Association of Suppression of Extracellular Signal-Regulated Kinase Phosphorylation by Epigallocatechin Gallate with the Reduction of Matrix Metalloproteinase Activities in Human Fibrosarcoma HT1080 Cells

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Matrix metalloproteinases (MMPs) play a crucial role in the process of cancer invasion and metastasis. Previous findings suggested that epigallocatechin gallate (EGCG), a main flavanol of green tea, caused decreased levels of MMP-2 and MMP-9 activities to be secreted into culture medium. To obtain further information on EGCG-mediated regulation of these MMPs, the effects of EGCG on enzyme activity, mRNA expression, and mitogen-activated protein kinase (MAPK) activities in human fibrosarcoma HT1080 cells were examined. EGCG was confirmed to suppress the gelatin-degrading activities due to MMP-2 and MMP-9 in the culture medium. This suppression of enzyme activities by EGCG was consistent with the decreased levels of MMP-2 and MMP-9 mRNAs. EGCG-mediated suppression was also observed for MT1-MMP mRNA. EGCG-mediated suppression of the level of MMP-9 transcript was correlated with its suppression of MMP-9 promoter activity. EGCG inhibited the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), which are the members of an MAPK family necessary for MMP-9 up-regulation. EGCG also suppressed p38 MAPK activity but gave no effects on stress-activated protein kinase/c-Jun N-terminal kinase activity. These findings suggest that suppression of ERK phosphorylation by EGCG is involved in the inhibition of expression for MMP-2 and MMP-9 mRNAs, leading to the reduction of their enzyme activities of the cancer cells. Methyl derivatives, epigallocatechin-3-*O*-(3-*O*-methyl) gallate and epigallocatechin-3-*O*-(4-*O*-methyl) gallate, exhibited effects similar to, but weaker than, those of EGCG, suggesting the important role of an unsubstituted triphenolic ester structure in these activities.

KEYWORDS: Tea catechin; EGCG; O-methylated EGCG; MMPs; MAPK; ERK; HT1080; human fibrosarcoma; cancer metastasis

INTRODUCTION

Many animal studies have shown that tea and tea components have anticancer activities (*1–4*). Green tea and black tea catechin compounds such as epigallocatechin gallate (EGCG) and theaflavin have been investigated most intensively to reveal the molecular basis for their antitumor activities (*1–4*).

Matrix metalloproteinase-2 (MMP-2 or gelatinase-A) and MMP-9 (gelatinase-B) are the members of a unique family of zinc-binding endopeptidases that are responsible for degradation of the extracellular matrix components and play a crucial role in the process of cancer invasion and metastasis (*5–8*).

In our previous study, we demonstrated that ester-type catechins from tea leaves inhibited cell invasion of human fibrosarcoma HT1080 cells and caused the reduced levels of MMP activities in the culture medium (*9*). However, the mechanism by which EGCG regulates MMPs remains to be determined.

In the present work, we examined the effects of EGCG on MMP expression and mitogen-activated protein kinase (MAPK) activities in these cells. MAPKs are known to regulate MMP expression (*10–24*). We also examined the effects of methyl derivatives of EGCG to gain an initial insight into the structure–

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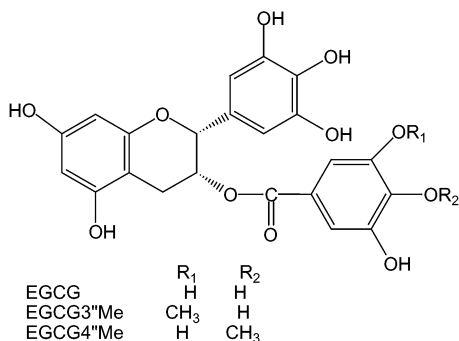# University of Shizuoka.
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Figure 1. Structures of EGCG and its derivatives from tea leaves.

	R_1	R_2
EGCG	H	H
EGCG3″Me	CH$_3$	H
EGCG4″Me	H	CH$_3$

Figure 2. Mapping of promoter region of MMP-9 gene.

function relationship in these activities. Our findings suggest that EGCG causes a reduction of MMP activities in the culture medium by inhibiting gene expression of MMPs in association with suppression of phosphorylation of extracellular signal-regulated kinase (ERK) in HT1080 cells. In addition, the importance of a triphenolic ester structure in catechins is also suggested for these activities.

EXPERIMENTAL PROCEDURES

Tea Catechins. (−)-Epigallocatechin gallate (EGCG), epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3″Me), and epigallocatechin-4-*O*-(4-*O*-methyl) gallate (EGCG4″Me) were purified to >98% from tealeaves (*25*). EGCG3″Me and EGCG4″Me contents in tea (cultivar Benifuki or Tong Ting oolong tea) were 1−2 and 0.1−0.2% dry weight, respectively. These samples were dissolved in phosphate-buffered saline (pH 7.4; PBS). The structures of these catechins are shown in **Figure 1**.

Reagents. TriZol RNA isolation solution, Dulbecco's minimum essential medium (DMEM), and fetal bovine serum were obtained from Gibco BRL (Grand Island, NY).

Cells and Cell Culture. Human fibrosarcoma HT1080 (IFO50354) cells were obtained from the Institute for Fermentation, Osaka Research Communications (Osaka, Japan). HT1080 cells were routinely cultured in DMEM supplemented with 5% heat-inactivated fetal bovine serum at 37 °C under humidified 5% CO$_2$/95% air. When required, the cells were cultured in a serum-free medium, the conditioned medium was collected, and the cells were rinsed once with PBS.

Gelatin Zymography. The gelatinase activity was examined by gelatin zymography using the protocol described previously (*26, 27*). Briefly, the conditioned medium of HT1080 cells cultured in serum-free DMEM was centrifuged, and an aliquot of the supernatant was loaded with a nonreducing sample buffer onto a 10% sodium dodecyl sulfate (SDS)−polyacrylamide gel containing 1 mg/mL gelatin and electrophoresed. After removal of the SDS from the gels by washing with 2.5% Triton X-100 solution for 30 min twice and with 10 mM Tris-HCl buffer (pH 8.0) for 30 min, gels were incubated in 50 mM Tris-HCl (pH 8.0) containing 0.5 mM CaCl$_2$ and 0.1 mM ZnCl$_2$ at 37 °C for 18 h to activate MMPs. The gels were stained with 1% Coomassie Blue R-250 in 10% methanol and 5% acetic acid and subsequently destained with 10% methanol and 5% acetic acid. The gelatinolytic activity of each gelatinase was detected as an unstained band against a blue background. The relative amounts of MMP-9 and MMP-2 were quantified by NIH Image Analysis software (v. 1.62).

RNA Analysis. For analyzing mRNA levels of MMPs, we used Northern blot analysis and a reverse transcription Polymerase Chain Reaction (RT-PCR)/Southern hybridization assay. Cultured HT1080 cells were washed with DMEM, further incubated for 24 h, and then cultured in the presence or absence of catechins for 8 h at 37 °C. Total RNA was prepared by modification of the guanidine thiocyanate procedure using a TriZol reagent.

For Northern blot analysis, total RNA (10 μg) from HT1080 cells was electrophoresed on formaldehyde−agarose (1%) gel and transferred to Hybond-N$^+$ membranes (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) according to the supplier's instructions. Hybridization
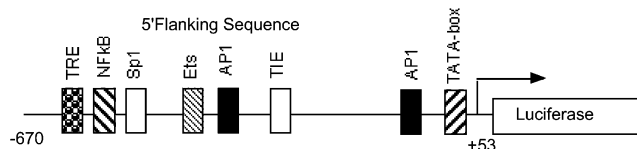
was carried out with digoxigenin-labeled cDNA probes at 42 °C for 20−26 h in 50% formamide, 5× SSC, 5× Denhardt's solution, 0.1 mg/mL denatured herring sperm DNA, 50 mM HEPES (pH 6.8), 5 mM EDTA, and 0.5% SDS, successively, followed by washing with 0.1% SDS in 0.1× SSC at 50 °C for 30 min. The cDNA probes used for hybridization were human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragment (bases 86−1068 of GenBank accession X0110), human MMP-9 fragment (bases 24−670 of GenBank accession J05070), human MMP-2 fragment (bases 296−947 of GenBank accession J03210), and human MT1-MMP fragment (bases 1468−2603 of GenBank accession NM004995). cDNA probes were labeled by the random prime method using a digoxigenin DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland). To normalize for RNA loading and transferring, hybridization with a cDNA probe for GAPDH was included.

For RT-PCR/Southern hybridization analysis, total RNA was reverse-transcribed to synthesize cDNA using a commercial kit [TaKaRa RNA PCR kit (AMV)] according to the manufacturer's protocol. PCR was then carried out in 50 μL of reaction volumes containing RNA PCR buffer, 2.5 mM MgCl$_2$, 0.2 μM of each primer, and 2.5 units of TaKaRa Taq polymerase. Samples were pre-denatured at 94 °C for 2 min, followed by amplification at 94 °C for 30 s, at 60 °C for 30 s, and at 72 °C for 1.5 min for 14 cycles, followed by a final 10 min extension step at 72 °C. The primers for GAPDH were 5′-CATTTGCAGGGGG-GAGCCA-3′ and 5′-TACTCCTTCGAGGCCATG-3′ with an expected amplified product of 677 bp. The MMP-2 primers were 5′-CGCAAGC-CCAAGTGGGACAAGAA-3′ and 5′-CGGAAGTTCTTGGTGTAG-GTGTA-3′ with an expected amplified product of 953 bp. The MT1-MMP primers were 5′-ACATCAAAGTCTGGGAAGGGAT-3′ and 5′-ATCCAAGGCTAACATTCGAGAG-3′ with an expected amplified product of 1136 bp. For Southern blot hybridization, reaction products were separated on 1% agarose gel, and PCR products were transferred onto nylon membranes (Hybond-N$^+$) and hybridized with digoxigenin-labeled probes as described above. All results are representatives of at least three independent experiments.

MMP-9 Transcription Analysis. HT1080 cells cultured as above were transiently transfected by electroporation with a pGL3-basic vector (GenBank accession U47295; Promega, Madison, WI), containing −670 to +57 bp of the 5′ regulatory region of the human MMP-9 gene (*28*) linked to the luciferase gene (**Figure 2**). After 24 h, the culture medium was changed to the serum-free medium, and HT1080 cells were incubated in the absence or presence of catechins after another 8 h. Luciferase activity was measured as described previously (*29*). To normalize for vector electroporation, the pRL-CMV vector (GenBank accession AF025843; Promega) was transfected simultaneously.

The results are shown as means ± SD in triplicate experiments. The significance of the difference between two means was evaluated on the basis of Duncan's multiple-range test, and $P < 0.05$ was taken as significant.

Immunoblotting. Antibodies against the following antigens were obtained from New England Biolabs (Boston, MA): ERK1/2, p38 MAPK, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), phospho-ERK1/2, phospho-p38 MAPK, and phospho-SAPK/JNK. HT1080 cells were cultured in the presence or absence of EGCG for 1 h after preincubation for 24 h in the serum-free medium. Cells separated by centrifugation were lysed in 1% Nonidet P-40 lysis buffer with phosphatase inhibitors and proteinase inhibitors (*30*). The lysates were centrifuged, supernatants were separated by 10% SDS−polyacrylamide gel electrophoresis, and proteins were blotted onto PVDF membranes (Millipore, Bedford, MA) electrically. PVDF membranes were blocked with a 5% fatfree dry milk in 0.2% Tween-20 in PBS and incubated with primary antibody for 1 h, washed with 0.2% Tween-20 in PBS, and then incubated with horseradish peroxidase-conjugated

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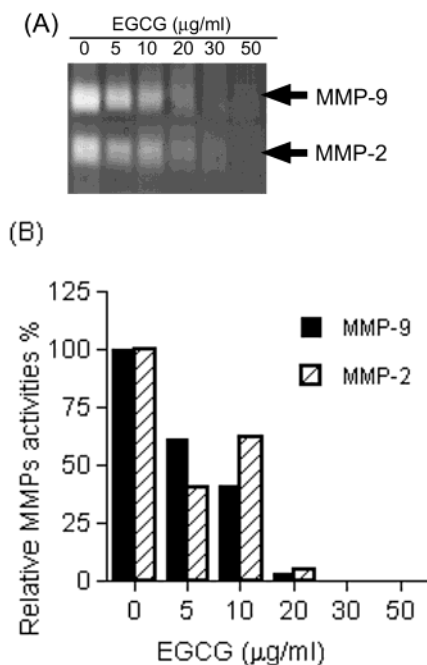
Figure 3. Gelatinolytic activities of MMP-2 and MMP-9 in conditioned media from human fibrosarcoma HT1080 cells treated with EGCG. Subconfluent monolayers of HT1080 cells were cultured for 24 h in serum-free DMEM in the presence of various concentrations of EGCG. The conditioned media were analyzed by gelatin zymography. Clear zones of degradation at 92 kDa (MMP-9), 72 kDa, 68 kDa, and 62 kDa (MMP-2) are seen. The relative enzyme activities of MMP-2 and MMP-9 are expressed as percent of untreated sample (B).

secondary antibody. Visualization of immunoreactive proteins was performed with enhanced chemiluminescence reagents (ECL kit; Amersham Pharmacia Biotech.).

RESULTS

Effects of EGCG on MMP-2 and MMP-9 Activities in the Culture Medium. Conditioned medium from HT1080 cells cultured in the serum-free medium contained gelatinolytic activities almost exclusively due to MMP-2 and MMP-9 as described previously (*9*). The gelatin lysis activity in the zymogram was inhibited in a dose-dependent manner by EGCG treatment (**Figure 3**) in accordance with our previous findings (*9*). The values of half-maximal effective inhibitory concentration (IC$_{50}$) for MMP-2 and MMP-9 were approximately 7.5 and 7.5 μg/mL, respectively (**Figure 3B**).

EGCG-Mediated Down-Regulation of MMP mRNA Levels. To examine whether EGCG affects the steady-state levels of MMP-9, MMP-2, and MT1-MMP mRNAs, Northern blot analysis of RNA from HT1080 cells was performed. The amounts of transcripts for MMP-9, MMP-2, and MT1-MMP were reduced with increasing EGCG concentration in a dose-dependent manner (**Figure 4A**). IC$_{50}$ values for the MMP-2 and MMP-9 mRNA levels were 2 and 0.1 μg/mL, respectively.

The results of the RT-PCR/Southern blot hybridization method for MMP-2 and MT1-MMP (**Figure 4B**) revealed again the inhibition of mRNA expression for MMP-2 and MT1-MMP by EGCG, confirming the results of the Northern blot analysis.

EGCG-Mediated Suppression of MMP-9 Promoter Activity. It was reported that EGCG affects transcription of MMP-2 and MT1-MMP (*31*). To examine the effect of EGCG on MMP-9 transcription, HT1080 cells were transiently transfected with a luciferase reporter construct containing −670 to +57 bp of the 5′ regulatory region of the human MMP-9 gene. As
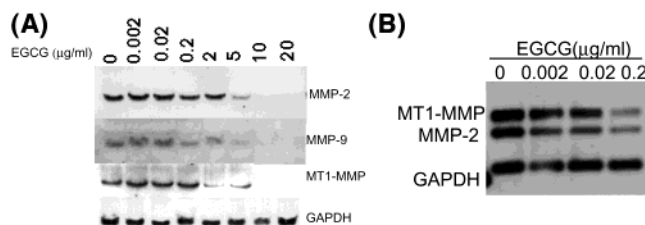
Figure 4. EGCG-mediated suppression of MMP-2, MMP-9, and MT1-MMP transcripts in HT1080 cells. HT1080 cells were incubated for 8 h in serum-free DMEM in the presence or absence of EGCG. After incubation, total RNA was isolated, and aliquots (10 μg) were analyzed for the levels of MMP-2, MMP-9, MT1-MMP, and GAPDH mRNAs by Northern blot hybridization (A) or RT-PCR/Southern hybridization (B).
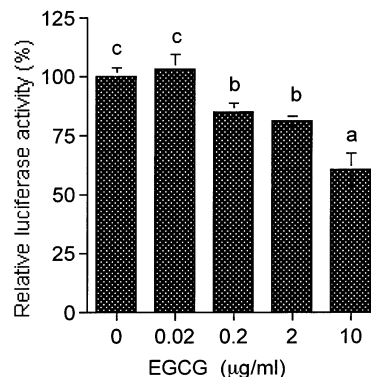
Figure 5. Effects of EGCG on a reporter construct containing the promoter region of human MMP-9 gene. Results are expressed as relative luciferase activity (%) (*n* = 3, mean ± SD). Values with different letters are significantly different at *P* < 0.05.

summarized in **Figure 5**, concentrations of EGCG ranging from 0.2 to 10 μg/mL significantly suppressed luciferase activity. This finding suggests that a major target of EGCG action is the level of gene transcription.

Inhibition by EGCG of ERK1/2 Phosphorylation. To examine whether the activities of MAPKs are down-regulated by EGCG, we analyzed the phosphorylation of MAPKs in HT1080 cells after treatment with EGCG (0.1−10 μg/mL) for 1 h. As shown in **Figure 6A**, EGCG inhibited ERK1 and ERK2 phosphorylation activities with a greater effect on ERK1. Densitometric determination indicated that the treatment of HT1080 cells with 1 μg/mL EGCG resulted in a 50% reduction in the amount of phosphorylated ERK1. EGCG also inhibited p38 MAPK activity at 1 μg/mL (**Figure 6B**), whereas it did not affect SAPK/JNK activity significantly (**Figure 6C**).
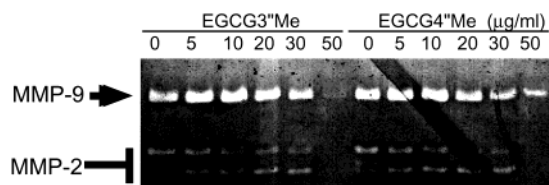
Activities of *O*-Methyl Derivatives of EGCG. Two *O*-methyl derivatives were examined for their effects on the MMP-2 and MMP-9 enzyme activities in the culture medium of HT1080 cells and on their mRNA levels. The results indicated that these derivatives showed inhibitory activities similar to, but weaker than, those of EGCG (**Figures 7** and **8**).

DISCUSSION

In this study, we obtained findings showing that EGCG causes the decreased levels of enzyme activities of MMP-2 and MMP-9 secreted into the serum-free conditioned medium by HT1080 cells in a concentration-dependent manner, confirming our previous observation (*9*). This suppression of MMP activities was correlated with the results of Northern blot analysis demonstrating that EGCG caused the decrease in the MMP-2 and MMP-9 mRNA levels dose-dependently.

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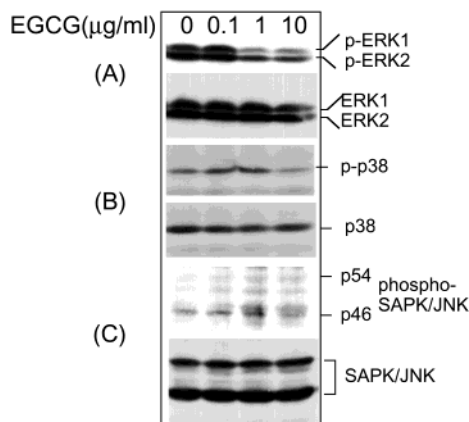
Figure 6. Phosphorylation level of ERK (A), p38 MAPK (B), and SAPK/JNK (C) in HT1080 cells treated with EGCG. HT1080 cells were treated with EGCG (0.1–10 μg/mL) for 1 h. Whole cell lysates were separated on 10% SDS–polyacrylamide gel electrophoresis, and active phosphorylated proteins were detected by the immunoblot analysis with phospho-ERK, phospho-p38 MAPK, and phospho-SAPK/JNK antibodies. Data are representative of three independent experiments.

Figure 7. Gelatinolytic activities of MMP-2 and MMP-9 in the conditioned media from human fibrosarcoma HT1080 cells treated with methylated derivatives of EGCG. Subconfluent monolayers of HT1080 cells were cultured for 24 h in serum-free DMEM in the presence of various concentrations of EGCG3″Me and EGCG4″Me. The conditioned media were then analyzed by gelatin zymography.
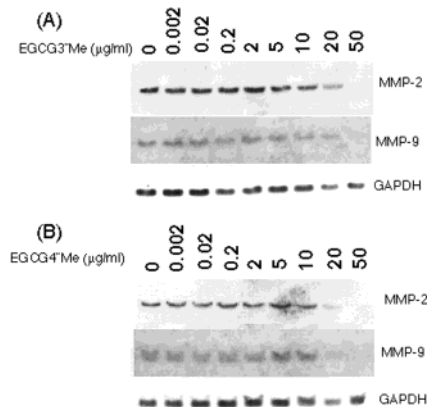
Figure 8. Effect of O-methylation of EGCG on suppression of MMP-2 and MMP-9 transcripts in HT1080 cells. HT1080 cells were incubated for 18 h in serum-free DMEM in the presence of EGCG3″Me (A) or EGCG4″Me (B). After incubation, total RNA was isolated, and aliquots (10 μg) were analyzed for the levels of MMP-2, MMP-9, and GAPDH mRNAs by Northern blot hybridization.

EGCG was also found to inhibit transcription of MT1-MMP. Because MT1-MMP is responsible for activation of MMP-2 (*32*), it is possible that this may contribute to the decreased level of the MMP-2 activity in the cultured medium.

Very recently, Annabi et al. (*31*) reported that the addition of EGCG inhibited the MT1-MMT-dependent proMMP-2 activation in U-87 glioblastoma cells. The inhibitory effect of

EGCG on MT1-MMP was also demonstrated by the down-regulation of MT1-MMP transcript levels and by the inhibition of MT1-MMP-driven cell migration of transfected COS-7 cells. The present findings are basically consistent with these results.

In this study, luciferase assay findings suggested that EGCG also suppressed the transcription of MMP-9 gene by inhibiting the promoter activity in accordance with the decreased level of mRNA for MMP-9 in HT1080 cells.

In the next experiment, we examined the effects on ERKs, p38 kinase activities, and SAPK/JNK. The results indicated that EGCG inhibited the activation of ERK1/2. EGCG also caused the suppression of p38 MAPK activity, but not SAPK/JNK activity. These protein kinases are MAPK family enzymes associated with MMP-9 gene expression (*10*, *11*, *13*, *15*, *17*, *21*−*24*) and the protein expression of MMP-9 (*12*, *14*, *16*, *18*−*20*).

Most previous studies support the notion that inhibition of MAPK suppresses MMP expression. For example, Reddy et al. (*15*) reported that when SKBR-3 cells are exposed to MAPK inhibitors such as apigenin or MAPK antisense phosphorothioate oligodeoxynucleotides, epidermal growth factor-induced cell proliferation, MMP-9 induction, and invasion through the reconstituted basement membrane are significantly reduced. Simon et al. (*13*) demonstrated that phorbol ester-enhanced MMP-9 secretion and in vitro invasiveness are associated with a strong activation of the p38 MAPK. Kurata et al. (*33*) reported that constitutive activation of MAP kinase kinase is critical and sufficient for the activation of MMP-2.

Furthermore, in this connection, several studies have provided evidence suggesting that EGCG inhibits MAPK, resulting in down-regulation of MMPs. For example, Chung et al. (*22*) showed that EGCG treatment decreased the levels of phospho-ERK1/2 and phospho-MEK1/2 time-dependently in mouse epidermal JB6 cells. Katiyar et al. (*23*) demonstrated that pretreatment of human epidermal keratinocytes with EGCG inhibited ultraviolet-B-induced hydrogen peroxide production and hydrogen peroxide-mediated phosphorylation of the MAPK signaling pathway. In addition, EGCG was reported to be an inhibitor of ultraviolet-B-induced p38 MAPK activation in human keratinocytes (*34*). The present results are compatible with these previous observations.

In contrast, in the case of human squamous cell carcinoma UM-SCC-1 cells, MAPK pathway inhibitors were reported to abrogate retinoic acid-mediated down-regulation of MMP-9 activity and invasion (*35*). Chen et al. (*17*) reported the activation of ERK1 and JNK1 by green tea polyphenol in human hepatoma HepG2 cells. More recently, Park et al. (*24*) reported that EGCG-mediated cyclooxygenase-2 expression and prostaglandin E2 production were associated with the activation of ERKs, SAPK/JNK, and p38 kinase.

In the case of human epidermoid carcinoma A431 cells, no apparent inhibition of MAPK-ERK1/2 activation was observed upon treatment of the cells with EGCG (*21*).

These differences may be explained, at least partly, by the difference in the cell types used.

Two methylated derivatives of EGCG, EGCG3″Me and EGCG4″Me, were newly identified as antiallergic compounds in Taiwanese oolong tea and certain varieties of green tea, and the antiallergic activities of these derivatives are higher than that of EGCG (*25*, *36*). Our previous comparison of tea catechins suggested a gallate ester group plays an important role in the reduction of MMP activities of catechin-treated HT1080 cells (*9*). The present findings showed that these methylated deriva-

tives also showed effects that reduce the enzyme activities of these MMPs in the culture medium. These decreases in the enzyme activities were in accordance with their down-regulation of the expression of mRNAs for these MMPs. However, their weaker activities realtive to that of EGCG suggest the importance of the unsubstituted triphenolic structure in a gallate ester group in these activities. The precise structure−function relationship required remains to be determined.

On the other hand, green tea catechins are known to inhibit directly enzyme activities of MMP-2 and MMP-9 (*9, 37−40*). In addition to these direct actions of EGCG on MMP, the present findings suggest that suppression of activation of MAPKs including ERK by EGCG causes the decreased level of MMP expressions in human sarcoma HT1080 cells. This would also contribute to the decreased invasion of these cells through the reconstituted basement membrane in the presence of EGCG as observed previously (*9*).

ABBREVIATIONS USED

DMEM, Dulbecco's minimum essential medium; EGCG, epigallocatechin gallate; EGCG3″Me, epigallocatechin 3-*O*-(3-*O*-methyl) gallate; EGCG4″Me, epigallocatechin 3-*O*-(4-*O*-methyl) gallate; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; SDS, sodium dodecyl sulfate.

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