) to determine whether EGCG can also inhibit HGF-induced motility. HGF significantly enhanced the migration and proliferation of KB cells. However, as shown in Fig. 2A, HGF-induced cell motility was blocked by EGCG, dose-dependently ($P<.01$).

A Transwell chamber invasion assay was performed with the KB cell line to determine whether HGF contributed to cell invasiveness and whether EGCG was capable of inhibiting HGF-induced invasion. HGF treatment significantly increased the number of cells that invaded dose-dependently compared with untreated controls. Fig. 2B demonstrates that this invasiveness, induced by HGF, was inhibited by EGCG in a dose-dependent manner ($P<.01$).

3.3. Effect of EGCG on the expression of HGF-induced MMP-2 and MMP-9 mRNAs in KB cells

To confirm whether HGF could induce MMP gene expression in KB cells, we performed RT-PCR after treatment with HGF (30 ng/ml). MMP-9 mRNA expression increased slightly after HGF treatment for 24 h; however, the expression of MMP-2 mRNA could not be increased (Fig. 2C). Next, we examined whether EGCG could influence HGF-induced MMP-2 and MMP-9 mRNA expression in KB cells and found that MMP-2 and MMP-9 gene expression was decreased by EGCG in a dose-dependent manner (Fig. 2C).

3.4. Effect of EGCG on apoptosis in KB cells

We used the TUNEL assay with an *in situ* cell detection kit as a method of evaluating apoptosis. Apoptosis is characterized by TUNEL staining. EGCG-induced nuclear condensation, DNA fragmentation and perinuclear apoptotic bodies in the KB cells were detected by Hoechst 33258 staining [25]. When KB cells were treated with EGCG in the absence of HGF, the TUNEL assay revealed numerous TUNEL-positive cells (Fig. 3). Even in the presence of HGF, staining of KB cells following EGCG treatment revealed numerous TUNEL-positive cells (Fig. 3).

3.5. Effect of EGCG on HGF-induced c-Met expression and HGF-activated AKT and ERK downstream pathways in KB cells

To examine whether HGF and c-Met were expressed in KB cells, we performed RT-PCR and Western blotting to detect mRNA and protein of HGF and c-Met, respectively. Fig. 4A shows that the expression levels of the HGF receptor c-Met mRNA and protein were detected by RT-PCR and Western blotting, respectively; however, expression of HGF was not detected by these methods. The mechanism by which EGCG inhibits growth factor signaling is unclear. Several published studies demonstrated that EGCG directly inhibited the enzymatic activities of AKT, ERK and DNA methyltransferase *in vitro* [26,27], while other

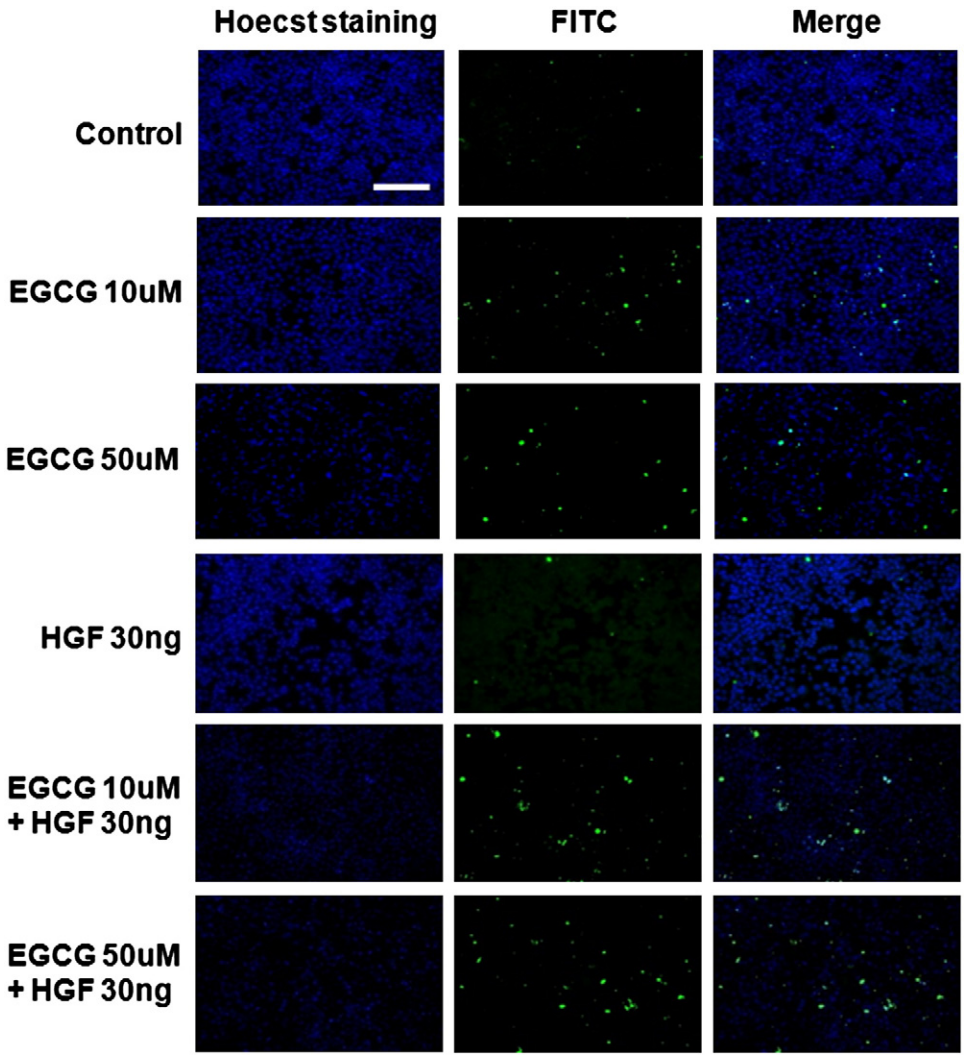

Fig. 3. Effect of EGCG on apoptosis in KB cells. Apoptosis in KB cells was determined by the TUNEL method using an *in situ* cell detection kit.

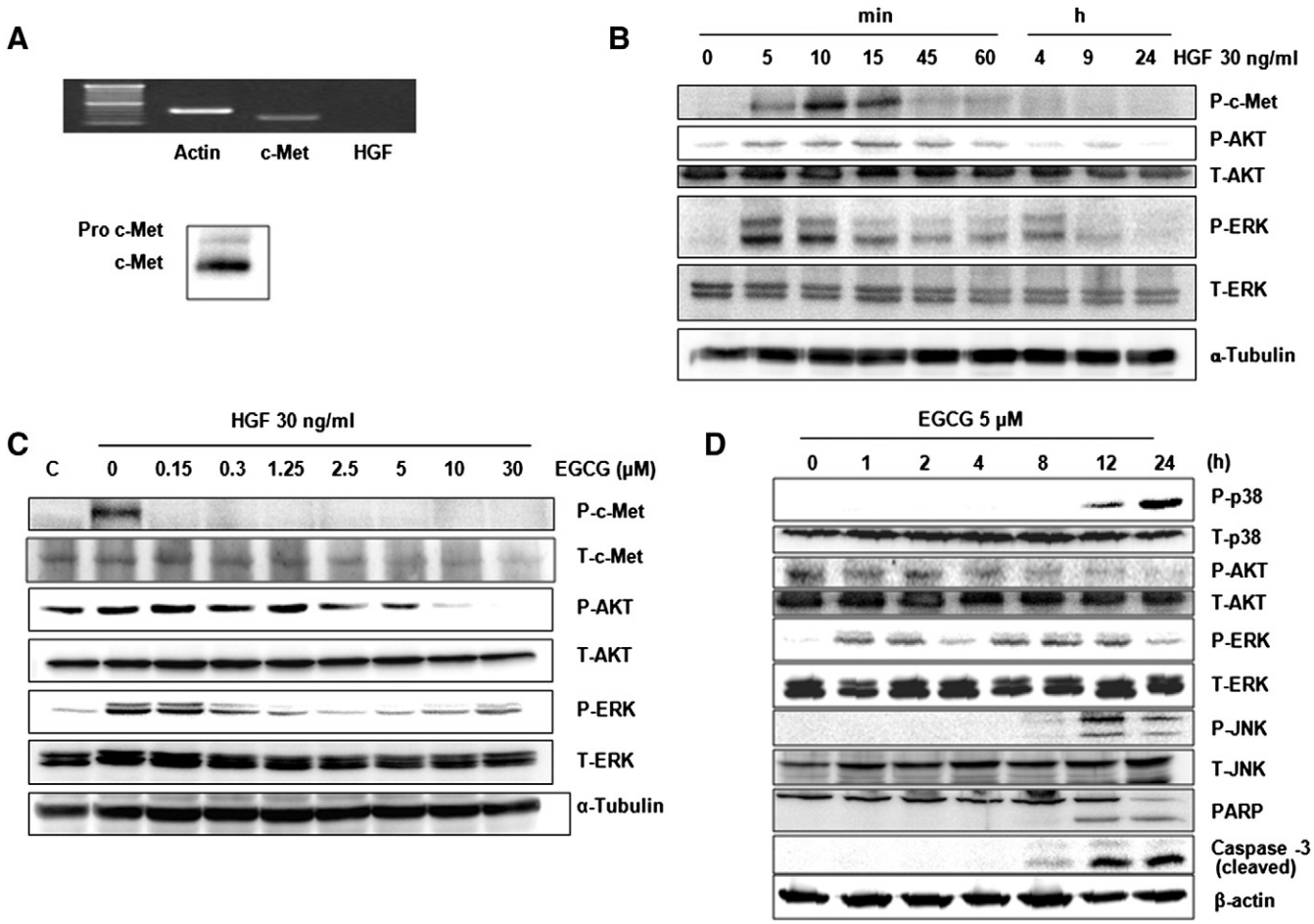

Fig. 4. Effect of EGCG on HGF-induced c-Met expression and HGF-activated AKT and ERK downstream pathways in KB cells. (A) The expression of c-Met mRNA and protein by RT-PCR and Western blotting in KB cells. (B) Effects of HGF on activation of Met, AKT and ERK. KB cells were deprived of serum overnight and then incubated for 24 h. Cells were then stimulated with 30 ng/ml of HGF for the indicated times. (C) Suppression of the HGF-activated Met, AKT and ERK pathway by EGCG. KB cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of HGF (30 ng/ml) together with the indicated concentrations of EGCG. Cell lysates were subjected to Western blot analysis with antibodies to phosphorylated forms of Met, AKT and ERK, as well as antibodies to total Met, AKT and ERK. (D) Effect of EGCG on c-Met, AKT, ERK, p38, JNK, cleaved caspase-3 and PARP phosphorylation in KB cells. P, phosphorylated; T, total.

studies indicated that this catechin could block the binding of growth factor with its receptor. First, to assess the downstream signaling events induced by HGF in KB cells, we treated the cells with 30 ng/ml of HGF, and cell lysates were prepared at regular intervals for up to 24 h and then analyzed for AKT and ERK activities. As shown in Fig. 4B, c-Met, AKT and ERK phosphorylations were increased in the HGF (30 ng/ml)-treated cells, with strong activation within 15 min. Next, to determine whether EGCG could block HGF signaling at the level of c-Met activation, we incubated KB cells for 30 min with increasing concentrations of EGCG (0.15–30 μM) in the presence of HGF (30 ng/ml), and protein lysates were prepared. Western blot analysis revealed that concentrations of EGCG as low as 0.15 μM completely blocked Met activation, while the phosphorylations of AKT and ERK decreased or disappeared at concentrations of 30 and 0.3 μM EGCG, respectively (Fig. 4C). Additionally, inhibition of phosphorylated AKT and ERK by EGCG was associated with marked increase of the phosphorylation of p38 and JNK, cleaved caspase-3 and PARP (Fig. 4D).

3.6. Effect of EGCG on tumorigenicity and apoptosis in a syngeneic mouse model (C3H/HeJ mice, SCC VII/SF cells)

To determine whether these results could be translated *in vivo*, we used an immunocompetent syngeneic mouse model (C3H/HeJ mice, SCC VII/SF cell) and randomly divided the mice into four equal groups (control and 25, 50 and 75 mg/kg) after injection of SCC VII/SF cells. Administration of EGCG significantly affected tumor growth vs. controls, and the observed differences in tumor development in the EGCG 50 mg/kg group compared with the EGCG 25 mg/kg group were significant ($P<.05$; Fig. 5A and B). Thus, EGCG seemed to produce dose-dependent tumor growth inhibition *in vivo*. However, administration of 75 mg/kg of EGCG to syngeneic C3H/HeJ mice resulted in poor oral intake, weight loss and lethargy, and four mice (of five) died within 3 weeks.

An immunohistochemical study of caspase-3 revealed that as the dose of EGCG increased, from 25 to 75 mg/kg, the number of caspase-3-positive cells tended to increase in a dose-dependent manner (Fig. 6A). Additionally, PCNA is widely used as a cell replication marker. An immunohistochemical study of PCNA also revealed that as the dose of EGCG increased, the number of PCNA-positive cells decreased (Fig. 6B). Western blotting of c-Met and HGF was performed on tumor specimens of C3H/HeJ syngeneic mice. We observed a strong reduction of *in vivo* c-Met phosphorylation and HGF expression in proteins that were extracted from tumors in EGCG (25 mg/kg)-treated vs. control mice (Fig. 6C).

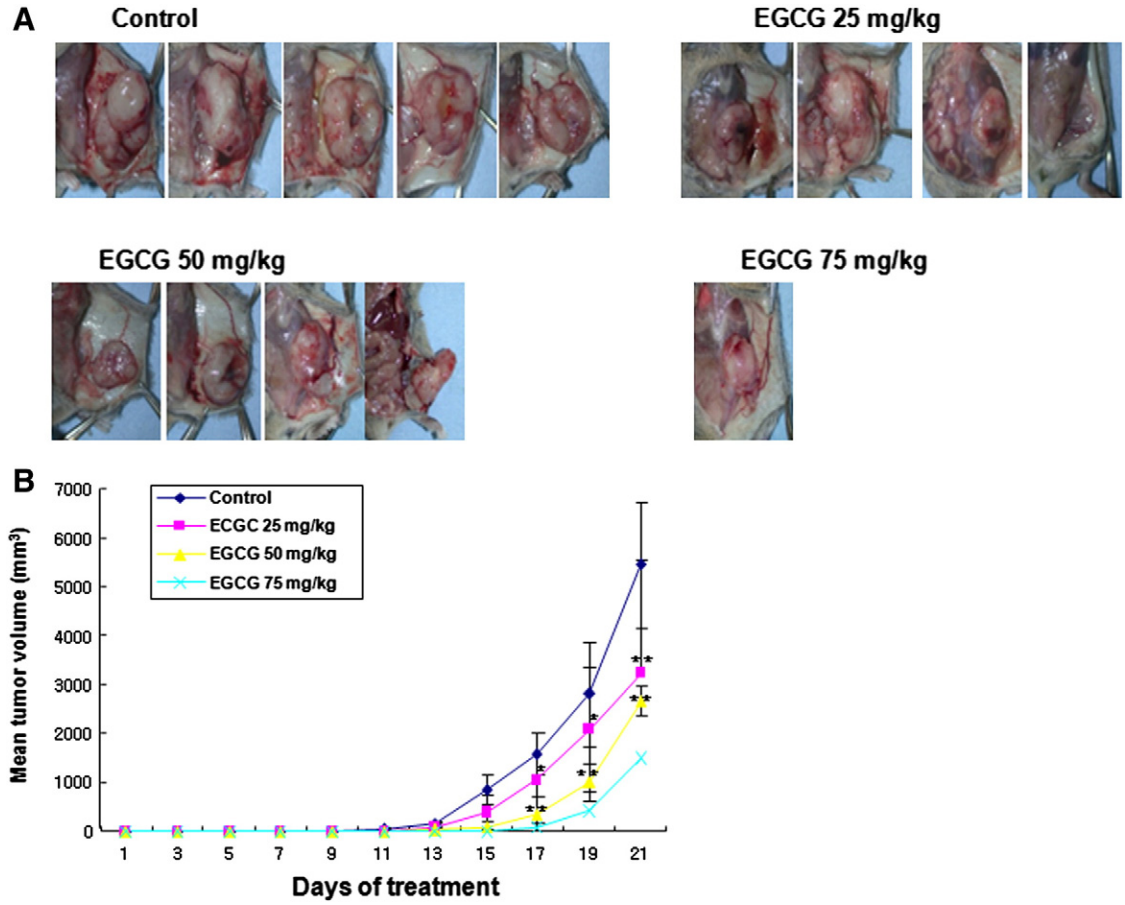

Fig. 5. Effect of EGCG on antitumor activity in a syngeneic mouse model (C3H/HeJ mice, SCC VII/SF cell). (A and B) Syngeneic C3H/HeJ mice were randomly divided into four equal groups. Three groups of animals received an intraperitoneal injection of EGCG (25, 50, 75 mg/kg), and the other group received D.W. alone and served as the control group. Animals were killed, and tumors were collected and weighed 22 days after implantation. $^{*}P<.05$ and $^{**}P<.01$, compared with the no-treatment control group.

4. Discussion

The HGF/Met signaling pathway is deregulated in most cancers and is associated with a poor prognosis [4,9–11]. However, little information is available regarding HGF/Met signaling pathways in oral cancer cells. We found that overexpression of HGF and c-Met was noted in more than half of oral cancer cases by immunohistochemical staining and that c-Met staining correlated significantly with lymph node metastasis, tumor stage and recurrence (data not shown). In a previous study, we demonstrated that HGF increased c-Met tyrosine phosphorylation and that HGF-induced phosphorylated c-Met expression was attenuated by EGCG in FaDu cells, a hypopharyngeal cancer cell line [28]. Based on these previous results, we tested whether HGF could induce oral cancer progression and if EGCG could inhibit HGF-induced invasion and metastasis in oral cavity cancer *in vitro* and *in vivo*.

Recently, many studies of different diet-derived compounds have revealed that such agents can inhibit one or more stages of the carcinogenic process [29,30]. The most important point of cancer biology is disrupted intracellular signaling networks, which transmit aberrant signals, resulting in abnormal cellular function. The study of plant polyphenols as anticancer agents has increased substantially, due in part to their profound effects *in vitro* and *in vivo* on tumor cell signaling pathways regulating growth and apoptosis. Additionally, epidemiological studies have revealed promising preventive and therapeutic roles for polyphenols. For example, some studies have demonstrated an inverse correlation between urinary tea polyphenols and gastric cancer, a reduction in colon cancer incidence in individuals who consumed tea and an improved prognosis of stages I and II breast cancer in those patients who drank five or more cups of green tea [4,31,32]. EGCG, the major catechin found in green tea, has been shown to inhibit proliferation and induce apoptosis in many cell lines [17]. *In vivo* mouse studies have established that EGCG can function as a chemopreventive agent against UV-induced and chemically and genetically induced carcinogenesis [33,34]. Recently, it has been shown in numerous reports that EGCG has multiple targets, including receptor tyrosine kinases, such as epidermal growth factor receptors and c-Met, MMPs and other signaling enzymes. A few mechanisms have been proposed to explain the various anticancer activities of tea polyphenols, including inhibiting the production of reactive oxygen species necessary for receptor signaling, directly inhibiting receptor activation, inhibiting the downstream signaling pathways and altering fluidity of membranes [20].

In this study, our results suggest that whole green tea extract and its principal constituent, EGCG, were the most effective in suppressing the proliferation of KB cells in the MTT assay. Moreover, whole green tea extract, as a mixture, was needed at a lower concentration when compared with EGCG, to induce similar cytotoxic effects in this cancer cell line. This may be explained by synergistic effects of combinations of different green tea catechins on cell growth and apoptosis [35]. EC had almost no effect on cell growth or the induction of apoptosis [35,36], but a significant synergistic effect on the induction of apoptosis was observed when EC was combined with

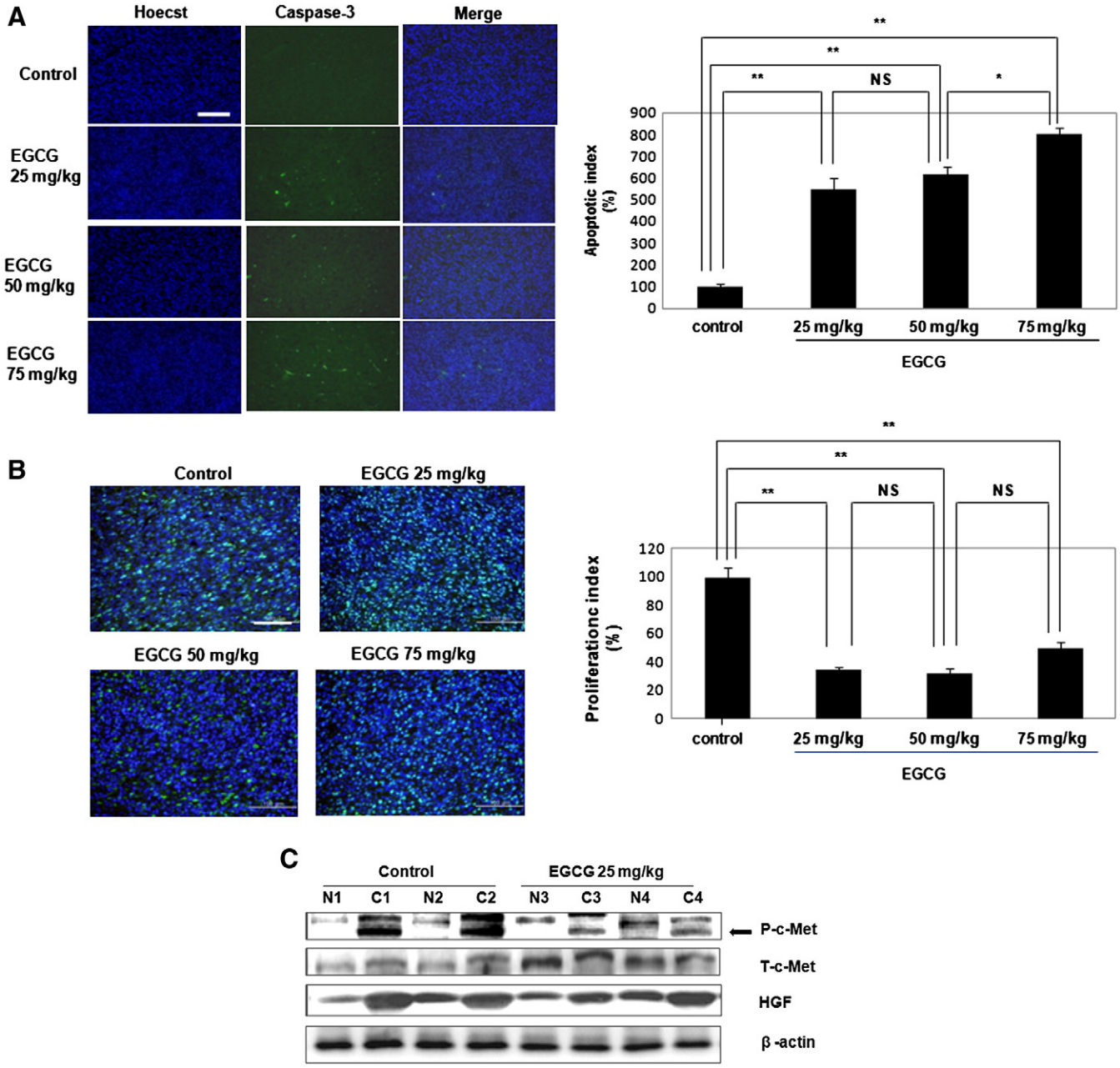

Fig. 6. Effect of EGCG on apoptosis and c-Met expression in a syngeneic mouse model (C3H/HeJ mice, SCC VII/SF cell). (A) Caspase-3 immunohistochemistry and (B) PCNA immunohistochemistry were performed on paraffin-embedded mouse tumor sections collected on polylysine-coated slides. (C) Western blotting of c-Met and HGF was performed on tumor specimens and normal soft tissue samples of C3H/HeJ syngeneic mice of the control and EGCG 25 mg/kg groups, respectively. $^{*}P<.05$ and $^{**}P<.01$. NS, statistically not significant. The scale bar represents 50 μm. N, normal soft tissue far from cancer of syngeneic mouse; C, cancer tissue of syngeneic mouse.

other catechins. Furthermore, Horie *et al.* [35] found that catalase specifically canceled the synergistic effects of EC and EGCG, suggesting that the synergism involved production of reactive oxidative species, such as hydrogen peroxide.

Our previous study suggested that serum HGF level was significantly correlated with tumor progression in head and neck squamous cell carcinoma (HNSCC) and that the mean serum HGF level was significantly higher in patients with HNSCC than in healthy controls [36]. Thus, we thought that the antitumor effect of EGCG under HGF treatment was important and may reflect a more real *in vivo* environment. Additionally, one of the accepted mechanisms of EGCG's actions is via *in vivo* inhibition of growth factor signaling [17]. Supporting this idea, a previous study revealed that oral administration of green tea catechins inhibited IGF signaling and reduced the activity of the PI3K and MAPK pathways in a prostate tumor mouse model [37].

As shown in Results, we found that EGCG significantly inhibited HGF-induced migration and invasion. Moreover, it is important to note that HGF altered cell morphology, including lamelliopodia and filopodia, which are found primarily in mobile cells. Specifically, EGCG effectively inhibited the appearance of lamelliopodia and filopodia.

We also investigated the effect of EGCG in the presence and that in the absence of HGF in the TUNEL assay, an early marker of apoptosis [25]. Even in the presence of HGF, as the dose of EGCG increased, the number of TUNEL-positive cells increased dose-dependently.

Interactions between cells with the extracellular matrix that promote adhesion and migration are believed to be essential for invasion, migration and metastasis of tumors [19]. MMP plays an important role in the processes of cancer invasion and metastasis. The progression of human tumors involves the MMP family; in particular, MMP-2 and MMP-9 seem to play important roles in tumor invasion and metastasis. They are involved in the turnover of basement membrane collagen under basal conditions and of other matrix proteins during angiogenesis, tissue remodeling and repair. Thus, the impact of EGCG on MMP involved in extracellular matrix degradation was investigated. We demonstrated that HGF-induced expression of MMP-2 and MMP-9 in KB cells was reduced by EGCG treatment in a dose-dependent manner. Similar results were found in that EGCG is a potent inhibitor of the expression and activity of MMP-2 and MMP-9 in HT1080 cells [19,38,39] and oral cancer cell line OEC-M1 cells [40]. Thus, inhibition of the migration or invasion mediated by MMP-2 or MMP-9 may be a key feature in the prevention of cancer invasion or metastasis. The present results suggest that EGCG inhibits the migration and invasion of KB cells by inhibiting the activation of MMP-2 and MMP-9 enzymes.

The potentially multiple mechanisms by which EGCG can accomplish these numerous effects are currently unclear. EGCG is not a select protein inhibitor and in fact can block the enzymatic activity of many proteins, including DNA methyltransferase, AP-1, matrix MMPs and FGF-R [4,17,27]. Because deregulation of the MAPK and PI3K pathways is frequently seen in a variety of human cancers, modulation of MAPKs and PI3K by EGCG may provide novel strategies for the prevention or treatment of cancer. A recent study suggested that the inhibition of several growth factor receptors, including epidermal growth factor receptors, IGFR, AKT and ERK1/2, was significant because these kinases play key roles in cancer cell proliferation [26]. EGCG has also been suggested to have the ability to directly inhibit AKT and ERK1/2 activity [26]. Thus, EGCG may inhibit cell proliferation by direct interaction with kinases at multiple levels in signaling cascades. This is consistent with recent reports in other systems [26,41,42]. In our study, EGCG could repress the HGF-induced increase in Met phosphorylation and block activation of the downstream kinases AKT and ERK. Inhibition of p-AKT and p-ERK by EGCG was associated with marked increases in the phosphorylation of p38 and JNK, cleaved caspase-3 and PARP. Thus, we suggest that EGCG could have anticancer effects through the activation of the JNK pathway.

Several studies have shown the antitumor activity of EGCG in a xenograft model [43–47]. The mechanisms underlying the anticancer effects of EGCG seem to be complex. EGCG can bind to target molecules and trigger signaling cascades, many of which are interconnected. Here, we showed the effects of EGCG treatment *in vivo* in a syngeneic mouse model (C3H/SCC7) and in *in vitro* cell culture models involving the KB cell line.

These *in vivo* results support the *in vitro* findings that EGCG significantly inhibited HGF-induced tumor growth and invasion in oral cancer cell lines through suppression of the HGF/c-Met signaling pathway. Tissue caspase-3 and PCNA staining revealed a large amount of apoptosis in EGCG-treated tumors. We also observed that EGCG induced a strong reduction of *in vivo* c-Met phosphorylation and HGF expression by Western blotting.

Numerous reports have concluded that EGCG inhibits a variety of growth factor receptors and signaling proteins. Therefore, EGCG may also have important preclinical ramifications as a multimodality inhibitor in various cancers. To our knowledge, this is the first reported study to demonstrate direct, selective antiproliferative/proapoptotic effects of EGCG against HGF/Met-induced progression of oral cancer cells *in vitro* and *in vivo*. This raises the possibility that EGCG may have chemopreventive and even therapeutic potentials for human oral cancer. Based on these findings, we believe that EGCG, either alone or in combination with standard therapy, could be a novel strategy for the management of oral cancer, mediated via modulating HGF/Met signaling.

Acknowledgments

This study was supported by a faculty research grant from the Yonsei University College of Medicine (6-2007-0185) and CCRB through the GRRC project of the Gyeonggi Provincial Government, Republic of Korea. The language in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/IuY2ur.

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