Modifying Effects of Dietary Factors on (−)-Epigallocatechin-3-gallate-induced Pro-matrix Metalloproteinase-7 Production in HT-29 Human Colorectal Cancer Cells

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Received April 13, 2007; Accepted June 18, 2007; Online Publication, October 7, 2007
[doi:10.1271/bbb.70213]

(−)-Epigallocatechin-3-gallate (EGCG), one of the main constituents of green tea, has been reported to function as an antioxidant with chemopreventive potential. In contrast, we have recently reported that EGCG enhanced pro-matrix metalloproteinase (MMP)-7 in HT-29 human colon cancer cells via spontaneous superoxide generation. In the present study, we examined the effects of dietary antioxidants on both spontaneous and EGCG-upregulated proMMP-7 production in HT-29 cells. Benzyl isothiocyanate (BITC), curcumin (CUR), gallic acid (GA), and N-acetyl-L-cysteine (NAC) reduced that production, while each alone did not have any effect on spontaneous production. None of the dietary factors suppressed EGCG-induced hydrogen peroxide generation in the media tested, whereas BITC, GA, and NAC inhibited the EGCG-enhanced activator protein (AP)-1 transcription activity by 126%, 77%, and 97%, respectively. Although CUR abolished the EGCG-upregulated MMP-7 mRNA expression, it unexpectedly enhanced the AP-1 activity by 502%, suggesting that this factor may disrupt the MMP-7 mRNA stabilization process. Together, our results indicate that dietary antioxidants modulate EGCG-induced MMP-7 production through different mechanisms.

Key words: (−)-epigallocatechin-3-gallate; matrix metalloproteinase (MMP)-7; dietary factor; antioxidant; HT-29

(−)-Epigallocatechin-3-gallate (EGCG) and green tea polyphenols (GTP) are known to be effective antioxidants and are recognized as promising candidates for cancer chemoprevention. However, there is an increasing body of evidence showing that GTP, including EGCG, also act as pro-oxidants. Matrix metalloproteinase (MMP)-7 secreted by cancer cells plays a pivotal role in the early stages of colon cancer, and also promotes their invasion and metastasis. In our previous study, EGCG enhanced proMMP-7 production in HT-29 human colon cancer cells by inducing oxidative stress. It has been revealed that the natural polyphenols, caffeic acid and sesamol, were tumorigenic toward rat forestomach epithelia, and catechol and p-methyleacetechol have been shown to induce neoplasia in rat glandular stomach epithelial specimens. Further, the synthetic antioxidant, butylated hydroxyanisole, has been found to have carcinogenic potential in rat and hamster forestomach epithelia. However, it is puzzling that β-carotene, a representative natural antioxidant, does not decrease cancer risk with supplemental consumption, whereas its combination with the antioxidants, vitamin E and selenium, significantly reduced cancer mortality in a Chinese population. Antioxidants used in combination, in which several compounds with different modes of actions are used rather than a single agent, is emerging as a new trend in chemoprevention strategies. For example, sulindac alone, a promising cancer-preventive agent for colon cancer, had adverse effects, although its combination with EGCG synergistically suppressed aberrant crypt focus formation without notable side-effects. Further, a combination of EGCG with curcumin (CUR), a dietary pigment from turmeric, synergistically inhibited the proliferation of oral cancer cells. In addition, genistein (GEN), a soy isoflavone, compensated for the adverse effects of EGCG, and was found to potentiate interferon-γ and lipopolysaccharide (LPS)-induced prostaglandin E2 production in macrophages. Based on this background, we investigated the effects of EGCG in combination with selected dietary factors on the spontaneous and EGCG-induced proMMP-7 production in HT-29 human colorectal cancer cells.
Materials and Methods

Reagents. EGCG and all other chemicals (Osaka, Japan), unless specified otherwise. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Antibodies were purchased from the following sources: anti-h proMMP-7 (F-82), Dako (Glostrup, Denmark). Oligonucleotide primers were synthesized by Proligo (Kyoto, Japan); goat anti-β-actin antibody, Santa Cruz Biotechnology (Santa Cruz, CA, USA); and horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-goat IgG, Dako (Glostrup, Denmark). Oligonucleotide primers were synthesized by Promega (Madison, WI, USA). A QIAGen (Hilden, Germany), and an RNA PCR kit (ver. 2.1, AMV) came from Takara Bio (Shiga, Japan).

Cell culture. HT-29 human colorectal cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS, l-glutamine (330 μg/ml), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

Western blotting. The cells (3 x 10⁵ cells/1.7 ml in a 35-mm dish) were incubated for 24 h, then washed twice with PBS and cultured in serum-free DMEM. After being pre-treated with 0.05% dimethylsulfoxide (DMSO) (v/v) or the dietary factors [all-trans-retinoic acid (ATRA, 20 μM), ascorbic acid (AA, 50 μM), benzyl isothiocyanate (BITC, 10 μM), CUR (20 μM), gallic acid (GA, 10 μM), [6]-gingerol (GIN, a major pungent component of ginger, 100 μM), GEN (50 μM), resveratrol (RES, a trihydroxystilbene present in red wine, 50 μM), capsacin (CAP, a pungent ingredient of hot red and chili peppers, 100 μM), and N-acetyl-L-cysteine (NAC, 1 mM)] for 30 min, the cells were exposed to 25 μM of EGCG or the vehicle alone (0.05% DMSO, v/v) for 1 h. After washing, the cells were incubated for another 23 h in serum-free DMEM. Negative control cells were treated only with 0.1% DMSO, which had no effect on the assay system (data not shown). The cells were lysed in a lysis buffer [a protease and phosphatase inhibitor cocktail (Takara Bio, Shiga, Japan), 10 mM Tris at pH 7.4, 1% sodium dodecyl sulfate (SDS), and 1 mM sodium vanadate (V)]. The protein concentration was determined by a DC protein assay (Bio-Rad Laboratories, Tokyo, Japan), with γ-globulin being used as the standard. Denatured proteins (10 μg of proMMP-7 and β-actin) were separated by using SDS–polyacrylamide gel electrophoresis on 10% polyacrylamide gel and transferred on to Immobilon-P membranes (Millipore, MA, USA). After blocking overnight at 4 °C in Block Ace (Dainippon Pharmaceutical, Osaka, Japan), the membranes were first incubated with each mouse anti-hMMP-7 and goat anti-β-actin, while the second incubation was performed with HRP-conjugated secondary anti-mouse IgG and anti-goat IgG (each 1:1000). The blots were developed by using the ECL Advance western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK), and the intensity of each band was analyzed by using NIH Image. The relative levels of intracellular proMMP-7 were corrected by employing β-actin as the internal standard.

Quantification of H₂O₂. The concentrations of H₂O₂ in the cell culture media were measured by the ferrous ion oxidation-xylene orange method with Bionycin H₂O₂-560 (OXIS International, Portland, OR, USA), according to the manufacturer’s instructions. The cells were inoculated on to 6-well plates at a density of 3.4 x 10⁵ cells/1.7 ml and incubated for 24 h. After being washed twice with PBS, the cells were incubated in 1.7 ml of serum-free DMEM. The cells were then pre-treated with the vehicle (0.05%, v/v) or a dietary factor (ATRA, BITC, CUR, GA, or NAC) for 30 min, then exposed to 25 μM of EGCG or the vehicle (0.05%, v/v), and incubated for an additional 30 min. The media were then collected into micro-tubes which were centrifuged at 1200 x g for 1 min and tested with the kit. Visible absorption at 550 nm was measured by a UV-spectrophotometer (Shimadzu, Kyoto, Japan). A standard curve was prepared from the absorbance values from known concentrations of H₂O₂ (0–100 μM). The background value of the vehicle was subtracted from the value for each sample.

Reporter assay. An activator protein (AP)-1 promoter/luciferase vector (0.16 μg; BD Bioscience Clontech, Tokyo, Japan) together with 0.16 μg of a control pRL-TK vector (Promega, Madison, WI, USA) were transiently transfected into 2 x 10⁵ HT-29 cells/well in a 24-well plate using the Lipofectamine/Plus reagent™ (Invitrogen), according to the instructions provided by the manufacturer. After 6 h, the cells were pre-treated for 30 min with DMSO or selected dietary factors dissolved in DMSO (0.05%, v/v), before being exposed to DMSO (0.05%, v/v) or 25 μM of EGCG for 1 h. After being washed twice with PBS, the cells were incubated for another 17 h and lysed. The luciferase activity was determined with a Dual-luciferase Reporter assay kit™ from Promega and a luminometer (Berthold Detection System, Pforzheim, Germany), normalized against the protein amount and expressed as number fold induction over the control cells.

Reverse transcription-polymerase chain reaction. HT-29 cells were inoculated on to a 60-mm dish at a density of 1 x 10⁶ cells/ml and incubated for 24 h. The cells were washed twice with PBS, cultured in serum-free DMEM, and then pre-treated with DMSO (0.05%, v/v) or a dietary factor for 30 min, before DMSO or 25 μM of EGCG dissolved in DMSO (0.05%,
v/v) was added to the cells, and the culture incubated for 1 h. The cells were washed twice with PBS, incubated in serum-free DMEM for 5 h, and then harvested. Total RNA was extracted by using QIAshredder™ and RNAase-free DNase sets, and an RNeasy Mini kit™. A glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) transcript served as the internal control. cDNA was synthesized by using 1 μg of total RNA and an RNA PCR kit (AMV), PCR amplification was then performed with a thermal cycler (PTC-100™, MJ Research, Watertown, MA, USA) using MMP-7 sense (5'-TCTTT-TggCCTA CCTATAACTgg-3') and antisense (5'-CTAgCTgCTACCAT CCgTC-3') primers (0.15 μM each, product size of 420 bp), and GAPDH sense (5'-gTgA-AggTCggAgTCAACg-3') and antisense (5'-ggTgAA-gACgCCAgTggA CTC-3') primers (0.05 μM each, product size of 300 bp). The PCR conditions consisted of 23 cycles, with 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C, and 30 s of primer extension at 72 °C. Amplified cDNA was electrophoresed on 2% agarose gel and an image analysis was performed by using NIH Image.

Statistical analysis. Each experiment was performed at least 3 times, and the data are shown as the mean ± standard deviation (SD) where applicable. GraphPad InStat 3.05 (San Diego, CA, USA) was used for the statistical analysis. Statistical significance was tested by ANOVA and the Dunnet test, with p < 0.05 as the criterion considered to indicate significance.

Results

Effects of dietary factors on proMMP-7 production with or without EGCG

To investigate the effects of dietary factors on EGCG-induced intracellular proMMP-7 production, HT-29 human colorectal cells were pre-treated with the vehicle or each dietary factor separately for 30 min, and then exposed to 25 μM of EGCG or the vehicle. After 1 h, the cells were washed and incubated for an additional 23 h. The concentration of each test sample was determined to be the non-toxic maximum (data not shown). Twenty-five μM of EGCG enhanced the production of intracellular proMMP-7 protein by 6-fold (Fig. 1A), as previously reported,9) whereas 20 μM of ATRA, a vitamin A metabolite,22) but not the other dietary factors, enhanced that production by 2-fold (Fig. 1A). On the other hand, separate pre-treatments with 1 mM NAC, 10 μM of BITC, 10 μM of GA, and 20 μM of CUR reduced the EGCG-induced protein production in the range of 61% to 78%, as compared with the EGCG-treated cells (Fig. 1B). Similar results were seen for extracellular proMMP-7 protein (data not shown).

Effects of selected dietary factors on EGCG-generated H₂O₂ in the cell culture systems

Free radicals are known to induce MMP expression and activity,23,24) via an oxidative mechanism,9) while EGCG has been reported to generate H₂O₂ in cell-free and cellular systems.25) We thus examined the effects of proMMP-7-suppressive dietary factors on EGCG-induced H₂O₂ generation. Cells were pre-treated separately with each dietary factor for 30 min, before being exposed to 25 μM of EGCG or the vehicle for an additional 30 min. Only EGCG and none of the selected dietary factors generated H₂O₂, and none attenuated EGCG-induced H₂O₂ generation under the present conditions (Fig. 2).

Effects of dietary factors on EGCG-induced AP-1 transcriptional activity

EGCG-induced MMP-7 production is dependent on AP-1 transcription activity.9) We transiently transfected the AP-1 promoter/luciferase and control vectors into HT-29 cells, and pre-treated them with each compound or the vehicle for 30 min, before exposing them to 25 μM of EGCG or the vehicle for 1 h. After being washed, the cells were incubated for another 17 h with each individual dietary factor. As shown in Fig. 3, ATRA without the EGCG treatment enhanced the AP-1 transcription activity by 2.1-fold, whereas none of the other dietary factors had any significant effect. Furthermore, EGCG alone enhanced the AP-1 transcription activity by 1.7-fold, as previously reported.9) ATRA increased the EGCG-upregulated AP-1 transcription activity by 154%, whereas BITC, NAC, and GA markedly suppressed it by 70% or more. To our surprise, CUR significantly enhanced the EGCG-induced AP-1 transcription activity by 502%, whereas it decreased the EGCG-induced proMMP-7 protein production (Fig. 1B).

Effects of dietary factors on EGCG-induced MMP-7 mRNA expression

We investigated the effects of the dietary factors on spontaneous and EGCG-induced MMP-7 mRNA expression in HT-29 cells. The vehicle-treated (6 h) cells expressed MMP-7 mRNA at a low level (Fig. 4), while EGCG upregulated that expression by 2.7-fold as compared with the vehicle-treated cells after 6 h, this result being consistent with our previous report.9) However, none of the other dietary factors alone had any significant effect on MMP-7 mRNA expression as compared to the vehicle-treated cells at 6h. Furthermore, NAC, CUR, BITC or GA, although not significantly different, each markedly suppressed the EGCG-upregulated MMP-7 expression, as compared with the EGCG-treated cells (Fig. 4).

Discussion

Some potential antioxidants have paradoxical activities. For instance, the scavenger of free radicals,26) NAC, induces nitric oxide generation.27) Furthermore, while AA (vitamin C) and the cruciferous vegetable constituent, BITC, are known to be effective antiox-
Fig. 1. Effects of Dietary Factors on Spontaneous and EGCG-Enhanced ProMMP-7 Protein Production in HT-29 Human Colon Cancer Cells.

The cells were pre-treated separately with each dietary factor or the vehicle (DMSO, 0.05%) for 30 min, and then by exposure to the vehicle (A) or 25 μM of EGCG (B) for 1 h. The cells were then washed twice with PBS and incubated for an additional 23 h, after which western blotting for proMMP-7 was performed as described in the Materials and Methods section. Each lane was loaded with 10 μg of the cell lysate. The proMMP-7 protein was normalized by β-actin as the internal standard. *p < 0.01 vs DMSO+DMSO by one way ANOVA with the dunnet test. Each value is shown as the mean ± SD of results from three independent assays, with one representative result shown for each.
Fig. 2. Effects of Dietary Factors on Spontaneous and EGCG-Induced H$_2$O$_2$ Generation in HT-29 Cells.

The cells were pre-treated separately with each dietary factor or the vehicle (DMSO, 0.05%) for 30 min, and then by exposure to the vehicle or 25 µM of EGCG for 30 min. The medium was harvested and H$_2$O$_2$ quantified as described in the Materials and Methods section. *$p < 0.01$ vs DMSO+DMSO by one way ANOVA with the Dunnett test. Each value is shown as the mean ± SD of results from three independent assays.

Fig. 3. Effects of Dietary Factors on Spontaneous and EGCG-Enhanced AP-1 Transcription Activity.

AP-1 promoter/luciferase and control vectors were transfected into HT-29 cells. The cells were pre-treated separately with each tested dietary factor or the vehicle for 30 min, and then by exposure to the vehicle or 25 µM of EGCG for 1 h. After washing with PBS, cells were incubated for an additional 17 h in serum-free DMEM. The cells were harvested, and the AP-1 activity was assayed by using a Dual-luciferase Reporter assay kit™ (Promega) with a luminometer, as described in the Materials and Methods section. Data were analyzed by using one way ANOVA and the Dunnett test and are expressed as the results of three independent assays. *$p < 0.05$, *$p < 0.01$ vs DMSO+EGCG.
idants and chemopreventive agents, they have also been reported to induce carcinogenesis in experimental models. In addition, EGCG, a potential chemopreventive agent, enhanced proMMP-7 production in HT-29 human colon cancer cell lines in our previous study. These results suggest the potential of natural agents alone to increase the risk of cancer or onset of the disease. In fact, misuse of /C12-carotene for intervention has revealed the need for more careful evaluation of drug safety before using an antioxidant at a high dose for chemoprevention.

Meanwhile, antioxidants in combination is a new trend in chemopreventions, and the findings of several combinatorial trials have already been presented. For example, synthetic drugs combined with natural compounds or preparations, as well as other types, have been reported. The results from several combination studies with natural antioxidants, including EGCG and CUR, EGCG and GEN, and several food factors, have recently been reported and suggest that combinations of different types of chemicals at low concentrations may be an effective chemopreventive strategy. We investigated in the present study the effects of combinations of dietary antioxidants with EGCG to elucidate whether they could compensate for the inverse effect of EGCG. The present western blotting results show that several dietary factors (NAC, BITC, GA and CUR) had a suppressive effects toward EGCG-enhanced proMMP-7 production, while none modulated spontaneous proMMP-7 protein production (Fig. 1A and B). Furthermore, ATRA with or without EGCG did not reduce proMMP-7 protein production. That result is not consistent with the report of Adachi, who found that ATRA reduced the invasion by HT-29 and BM314 human colon cancer cells through down-regulated spontaneous MMP-7 secretion. This discrepancy may be due to the different doses of ATRA used. We also investigated the effects of the selected dietary factors on spontaneous and EGCG-induced H2O2 generation. EGCG has pyrogallol and catechol moieties. Its catechol structure is readily converted into ortho-quinine, thereby liberating superoxide, this superoxide being subsequently converted to H2O2 in a medium. GA, a phenolic phytochemical, has also been reported to generate H2O2 in a cell culture medium. However, in the present study, no dietary factors, including GA, exhibited any significant effect on spontaneous or EGCG-induced H2O2 generation (Fig. 2). Those results may be attributable to differences in the incubation times and doses. AP-1 is an important transcription

![Fig. 4. Effects of Dietary Factors on Spontaneous and EGCG-Enhanced MMP-7 mRNA Expression. The cells were pre-treated with each tested dietary factor or the vehicle for 30 min, and then by exposure to the vehicle or 25 μM of EGCG for 1 h. The cells were then washed twice with PBS and incubated for an additional 5 h. GAPDH was used as the internal control. a: p < 0.01 vs DMSO+DMSO; b: p < 0.01 vs DMSO+EGCG by one way ANOVA with the dunnet test. Each value is shown as the mean ± SD of results from three independent assays, with one representative result shown for each.](image-url)
factor that contributes to MMP-7 expression. EGCG alone enhanced the AP-1 DNA binding activity, as also shown in previous studies, and BITC, GA and NAC suppressed that activity in the present experiments (Fig. 3). These results suggest that the inhibitory effects of those dietary factors on EGCG-induced MMP-7 may be associated with down-regulation of the AP-1 transcription activity. To support this hypothesis, it has been reported that BITC and NAC suppressed the AP-1 transcription activity, while NAC and BITC similarly inhibited tumor necrosis factor-α-induced MMP-9 expression by down-regulating the nuclear factor-kappaB (NF-κB) and AP-1 transcription activities. In contrasting results, CUR enhanced the EGCG-induced AP-1 transcription activity, whereas it markedly suppressed the EGCG-enhanced MMP-7 mRNA expression (Figs. 3 and 4). The mRNA expression of some, if not all, proinflammatory genes is regulated by a post-transcriptional mechanism. Along a similar line, our group has recently reported that zernobume abrogated LPS-induced cyclooxygenase-2 mRNA expression, while it did not show any suppression of the transcriptional activation of NF-κB and AP-1. This raises the possibility that CUR might have suppressed MMP-7 mRNA expression via a post-transcriptional mechanism, though further detailed investigation is necessary to confirm this.

In conclusion, our results provide partial insight into the action mechanism of ATRA, BITC, CUR, GA, and NAC toward EGCG-enhanced MMP-7 production. BITC, GA, and NAC were each shown to suppress the EGCG-induced AP-1 transcriptional activities, while CUR may have disrupted EGCG-induced MMP-7 mRNA stabilization without perturbing the EGCG-enhanced AP-1 activity. On the other hand, ATRA did not decrease spontaneous or EGCG-enhanced MMP-7 production. We therefore found that the interactions of dietary factors with EGCG differed. Additional investigations are necessary to elucidate the mechanism of action of these agents, although a combination strategy based on their distinct molecular mechanisms may be warranted.

Acknowledgments

This research was supported by grant aid from the Ministry of Agriculture, Forestry and Fisheries of Japan (to A.M.).

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