Inhibitory Effect of Green Tea Polyphenols on Membrane-Type 1 Matrix Metalloproteinase, MT1-MMP1)

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Matrix metalloproteinases (MMPs), especially membrane-type 1 matrix metalloproteinase (MT1-MMP), which generates an active form of MMP-2 from proMMP-2, are deeply involved in angiogenesis as well as in tumor cell migration and metastasis. To obtain a specific inhibitor for MT1-MMP, we screened a number of natural and synthetic compounds using recombinant human MMP-2, MMP-7, and soluble MT1-MMP in a fluorogenic peptide cleavage assay. (−)-Epigallocatechin 3-O-gallate (EGCG) followed by (−)-epigallocatechin 3,5-di-O-gallate and epiteaflagallin 3-O-gallate, was found to have potent and distinct inhibitory activity against MT1-MMP. Therefore, we investigated the effect of EGCG on the suppression of MMP-2 activation as determined by gelatin zymography, and observed that the active form of MMP-2 in the conditioned medium of human umbilical vein endothelial cells was decreased in the presence of EGCG. The results suggest the possibility that tea polyphenols suppress tumor growth through the suppression of angiogenesis.

Key words membrane-type 1 matrix metalloproteinase (MT1-MMP); green tea; catechin; angiogenesis

Angiogenesis has been revealed to be a critical event for solid tumor growth and metastasis.1,2) Thus, inhibition of this process causes suppression of tumor growth by cutting off the supply of oxygen and nutrients. Many antiangiogenic agents have been developed toward this end, and some of them are now in preclinical and clinical trials.3,4) Angiogenesis is initiated by the release of certain factors from tumor cells, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF).5) These factors activate the endothelial cells of pre-existing blood vessels, and promote the migration and growth of these cells.6) Degradation of the basement membrane is a prerequisite for the migration of endothelial cells into the tumor tissue, and is mediated by matrix metalloproteinases (MMPs).7) MMP-2 (type IV collagenase/gelatinase A) and MMP-9 (gelatinase B), in particular, are responsible for tumor angiogenesis.8,9) MMP-2 is secreted from various tumor cells in a pro-enzyme form, i.e., as an enzymologically inactive zymogen, and is activated by processing mainly by membrane-type 1 MMP (MT1-MMP).10,11) Therefore, suppression of MT1-MMP activity at the tumor site is an interesting approach from the viewpoint of angiogenesis suppression; and so a potent inhibitor of MT1-MMP would be expected to be a useful antitumor agent by blocking angiogenesis.

In the present study, we first established a novel method using a fluorophore-conjugated MMP substrate to detect various MMP activities. Then, we screened approximately 170 compounds for specific inhibition of MT1-MMP activity. As a result, we found that tea polyphenols, especially (−)-epigallocatechin gallate (EGCG), had potent inhibitory activity against MT1-MMP. The cancer chemopreventive activity of green tea, from the leaves of *Camellia sinensis*, has been epidemiologically suggested, and tea polyphenols have been shown to be the active substances.12) In fact, green tea polyphenols cause dysregulation of mitotic signaling cascades and induce apoptosis of tumor cells.13–15) Therefore, the screening result that EGCG specifically inhibited MT1-MMP is reasonable and interesting. Moreover, we demonstrated that EGCG actually inhibited activation of MMP-2 from proMMP-2. The present results suggest that another aspect of the antitumor activity of EGCG is the inhibition of angiogenesis via the blocking of MMP-2 activation.

MATERIALS AND METHODS

Preparation of Recombinant Human MMPs Recombinant human MMP-2, MMP-7 (matrilysin), and soluble MT1-MMP were prepared according to the method described previously.16) In brief, the cDNAs for procatalytic domains of human MMP-2, MMP-7, and MT1-MMP were prepared by polymerase chain reactions using sets of primers (5′ primer, GGCGGATCCATGCTGCCGTCGCCCATCRTC, 3′ primer; GCCGTGACTACAATTGCTTGGTGTGCAGAT; 5′ primer, GGCGGATCCATGCTCGCCTCCCTCGGCTCG, 3′ primer, GGCAGATCCATGTCGCTCGCTCCCTCGGCTCG, 3′ primer, GGCGGATCCATGTCGCTCGCTCCCTCGGCTCG, 3′ primer, GGCAGATCCATGTCGCTCGCTCCCTCGGCTCG, respectively) based on the reported sequences, and the templates (pSG-GeLa having a 3.3 KbdDNA fragment of MMP-2, poly(A)+ RNA isolated from a human rectal carcinoma cell line [CaR-1], and pME18S-MTMMP having a 3.5 KbdDNA fragment of MT1-MMP, respectively). The resulting PCR fragments were inserted into the bacterial expression plasmid pTH-72, having a tandem repeat of the T7 promoter and a hexahistidine-Tag encoding sequence. The expression, purification, and refolding of the human recombinant MMPs were performed by the following procedures: Human recombinant proMMPs were produced in E. coli strain BL21 (DE3) transfected with the corresponding expression plasmids derived from pTH-72, solubilized in 8 M urea/10 mM

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Tris–HCl/100 mM Na-phosphate (pH 8.0)/100 mM β-mercaptoethanol, purified with Ni-NTA resin (QIAGEN Inc., U.S.A.), and refolded by using the urea concentration.

**Microplate Assay for MMPs** MMP substrate peptide, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[N-F(2,4-dinitrophenyl)-I-2,3-diaminopropionyl]-Ala-Arg-NH$_2$(17) was purchased from Peptide Institute Inc. (Osaka, Japan). Sixty microliters of reaction mixture containing an appropriate amount of rhMMPs and 20 μl of test sample solution was pre-incubated in each well of a microplate at 37°C for 15 min, and then 120 μl of fluorogenic substrate peptide (2.5 μM as final concentration) was added to initiate the proteolytic reaction. The fluorescence intensity was measured at 340 nm for excitation and 400 nm for emission every 15 min, and then 120 μl of fluorogenic substrate peptide (2.5 μM as final concentration) was added to initiate the proteolytic reaction. The fluorescence intensity was measured at 340 nm for excitation and 400 nm for emission every 15 min for a 2 h of period without interrupting the reaction by using a fluorescence microplate reader (MTP-100F, Corona Electric, Tokyo). The IC$_{50}$ values were calculated based on the resultant data at 2 h.

**Gelatin Zymography** Human umbilical vein endothelial cells (HUVECs, BioWhittaker, Walkersville, MD, U.S.A.) were cultured in endothelial cell growth medium (EGM, BioWhittaker) in a CO$_2$ incubator. HUVECs were seeded onto 6-well plates (1.0 × 10$^5$ cells/well) precoated with 0.1% gelatin (Difco Laboratories, MI, U.S.A.). After a 24 h incubation, the medium was changed to serum-free endothelial cell basal medium (EBM, BioWhittaker) and EGCG solution was added to some of the wells. After a 24 h incubation, the medium conditioned by the HUVECs was centrifuged at 220 g for 5 min and mixed with sample buffer containing 1.25 mM Tris–HCl (pH 6.8), 40% glycerol, 8% SDS, and

### Table 1. Inhibitory Activity of Polyphenols on MMPs (IC$_{50}$, μM)

<table>
<thead>
<tr>
<th>Flavans-3-ols</th>
<th>rhMT1-MMP</th>
<th>rhMMP-2</th>
<th>rhMMP-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>(−)-Epiafzelechin</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>(−)-Epiafzelechin 3-O-gallate</td>
<td>1.8</td>
<td>&gt;100</td>
<td>30</td>
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<tr>
<td>(−)-Epicatechin</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>(−)-Epicatechin 3-O-gallate</td>
<td>9.4</td>
<td>&gt;100</td>
<td>25</td>
</tr>
<tr>
<td>(−)-Epicatechin 3-O-(3′-O-methyl)-gallate</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>(−)-Epicatechin 3-O-(4′-O-methyl)-gallate</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>98</td>
</tr>
<tr>
<td>(−)-Epigallocatechin</td>
<td>20</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>(−)-Epigallocatechin 3-O-gallate</td>
<td>0.019</td>
<td>&gt;100</td>
<td>1.6</td>
</tr>
<tr>
<td>(−)-Epigallocatechin 3,5-di-O-gallate</td>
<td>0.022</td>
<td>23</td>
<td>0.25</td>
</tr>
<tr>
<td>(−)-Epigallocatechin 3′,3′- and 3,4′-di-O-gallate (mixture)</td>
<td>0.044</td>
<td>17</td>
<td>0.45</td>
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<tr>
<td>(−)-Epigallocatechin 3-O-p-coumaroate</td>
<td>4.6</td>
<td>62</td>
<td>15</td>
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<tr>
<td>(+)-Catechin</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>(−,−)-Gallocatechin</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>8.0</td>
</tr>
<tr>
<td>(−)-Catechin 3-O-rhamnside</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>8-C-Ascorbyl (−)-epigallocatechin 3-O-gallate</td>
<td>0.036</td>
<td>25</td>
<td>0.46</td>
</tr>
<tr>
<td>8-C-Ascorbyl (−)-epigallocatechin</td>
<td>0.046</td>
<td>8.0</td>
<td></td>
</tr>
</tbody>
</table>

**Theaflavins and theaflagallin**

| Epitheflagallin 3-O-gallate | 0.025 | 16 | 0.25 |
| Theaflavin                | 5.4   | >100 | 8.0 |
| Theaflavin 3′-O-gallate   | 0.07  | 23  | 0.35 |
| Theaflavin 3,3′-di-O-gallate | 0.041 | 19  | 0.30 |

**Proanthocyanidin**

| Procyanidin B-2          | 0.90  | >100 | 0.70 |
| Procyanidin B-2 3′-O-gallate | 0.90   | >100 | 6.5  |
| Procyanidin B-2 3,3′-di-O-gallate | 0.25   | 39   | 0.15 |
| (−)-Epicatechin(4β-8) (−)-epigallocatechin 3-O-gallate | 0.31  | 50   | 0.20 |
| (−)-Epicatechin 3-O-gallate(4β-8) (−)-epigallocatechin 3-O-gallate | 0.48  | 29   | 0.49 |
| (−)-Epicatechin(4β-8) (−)-epicatechin 3-O-gallate | 0.42  | 29   | 0.66 |
| (−)-Epigallocatechin 3-O-gallate(4β-8) (−)-epicatechin 3-O-gallate | 0.4   | 55   | 1.0  |
| Prodelphinidin B-2        | 0.46  | >100 | 0.26 |
| Prodelphinidin B-2 3′-O-gallate | 0.15   | >100 | 0.60 |
| Prodelphinidin B-2 3,3′-di-O-gallate | 0.27   | 23   | 0.33 |
| (−)-Catechin(4α-8) (−)-epigallocatechin | 2.0   | 100  | 6.0  |
| (−)-Catechin(4α-8) (−)-epigallocatechin 3-O-gallate | 0.36  | 42   | 0.55 |
| (−,−)-Gallocatechin(4α-8) (−)-epicatechin | 2.1   | >100 | 5.0  |
| Prodelphinidin B-4        | 2.6   | >100 | 2.6  |
| Prodelphinidin B-4 3′-O-gallate | 0.68  | 56   | 0.70 |
| (−)-Epicatechin 3-O-gallate(4β-6) (−)-epigallocatechin 3-O-gallate | 10    | 21   | 0.38 |
| Procyanidin B-5 3,3′-di-O-gallate | 0.070 | 25   | 0.15 |
| (−)-Epicatechin 3-O-gallate(4β-6) (−)-epigallocatechin 3-O-gallate | 15    | 26   | 1.0  |
| (−)-Epigallocatechin 3-O-gallate(4β-6) (−)-epicatechin 3-O-gallate | 30    | 25   | 2.0  |
| Prodelphinidin B-5 3,3′-di-O-gallate | 0.27  | 18   | 0.28 |
| Procyanidin B-3           | 5.7   | >100 | 1.0  |
| (−)-Gallocatechin(4α-8) (−)-catechin | 33    | >100 | 1.5  |
| Procyanidin C-1           | 0.25  | 52   | 0.85 |
| Prodelphinidin A-2 3′-O-gallate | 0.28  | 40   | 0.33 |
| (−)-Catechin(3-O-rhamnopyranosyl)-(4α-8) (−)-catechin | 80    | 100  | 68   |
0.0096% (w/v) bromophenol blue at the ratio of 3 to 2. These samples were then loaded onto a 10% acrylamide gel containing 0.1% (w/v) gelatin. Electrophoresis was carried out at the constant current of 20 mA, and Tris–glycine buffer (pH 8.3, 25 mM Tris, 192 mM glycine and 0.1% SDS) was used as the electrophoresis buffer. After electrophoresis, the gels were soaked in 2.5% Triton X-100 and rinsed in 10 mM Tris–HCl (pH 8.0) buffer for 30 min. They were then incubated at 37 °C for 16 h in incubation buffer containing 50 mM Tris–HCl (pH 8.0) and 0.5 mM CaCl2. Finally, the gels were stained for 10 min in 1% (w/v) Coomassie Brilliant Blue R-250 (Tokyo Kasei Industrial, Tokyo) in 10% methanol and 5% acetic acid, and destained overnight in the same solution without the Coomassie Brilliant Blue R-250 dye. Enzyme activity was quantified from the gel images with a MacBas2.0 (Fuji Film, Tokyo).

RESULTS AND DISCUSSION

We first established a fluorogenic peptide cleavage assay system using a coumarin derivative-conjugated MMP substrate, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[N\textsubscript{3}-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH\textsubscript{2} (17) and soluble human recombinant MMPs (rhMMPs) to screen for MMP inhibitors. When this substrate is hydrolyzed by the enzyme, the resulting (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-OH has a strong fluorescence, thus making the MMP-inhibiting activity easily detectable. We screened hot-water or ethanol extracts of 25 herbs using this method and analyzed the active fractions by HPLC. Since polyphenols showed strong inhibitory activity against MMPs, we next examined the inhibitory activity of polyphenols against rhMMP-2, rhMMP-7, and soluble rhMT1-MMP, the \( K_m \) and \( V_{max} \) of which against the substrate are 2.20 \( \mu \)M, 0.572 pmol/min, 3.04 \( \mu \)M, 1.24 pmol/min, and 8.74 \( \mu \)M, 2.65 pmol/min, respectively. The polyphenols tested were 17 flavonoids, 4 theaflavins and theafagallin, and 25 proanthocyanidin. Table 1 and Fig. 1 summarize the inhibitory activity of polyphenols, and indicates that (−)-epigallocatechin 3-O-gallate (EGCG) had a potent and specific inhibitory activity against MT1-MMP followed by (−)-epigallocatechin 3,5-di-O-gallate and theaflagallin 3-O-gallate. Figure 1 also shows that a potent inhibitor of MT1-MMP is not potent against MMP-2 and MMP-7. This means that the mode of interaction of polyphenols is quite different among MMPs.

Based on the evidence shown above, EGCG is a suitable candidate for the specific inhibition of tumor angiogenesis in which MT1-MMP-mediated MMP-2 activation is required. Therefore, we next confirmed the inhibitory activity of EGCG toward MT1-MMP by determining the activation of MMPs from proforms. Conditioned medium of HUVECs grown in the presence of EGCG or not was prepared, and gelatin zymography was performed (Fig. 2). HUVECs secreted mainly proMMP-2 and a small amount of proMMP-9 among the MMPs. The total amount of proMMP-2/MMMP-2 in the HUVEC culture medium decreased only a little with...
an increase in the concentration of EGCG. In contrast, the activated form of MMP-2 was significantly decreased in the presence of EGCG in a dose-dependent manner. These results suggest that the activation process of proMMP-2 to MMP-2, which is mediated by MT1-MMP, had been inhibited by EGCG.

The chemopreventive effects of green tea on cancer have been widely investigated, although the site of action is still unclear. The findings of this study suggest that EGCG suppressed tumor growth through the inhibition of MT1-MMP, which plays an important role in the angiogenic process. In fact, the expression and activation of MMPs were inhibited by EGCG. For instance, the inhibition of urokinase-type plasminogen activator, which is also involved in the degradation of the ECM, was inhibited by EGCG. In addition, EGCG suppressed mRNA expression of MMP-2 and -9 in HT1080 fibrosarcoma cells. In this study, we selected EGCG through the screening of natural and synthetic substances, as it inhibited MT1-MMP specifically. Furthermore, although most previous studies focused on the MMPs in tumor cells, we focused on MMP-activation in endothelial cells instead of tumor cells. Our present study clearly indicates that EGCG suppressed MMP activation. The antiangiogenic effect of EGCG was observed in a VEGF-stimulated angiogenesis model using chick chorioallantoic membrane after treatment with green tea. Angiogenesis is a complex process that involves the degradation of basement membrane, migration of endothelial cells toward a tumor mass, and proliferation of the cells to form neovasculature. It is also deeply involved in blood borne metastases. Thus, an antiangiogenic agent may provide hope for tumor treatment and metastasis suppression.

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