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Epigallocatechin-3 Gallate Inhibits Invasion, Epithelial−Mesenchymal Transition, and Tumor Growth in Oral Cancer Cells

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ABSTRACT: Epithelial to mesenchymal transition (EMT) is critical for the progression, invasion, and metastasis of epithelial tumorgenesis. Here, we provided molecular evidence associated with the antimetastatic effect of green tea polyphenol epigallocatechin-3 gallate (EGCG) in an oral squamous cell culture system by showing a nearly complete inhibition on the invasion ($P < 0.001$) of squamous cell carcinoma-9 (SCC-9) cells via a reduced expression of matrix metalloproteinase-2 ($P < 0.001$) and urokinasetype plasminogen activator ($P < 0.001$). EGCG exerted an inhibitory effect on cell migration ($P < 0.001$), motility ($P < 0.001$), spread, and adhesion ($P < 0.001$). We performed Western blot to find that EGCG inhibited p-focal adhesion kinase (p-FAK), p-Src, snail-1, and vimentin, indicating the anti-EMT effect of EGCG in oral squamous cell carcinoma. EGCG was also sufficient to inhibit phorbol-12-myristate-13-acetate-induced cell invasion and matrix metalloproteinase-9 expression, as evidenced by its inhibition on the tumor growth of SCC-9 cells in vivo via cancer cell xenografted nude mice mode. These results suggested that EGCG could reduce the invasion and cell growth of tumor cells, and such a characteristic may be of great value in developing a potential cancer therapy.

KEYWORDS: EMT, EGCG, invasion, MMP, u-PA

INTRODUCTION

The tongue and the floor of the mouth are the most common sites of origin in the Western world. Nearly 50% of patients with oral squamous cell carcinoma (OSCC) present with pathologic or clinical evidence of lymph node metastasis and a 5-year survival rate of less than 50%.[1,2] Death as a result of oral cancer is often the result of local recurrence or regional or systemic metastasis. Thus, metastasis is a major problem in successful cancer treatment.

The metastasis of solid tumor is known to be the primary cause of human cancer death and is a complex multistep process involving a group of proteolytic enzymes, such as matrix metalloproteinases (MMPs), serine proteinases, and cathepsins, which participate in the degradation of environmental barriers such as basement membrane and extracellular matrix (ECM).[3,4] Among these enzymes, matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), which are a family of zinc-dependent endopeptidases, are deeply involved in the invasion and metastasis of various tumor cells.[5,6] Urokinase-plasminogen activator (u-PA), a serine protease, converts inactive plasminogen into plasmin and is found in cellular structures at the leading edge of migrating cells that are involved in migration and invasion of OSCC.[7]

Epithelial to mesenchymal transition (EMT), the process whereby epithelial cells transform into mesenchymal cells, which has been recognized for several decades as a critical feature of embryogenesis, has more recently been shown to be relevant for tumorgenesis. During EMT, cancer cells lose expression of proteins that promote cell−cell contact such as E-cadherin and acquire mesenchymal markers such as snail, slug, vimentin, fibronectin, and N-cadherin, which promote cell invasion and metastasis.[8]

Green tea is one of the most popular beverages in the world, and it has received considerable attention because of its many scientifically proven beneficial properties on human health. The beneficial effects of tea polyphenols in inhibition of cancer cell invasion and tumor growth have been reported in many studies.[9−11] A number of epidemiological studies have revealed the consumption of green tea inhibits growth of various tumor types.[12] The most abundant polyphenol in green tea is epigallocatechin-3-gallate (EGCG), which has been shown to inhibit proliferation and induce apoptosis of various cancer cells via activation of caspase cascades.[13] EGCG has been reported to be able to inhibit the invasion of human breast cancer MCF-7 cells

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via suppressing the activities of MMPs.[14] In previous studies, EGCG could inhibit the invasion of human OC2 oral cancer cells via decreased MMPs and u-PA expression[15] and reverse epithelial to mesenchymal transition in DMBA-treated NF-kappaB c-Rel-driven mammary tumor cells.[16] EGCG treatment of Her-2/neu-driven mammary tumor cells alters the expression of sereral regulators in the EMT pathway; the epithelial genes E-cadherin, MTA3, gamma-catenin, and estrogen receptor alpha were up-regulated by EGCG, whereas the proinvasive snail gene was down-regulated.[17] However, little is known about the precise molecular mechanisms of EGCG on metastasis of oral cancer. The processes of proliferation, metastasis, and angiogenesis are vital for cancer progression and could be targeting subjects for anticancer drug discovery. Therefore, the purpose of the present study was to characterize the inhibitory effects of EGCG on cell invasion and migration of SCC-9 cells, while the changes of cell physiology and precise molecular events involved in cell invasion and EMT and the effect on tumor growth in vivo were also investigated.

MATERIALS AND METHODS

Materials and Chemicals. EGCG, phorbol-12-myristate-13-acetate (PMA), 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's modified Eagle medium (DMEM), and F-12 Ham's were obtained from Sigma Chemical Co. (St. Louis, MO). Matrigel and BD Falcon cell culture inserts were purchased from BD Biosciences (Bedford, MA). Monoclonal antibodies against p-focal adhesion kinase (p-FAK; pY397) were purchased from BD Biosciences (Bedford, MA), and a rabbit polyclonal antibody against p-Src (Tyr 416) was purchased from Cell Signaling Technology (Dancers, MA). A rabbit polyclonal antibody against p-paxillin and mouse monoclonal antibodies against MMP-2 and u-PA were obtained from Millipore (Burlington, MA); gout polyclonal antibodies against RhoA and β-actin and rabbit polyclonal antibodies against vimentin and TIMP-2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The Immobilon Western Chemiluminescent HRP substrate kit was obtained from Millipore (Burlington, MA).

Cell Culture. SCC-9, a human tongue SCC, was cultured in Dulbecco's modified Eagle's medium supplemented with nutrient mixture F-12 Ham's (Life Technologies, Grand Island, NY), 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 400 ng/mL hydrocortisone.

Determination of Cell Viability (MTT Assay). Cells were incubated with 0.5 mg/mL MTT in culture medium for an additional 4 h; the blue formazan crystals of viable cells were dissolved by lysis buffer and measured spectrophotometrically at 570 nm.[18]

Transwell Cell Invasion and Motility Assays. SCC-9 cells were pretreated with EGCG at an indicated concentration (0, 5, 10, 15, and 20 μM) for 24 h. Otherwise, cells were pretreated with PP2 (Src inhibitor) for 30 min followed by incubated with or without 10 μM EGCG for an additional 24 h. Afterward, cells were harvested and seeded to BD Falcon cell culture inserts at 10^4 cells/well in serum-free medium, and then incubated for 24 h at 37 °C. For the invasion assay, 100 μL Matrigel (1 mg/1 mL) was applied to 12-μm-pore-size polycarbonate membrane filters, with the bottom chamber of the apparatus containing standard medium. Following incubation, the filters were then air-dried for 5 h in a laminar flow hood. The invaded cells were fixed with methanol and stained with Giemsa. Cell numbers were counted using a light microscope, while motility assay was carried out as described for the invasion assay, with no coating of Matrigel.[19]

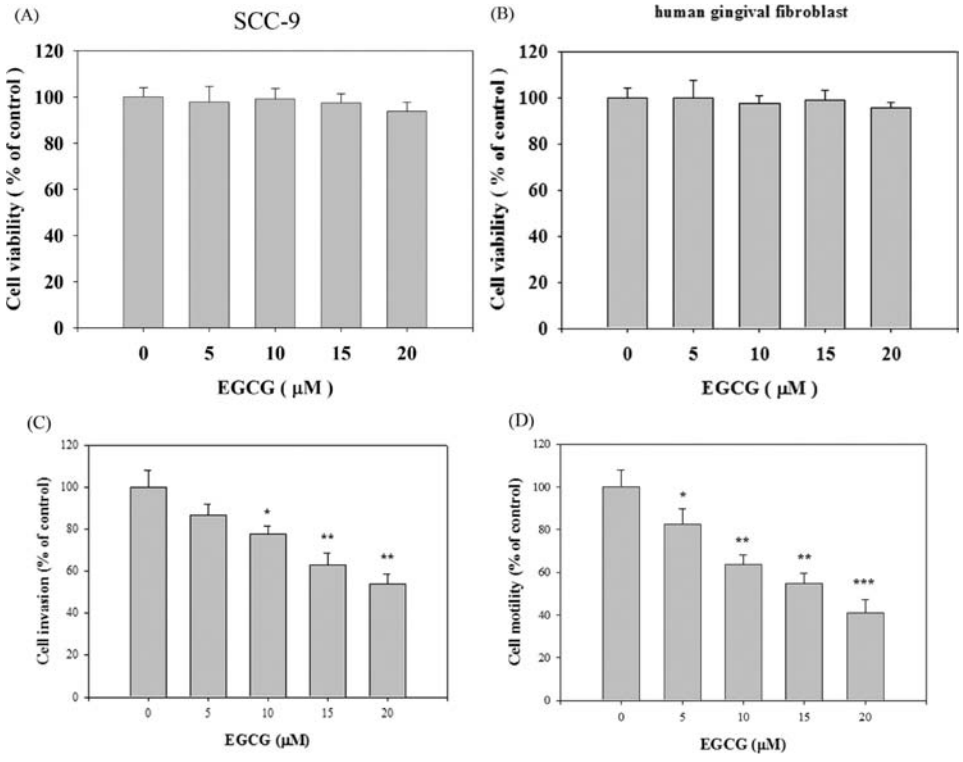

Figure 1. The effect of EGCG on cell viability, cell invasion, and cell motility. Oral squamous cell lines, SCC-9 cells (A), and normal human gingival cells (B), were treated with EGCG for 24 h, and then subjected to MTT assay for cell viability. SCC-9 cells were pretreated with EGCG at an indicated concentration for 24 h, and then equal numbers of cell were subjected to analyses for cell invasion (C) and cell motility (D) as described in Materials and Methods. The experiments were repeated twice, each in triplicate. Data represented the mean ± SD of at least three independent experiments. Statistical significance was determined by using Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Wound Healing Migration Assay. For cell migration assay, SCC-9 cells were seeded into a 12-well culture dish and grown in culture media containing 10% FBS to a nearly confluent cell monolayer (>95% confluence). Wounds were introduced to the confluent monolayer of cells with a sterile 200 μL plastic pipet tip to create a denuded area. Floating cells were removed by washing twice with PBS, replaced with culture media containing 1% FBS, and then EGCG was added. The cells were incubated at 37 °C, and cell movement into the wound area was photographed at 0 and 24 h using a microscope. Cell motility into a wound was expressed as percentage of cell migration.[20]

Cell−Matrix Adhesion Assay. After a 24 h treatment with EGCG, cells were plated on 24-well dishes coated with type I collagen (10 μg/mL) and cultured for 60 min. Nonadherent cells were removed by PBS washes, and adherent cells were fixed in ethanol. After being stained with 0.1% crystal violet, fixed cells were lysed in 0.2% Triton X-100, and the absorbance was measured at 550 nm.[20]

Cell Spreading Assay. After a 24 h treatment with EGCG, cells were plated in type I collagen (10 μg/mL) coated 24-well plates and cultured in culture media with or without EGCG for 2 h. Cell morphology was photographed for morphological studies.

Determination of MMPs and u-PA by Zymography. In gelatin zymography, collected media were subjected to 0.1% gelatin−8% SDS polyacrylamide gel electrophoresis (SDS-PAGE) to determine the MMPs. After electrophoresis, gels were washed with 2.5% Triton X-100 and then incubated in reaction buffer for 16 h at 37 °C. Gel was then stained with Coomassie brilliant blue R-250. Visualization of u-PA activity was performed by casein zymography. Briefly, 2% w/v casein and 20 μg/mL plasminogen were added to 8% SDS-PAGE gel, and then performed as described in the gelatin zymography.[19]

Reverse Transcription−Polymerase Chain Reaction (RT-PCR). For reverse transcription, 2 μg of total RNA was used as templates in a 20 μL reaction containing 4 μL of dNTPs (2.5 mM), 2.5 μL of oligo dT (10 pmol/μL), and 200U RTase. The appropriate primers 5′-GGCCCTGTCACTCCTGAGAT-3′ and 5′-GGCATCCAGGTTATCGGGGA-3′ for MMP-2 (473bp), 5′-TTGCGGCCATCTACAGGAG-3′ and 5′-ACTGGGGATCGTTATACATC-3′ for u-PA (351bp), 5′-GGCGTTTTGCAATGCAGATGTAG-3′ and 5′-CACAGGAGCCGTCACTTCTCTTG-3′ for TIMP-2 (496bp), and 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and 5′-AGCCTTCTCCATGGTTGGTGAAGAC-3′ for GAPDH (305bp) were used for PCR

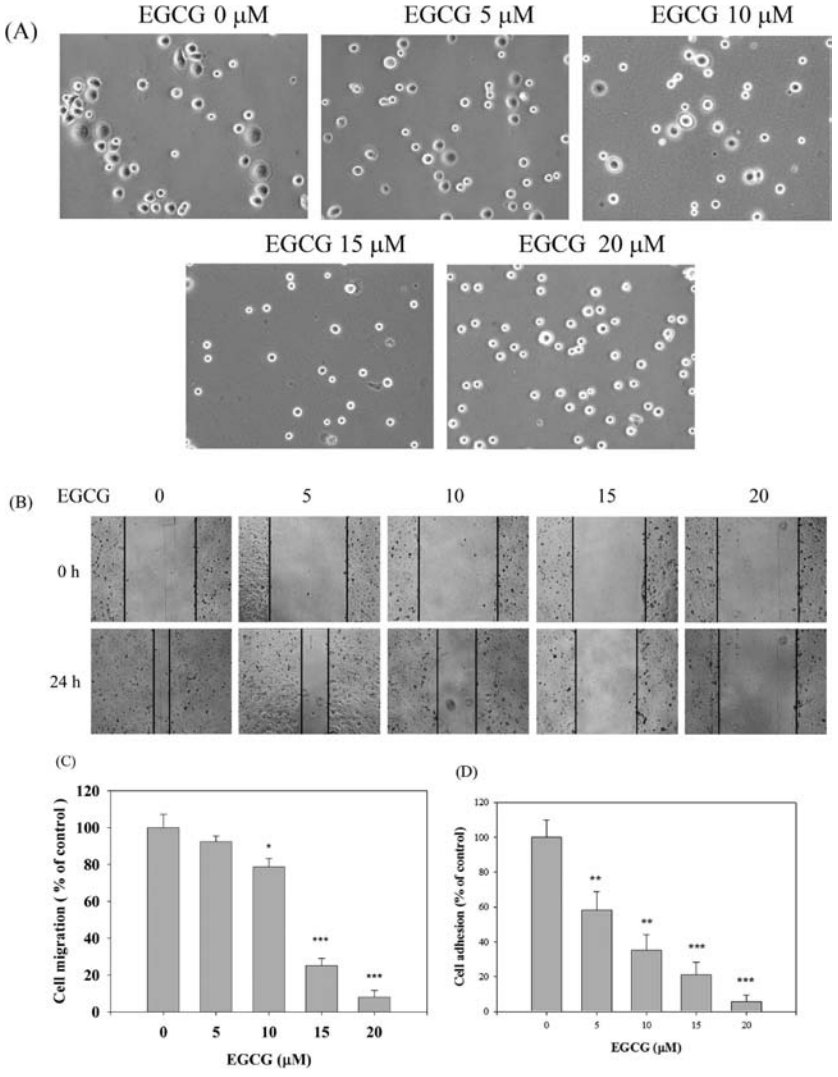

Figure 2. The effects of EGCG on cell spreading, cell migration, and cell adhesion. (A) SCC-9 cells were treated with EGCG for 24 h, and then subjected to analyses for cell spread. (B) Cells were subjected to analyses for cell migration by wound healing assay. (C) Determined migration ability of SCC-9 was subsequently quantified with that of control being 100% (without EGCG for 24 h). (D) Cells were treated with EGCG for 24 h, and then subjected to analyses for adhesion. Data represented the mean ± SD of at least three independent experiments. Statistical significance was determined by using Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

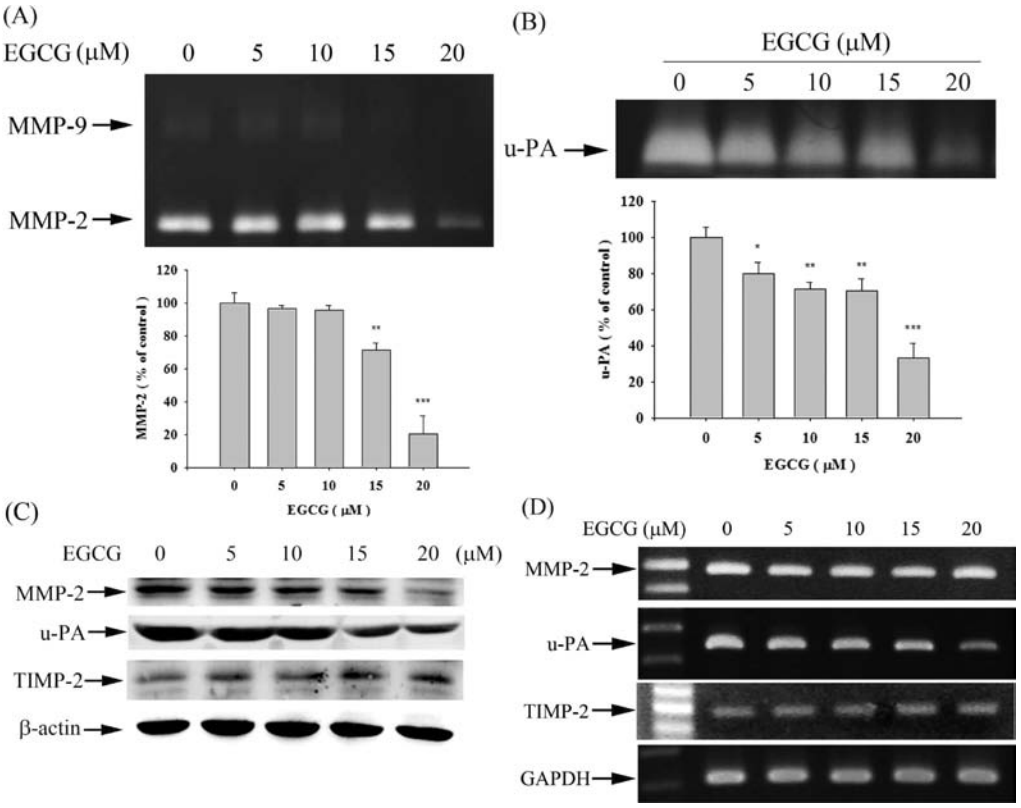

Figure 3. Inhibitory effects of EGCG on the protein and mRNA levels of proteases and their endogenous inhibitors. The condition media of treated cells were analyzed for the activities of MMP-2 (A) and u-PA (B) by gelatin zymography and casein zymography, and that total cell lysates were prepared and subjected to Western blot to analyze the expressions of MMP-2, u-PA, and TIMP-2 (C) as described in Materials and Methods. For mRNA levels (D), total RNAs were extracted and subjected to a semiquantitative RT-PCR for MMP-2, u-PA, and TIMP-2 with GAPDH being an internal control. Similar results were obtained from three repeated and independent experiments. The statistical significance of results was analyzed by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

amplifications. PCR was performed using Platinum Taq polymerase (Invitrogen) with the condition: 25 cycles of 94 °C for 1 min, 55 °C (u-PA) or 63 °C (MMP-2, TIMP-2, and GAPDH) for 1 min, 72 °C for 2 min, followed by 10 min at 72 °C.[5]

Immunofluorescence Staining. Cells were cultured on sterile glass coverslips in six-well plates. The slides were incubated overnight at 4 °C with antifocal adhesion kinase (FAK) antibody, followed by incubation with FITC-conjugated goat antimouse Ig at room temperature for 1 h. Slides were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) and analyzed by a microscopy.

Immunoblotting. Samples of cell lysates were separated in a 12.5% polyacrylamide gel and transferred onto a nitrocellulose membrane as previously described (Chu et al., 2004). The blot was subsequently operated with standard procedures and probed with primary and secondary antibodies. The protein expression was detected by chemiluminescence using an Immoblon Western Chemiluminescent HRP Substrate kit.[18]

Measurement of Tumor Growth in Nude Mice. For the nude mice xenograft model, 5−6 weeks old immuno-deficient nude mice (BALB/c *nu/nu* mice) weighing 18−22 g were used. The mice were housed with a regular 12 h light/12 h dark cycle and ad libitum access to standard rodent chow diet (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO) and were kept in a pathogen-free environment at the Laboratory Animal Unit. SCC-9 cells (1×10^7 cells/0.2 mL/mouse) were injected subcutaneously into the right front axilla. Eight days postimplantation, the mice were randomly divided into three groups ($N = 5$ for each group) and fed by oral gavage with saline (control) and EGCG (10 and 20 mg/day/kg) suspended in saline. The day of cell implantation was designated day 0. The average tumor volume at the start of treatment was approximately 211 mm^3, and tumors of the treated group and control group were measured daily using vernier calipers to measure the long and short dimensions after 8 days of cell injections. Body weight was assessed daily after cell injection. After 45 days, the animals were euthanized, and the primary tumors were isolated and weighed.[21]

Statistical Analysis. Statistical significances of difference throughout this study were calculated by Student's *t* test (SigmaStat 2.0, Jandel Scientific).

RESULTS

Effect of EGCG on Cell Viability. The cell growth inhibitory effects of EGCG on SCC-9 cells were determined. Results of MTT assay showed that a 24 h treatment of EGCG at various concentrations has no cytotoxicity on SCC-9 cells (Figure 1A). Results from the same procedures performed on a normal human gingival fibroblast revealed that this compound did not have any significant cytotoxicity on these cells (Figure 1B).

Inhibition on Cell Invasion and Cell Motility of SCC-9 Cells by EGCG. To screen for the preventive effectors against cancer metastasis, the inhibitory effect of EGCG on invasion and motility of SCC-9 cells was examined with Transwell. The result was shown that EGCG significantly reduced the invasion ($P < 0.001$) and motility ($P < 0.001$) of SCC-9 cells in a concentration-dependent manner (Figure 1C,D).

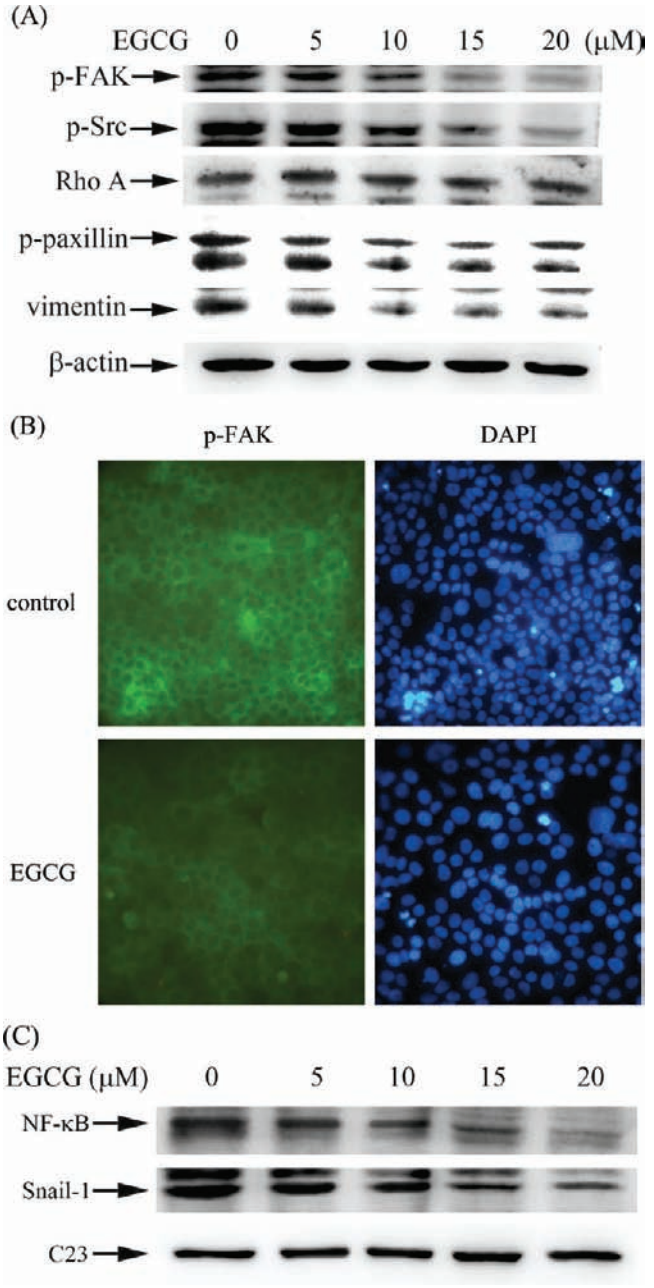

Figure 4. Inhibitory effects of EGCG on the phosphorylation of FAK and Src. (A) Western blot analysis of p-FAK, p-Src, Rho A, p-paxillin, and vimentin with β-actin being an internal control in SCC-9 cells after 24 h of treatment with EGCG. (B) Immunofluorescence analysis of p-FAK in SCC-9 cells with or without EGCG adding. (C) Nuclear extracts were subjected to SDS-PAGE followed by Western blotting with anti-NF-κB, antisnail-1 antibodies with anti-C23 being an internal control. Similar results were obtained from three repeated and independent experiments.

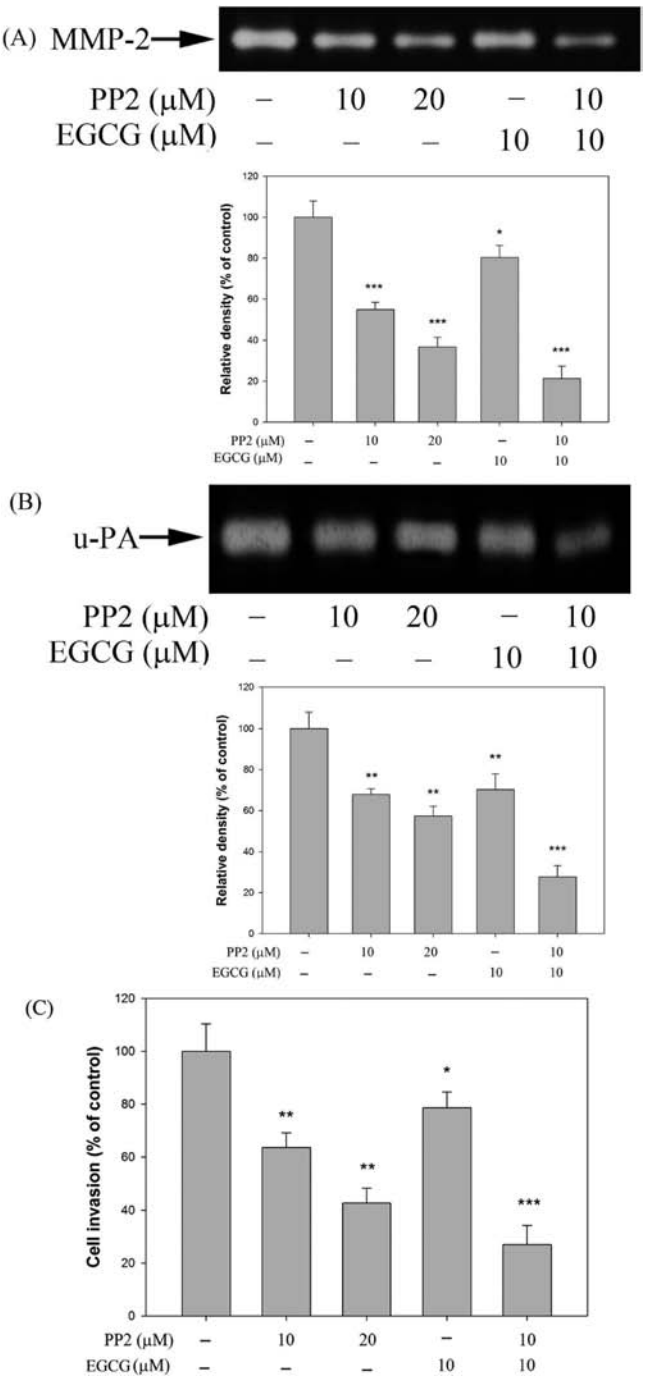

Figure 5. Effects of Src inhibitor (PP2) and EGCG on the activities of MMP-2 and u-PA and on cell invasion. Cells were pretreated with PP2 for 30 min and then incubated in the presence or absence of EGCG for 24 h. For zymography, condition media were subjected to gelatin zymography and casein zymography to analyze the activities of MMP-2 (A) and u-PA (B). For in vitro invasion assay, the cells with the indicated pretreatment were subjected to in vitro invasion assay as described in Materials and Methods (C). Data represented mean ± SD, with that of the control being 100%, and the statistical significant of results were analyzed by Student's *t* test. The experiments were repeated twice, each in triplicate (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Inhibition on Cell Spreading, Migration, and Adhesion of SCC-9 Cells by EGCG. The majority of control (without EGCG) cells exhibited spread morphology within 2 h of plating. By contrast, cells that were treated with EGCG (20 μM) remained round at the same time point (Figure 2A). Next, a wound healing migration assay was performed to assess whether EGCG affects cell migration. Incubation of SCC-9 with 1% FBS produced a marked cell migration in the wound area 24 h after wounding, whereas wounds treated with EGCG showed significant delays in

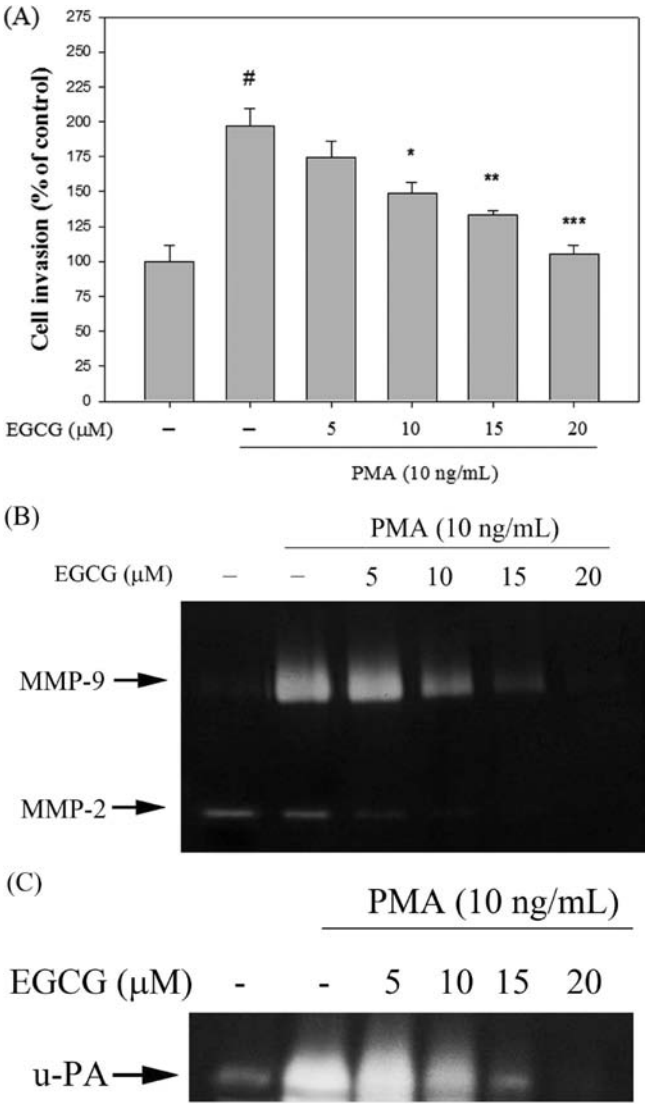

Figure 6. Concentration-dependent inhibitory effect of EGCG on PMA-induced cell invasion, MMP-9, and u-PA activity. Cells were pretreated with various concentration of EGCG for 1 h and then cultured in the presence of 10 ng/mL PMA for 24 h. (A) Cells were then subjected to analyses for invasion. Condition media were collected for analysis of (B) MMP-2 and MMP-9, (C) u-PA activities by gelatin zymography and casein zymography, respectively. The quantitative data were presented as means ± SD of three independent experiments (#, $P < 0.001$ as compared to control; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ as compared to PMA-treated group).

wound healing under the same conditions. The results showed that EGCG inhibited cell migration in a dose-dependent manner (Figure 2B,C). Cancer cells, invading the host tissue, break from their cell−cell contacts and make new contact with the ECM. Therefore, EGCG was tested to determine their effects on the cell−matrix adhesion. The results showed that EGCG significantly reduced the cell−matrix interactions of SCC-9 cells (Figure 2D).

Inhibitory Effect of EGCG on MMP and u-PA Activity. To clarify whether MMPs and u-PA were involved in inhibiting the invasion and migration of SCC-9 cells by EGCG, the effects of EGCG on MMP-2 and u-PA activities were investigated by gelatin and casein zymography under conditions of serum starvation, respectively. EGCG reduced the activities of MMP-2 ($p < 0.001$) in gelatin zymography (Figure 3A), and the activity of u-PA ($p < 0.001$) in casein zymography (Figure 3B). EGCG also reduced the levels of MMP-2 and u-PA in Western blot in a dose-dependent manner (Figure 3C).

Furthermore, semiquantitative RT-PCR analysis was performed to test if the regulatory effects of EGCG on proteases were on the mRNA levels. Using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control, the mRNA level of u-PA was significantly reduced in SCC-9 cells, while MMP-2 were not significantly affected (Figure 3D).

Effect of EGCG on the Expression of TIMP-2. The physiological activity of MMP-2 greatly related to that of TIMP-2, specific endogenous inhibitors. Western blot and RT-PCR were used to examine the effect of EGCG on the expression of TIMP-2. The results showed that the protein level (Figure 3C) and mRNA level (Figure 3D) of TIMP-2 did not change.

Inhibition of FAK and Src Phosphorylation by EGCG. EGCG significantly inhibited the activation of FAK and Src in SCC-9 cells in a dose-dependent manner, with a slight decrease on p-paxillin and vimentin, whereas it has no significant effect on RhoA expression (Figure 4A). Moreover, immunofluorescence staining confirmed that p-FAK expression was reduced in EGCG-treated cells (Figure 4B). In Western blotting, EGCG significantly inhibited the levels of NF-κB and snail-1 in nuclear extracts of SCC-9 cells in a dose-dependent manner (Figure 4C).

Inhibitory Effect of PP2 on SCC-9 Cell Invasion and Protease Secretion. We have shown that treatment of EGCG to SCC-9 cells inhibited the activities of MMP-2 and u-PA as well as cell invasion, while also inhibiting the phosphorylation of Src. To further delineate the involvement of Src pathway in SCC-9 cell invasive behavior, we investigated the effects of the specific inhibitor PP2, for Src phosphorylation, on SCC-9 cells. The results showed that sole treatment with PP2 led to inhibitions of MMP-2 ($p < 0.001$) (Figure 5A) and u-PA ($p < 0.001$) (Figure 5B) activity, in a way similar to that of EGCG. In addition, the combined treatment of inhibitor with EGCG could further decrease the MMP-2 or u-PA activity. Furthermore, a similar trend for inhibition of the invasion of SCC-9 cells by sole treatment ($p < 0.01$) and combination treatment ($p < 0.001$) was also observed (Figure 5C). Therefore, the inhibition of Src signaling pathways may result in reduced activities of MMP-2 and u-PA, as well as tumor cell invasion in SCC-9 cells.

EGCG Inhibits Phorbol-12-myristate-13-acetate (PMA)-Induced Invasion of SCC-9 Cells by Suppressing the MMP-9 and u-PA Expression. Quantitative analyses by a cell invasion assay showed that the invasion of SCC-9 cells was increased by∼1.9-fold upon the PMA (a tumor promoter) treatment and the PMA-induced invasion was reduced by EGCG treatment in a dose-dependent manner (Figure 6A). EGCG also reduced PMA-induced MMP-9 (Figure 6B) and u-PA (Figure 6C) activity of SCC-9 cells in a dose-dependent manner.

Antitumor Effects of EGCG in Vivo. To verify the in vivo antitumor effects of EGCG, SCC-9-bearing nude mice were treated with saline or EGCG. Small solid tumors were observed 8 days after the cell inoculation, and a 3.4- and 5.8-fold reduction in EGCG-treated (10 and 20 mg) animals was seen on day 44, respectively, as compared to control animals (Figure 7A,B). Moreover, by day 45, EGCG feeding induced a 5.7-fold reduction in tumor weight (Figure 7C) without any apparent signs of toxicity as evidenced by body weight monitoring (Figure 7D) throughout the experiment.

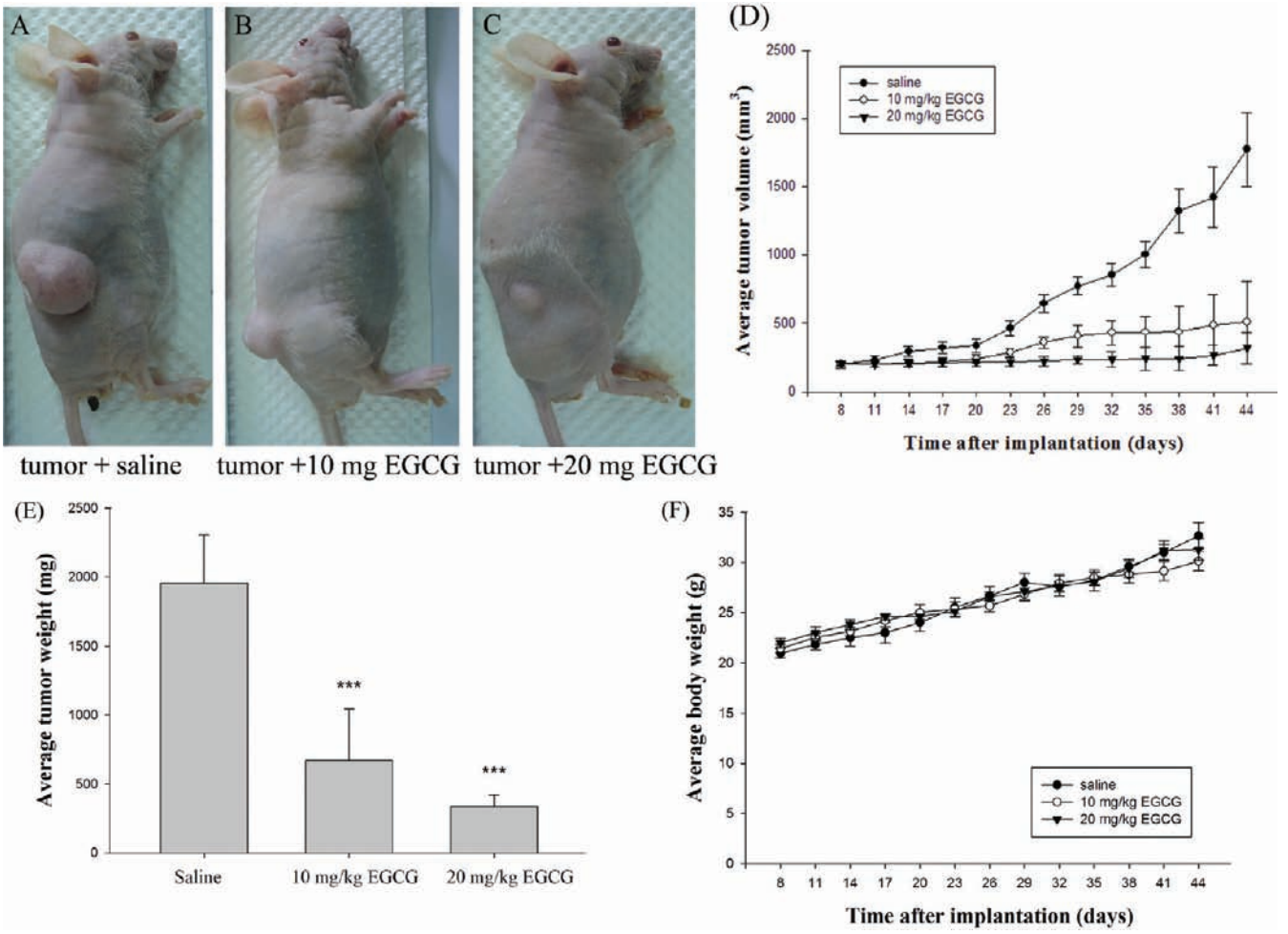

Figure 7. The in vivo antitumor effects of EGCG. After subcutaneous implantation of SCC-9 cells, BALB/c *nu/nu* mice were treated with saline or EGCG and then analyzed for the growth of tumor. (A) Control (saline), (B) 10 mg/kg/day EGCG, (C) 20 mg/kg/day EGCG. (D) Average tumor volume, (E) tumor weight, and (F) body weight. The values represented the means ± SD. Comparisons were performed by Student's *t* test (***, $P < 0.001$).

DISCUSSION

Epidemiological studies have shown that the ingestion of green tea may decrease the risk of cancer. For example, consumption of green tea was closely associated with reduced numbers of axillary lymph node metastases among breast cancer patients.[22] The cancer chemopreventive property of green tea has mainly been attributed to the most prevalent polyphenol, EGCG, which has potent antioxidant properties. Here, we demonstrated that EGCG could significantly inhibit the invasion, motility, migration, and secretion of MMP-2 and u-PA of SCC-9 oral cancer cells via attenuation of p-FAK and p-Src. EGCG also inhibited cell spreading, cell−matrix adhesion, and PMA-induced cell invasion and proteases activities.

The invasion of cancer cells is a complicated process involving a group of proteolytic enzymes, which participate in the degradation of environmental barriers such as ECM and basement membrane. Among these enzymes, MMPs and u-PA are deeply involved in the invasion and metastasis of various tumor cells.[23] The expressions of MMP or u-PA gene in various tumor cells were known to regulate at a transcriptional, posttranscriptional, or protein level. Therefore, MMP or u-PA protein expressions and their regulatory pathways were considered as promising targets for anticancer drugs and chemopreventive agents.[24] Here, it was demonstrated that EGCG markedly decreased the expression levels of MMP-2 and u-PA and PMA-induced MMP-9 and u-PA activity on SCC-9. To further evaluate if the significant regulatory effects of EGCG on MMP-2 and u-PA are on the mRNA level, a RT-PCR analysis was performed. After treatment with the EGCG, the mRNA level of u-PA was significantly reduced in a dose-dependent manner, while that of MMP-2 remained unchanged (Figure 3D). The results showed that EGCG treatment might regulate the expression of u-PA, at least partly, on the transcriptional level. Otherwise, EGCG treatment significantly inhibited MMP-2 protein expression of SCC-9 cells without affecting mRNA expression, and it was suggested that it may through translational or post-translational level regulation, such as reducing protein biosynthesis, decreasing protein stability, or increasing protein degradation.[25] However, the regulation of EGCG on MMPs expression in SCC-9 cells is needed to further define in more detail. The 5′ flanking region of the MMP-9 gene contains several functional regulatory motifs that can bind with several well-characterized transcription factors, including NF-κB, activator protein-1 (AP-1), stimulatory protein-1, or polyoma virus enhancer activator-3.[26] Through one or more of these binding sites on the specific response elements on the

promoter region of target genes, the expression of MMP-9 is regulated by various physical stimulators or chemical, including cytokines (e.g., tumor necrosis factor-a), growth factors (e.g., epidermal growth factor), oncogenes (e.g., Ras), or PMA.[27] Among these stimulators, PMA can act as a tumor promoter to induce MMP-9 expression in several cancer cells, including lung carcinoma, hepatocellular carcinoma, and oral squamous cell carcinoma. In the present study, EGCG was sufficient to inhibit PMA-induced cell invasion and MMP-9 expression in SCC-9 cells.

EMT has been observed to play a main role in invasion and metastasis of epithelial tumors. This process is mainly coordinated by the disappearance of E-cadherin with the concomitant appearance of vimentin and secretion of MMPs. In the present study, EGCG treatment showed the suppression of vimentin expression. FAK is a nonreceptor tyrosine kinase, which participates in many important cellular processes such as cell adhesion and migration. FAK is one of the Src's major binding partners, and the Src signaling pathway is an important component of adhesion changes associated with the EMT in head and nick squamous carcinoma.[28] The diversity of responses to FAK/Src on cell−cell adhesion has been well documented in many cell types. It is reported that FAK may regulate cell migration and invasion through distinct pathways by promoting the dynamic regulation of focal adhesion and peripheral actin structures, as well as the MMPs-mediated ECM degration.[29] Activation of FAK has been shown to play a crucial role in tumor morphogenesis, invasion, and metastasis. We demonstrated here that phosphorylation of FAK and Src was reduced in a dose-dependent manner by an EGCG treatment.

In conclusion, these findings suggested that the inhibition on invasion by EGCG may be through a down-regulation of MMPs and u-PA expression of oral cancer cells, as well as EGCG as evidenced by its inhibition on the growth of SCC-9 xenograft nude mice. These results suggest that EGCG may be useful as an effector for the prevention of cancer metastasis, in addition to supporting the role of green tea as an oral cancer chemopreventive agent.

■ ABBREVIATIONS USED

EMT, epithelial−mesenchymal transition; PMA, phorbol-12-myristate-13-acetate; FAK, focal adhesion kinase; ECM, extracellular matrix; EGCG, epigallocatechin-3-gallate; MMP, matrix metalloproteinase; u-PA, urokinase-type plasminogen activator; TIMP-2, tissue inhibitor of matrix metalloproteinase-2; AP-1, activating protein-1; NF-κB, nuclear factor-κB.

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