Tumor Gelatinases and Invasion Inhibited by the Green Tea Flavanol Epigallocatechin-3-Gallate

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BACKGROUND. Given the association of continued consumption of green tea with prevention of cancer development, metastasis, and angiogenesis, the effect of the main flavanol present, epigallocatechin-3-gallate (EGCG), on two gelatinases most frequently overexpressed in tumor and angiogenesis (MMP-2 and MMP-9) and on tumor cell invasion and chemotaxis was examined.

METHODS. Zymography, Western blotting, and enzyme-linked immunosorbent assay were used to analyze the effect of EGCG on MMP-2 and MMP-9 activity, whereas its effect on tumor cell invasion and chemotaxis was examined using modified Boyden chamber assays.

RESULTS. In a cell culture, dose-dependent, time-dependent, noncompetitive inhibition by EGCG of both gelatinases was found at concentrations 100 times lower than those reported to inhibit urokinase. Tumor cell invasion of a reconstructed basement membrane matrix, but not chemotaxis, was reduced by 50% with EGCG concentrations equivalent to that in the plasma of moderate green tea drinkers, and 2 orders of magnitude below those of tissue inhibitors of MMPs. Although higher concentrations of EGCG were associated with increased levels of both cell-associated gelatinases and their active TIMP-2, MMP-9 or increased gelatinase activity was found, and TIMP-1 and TIMP-2 inhibitors were up-regulated. Finally, concentrations of EGCG active in reconstituting p53 inhibition and inducing apoptosis of transformed cells were more than 100 times lower than those reported for normal cells.

CONCLUSIONS. Epigallocatechin 3-gallate is a potent inhibitor of gelatinases and an orally available pharmacologic agent that may confer the antiangiogenic and antimitastatic activity associated with green tea. Cancer 2001;91:1822-32.

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Several epidemiologic observations have led to the association of green-tea consumption with prevention of cancer development and metastasis.1 The beneficial properties of green tea and its components are increasingly reported in the scientific literature and in clinical trials.2 Several studies have reported that the consumption of green tea is associated with a lower risk of several cancers, particularly colorectal cancer.3 The benefits of green tea consumption include a reduction in the risk of cancer mortality,4 a reduction in the risk of cardiovascular disease,5 and a reduction in the risk of developing diabetes.6 The anti-inflammatory, antioxidant, and antiangiogenic properties of green tea have been reported to be responsible for the beneficial effects of green tea on health.7,8 Green tea contains polyphenols, most of which are flavonoids, commonly known as catechins, that are mostly lost through oxidation during the production of black tea. Polyphenols interact with reactive oxygen species and have high complexation affinity to some metal ions and biologic molecules.9,10 Urokinase (uPA), one of the hydrodases implicated in degradation of extracellular matrix and tumor invasion, is directly inhibited by epigallocatechin-3-gallate (EGCG),11 the most prevalent flavanol of green tea. Taking this into account...
further could suggest that EGGG inhibits the enzy-
matic cascade beginning with uPA and ending in ac-
tivation of MMP-2 and MMP-9.1,4 The two matrix met-
alloproteinases most frequently overexpressed in cancer and angiogenesis and instrumental in cutting
through basement membrane barriers.14 However, in EGCG plasma concentrations (0.1–0.3 μM) after mod-
erate green tea consumption (2-3 cups)15 are much lower than those required for uPA inhibition (IC50 = 4 mM).

More recent in vivo experiments show that these plasma levels are instead sufficient to inhibit angio-
genesis,2 crucial for growth of all solid tumors. From
this, it was a short step to suspect that EGCG may be
a direct inhibitor of MMP-2 and MMP-9 gelatinases;
soon after, this was confirmed in preliminary stud-
ies.1,15 However, the molecular mechanisms respon-
sible for this activity remain to be elucidated.

Tumor invasion and angiogenesis are induced by
both tissue (TIMPs)13 and synthetic inhibitors of
MMPs. Some synthetic MMP inhibitors are currently
in clinical trials for cancer treatment but carry unde-
sirable side effects.14 Understanding the molecular
mechanisms by which EGCG interacts with and inhib-
its these enzymatic activities, thus restraining matrix
degradation and cell invasion, is therefore important
in exploiting its properties for cancer prevention and
treatment, and will serve as a basis for designing
even more effective and invasive drugs.

This study investigates the effects and interactions of
EGCG with gelatinases MMP-2 and MMP-9, in rela-
tion to both down-regulation of cell proliferation
and the expression of MMP inhibitors TIMP-1 and
TIMP-2 and potential activators MTI-MMP and uPA.
The inhibition of MMP-2 and MMP-9 occurs at much
lower concentrations than those of the TIMPS13 and
is achieved at flavonoid concentrations equivalent
to those in the plasma of moderate green tea drinkers.

MATERIALS AND METHODS

Cell Lines

SK-N-SE human neuroblastoma,15 HT1080 human fi-
brosaroma, Ekar26 cells, derived from fusion of
human umbilical vein endothelial cells with A311 hu-
man carcinoma,16 and NIT3 murine embryo fibro-
blasts were routinely grown in Dulbecco’s modified
Eagle’s medium (Flow Laboratories, UK) supple-
mented with 10% heat-inactivated fetal calf serum
(hi-PFC, Biochrom, Berlin, Germany), 100 U/mL pen-
ticillin, 100 μg/mL streptomycin (Labtech Eurobio, Cor-
sin, Italy), 5% CO2 in air at 37 °C.

All experiments were initiated with cells in log
phase of growth, at least at 25% confluence, and de-
signed to be completed before 80% confluence. One-
millimolar stock solutions of 1-(4-ethylpyridin-3-
gallate (EGCG) and of 1-(4-ethylpyridin-3-gallate (EGCG). Sigma. St. Louis, MO were freshly prepared in culture
medium and added to cultures at the reported con-
tentation. At the end of the incubation, the condi-
tioned media were clarified, aliquoted, and stored at
−80 °C, and the cells were harvested for apoptosis
evaluation, or counted for proliferation studies, or di-
rectly solubilized into the culture flask with 1 mL/25 cm2
of sodium dodecyl sulfate-polyacrylamide gel
electrophoresis (SDS-PAGE) sample buffer for West-
ern blot analysis.

FCS Treatment

Culture media were supplemented with either hi-FCS
or gelatin-free hi-FCS. The latter was used to detect
gelatinases secreted by cells in presence of serum and
prepared by adding 20% v/v of gelatin-Sepharose
(Amersham Pharmacia Biotech, Buckinghamshire, UK) to hi-FCS. After mixing gently at 4 °C for 2 hours
and centrifugation at 10,000 x g for 5 minutes, the super-
natant and pellet were analyzed by gelatin zymogra-
phy to verify removal of all gelatinases from the serum.

Binding of EGCG to Gelatinases and Gelatin

Increasing concentrations of EGCG (1 nM to 0.1 μM)
were mixed for 2 hours at 4 °C with 30 μL of gelatin-
Sepharose. The suspension was rinsed three
times at 4 °C with chilled phosphate-buffered saline
(PBS), and the pellet rinsed gently for an additional
3 times at 4 °C with 200 μL of HT1080 conditioned
medium (10%). After centrifugation at 10,000 x g for
5 minutes, the pellet was rinsed 3 times at 4 °C with
1 mL of chilled PBS and immediately processed for
zymographic analysis. Alternatively, the same procedure
was followed, mixing first EGCG with HT1080 condi-
tioned medium (in this case no final rinsing), and then
gelling gelatin-Sepharose.

Aliquots of gelatin-Sepharose were preincubated
with 100 μM EGCG, rinsed as above, and then incu-
bated 15 minutes in PBS with either 1) 2 M NaCl, 2) 2%
SDS, or 3) with NaOH to bring the pH to 11.0. The
supernatant then was recovered and analyzed with a
Perkin Elmer (Oak Brook, IL) spectrophotometer (ex-
cit. 330 nm, emis. max. 400 nm) and compared with
positive and negative controls.

Cell Proliferation

After harvesting with trypsin-ethylenediamine tet-
racetic acid solution (Biochrom), the cells were
counted under a phase-contrast microscope by using a
hemocytometer, and cell viability was determined
with Trypan blue exclusion. The results of two independent
observers were averaged and always showed standard deviation less than 10%.

**Apoptosis Evaluation**

After cryopreservation, the cells were washed once with FCS-containing medium. To detect necrotic cells or those undergoing apoptosis, we incubated cell suspensions with Annexin V-FITC, ApoAlert Ameoth V-FITC, and ApoAlert Propidium Iodide (Cisbio Bioassays, Stourbridge, CA) following specific protocol and analyzed them by flow cytometry (exc. 488 nm, emi. 530 nm). Cells positive for propidium iodide alone were considered necrotic; cells double positive and/or positive for annexin were considered to be undergoing apoptosis or apoptotic.

**Modified Boyden Chamber Assay**

The invasive behavior of tumor cells was tested using the modified Boyden chamber assay.57 Matrigel (Collaborative Res., Inc., Bedford, MA) or gelatin (Bio-Rad, Hercules, CA) was used in the matrix for cells to migrate through, toward a chemotactic gradient represented by culture medium conditioned by NIH 3T3 cells. Polyvinylpyrrolidone-free polycarbonate filters (0.8-μm pore size) (Corning Inc., Acton, MA) were coated with gelatin (5 mg/ml) for membranes or with Matrigel (15 μg/filter) for invasion. Cells were pre-cultured overnight in medium with and without ECGG at the concentration used in the assays; after seeding of 2 × 10^5 cells onto the filters, and 5-hour incubation in serum-free medium with and without ECGG, non-migrated cells on the upper surface of the filter were removed. The filter was rinsed in water and fixed with 10% ethanol for 30 seconds, stained with toluidine blue for 10 minutes, and examined at 680 μm. The cells that actively migrated to the undersurface of the filter were quantitated using National Institutes of Health Image 1.61 software. Results of triplicate experiments were averaged and always showed standard deviation less than 10%. Control experiments were performed in absence of chemotactic gradient.

**Zymographic Analysis**

Gelatinolytic activity in culture media was assayed as described.60 Without boiling the samples, zymography was performed by electrophoresis using 10–30 μl of medium in 0.1% gelatin-containing 8% polyacrylamide, in presence of SDS. After electrophoresis, the gels were washed twice for 15 minutes with 2.5% Triton X-100, incubated overnight at 37 °C in either Tris buffer (50 mM Tris-HCL, 200 μM NaCl, 10 mM CaCl2, pH 7.4) or glycine-buffer (as above but 50 mM glycine), with and without 100 μM ZnCl2.

For gelatinase inhibition assays, ECOG, EEC, ECG, gallic acid, and tannic acid (all from Sigma) were freshly solubilized in the Tris buffer used for developing the zymograms; the gel slab was cut into strips corresponding to the lanes and then put in different tanks containing the stated concentrations of inhibitors.

The gels then were stained for 30 minutes with 30% methanol/10% acetic acid containing 0.5% Coomassie Brilliant Blue R-250 and destained in the same solution without dye clear bands represent areas of gelatinolysis on the blue background. Culture medium conditioned by HT116 (DLD-1) melanoma cells was co-cultured as control to identify pre- and active gelatinolytic bands.57 Digestion bands were quantitated by an image analyzer system (Avis). GelDoc 2000 and Quantity One software (Bio-Rad).

**Activation of Gelatinases by Organosomodrugs**

To increase the activated/zymogen form ratio of MMP-2 and MMP-9, we followed the method described by Stiles-Simmon et al.57 HT116 conditioned medium was incubated at 37 °C with 1 μM final concentration of p-APMA (Sigma) for 2 hours, and analyzed directly by SDS-PAGE.

**Classification of Inhibition and Affinity**

To further characterize the type of inhibition exerted by ECOG on gelatinases, we used a MTS/ROCK MMP-2 immunoassay system (American Pharmacia Biotech), following the manufacturer's instructions, with slight modifications. The microwell plates—coated with an anti-MMP-2 antibodies—were saturated with the enzyme contained in 5 μl of medium conditioned by SK-N-SE human neuroblastoma cells after overnight incubation at 4 °C, and thereafter washed, the gelatinase was activated by trypsin with 0.25 mM p-APMA. The wells were then incubated at 37 °C in presence of increasing amount of the peptide substrate with 100 μM or without ECGG, and the intensity of the color developed by the dispersed substrate was measured at 490 nm after 4 hours. A double-reciprocal plot of the results allowed the type and constant (Ks) of inhibition to be deduced.

**Western Blotting**

After SDS-PAGE, samples were electroblotted to a Hybond-C Extra membrane (Amersham Pharmacia Biotech). The membrane was prehydrated at 5000 psi temperature (RT) for 2 hours in PBS with 0.1% Tween 20 (PBS-T containing 1% non-fat dried milk (Sipagins solution), and hybridized at RT for 1 hour in PBS-T containing 3% BSA and the first antibody. As first antibody, the following purified polyclonal anti-human
MMP-2 (1:200). MAB anti-human TIMP-1 (1:500), TIMP-2 (1:500), and TIMP-3 (1:500). The horseradish peroxidase-labeled anti-mouse (1:500). The bridge site, anti-mouse (1:1000), anti-rabbit (1:2000), and anti-goat (1:1000) were incubated for 1 hour at RT with 0.125 μM/mL of ECL detection solution and exposed to Hyperfilm MP (Eastman Kodak Company) for 30 seconds up to 5 minutes.

Preparation and Dot Blotting

Aliquots of gelatinase-containing HT1080 conditioned medium were incubated 1 hour at RT in the presence of 100 μM ECGG or taurine acid (YPHARM, Sigma). After centrifugation at 5 minutes at 15,000 g, equal aliquots of supernatant were directly absorbed to a Hyperfilm. A 100 mmol/L Tris-HCl pH 6.8 (100 μL) was added to the gelatinase-containing samples. The resultant gelatinase activity was measured by gelatin zymography. The reaction was initiated by the addition of gelatinase-containing medium to gels consisting of 1% gelatin and 0.1% Triton X-100. The gelatinase activity was measured by gelatinase-containing extracts to the gels after incubation for 1 hour at 37°C. The gelatinolytic activity was detected by staining with Coomassie Brilliant Blue R-250.

Results

Effect of ECGG and ECG Flavonoids on MMP-2 and MMP-9

To compare the inhibition exerted on MMP-2 and MMP-9 gelatinase by the two most abundant flavonoids in green tea, ECGG and ECG, HT1080-conditioned medium was analyzed by gelatin zymography in the presence of increasing concentrations of flavonoids. As shown in Figure 1A, in which the sum of gelatinase and activated forms of each gelatinase is plotted, ECGG inhibited MMP-2 and MMP-9 to a dose-dependent manner (lowest registered values of ICP99% and 13 μM, respectively). ECGG inhibited gelatinase activity with an ICP50 approximately 20- and 30-fold higher, and at low concentrations (up to 6 μM) may have even slightly increased proteolytic activity.

After activation with an organoselenium (to balance the zymogen/activated form ratio) and zymography in presence of ECGG, separate analysis of the two forms of each gelatinase revealed no remarkable differences in inhibition, although the MMP-9 zymogen was found to be more sensitive to the flavonoids than its activated counterpart (Fig. 1A).

The inhibition exerted by ECGG is noncompetitive, as determined by double-reciprocal plot (Fig. 2), in which the plots show a common -1/km on the abscissa. The calculated K, was 22 μM.

Because polyphenols can interact with metal ions that are critical for MMP activity, zymographies of conditioned medium were developed in buffer containing an excess of ZnCl2. As shown in Figure 3, addition of 100 μM ZnCl2 to the Tis buffer did not lower the inhibition exerted by ECGG on MMP-2 and MMP-9, but instead increased it several times. Addition of ZnCl2 to the glycine buffer increased MMP-2

FIGURE 1. Analysis of gelatinase by gelatin zymography on MMP-2 and MMP-9 extracts. The reaction was initiated by the addition of MMP-2 and MMP-9 gelatinase to the radiolabeled gelatin substrate (100 μM ECGG or taurine acid (YPHARM, Sigma)). After centrifugation at 5 minutes at 15,000 g, equal aliquots of supernatant were directly absorbed to a Hyperfilm. A 100 mmol/L Tris-HCl pH 6.8 was added to the gelatinase-containing samples.
activity, however, EGGG inhibition remained effective. These data indicate that an excess of Zn^{2+} ions does not affect EGGG inhibition of MMP activity, suggesting the MMP inhibition by EGGG does not involve chelation of Zn^{2+} ions.

To determine whether EGGG binds to the enzyme, the substrate, or both, we first incubated the flask with 1°C gelatin containing medium and then with gelatin-Sepharose, or vice versa (in which case the unbound EGGG was rinsed off before adding the medium). The pellet then was analyzed by gelatin

zymography. Although incubation of the enzyme with EGGG at concentrations greater than 1 μM strongly reduced the recovery of both MMP-2 and MMP-9, enzyme recovery after incubation of the gelatin-Sepharose with EGGG, followed by washing, remained fairly constant at all EGGG concentrations used (Fig. 4A). This suggests a preferential binding of EGGG to gelatin rather than to gelatin. Finally, incubation of the gelatin-Sepharose with 100 μM EGGG, washing, and analysis of the eluates after chemically induced dissociation (2 M NaCl, 2% SDS, pH 11.5) failed to detect the flavanol in solution (not shown).

To determine whether the inhibition exerted by EGGG is due to precipitation of the enzyme-, we incubated gelatinase-containing samples with the flavanol, or with tannic acid (galloylmalic) as positive control. Although the addition of 100 μM tannic acid completely precipitated the gelatinase antigen, in the presence of 100 μM EGGG, the enzyme remained in the supernatant (Fig. 4B). In the zymographic assay, tannic acid inhibited the gelatinolytic activities at concentration even lower than EGGG, but no inhibition was exerted by gallic acid (not shown).

Effect of EGGG on Cells

Effects of EGGG on cell proliferation, gelatinase secretion, and invasion first were studied on SK-N-BE human neuroblastoma cells, which show a simple pattern of secreted gelatinolytic activity, mostly MMP-2, as shown. In Figure 5, within a 37-hour period low doses of EGGG inhibited proliferation in a dose-dependent manner with an IC_{50} of 2.4 μM. At concentrations greater than 16 μM, the cells became round, detached from the plastic surface, and eventually shrunk. Evaluation by specific antibody and cytofluo-
zymography of exposed phospho-tyrosine indicated that most cells underwent apoptosis at these high concentrations (not shown). The effect of ECGC on proliferation also was studied on other cell lines over a 30-hour period and revealed an IC_{50} in the same range for other transformed cells. 15 μM for HT1080 human fibrosarcoma cells and NIH 3T3 mouse embryo fibroblasts and 0.3 μM for EAhy926 endothelial cells (not shown).

Zymography of SK-N-BE media, conditioned overnight with increasing concentrations of ECGC. In absence of FCS, showed an unmodified level of MMP-2 up to 2 μM ECGC, then a progressive decrease at concentrations higher than 5 μM (with an IC_{50} of 20 μM), corresponding to induction of apoptosis. In contrast, the invasive capacity through a reconstituted basement membrane (Matrigel) toward a chemotactic-activating gradient of chemotactic-activating medium of ECGC in the very low dose range of 0.05–0.1 μM, with an IC_{50} of 0.08 μM (Fig. 3). No effect was detected at the same concentrations on chemokinesis (not shown).

Effects of the molar concentration of gelatinase secretion were studied further on HT1080 cells, because these concomitantly express a relatively balanced MMP-2/MMP-9 ratio compared with SK-N-BE. To avoid starvation and overestimation of gelatinases, we cultured the cells in medium supplemented with gelatine-free FCS. The efficacy of venum gelatinase removal by gelatin-Sepharose was demonstrated in the control studies of Figure 6 (panel A). A progressive increase in the release of both MMP-2 and MMP-9 was registered from 2 to 32 hours, both in the presence and absence of 1 μM flavan (Fig. 6, panel A). Even though only 40% of the control cells were present after 32 hours with ECGC, increased secretion of MMP-2 and MMP-9 per cell was confirmed by loading volumes of conditioned medium corresponding to an equivalent number of cells after 32 hours (Fig. 6, panel B). The two gelatinases were mainly in the zymogen form, and the flavan did not appreciably modify the zymogen/activated form ratio.

The levels of uPA and MT1-MMP were then investigated in HT1080 cells cultured 32 hours with 10% gelatine-free FCS with 1 μM ECGC. Western blotting of the cell extract showed that, as compared with the control, the presence of the flavonoid results in a moderate reduction in the level of cell-associated uPA and at the same time a substantial increase in MT1-MMP (Fig. 7). Under the same conditions, an increase also is registered in the levels of cell-associated MMP-2, MMP-9, TIMP-1, and TIMP-2 in particular (Fig. 7).

To study whether the interaction with basement membrane molecules modifies the release of gelatinase by cells in presence of ECGC, we seeded HT1080 cells on Matrigel with and without the flavanol. Zymography of the medium supplemented with gelatin-
nase-free serum and conditioned for 5 hours revealed the presence of mostly MMP-2, whereas in the medium conditioned for 48 hours, the level of MMP-2 sub- stantially decreased and was suppressed by that of MMP-4, independently of the flavan (Fig. 6). No active gelatinase release from Matrigel was registered in absence of cells. A substantial decrease in cell invasion into the Matrigel matrix at 48 hours was evident in the presence of EGGC.

**DISCUSSION**

Micromolar concentrations of the major flavan of green tea, EGGC, inhibit a dose-dependent proliferation-independent manner the activity of both baseline membrane collagen- and gelatin-degrading metalloproteinases, MMP-2 and MMP-9. In the experimental conditions used, the effect of this flavan is exerted at an apparent lower concentration on MMP-2 than on MMP-3, with moderate differences for zymogen and activated forms in later. The degree of inhibition we measured (K_i = 22 µM, for MMP-2) is in the range of that recently reported by others as IC_{50} and the moderate differences may be attributable to the different methodologic approaches (capture ELISA, zymography, fluorometric assays) and substrates (gelatin, synthetic peptide). The noncompeting nature of the inhibition, revealed by the double-reciprocal plot, is not surprising given the different structures of EGGC and the substrate of the gelatinases.

**FIGURE 6.** Zymograms of MMP-2 and MMP-9 gelatinases immobilized by nitrocellulose. The cells were cultured 2, 8, and 24 hours with 0.1, 1, and 50 mM EGGC in gelatinase-free 1× FCS-supplemented medium. At 24 hours, the cells were conditioned by an equal number of adherent cells (a), whereas in the cell invasion model conditioned by an equal number of cells at the end of the experiment (b) in presence (c) or absence of EGGC. The gelatinolytic activity was detected by zymography. The plot refers to densitometric evaluation of gels after bands in lane (open and filled squares) and (a) MMP-2 and (b) MMP-9, respectively.

The inhibition exerted in vitro on tumor cell invasion occurred at concentrations of EGGC similar to those in the plasma of moderate green tea drinkers (0.1–0.3 µM), which recently have been shown to inhibit angiogenesis in vivo. EGGC thus is much more effective in vitro (two orders of magnitude) than either the natural tissue inhibitor TIMP-2 or even some synthetic MMP inhibitors currently in clinical trials whose use must also come to terms with undesirable side effects.

Tumor cell proliferation was restrained at concentrations of EGGC approximately five times higher than values at which invasion is inhibited, thus indicating separable cellular targets of EGGC action. It has been suggested that EGGC exerts its growth-inhibitory effects either through modulation of the activities of several key (G) regulatory proteins such as cyclin-de- pendent kinases G2k2 and Cdk4 or through induction of Cdk inhibitor p21 and p27. In either case, compared with RGF-2–stimulated bone capillary1 and first passage human umbilical vein (unpublished data) normal endothelial cells, the proliferation in the transformed cells we examined was greater than 100 times more sensitive to the flavan D₅₀ = 100 and 0.4 µM, respectively. In addition, apoptosis was induced at much lower EGGC concentrations in cancer cells than
up-regulated without a detectable increase in activation, and more cell-associated gelatinases were found. Nevertheless, higher levels of their tissue inhibitors TIMP-1 and especially TIMP-2 were also cell-associated, thus potentially counterbalancing the enzyme action. It is noteworthy that the pattern of gelatinases in the medium varied according to the type of substrate the cells were interacting with. On plastic, MMP-2 always prevailed, whereas on recombinant extracellular matrix, after a period in which MMP-2 dominated, the main gelatinase was MMP-9, and the previously secreted MMP-2 decreased substantially. This is very likely attributable to both a shift in gelatinase secretion induced by cell-matrix interaction (reduction of MMP-2, increase of MMP-9) and the experimentally proven difference (data not shown) in affinity of the two gelatinases for Matrigel molecules (the lower the affinity, the more enzyme in solution).

In presence of EGCG, MMP-2 continues to be expressed by SK-N-BE: cells independently of proliferation up to the cytotoxic threshold, beyond which most cells follow an apoptotic pathway. It therefore is more reasonable to attribute inhibition of invasion and angiogenesis to direct inhibition of gelatinase activity rather than to down-modulation of proliferation and/or gelatinase expression. This inhibited activity may set off a cellular feedback response that results in up-expression of both secreted and membrane-bound gelatinases along with over-expression of the TIMPs and the membrane-bound activator MTI-MMP. Then, direct inhibition of MTI-MMP by EGCG could explain why the level of activated form of gelatinase was found to be low, despite the up-regulation of MTI-MMP. This possibility is realized by other antioxidant and anti
angiogenic compounds that directly inhibit both the two gelatinases and MT1-MMP.

These results may play a part in resolving an apparent contradiction: certain MMPs, such as MMP-3, -7, -9, and -12, in addition to their critical role in tumor and endothelial cell invasion, also can contrib-
ute to the generation of angiostatin by cleavage of plasminogen.

Angiostatin is an angiogenesis inhibitor originally reported to be a factor produced by primary tumors that inhibits the growth of metastases. However, its effects on the primary tumor vasculature are not clear, and its efficacy as an anti-tumor therapy appears to be limited to the use of high pharmaceutical doses or localized gene therapy. Our results suggest that EGCG, as a direct inhibitor of MMPs, inhibits angiogenesis and metastasis upstream of the action of angiogenesis inhibitors derived from the action of MMPs. Nevertheless, combined pharmacologic use of flavonoid and angiostatin or similar angiogenesis inhibitors could result in a synergistic effect.

As mentioned, EGCG also is reported to block uPA. However, this occurs only at concentrations approximately 500 times greater than the values reported here for gelatinases, far exceeding any reasonable tea-drinking level. Down-regulation of cell-bound uPA by a much lower concentration of EGCG (1 μM) on HT115 human fibrosarcoma cells now reveals that the flavonoid exerts its effect on uPA upstream of direct inhibition of enzymatic activity. Nevertheless, because no FGGG-induced up-regulation was registered in the synaptic/cellular form ratio of gelatinases, the uPA–plasminogen–plasmin activation cascade on MMP-2 and MMP-9 probably is not involved in this model system.

The registered effects on proteases are not exclusive to EGCG. Green tea contains a family of flavonoids of which catechins, EGCG, ICG, and ECG are the prevalent members, present in the ratio of approximately 18.5:2. The latter two also exert inhibitory activity toward the two gelatinases, but whereas ECG is effective at concentrations 20–30 times higher than those used in the experiment, EGCG has been reported to block gelatinases at concentrations close to those reported here for EGCG.

This fits with the suggestion that both the flavonoid skeleton and the galloyl moiety are necessary for the inhibitory activity. In fact, gallic acid itself lacks any in-gel inhibitory activity on gelatinases in the same range of concentration, but tannic acid (gallo-
atin) shows an even stronger inhibition than EGCG; nevertheless, although the gallo-tannin–gelatinase in-
teraction leads to enzyme precipitation, its presence on the flavonoid the galloyl remains in solution. This point therefore should be considered in designing new
drug-like compounds with galloyl moieties on a flavanol skeleton.

In the same model system (HT115 cells), ECG also inhibited the secretion of MMP-2 and MMP-9, but only at cytotoxic concentrations (40–200 μM). Although EGCG gave parallel results within this range, inhibition of MMP-2 and MMP-9 was observed only at low concentrations (more than two orders of magnitude) than those reported for FGGG in chemotaxis assays. This may indicate that the block of invasion is not due to cytotoxicity or cell motility impairment. Nevertheless, it has been suggested that the anti-
invasive activity may be due to binding with laminin, a glyco-protein of basement membrane, which in turn leads to a lower cell adhesion to the extra-
cellular matrix. Interaction of EGCG with laminin cannot be excluded in the chemotaxis assay (laminin is a component of Matrigel); to what extent EGCG affects adhesion to this glycoprotein or others is still to be determined.

Very little is known about the molecular mecha-
nisms by which EGCG blocks gelatinolytic activities. It has been suggested that the inhibition of gelatinases by EGCG is due to zinc chelation, polyphenols do, in fact, have high complexation affinity to metal ions, and zinc is essential for enzymatic activity.

Moreover, because addition of excess zinc ions with EGCG failed to restore full gelatinolytic activity during zymography, and instead improved the inhibition, this hypothesis is not likely. The assay in gelatin buffer confirms that the reduced Zn2+ availability may not be the expla-
nation, although gelatin is a good zinc chelator, addition of zinc does not lower the progressive inhibition exerted by EGCG.

Apart from possible chelation, direct EGCG-pro-
tein interaction has to be taken into account. The experimental results of tumor gelatinase binding to gelatin in presence of the flavonoid (Fig. 4)—run at 4°C to retain enzymatic activities—can be described ac-
cording to the following three equilibria

1. MMP + EGCG ⇌ MMP-EGCG
2. gelatin + EGCG ⇌ gelatin-EGCG
3. MMP-EGCG + gelatin ⇌ MMP-EGCG-gelatin

where the first is characterized by the higher affinity constant, and the third consider the formation of a ternary inactive complex. At high EGCG/MMP ratios, the lowering in gelatin-bound enzyme recovery indi-
cates that the free flavonoid competes with the flavanol–MMP complex for binding to gelatin. In fact, when the free EGCG was removed before adding the gelatinase, MMP recovery remained constant. Accord-
ing to the proposed model, a stronger binding of
EGCG to the enzymes rather than to the substrate also is suggested by the lack of EGCG release from cells, after incubation with the flavanol and chemically induced dissociation from the cytoskeleton, denaturation, pH-induced ionization of phenolic groups.

Serum mobility of genistinates less than 0.01 M/L is 10 times lower than that of EGCG in moderate green, tea drinks. These genistinates are generally more (reversibly) blocked by serum and tissue inhibitors, but even so, they may intercept EGCG. A close study of whether this inhibition of EGCG synthase and plasma self-retard the growth to which the inhibition of the in vivo tissue tumor MTPs is influenced.

These data show 1) that the EGCG concentration effectively in inhibiting MTP-2 and MTP-9 is at least 500-fold lower than that reported for up to 2% that even lower concentrations equivalent to that in plasma of moderate green, tea drinkers are effective in reducing tumor cell invasion by 50%; 2) that this reduction is obtained with an EGCG concentration two orders of magnitude lower than that reported for TIMPs as well as some in clinical trials synthetic inhibitors of MPP-2 and 4) that the inhibitory activity is not due to metabolizing properties of EGCG but correlates with direct binding of EGCG to gelsinates, in addition to having a preservative role, EGCG may be effective in combination with other agents as a dual-action clinical treatment blocking both tumor invasion and neoangiogenesis.

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