EGCG inhibits recepteur d'origine nantais expression by suppressing Egr-1 in gastric cancer cells

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Abstract. Abnormal accumulation and activation of the recepteur d'origine nantais (RON) has been implicated in epithelial tumor carcinogenesis. In the present study, we examined the effect of epigallocatechin-3-gallate (EGCG), the major green tea catechin, on the induction of RON and tumor growth in human gastric cancer. EGCG inhibited phorbol 12-myristate 13-acetate (PMA)-induced RON expression and reduced RON transcriptional activity. However, (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin (EC) did not affect RON expression. Experiments with deleted and site-directed mutagenesis of the RON promoter indicated that Egr-1 binding sites in the RON promoter may be the EGCG-response element acting as a *cis*-element in gastric cancer cells. EGCG also inhibited PMA-induced Egr-1 expression and DNA binding in a dose-dependent manner. Furthermore, gastric cancer cells pretreated with PMA showed markedly enhanced invasiveness, which was partially abrogated by EGCG and siRNA-targeted RON and Egr-1. EGCG significantly reduced tumor growth in an *in vivo* tumor model, whereas RON expression was downregulated. These results suggest that EGCG may exert at least part of its anticancer effect by controlling RON expression through suppression of Egr-1 activation.

Introduction

Epidemiological and preclinical studies have reported that green tea consumption reduces the risk of cancer development (1,2). The anticarcinogenic effects of green tea have been attributed to the biological activities of its polyphenol components. Green tea extract contains (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin (EC) (3). EGCG, the most abundant polyphenol in green tea, inhibits cell proliferation and induces apoptosis in tumor cells (4). In addition to its cancer chemopreventive activity, EGCG inhibits tumor invasion, which is a crucial step in the metastasis of all solid tumors. In a previous study, we demonstrated that treating mice with EGCG resulted in marked inhibition of vascularity and proliferation of human colon cancer xenografts in nude mice (5); however, the mechanisms involved have yet to be fully elucidated.

Although the incidence rates of gastric cancer have declined in several industrialized countries over the past few decades, gastric cancer remains the most common cancer of the digestive tract, with a poor prognosis and high mortality rate (6). Due to local tissue invasion and metastasis, radiation therapy and chemotherapy do not significantly affect the survival or the quality of life of patients with advanced gastric cancer. Thus, developing effective therapeutic strategies against gastric cancer could help improve treatment strategies.

Recepteur d'origine nantais (RON), a member of the c-MET family of scatter factor receptors, plays an important role in the occurrence, progression and metastasis of gastric cancer (7). RON is activated through ligand-dependent or -independent mechanisms, which lead to responses associated with tumor development and metastasis (8). Macrophage-stimulating protein is the only RON ligand that has been identified thus far (9). Upon ligand binding, RON dimerizes, autophosphorylates and transduces a variety of signals that regulate different downstream pathways, including Ras/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, c-Jun N-terminal kinase, β-catenin and nuclear factor-κB (10,11). Several human tumor tissues exhibit aberrant expression and activation of RON, including tumors of the breast, colon and prostate gland (12). It has been suggested that multiple regulatory elements are required for full RON promoter activity and gene expression (13). Since RON plays a central role in multiple processes involved in cancer progression and metastasis, it is an attractive target for molecular-based cancer therapy.

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In this study, we discovered that EGCG suppressed RON expression in cancer cells and inhibited tumor growth *in vivo*. We verified the EGCG-response elements in the RON promoter in order to investigate the mechanism behind EGCG-mediated regulation of RON.

Materials and methods

Cell culture and culture conditions. AGS human gastric cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and MKN28 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The TMK-1 human gastric cancer cell line was provided by Dr Eiichi Tahara (Hiroshima University, Hiroshima, Japan). The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37˚C in an atmosphere containing 5% CO_2. AGS cells pretreated with 30 μM EGCG for 1 h were exposed to 200 nM phorbol 12-myristate 13-acetate (PMA) for 8 h and the levels of RON were analyzed by western blot analysis to determine the effect of EGCG on the tumor promoter.

Western blot analysis. Cells were suspended in ice-cold RIPA-M buffer with 1% NP-40 and cell lysates were prepared as previously described (7). Cell lysate proteins (100 μg) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The blots were blocked for at least 1 h at room temperature in blocking buffer (5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20; TBST). Anti-RONβ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted in blocking buffer and incubated with the blots overnight at 4˚C. The bound antibodies were detected with a 1:3,000 dilution of horseradish peroxidase-conjugated secondary antibody, according to the instructions of the enhanced chemiluminescence kit (Amersham, Franklin Lakes, NJ, USA).

Construction of the RON promoter-reporter construct. A construct of the RON promoter fragment, ~3 kb in length, was synthesized from human genomic DNA (Promega, Madison, WI, USA) by polymerase chain reaction (PCR) using the primers 5'-GGTACCTAGCTGACC-3' (forward) and 5'-GGGCCAAATTTAAGC-3' (reverse). The amplified PCR products were ligated into the T&A Vector (RBC Bioscience, Saskatoon, SK, Canada), then digested with *Kpn*I and *Bgl*II. The products were ligated into the *Kpn*I and *Bgl*II sites of the pGL3-Basic Vector (Promega). A series of deletion constructs of the human RON promoter fragments was synthesized by PCR using the pRON-Luc plasmid as the template. The forward primer sequences were: 5'-CCAAGGGCCGGAAGA-3' (-128/+173, pGL3-RON-301), 5'-TCGGCTGAGCGCTAA-3' (-20/+173, pGL3-RON-193) and 5'-TCGTGCGTCCGCAGG-3' (+50/+173, pGL3-RON-123). One reverse primer, 5'-GGGCCA AATTTAAGC-3', was used to generate all three deletion constructs. The amplified PCR products were ligated into the T&A Vector and then digested with *Kpn*I and *Bgl*II. Subsequently, the products were ligated into the *Kpn*I and *Bgl*II sites of the pGL3-Basic Vector. Site-directed mutagenesis was utilized to mutate potential transcriptional Egr-1 elements in the promoter region. Mutant promoter constructs were generated using the pGL3-RON-301 construct as a template. The primers used for mutagenesis (mutations underlined) were: TCCGCCCCGCC to TCCATATGCC (pGL3-Mt1) and CCCGCCCCCA to CCCAATTCCA (pGL3-Mt2). The mutated nucleotide sequences of all mutant constructs were confirmed by DNA sequencing.

RON promoter-reporter assay. Transcriptional regulation of RON was examined by transient transfection of a RON promoter-luciferase reporter construct (pGL3-RON). Gastric cancer cells ($5x10^5$) were seeded and grown until they reached 60-70% confluence and pGL3-RON wild-type and deletion mutants were transfected into the cells using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The significance of the Egr-1 binding site as an EGCG-response element in the regulation of RON was examined by co-transfecting the cells with pGL3-RON and Egr-1 expression plasmids containing full-length complementary DNA (cDNA) coding for human Egr-1 (a gift from Dr Young Han Lee, Konkuk University, Seoul, Korea). The pRL-null plasmid encoding *Renilla* luciferase was included in all the samples to monitor transfection efficiency. At 24 h post-transfection, the levels of firefly and *Renilla* luciferase activity were measured sequentially from a single sample using the Dual-Glo Luciferase Assay system (Promega). Firefly luciferase activity was normalized to *Renilla* activity and the relative amount of luciferase activity in the untreated cells was designated as 1.

Reverse transcription-PCR. Total RNA was extracted from AGS cells using TRIzol reagent (Invitrogen). One microgram of total RNA was used for first-strand cDNA synthesis using random primers and superscript reverse transcriptase (Invitrogen). The cDNA was subjected to PCR amplification with the *Egr-1* and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer sets. The specific primers sequences were: *Egr-1*, sense, 5'-CAGTGGCCTAGTGAGCATGA-3' and antisense, 5'-CCGCAAGTGGATCTTGGTAT-3' (786 bp); GAPDH, sense, 5'-TTGTTGCCATCAATGACCCC-3' and antisense, 5'-TGACAAAGTGGTCGTTGAGG-3' (836 bp). The PCR conditions were: denaturation at 94˚C for 20 sec, annealing at 53˚C for 20 sec and extension at 72˚C for 50 sec.

Chromatin immunoprecipitation assay. AGS cells ($2x10^6$), grown in 6-well plates, were cross-linked with 0.5% (v/v) formaldehyde at 37˚C for 5 min. The cells were sonicated for 3x20 sec prior to centrifugation at 16,000 x g for 15 min at 4˚C. The specific DNA-bound transcription factor complexes were precipitated with 20 μl anti-Egr-1 at 4˚C overnight prior to the addition of Protein A agarose beads. The proteins were removed from the DNA by digestion with 10 μg/ml Proteinase K at 65˚C for 30 min. The DNA was recovered from the solution using the QIAquick PCR Purification kit (Qiagen Inc., Valencia, CA, USA) and eluted in 50 μl sterile water. Eluted DNA (20 μl) was subjected to PCR with forward, -5'-AGGAGCCAGGCCTCCAAGGGC-3' and reverse, -5'-TCCCGACAGCCCCAAGATAGC-3' primers, which flank the Egr-1 binding sites.

Small interfering RNA transfection. Gene silencing was performed using human Egr-1 (sc-29303; Santa Cruz Biotechnology) and human RON sequence-specific duplex small interfering RNA (siRNA) (sc-36434). Briefly, 20 nM of siRNA oligonucleotides and 2 μl of Lipofectamine RNAiMAX (Invitrogen) were mixed with 100 μl of Opti-MEM serum-free medium (Hyclone, Logan, UT, USA) for each transfection reaction in two separate tubes and incubated for 5 min at room temperature. Subsequently, the contents of the two tubes were combined and allowed to form siRNA-Lipofectamine complexes for 30 min at room temperature. A 900 μl volume of AGS cells cultured in serum-free medium was combined with the siRNA-Lipofectamine mix, plated in the wells of a 6-well tissue culture dish and placed in a 37˚C, 5% CO_2 incubator for 5 h. The medium was replaced with normal growth medium.

Matrigel invasion assay. The cell invasion assay was carried out using BioCoat Matrigel Invasion Chambers (Becton-Dickinson, Bedford, MA, USA) with 10% FBS as the chemoattractant in the lower chamber. AGS cells (10^5) in 300 μl were allowed to invade the Matrigel for 24 h. The non-invading cells on the upper surface of each membrane were removed from the chamber and the invading cells on the lower surface of each membrane were stained with the Diff-Quick Stain kit (Becton-Dickinson). Following two washes with water, the chambers were allowed to air-dry. The number of invading cells was counted using a phase-contrast microscope.

Animal model. Eight-week-old male athymic nude mice (BALB/c*nu*/*nu*; Charles River, Sulzfeld, Germany) were used for the experiments, as approved by the Institutional Animal Care and Use Committee of the University of Regensburg and the regional authorities. Experiments were conducted according to the Guidelines for the Welfare of Animals in Experimental Neoplasia by the United Kingdom Coordinating Committee on Cancer Research. The effects of EGCG inhibition on the growth of TMK-1 human gastric cancer cells were investigated in a subcutaneous xenograft tumor model. TMK-1 cells ($1x10^6$) were injected subcutaneously into the right flank of nude mice. Mice were randomized (n=8 per group) and assigned to treatment groups. Intraperitoneal injections of EGCG (1 /mg/mouse, twice/week) were initiated on Day 1. The control group (n=8) was treated with the same dosage of EC. Tumor diameters were measured every other day and tumor volumes were calculated (width^2 x length x 0.5). The experiment was terminated on Day 27 following tumor cell injection, and the tumors were then excised, weighed and prepared for western blot analyses.

Results

Effect of EGCG on PMA-induced RON expression in gastric cancer cells. To examine whether green tea catechins inhibit PMA-induced RON expression in gastric cancer cells, AGS human gastric carcinoma cells were pretreated with 30 μM catechins for 1 h prior to an 8-h incubation with 200 nM of PMA, and the RON protein levels were measured by western blot analysis. EGCG at 30 μM inhibited PMA-induced RON protein expression. However, other tea catechins such as EC, ECG and EGC inhibited RON expression only slightly at the same concentrations (Fig. 1A). We then examined the effect

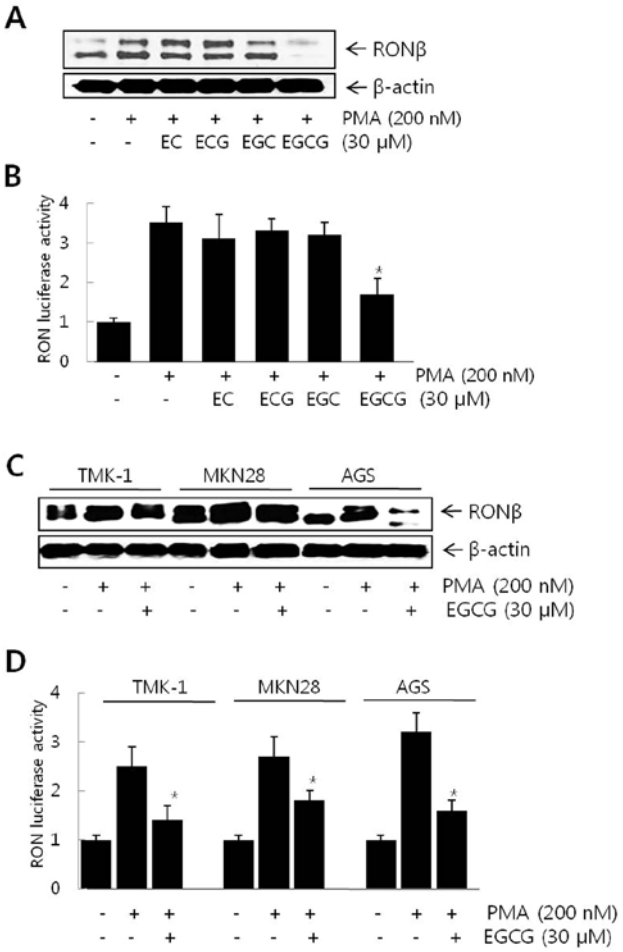

Figure 1. Effect of EGCG on PMA-induced RON expression in gastric cancer cells. (A) AGS cells were exposed to 200 nM of PMA for 8 h and the levels of RON were determined by western blot analysis following pretreatment with 30 μM of EC, ECG, EGC and EGCG for 1 h. (B) AGS cells were transiently transfected with the pGL3-RON reporter (pRON-Luc, -128/+173) construct. Following pretreatment with 30 μM of EC, ECG, EGC and EGCG for 1 h, the transfected cells were exposed to 200 nM of PMA for 8 h and luciferase activity was determined using a luminometer. (C) Following pretreatment with 30 μM of EGCG for 1 h, TMK-1, MKN28 and AGS cells were exposed to 200 nM of PMA for 8 h and the levels of RON were determined by western blot analysis. (D) TMK-1, MKN28 and AGS cells were transiently transfected with the pGL3-RON reporter (pRON-Luc, -128/+173) construct. Following pretreatment with 30 μM of EGCG for 1 h, the transfected cells were exposed to 200 nM of PMA for 8 h and luciferase activity was determined using a luminometer. Data are the means ± standard deviation from triplicate measurements. *P<0.05 vs. PMA.

of EGCG on transcriptional regulation of the RON gene induced by PMA. AGS cells were transiently transfected with the promoter-reporter construct (pGL3-RON) of the human RON gene fused to the luciferase gene. AGS cells transfected with pGL3-RON exhibited an ~3.5-fold increase in promoter activity following PMA treatment (Fig. 1B). When the transfected cells were pretreated with 30 μM catechins prior to PMA treatment, only EGCG significantly inhibited PMA-induced RON promoter activity (Fig. 1B). Catechins at the same concentrations did not affect cell viability (data not shown). TMK-1, MKN28 and AGS gastric cells were used to explore whether EGCG was able to inhibit RON expression in various gastric cancer cells. As shown in Fig. 1C and D, EGCG inhibited PMA-induced RON expression and promoter

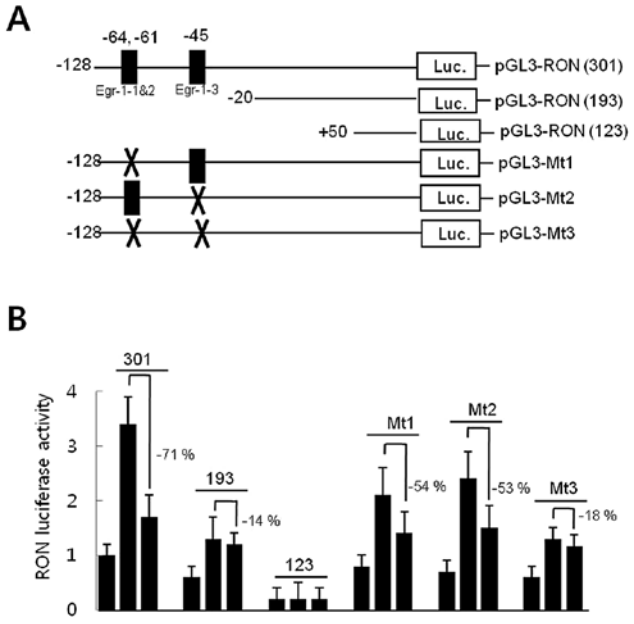

Figure 2. EGCG-response elements in the RON promoter in gastric cancer cells. (A) Schematic representation of Egr-1 binding sites and deleted and mutated regions in the human RON promoter. (B) AGS cells were transiently transfected with a series of 5'-deletion constructs of the RON promoter-reporter plasmid or site-specific mutants for Egr-1 (Mt1, Mt2 and Mt3) derived from the -128/+173 construct. Following pretreatment with 30 μM of EGCG for 1 h, the transfected cells were exposed to 200 nM of PMA for 8 h and luciferase activity was determined using a luminometer. Data are the means ± standard deviation from triplicate measurements.

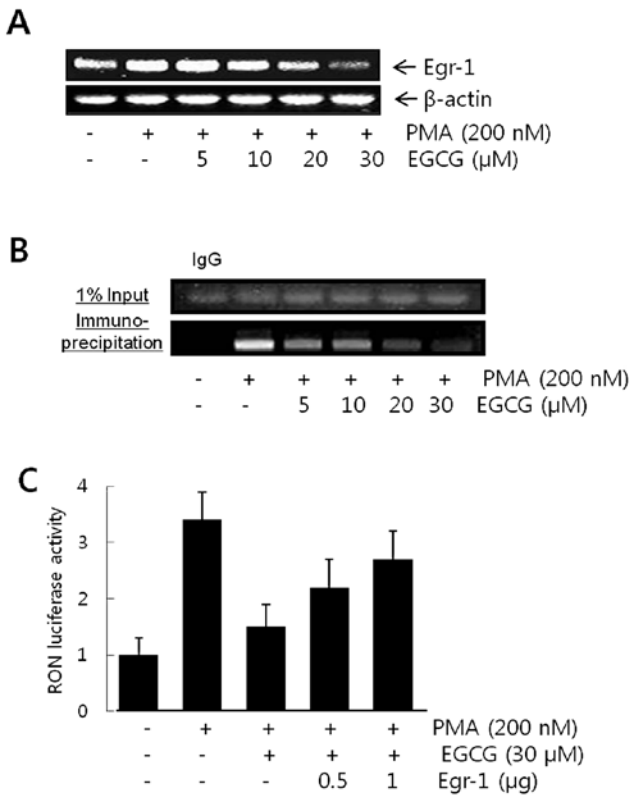

Figure 3. The role of Egr-1 in the inhibition of RON by EGCG in gastric cancer cells. (A) Following pretreatment with 30 μM of EGCG for 1 h, AGS cells were exposed to 200 nM of PMA for 8 h and Egr-1 mRNA levels were determined by RT-PCR. (B) A chromatin immunoprecipitation assay was performed with anti-Egr-1 antibodies at the RON promoter in cells treated with EGCG. Following pretreatment with 0-30 μM of EGCG for 1 h, the AGS cells were exposed to 200 nM of PMA for 8 h and were cross-linked, lysed and immunoprecipitated with anti-Egr-1 along with normal rabbit IgG (negative control). The precipitated DNA was subjected to regular PCR with primers specific for Egr-1 (-407/-112). (C) AGS cells were transiently cotransfected with the pGL3-RON reporter (pRON-Luc, -128/+173) construct and different concentrations of Egr-1 expression plasmids. Following pretreatment with 30 μM of EGCG for 1 h, the transfected cells were exposed to 200 nM of PMA for 8 h and luciferase activity was determined using a luminometer. Data are the means ± standard deviation from triplicate measurements.

activity in TMK-1, MKN28 and AGS cells. Collectively, these results demonstrate that EGCG suppressed the expression of the RON gene and reduced its promoter activity in human gastric cancer cells.

EGCG-response elements in the gastric cancer cell RON promoter. Promoter deletion analyses were performed to locate *cis*-response elements in the RON promoter in response to EGCG, in order to explore the underlying molecular mechanisms by which EGCG reduced RON gene promoter activity in AGS gastric cancer cells. AGS cells were transfected with pGL3-RON promoter-reporter constructs of different lengths (Fig. 2A). AGS cells were pretreated with 30 μM of EGCG for 1 h prior to an 8-h incubation with 200 nM of PMA, and luciferase activity was measured. As shown in Fig. 2B, EGCG significantly reduced luciferase activity by 71% in cells transfected with pGL3-RON (301). However, cells transfected with pGL3-RON (193) resulted in a decrease of 14% in response to EGCG, suggesting that the -128 to -20 DNA fragment in the RON promoter might contain the EGCG-response element(s). A computer-aided search revealed three consensus Egr-1 binding sites in the -128 to -20 DNA fragment of the RON promoter. Site-directed mutagenesis of the Egr-1 binding site was generated in the pGL3-RON plasmids (301) to examine the role of the Egr-1 binding site in the inhibition of RON promoter activity by EGCG (Fig. 2A). A construct containing a mutation in the core sequence of the Egr-1-1 and -2-binding motif (CCCGCCCCCA to CCCAATTCCA, pGL3-Mt1) and the Egr-1-3-binding motif (TCCGCCCGCC to TCCATATGCC, pGL3-Mt2) reduced PMA responsiveness to ~54 and 53%, respectively, of that of the wild-type construct. However, a double-mutant promoter, containing mutations in Egr-1-1 and -2, as well as in Egr-1-3 (pGL3-Mt3), resulted in an 18% decrease in the response to EGCG, indicating that the presence of the wild-type Egr-1 binding sites was required for EGCG to reduce RON promoter activity (Fig. 2B). Collectively, these results suggest that the Egr-1 binding sites in the RON promoter might be the EGCG-response element acting as a *cis*-element to regulate promoter activity.

Role of Egr-1 in the inhibition of RON by EGCG in gastric cancer cells. Egr-1 mRNA level was determined by RT-PCR to verify the inhibitory mechanisms of EGCG on Egr-1 activity in AGS gastric cancer cells. As shown in Fig. 3A, EGCG dose-dependently reduced PMA-induced Egr-1 expression at the transcriptional level. A chromatin immunoprecipitation assay was performed to further determine whether EGCG inhibits Egr-1 binding to the putative Egr-1-binding sequence in the RON promoter. AGS cell chromatin was immunoprecipitated

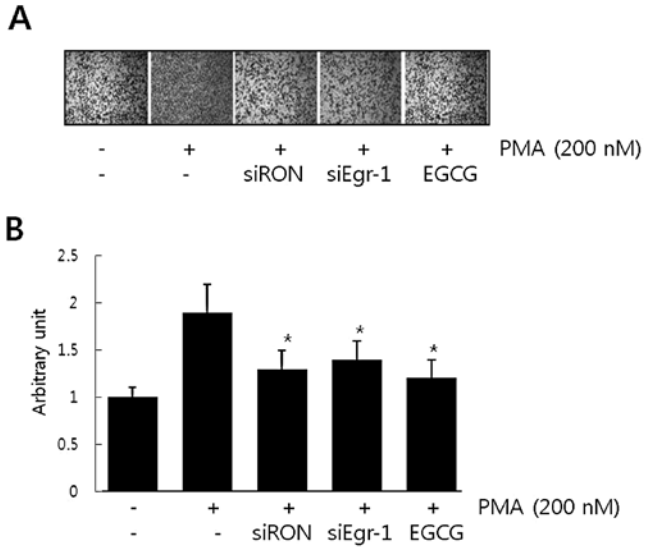

Figure 4. Effect of siRON, siEgr-1 and EGCG on PMA-induced gastric cancer cell invasion. AGS cells (10^5), transfected with RON siRNA and Egr-1 siRNA or pretreated with 30 μM of EGCG, were incubated with 200 nM of PMA in a Boyden Matrigel apparatus for 24 h. (A) Following incubation, the cells that invaded the undersurface of the chambers were counted using a phase-contrast light microscope after staining with a Diff-Quick Stain kit. (B) Data are the means ± standard deviation from triplicate measurements. *P<0.05 vs. PMA.

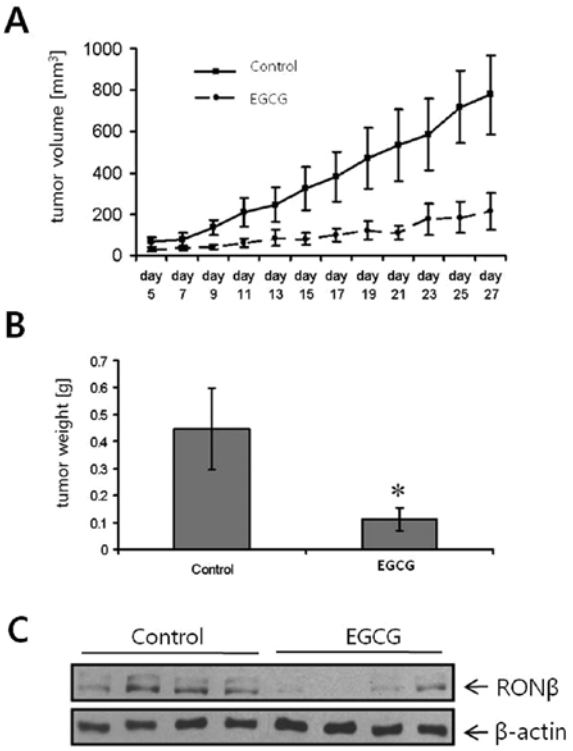

Figure 5. Effects of EGCG on tumor growth and RON expression *in vivo*. The effects of EGCG (1 mg/day/mouse, intraperitoneally) and EC (control) on (A) tumor growth and (B) tumor weight at 27 days following tumor-cell implantation were investigated in a subcutaneous xenograft model (n=8/group). Bars indicate standard error.*P<0.05 (8 mice/group). (C) RON levels in the gastric tumor tissues were determined by western blot analysis.

with rabbit anti-Egr-1 antibody and the resulting immunoprecipitates were analyzed by PCR using primers flanking the Egr-1-binding sequences (-407 to -112) of the RON promoter. An evident increase in DNA band intensity was observed in cells treated with PMA and anti-Egr-1 antibody, but not when normal rabbit IgG was used (Fig. 3B). When the cells were pretreated with 0-30 μM of EGCG prior to PMA treatment, the induction of Egr-1-DNA binding by PMA was inhibited in a dose-dependent manner (Fig. 3B). To explore the role of Egr-1 in regulating the RON promoter activity, AGS cells were co-transfected with the RON promoter-luciferase reporter and the Egr-1 cDNA expression plasmid (pEgr-1cDNA) at the indicated concentrations. As shown in Fig. 3C, forced expression of Egr-1 cDNA dose-dependently eliminated the EGCG inhibitory effect, suggesting that the increase in the abundance of cellular Egr-1 eradicated the inhibitory effect of EGCG on RON promoter activity in AGS cells.

Effect of EGCG, siRON and siEgr-1 on PMA-induced gastric cancer cell invasion. It has been suggested that RON expression is essential for the invasive phenotype of cancer cells. The role of PMA-induced RON in AGS cell invasion was evaluated in a modified Boyden invasion chamber. As shown in Fig. 4, cell invasiveness increased ~2-fold following incubation with PMA. However, cells transfected with RON siRNA and Egr-1 siRNA partially lost the Matrigel invasiveness induced by PMA. These results suggest that PMA-induced Egr-1 and RON in AGS cells stimulated AGS cell invasion. In addition, the effect of EGCG on AGS cell invasion stimulated by PMA was examined. Cells pretreated with 30 μM of EGCG also partially abrogated PMA-induced cell invasion. Our results suggest that treating AGS cells with EGCG reduced RON by regulating Egr-1 and that these events contributed to a reduction in AGS gastric cancer cell invasion.

Effects of EGCG on tumor growth and RON expression in vivo. The effects of EGCG on cancer growth *in vivo* were determined in a subcutaneous gastric (TMK-1) cancer xenograft model. Treatment with EGCG (1 mg in 0.1-0.2 ml PBS/day/mouse) inhibited gastric tumor growth (Fig. 5A). The control group was treated with the same dosage of EC. The potent growth-inhibitory effect was also mirrored by the weights of the excised tumors, which were significantly decreased in the EGCG-treated mice (Fig. 5B). Notably, *in vivo* RON expression levels were also substantially decreased in mice treated with EGCG, compared to controls (Fig. 5C). Mouse body weight did not differ among treatment groups (data not shown). We concluded that EGCG sufficiently inhibited gastric cancer cell growth *in vivo*. Our data provide the first evidence that the inhibition of tumor growth by EGCG may, in part, be mediated by impairing the RON system.

Discussion

We previously delineated the role of RON in the acquisition of the gastric cancer cell invasive phenotype and we also identified the critical regulatory elements that are necessary for oncogenic RON tyrosine kinase promoter activity and gene expression (14). Tyrosine kinase receptors regulate multiple processes involved in tumor progression and metastasis, making them attractive molecular therapy targets. RON is mainly transcribed at relatively low levels in normal epithelial cells. However, the levels of RON expression in malignant epithelial cells increase severalfold compared with those in

benign epithelium. This aberrant expression and activation of RON has been observed in human cancer and is responsible for various malignant behaviors in breast, colon and ovary cancer (12,15). Increased RON expression is strongly correlated with phosphorylation and tumor invasiveness, suggesting that increased RON expression plays a role in the progression of carcinomas to an invasive-metastatic phenotype (16).

In this study, we demonstrated that EGCG suppressed RON expression by regulating Egr-1 in gastric cancer cells and that these events may contribute to the reduction of tumor growth *in vivo*. Interest in green tea as an anticancer agent in humans has increased for several reasons: i) several epidemiological studies have reported that green tea lowers the risk of cancer, when consumed in large amounts (17); ii) green tea inhibits the development and progression of skin, lung, mammary gland and gastrointestinal tract cancer in animal models (18); iii) green tea extracts, including purified EGCG, stimulate apoptosis in various cancer cell lines, such as stomach, prostate, lymphoma and lung *in vitro* (5); and iv) green tea consumption is associated with few adverse events and it can be easily obtained at a low cost (19). The anticancer effects of green tea have been attributed to the biological activities of its polyphenol components. EGCG is the most abundant of the green tea polyphenols, accounting for >40% of the total polyphenolics (3). Several molecular mechanisms have been suggested for the observed anticancer effect of EGCG, including suppression of ligand binding to the epidermal growth factor receptor (EGFR) (20), inhibition of protein kinase C (21), lipoxygenase and cyclooxygenase activities (3), induction of apoptotic cell death and arrest of the tumor cell cycle (5).

The suppression of RON expression by EGCG occurred at the transcriptional level, as shown by the transient transfection study using the RON promoter-reporter construct (Fig. 1). It has previously been suggested that multiple regulatory elements are required for full RON promoter activity and a portion of the 5'-flanking region of the RON gene has been cloned (13). Similar to numerous tyrosine kinase receptor gene promoters, the RON promoter also lacks distinct TATA box and CCAAT sequences. However, it contains several GC boxes, seven Sp1-binding sites, four retinoblastoma control elements, three IL-6 response elements and two AP-2 elements (15). Putative Egr-1-binding motifs in the RON gene promoter region of gastric cancer cells have also been reported (14). The role of the Egr-1 signaling pathway in the inhibition of RON gene expression by EGCG was evaluated in the present study. Site-directed mutagenesis of Egr-1 binding sites resulted in a decrease in the effects of EGCG on RON expression (Fig. 2). In addition, forced Egr-1 expression eliminated the inhibitory effects of EGCG in a dose-dependent manner (Fig. 3). These results indicate that interruption of Egr-1 by EGCG played a critical role in suppressing RON gene promoter activity. A similar finding was reported by Fu and Chen (22), who observed that EGCG suppresses EGFR gene expression in rat hepatic stellate cells by reducing Egr-1 activity. They suggested that inhibition of Egr-1 transactivation activity and the EGFR gene promoter activity by EGCG occurs through an interruption in the ERK signaling pathway. By contrast, regarding the effect of EGCG on Egr-1 in human pulmonary epithelial cells, Moon *et al* reported that EGCG induced Egr-1 expression and mediated Egr-1 nuclear translocation via the ERK signaling pathway in pulmonary epithelial cells (23). Comparing these findings with the downregulation of Egr-1 by EGCG demonstrated by our results, it can be hypothesized that Egr-1 and ERK responses are differentially regulated by EGCG, depending on the tissue environment and external stimuli.

The Egr-1 transcription factor is an immediate-early response gene that is rapidly induced by various growth factors, cytokines and DNA-damaging agents, and modulates cell proliferation, differentiation, apoptosis and inflammation in a variety of cells (24,25). The role of Egr-1 in tumor development might be largely dependent on tissue type, since Egr-1 is highly expressed and plays an essential role in tumor growth and survival in various types of cancer (26,27). The exact mechanisms by which EGCG inhibits the activation of Egr-1 are unknown. One possible explanation is that EGCG inhibits the kinases that are involved in the activation Egr-1. Numerous studies have suggested a causal relationship between ERK activation and induction of Egr-1 gene expression (28,29). Activation of ERK induces Egr-1 gene expression mediated by the transcription factor Elk-1, an ERK substrate (29). Elk-1 binds to multiple serum response elements and their adjacent Ets motifs located in the Egr-1 gene promoter and stimulates promoter activity, leading to Egr-1 transcription (29). Previous studies suggested that MAPK is one of the target molecules in the signaling cascades regulated by EGCG. It was previously discovered that EGCG treatment suppresses ERK phosphorylation, resulting in the downregulation of vascular endothelial growth factor in HT29 human colon adenocarcinoma cells (5). Katiyar *et al* (30) demonstrated that pretreatment of human epidermal keratinocytes with EGCG inhibits ultraviolet B-induced hydrogen peroxide production and hydrogen peroxide-mediated phosphorylation of the MAPK signaling pathway. EGCG modulates multiple signaling pathways, and the tyrosine kinase receptor (30), which is a strong metal ion chelator (31). Since certain kinases depend on divalent cations for their activity, EGCG may inhibit the activity of receptor kinases by chelating divalent cations. On the other hand, the EGCG inhibitory effects could be considered 'non-specific'. It has been demonstrated that EGCG non-specifically binds proteins and modulates enzyme activity, leading to inhibition of cell cycle-related kinases, MAPK and the activity of receptor tyrosine kinases (32). Our results do not exclude the role of other signaling pathways in EGCG-induced suppression of RON gene expression.

EGCG administration inhibits carcinogenesis in several animal models. Our results suggest, for the first time, that EGCG may exert its anticancer effects by inhibiting RON, supporting a role for green tea in cancer chemoprevention. The suitability of RON as a therapeutic target has been demonstrated in an experimental study that used a novel function-blocking antibody (33). The growth-inhibitory effects of the RON antibody have been validated in preclinical tumor models and a comprehensive analysis was performed on RON expression in various human cancer entities. The authors concluded that inhibiting RON is a potentially useful target for human cancer therapy. However, another report revealed that the growth-inhibitory effects of RON inhibitors may be transient (34). In that study, Logan-Collins *et al* demonstrated that silencing RON reduces tumor growth and renders cancer cells susceptible to chemotherapeutic agents; however, downregulation of RON is lost over time. This transient effect was linked

to an increase in c-MET and EGFR expression, suggesting that these two oncogenic receptor systems provide an escape mechanism from RON silencing in cancer cells. The EGFR and c-MET findings are particularly relevant to our study, since both are possibly inhibited by EGCG. Thus, further studies are required to elucidate the detailed mechanism by which EGCG inhibits RON activity and to examine whether EGCG exerts the same effects in human cancer.

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