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Mechanistic Aspects of Green Tea as a Cancer Preventive: Effect of Components on Human Stomach Cancer Cell Lines

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It is now well accepted that (–)-epigallocatechin gallate (EGCG) inhibits carcinogenesis in the digestive tract in rodents. To understand the mechanisms of anticarcinogenesis, we first studied growth inhibition by EGCG in human stomach cancer cell lines established at Seoul National University (SNU cell lines). Inhibition by EGCG of [^{3}H]thymidine incorporation into eight SNU cell lines was examined, in relation to transforming growth factor-β (TGF-β) responsiveness. Various tea polyphenols derived from green tea and black tea induced growth inhibition and apoptosis of human stomach cancer cell line KATO III, and inhibition of tumor necrosis factor-α (TNF-α) release from the cells, in the order of (–)-epicatechin gallate (ECG), EGCG, (–)-epigallocatechin (EGC), teaflavins (TF) and (–)-epicatechin (EC). In addition, we demonstrated that EGCG inhibited *TNF-α* gene expression in KATO III cells, as well as okadaic acid-induced AP-1 and NF-κB activation. The inhibitory potencies of EGCG for AP-1 and NF-κB binding to DNA were different between KATO III cells and mouse fibroblast cell line BALB/3T3. Thus, EGCG and other tea polyphenols may interact with various transcription factors, in addition to AP-1 and NF-κB, in nuclei of various cells, resulting in inhibition of *TNF-α* gene expression and TNF-α release.

Key words: Stomach cancer — Apoptosis — TNF-α — AP-1 — NF-κB

One of the important features of (–)-epigallocatechin gallate (EGCG) and green tea as cancer preventives is the wide range of effective target organs, at least in rodent carcinogenesis experiments.[1,2] Since tea polyphenols are ingested orally in the daily life of Japanese, we are interested in studying how EGCG and other tea polyphenols actually induce inhibition of carcinogenic processes in the human digestive tract. We previously reported, in collaboration with Tetsuro Yamane, that a solution of 0.05% EGCG in drinking water inhibited *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced carcinogenesis of the glandular stomach in rats.[3]

Our colleagues (K. P. and S.-J. K.) first observed the inhibitory effects of EGCG on growth of human stomach cancer cell lines established by Seoul National University (SNU cell lines).[4] Since EGCG is one of the main polyphenols contained in green tea, we next examined the effects of (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC), (–)-epicatechin (EC) and a mixture of teaflavins (TF) derived from black tea on the growth of human stomach cancer cell line KATO III. Induction of DNA ladder formation by tea polyphenols was also studied in KATO III cells, since EGCG has been reported to induce apoptosis in human cancer cell lines.[5–7]

Based on our previous finding that okadaic acid, a tumor promoter in mouse skin and rat glandular stomach, induces tumor necrosis factor-α (TNF-α) release from target organs and KATO III cells,[8,9] we wanted to determine whether tea polyphenols also inhibit TNF-α release from KATO III cells. Moreover, whether the activation of AP-1 and NF-κB, which are directly involved in the expression of *TNF-α* gene,[10] is inhibited by EGCG, was also investigated by electrophoretic mobility gel-shift assay (EMSA). Mechanisms of action of tea polyphenols in human stomach cancer cell lines are discussed in terms of the inactivation of transcription factors.

MATERIALS AND METHODS

Chemicals EGCG was purified from Japanese green tea leaves; its purity was 99.7%. Other tea polyphenols, ECG, EGC, and EC, were purchased from Funakoshi Co., Ltd., Tokyo. TF, which is a mixture of TF derived from black tea, was a generous gift of the American Tea Association, New York, NY.

Cell lines and culture conditions SNU-1, -5, -16, -484, -601, -638, -668 and -719 are established cell lines from gastric carcinoma of individual patients (primary tumor or malignant ascites) at the Department of Surgery, Seoul National University.[11] These cell lines and KATO III cell

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line obtained from Japanese Cancer Research Resources Bank were cultured in RPMI 1640 medium with 10% fetal bovine serum and maintained at 37°C in 5% CO_2/air.

Inhibition of [^{3}H]thymidine incorporation in SNU cell lines by EGCG Eight SNU cell lines were cultured in 24-well plates at a density of 5×10^4 cells/well in 0.5 ml of assay medium (RPMI 1640 containing 0.25% fetal bovine serum), and various concentrations of EGCG (0.2–20 μg/ml) were added. After incubation for 48 h, cells were pulse-labeled with 18.5 kBq of [^{3}H]thymidine for 2 h at 37°C. Radioactivity of cells was measured as described previously.[11] The assay was done in triplicate.

Growth inhibition of KATO III cells by tea polyphenols KATO III cells (2×10^5/ml) in 24-well plates were incubated with each of the tea polyphenols at various concentrations (10–200 μM) for 1 and 3 days. Viable cells were identified by means of the trypan blue dye exclusion test and counted. The results were confirmed by an additional independent experiment.

Induction of apoptosis in KATO III cells by tea polyphenols KATO III cells (1×10^6) were treated with various concentrations of tea polyphenols (100–500 μM) for 48 h. The DNA isolated using an ApopLadder EX kit (TaKaRa Biomedicals, Tokyo) was subjected to 2% agarose gel electrophoresis, and the DNA ladder was visualized by ethidium bromide staining. The results were confirmed by two additional independent experiments.

Inhibition of TNF-α release from KATO III cells KATO III cells (2×10^5) were first incubated with tea polyphenols at various concentrations (1–500 μM). After 1 h, okadaic acid (50 nM final concentration) was added and incubation was continued for a further 24 h. The concentration of TNF-α in the medium was measured using an ELISA for human TNF-α (Amersham Pharmacia, Buckinghamshire, UK), as described previously.[9] Okadaic acid-induced TNF-α release without pretreatment was taken as 100%. Results are expressed as the means of two independent experiments.

Inhibition of *TNF-α* gene expression in KATO III cells KATO III cells (6×10^6) were treated with EGCG at various concentrations (10–500 μM); 1 h later, okadaic acid was added at 50 nM final concentration. After 14 h, total RNA was prepared according to the method of Chomczynski and Sacchi.[12] The level of TNF-α mRNA was determined with a GeneAmp RNA polymerase chain reaction (PCR) kit (Roche Molecular Systems, Inc., Branchburg, NJ). Total RNA (1 μg) was transcribed to cDNA by reverse transcriptase of murine leukemia virus at 37°C for 1 h, then cDNA was amplified in GeneAmp PCR reaction mixture containing 55 kBq of [α-^{32}P]dCTP, and 0.25 units of AmpliTaq Gold polymerase (Roche Molecular Systems, Inc.) as described previously.[9] The 5′ and 3′ primers for TNF-α and those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized as reported previously.[13, 14] Radioactivity of TNF-α mRNA was measured with a BAS 2000 Image analyzer (Fuji Photo Film Co., Ltd., Tokyo), and normalized with respect to GAPDH mRNA as a qualitative control. TNF-α mRNA was expressed in relative terms compared with that of the non-treated control. The results were confirmed by an additional independent experiment.

Effect of EGCG on okadaic acid-induced AP-1 and NF-κB activation KATO III cells and BALB/3T3 cells were first incubated with various concentrations of EGCG (1–500 μM). One hour later, KATO III cells were treated with 50 nM okadaic acid and BALB/3T3 cells were treated with 200 nM okadaic acid, for another 8 h. Nuclear protein was prepared by the published method with some modification.[15] EMSA was performed by incubating 4 μg of nuclear extract with 0.03 pmol of ^{32}P-end-labeled 22-mer double-stranded NF-κB oligonucleotide 5′-AGTTGAGGGGACTTTCCCAGGC-3′ or 21-mer double-

Table I. Inhibition of [^{3}H]Thymidine Incorporation by EGCG in SNU Cell Lines

Cell lines	[^{3}H]Thymidine incorporation (% of control)	Responsiveness to TGF-β[a)]	TGF-β type II receptor[a)]	
			Genetic changes	mRNA
SNU- 1	21.6	resistant		undetectable
SNU- 5	13.6	resistant	deleted	truncated
SNU- 16	32.4	sensitive		increased
SNU-484	3.8	resistant		undetectable
SNU-601	30.7	resistant	amplified	increased
SNU-638	13.4	resistant		undetectable
SNU-668	27.9	resistant	deleted	truncated
SNU-719	52.7	resistant	amplified	increased

SNU cell lines were treated with EGCG at a concentration of 10 μg/ml (21.8 μM) for 48 h. Representative results are shown.
a) Data from Reference No. 11.

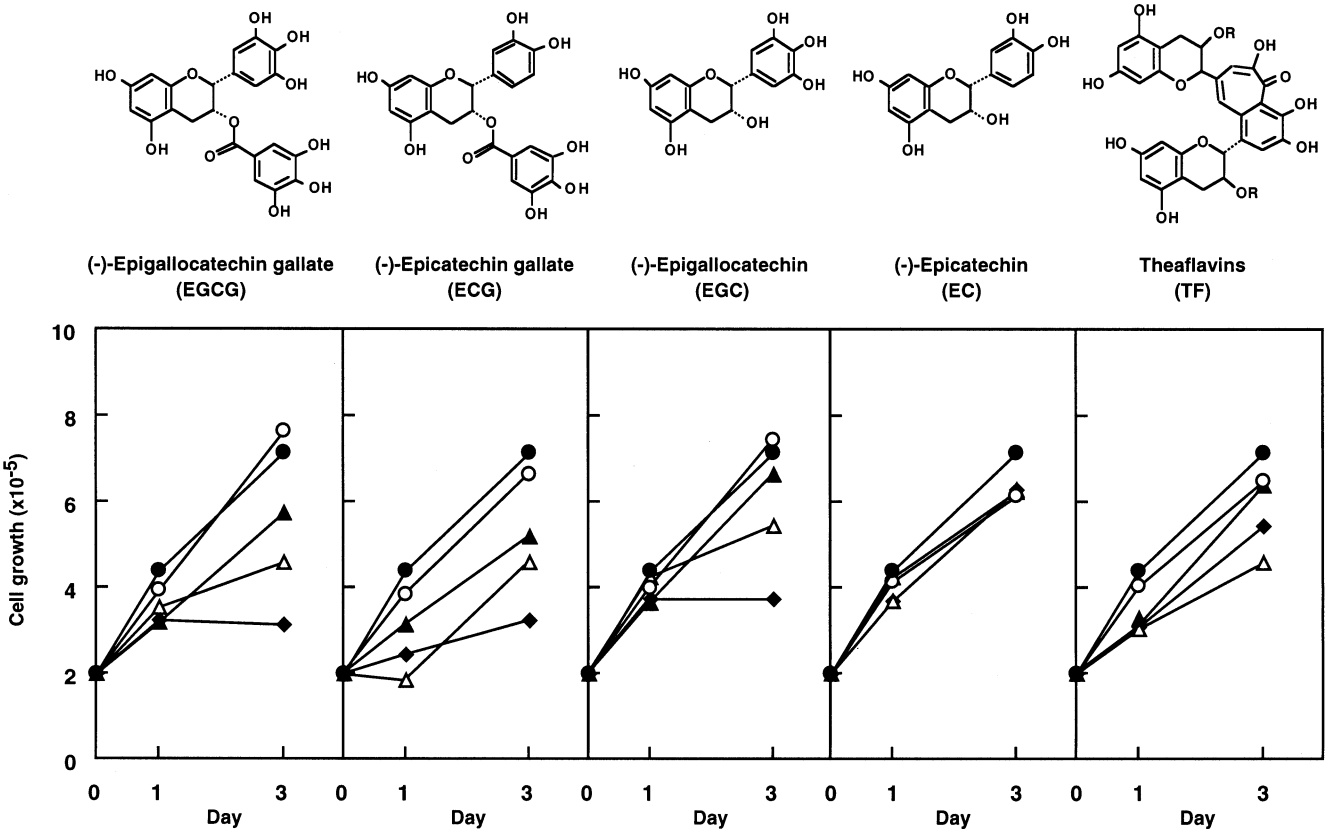

Fig. 1. Structures of tea polyphenols and their growth inhibition of KATO III cells. KATO III cells (2×10^5/ml) were treated with EGCG, ECG, EGC, EC and TF at various concentrations: control (○), 10 μM (●), 50 μM (▲), 100 μM (△), 200 μM (◆). The number of viable cells was counted, based on trypan blue dye exclusion. The results are means of duplicate determinations and were confirmed by two independent experiments. R of TF represents a galloyl moiety.

stranded AP-1 oligonucleotide 5′-CGCTTGATCAGT-CAGCCGGAA-3′ (Promega Corporation, Madison, WI), for 30 min at room temperature. The incubation mixture included 2 μg of poly(dI-dC) in binding buffer (0.1 mg/ml BSA, 10 m*M* Tris-HCl pH 7.5, 50 m*M* NaCl, 1 m*M* $MgCl_2$, 4% glycerol, 0.5 m*M* EDTA, and 0.5 m*M* DTT). The DNA-protein complex formed was separated from free oligonucleotides on polyacrylamide gel, and the radioactive band was quantitated with the BAS 2000 Image analyzer. The results were confirmed by two additional independent experiments.

RESULTS AND DISCUSSION

Growth inhibition of human stomach cancer cell lines by EGCG Growth of SNU cell lines was determined in terms of [^{3}H]thymidine incorporation. Treatment of EGCG at various concentrations (0.2 to 20 μg/ml) for 48 h inhibited [^{3}H]thymidine incorporation in eight SNU cell lines dose-dependently (data not shown). Table I summarizes the results for inhibition of [^{3}H]thymidine incorporation of eight SNU cell lines by EGCG at a concentration of 10 μg/ml (21.8 μM) for 48 h. EGCG inhibitory potencies varied from 3.8 to 52.7%, based on 100% in a control group without EGCG. Similar results were obtained with other cell lines as follows: EGCG at a concentration of 10 μg/ml (21.8 μM) inhibited growth of human lung adenocarcinoma cell line A549 to 3.4%, human prostate adenocarcinoma cell line PC-3 to 30.5%, and human hepatoma cell line PLC/PRF/5 to 12.3%. We have so far tested the effects of EGCG on ten human cancer cell lines: in only one (schwannoma cell line) did EGCG not inhibit growth. We previously hypothesized that EGCG had a sealing effect on the cell membrane.[1)] This was recently confirmed by us in experiments with liposomes in which various concentrations of EGCG had been dispersed, indicating that EGCG was incorporated into the surface region of the phospholipid bilayer membrane.[16)] In addition, we found [^{3}H]silver grains on the membrane when human lung cancer cell line PC-9 was treated with [^{3}H]EGCG *in vitro*.[17)]

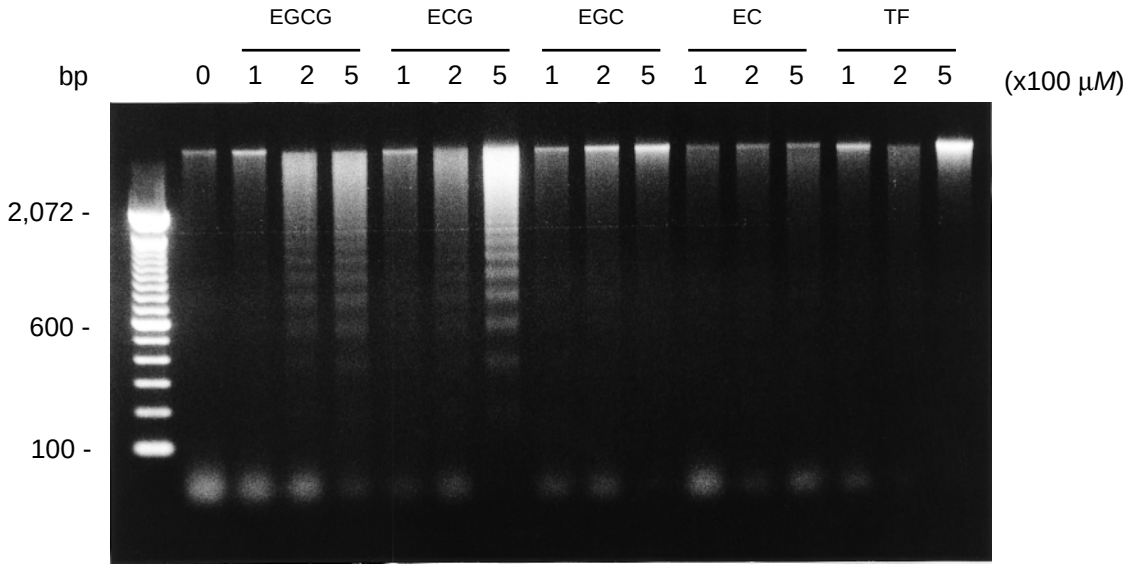

Fig. 2. Induction of apoptosis in KATO III cells by tea polyphenols. KATO III cells were treated with EGCG, ECG, EGC, EC, and TF at various concentrations for 2 days, and fragmented DNA was analyzed by electrophoresis, as described in "Materials and Methods." The results were confirmed by three independent experiments. The left lane shows DNA markers.

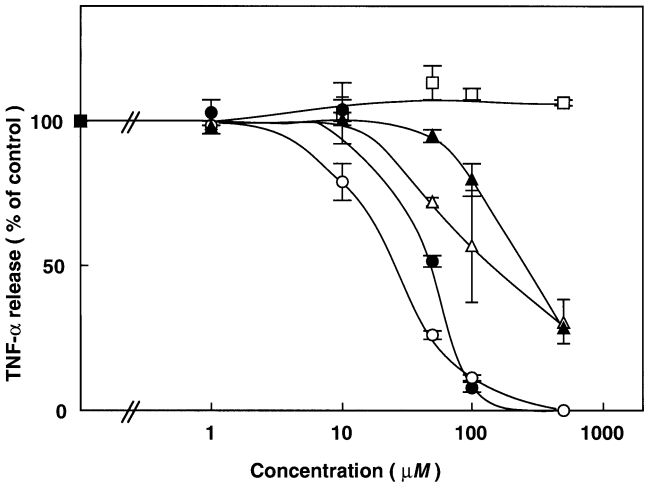

Fig. 3. Inhibition of TNF-α release from KATO III cells by tea polyphenols. Pretreatment with various concentrations of each tea polyphenol for 1 h was followed by treatment with 50 n*M* okadaic acid. After 24 h incubation, the concentration of TNF-α in the medium was determined using ELISA. ECG (○), EGCG (●), TF (△), EGC(▲) and EC (□). TNF-α release (300 pg/ml) induced by okadaic acid was taken as 100%. The data are means of two separate experiments performed in duplicate.

EGCG seems to bind to the cell membrane of various human cancer cell lines, resulting in inhibition of interaction of ligands with receptor proteins. Recently, we investigated the responsiveness of these SNU cell lines to transforming growth factor-β (TGF-β), from the viewpoint of genetic changes and expression patterns of *TGF-β type II receptor* gene (Table I).[11] EGCG was effective on TGF-β-sensitive as well as TGF-β-resistant SNU cell lines, suggesting that the effects of EGCG are not related to the TGF-β pathway.

Growth inhibition of KATO III cells by tea polyphenols mediated through induction of apoptosis Next we studied the inhibitory effects of various tea polyphenols contained in green tea and black tea. EGCG, ECG, EGC and EC are the main polyphenols in green tea, and their content is somewhat lower in black tea; TF are found only in black tea, and polymers of tea polyphenols are a product of advanced fermentation of tea leaves.[18] Fig. 1 shows growth inhibition of human stomach cancer cell line KATO III by five tea polyphenols. EGCG, ECG, EGC and TF dose-dependently inhibited the growth of KATO III cells, whereas EC (with no galloyl moiety) did not show any significant inhibition. The inhibitory potencies of these tea polyphenols were compared in terms of the concentrations required to achieve 50% inhibition of cell growth: ECG (140 μM) = EGCG (140 μM) > EGC (210 μM) > TF (240 μM), suggesting that green tea contains more effective compounds than black tea. Similar results were also obtained with PC-9 cells, as mentioned above.[17] In addition, treatment with tea polyphenols induced apoptosis, leading to DNA fragmentation: Fig. 2 shows that treatment with 100 to 500 μM EGCG for 2 days dose-

dependently induced DNA fragmentation in KATO III cells. It is important to note that ECG was approximately as potent in induction of DNA fragmentation as EGCG, whereas the effects of EGC and TF at a concentration of 500 *μM* were negligible. The results indicate that tea polyphenols are much less potent as inducers of DNA fragmentation than as inhibitors of cell growth.

Inhibition of TNF-α release from KATO III cells In our previous studies on tumor promotion, we demonstrated that TNF-α is an endogenous tumor promoter and a central mediator of tumor development.[8, 19] Thus, compounds which inhibit TNF-α release from the cells and *TNF-α* gene expression in the cells are potential anticancer agents.[20] We tested whether tea polyphenols inhibit TNF-α release into the medium from KATO III cells treated with 50 n*M* okadaic acid, a tumor promoter, for 24 h. As expected, ECG, EGCG, EGC and TF dose-dependently inhibited TNF-α release from KATO III cells induced by okadaic acid (Fig. 3). The IC_{50} values for 50% inhibition of TNF-α release were 26 *μM* for ECG, 48 *μM*

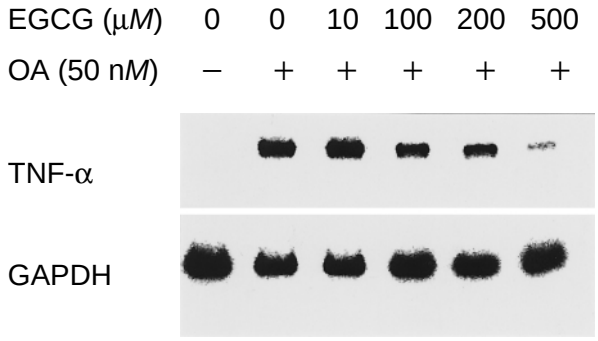

Fig. 4. Inhibition of *TNF-α* gene expression by EGCG in KATO III cells. Pretreatment with EGCG at various concentrations for 1 h was followed by treatment with 50 n*M* okadaic acid (OA). After 14 h incubation, *TNF-α* gene expression was measured by RT-PCR, as described in "Materials and Methods." The results were confirmed by two independent experiments.

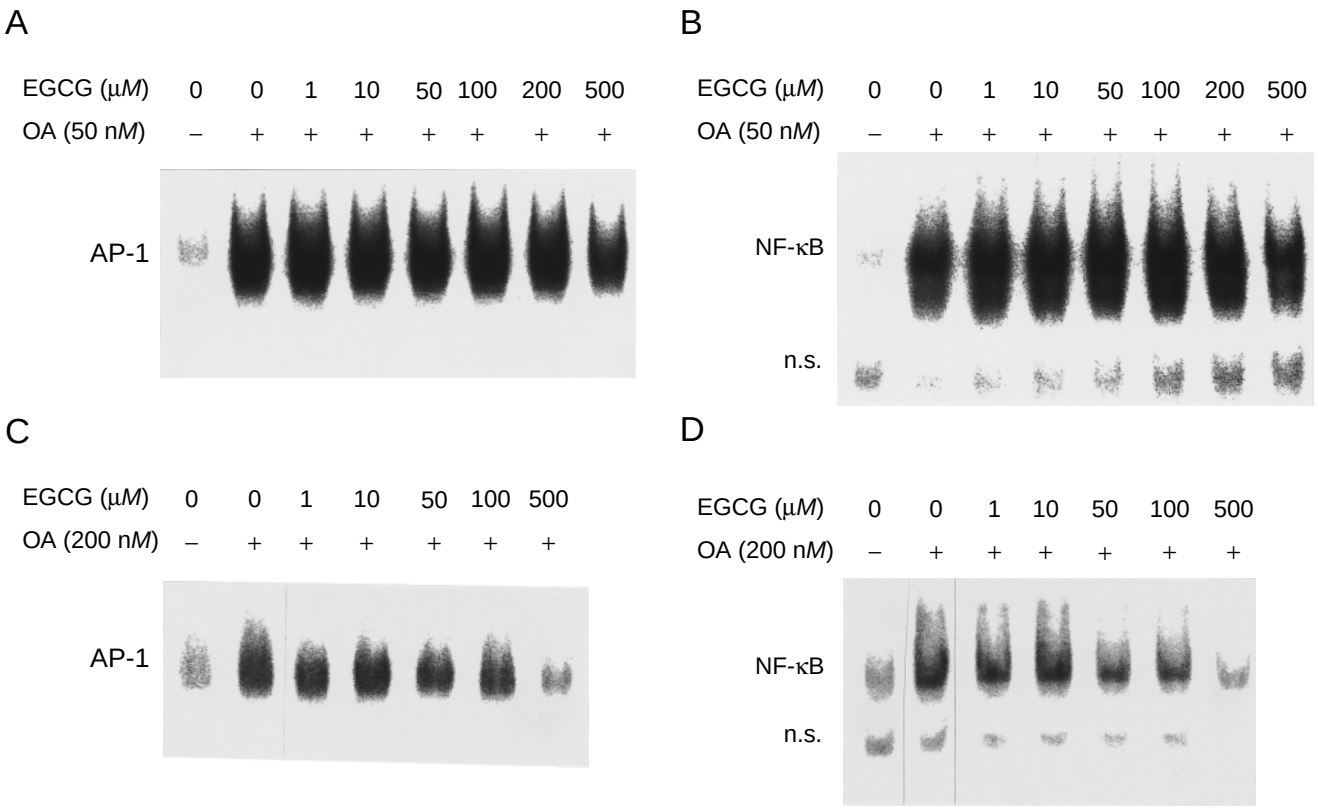

Fig. 5. Effect of EGCG on okadaic acid-induced AP-1 and NF-κB activation. Dose-response effects of EGCG on KATO III cells (A, B), and those of EGCG on BALB/3T3 cells (C, D). One hour after EGCG treatment, the cells were treated with okadaic acid (50 n*M* OA for KATO III cells, 200 n*M* OA for BALB/3T3 cells) for another 8 h. Nuclear extracts were subjected to EMSA with ^{32}P-labeled oligonucleotide probes for AP-1 (A, C) or NF-κB (B, D) binding site.[15] The results were confirmed by three independent experiments.

for EGCG, 115 μM for TF, 210 μM for EGC and >500 μM for EC. These results correlated well with those for cell growth inhibition.

Inhibition of *TNF-α* gene expression in KATO III cells KATO III cells without okadaic acid treatment expressed *TNF-α* gene at a marginal level; treatment with 50 n*M* okadaic acid for 14 h significantly increased *TNF-α* gene expression in the cells (Fig. 4). Pretreatment with EGCG at various concentrations (10–500 μM) reduced the mRNA levels dose-dependently, and showed 80% reduction at 500 μM. But EGCG did not have any inhibitory effect on GAPDH mRNA levels (control) (Fig. 4). We previously reported that, in addition to EGCG, cancer inhibitors such as sarcophytol A, canventol and tamoxifen inhibited *TNF-α* gene expression in BALB/3T3 cells induced by okadaic acid, suggesting that inhibition of *TNF-α* gene expression correlates well with inhibition of TNF-α release from the cells.[20] Based on this, we think that other tea polyphenols, such as ECG, EGC and TF, may also inhibit *TNF-α* gene expression in KATO III cells.

Effect of EGCG on okadaic acid-induced AP-1 and NF-κB activation *TNF-α* gene expression is regulated by several transcription factors, such as AP-1 and NF-κB.[10] Okadaic acid at 50 n*M* concentration significantly enhanced AP-1 and NF-κB binding to DNA by about 10-fold in KATO III cells, and 200 n*M* okadaic acid did so by 2.5-fold in BALB/3T3 cells (Fig. 5). Under these EMSA conditions, we investigated whether pretreatment with EGCG would inhibit okadaic acid-induced AP-1 and NF-κB activation. In KATO III cells, a high concentration (500 μM) of EGCG weakly inhibited AP-1 and NF-κB activation induced by 50 n*M* okadaic acid (Fig. 5, A and B). However, in BALB/3T3 cells treated with 200 n*M* okadaic acid, EGCG at concentrations of 50 μM and above more clearly inhibited both AP-1 and NF-κB binding to the DNA (Fig. 5, C and D). These results suggest that *TNF-α* gene expression in KATO III cells may be differently regulated by transcription factors than that in BALB/3T3 cells. Recently, cell-type specific regulation of *TNF-α* gene by C/EBP$_\beta$ (NF-IL6) and CRE/ATF was reported.[21, 22] It will be necessary to study the effects of EGCG on these new transcription factors. We previously presented data on [^{3}H]EGCG incorporation into PC-9 cells,[17] and [^{3}H]EGCG was also found in nuclei, suggesting that the interaction of EGCG with various proteins in nuclei, including transcription factors, results in partial inhibition of *TNF-α* gene expression and tumor development.

In conclusion, the mechanistic aspects of the effects of green tea should be discussed briefly. The tumor promoter okadaic acid induces expression of many genes through activation of AP-1 and NF-κB in cells, and we focused on a specific gene, *TNF-α* gene, because TNF-α is an endogenous tumor promoter. In our system, EGCG inhibited both *TNF-α* gene expression in the cells and TNF-α release from the cells, mediated through inhibition of AP-1 and NF-κB activation. Recently, it has been accepted that various cancer-preventive agents, such as tea polyphenols, caffeic acid phenethyl ester and curcumin inhibit AP-1 and NF-κB activation.[15, 23, 24] Furthermore we have shown that various cancer inhibitors also inhibit TNF-α release. All the results suggest that inhibition of AP-1 and NF-κB activation is a general but partial mechanism of cancer prevention. Thus, we conclude that green tea is effective in stomach through this mechanism of action, which is also applicable to a wide range of other target organs.

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