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

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BACKGROUND. Given the association of 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 cancer and angiogenesis (MMP-2 and MMP-9) and on tumor cell invasion and chemotaxis were examined.

METHODS. Zymography, Western blotting, and enzyme linked immunoadsorbent 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. A Zn^{2+} chelation-independent, dose-dependent, noncompetitive inhibition by EGCG of both gelatinases was found at concentrations 500 times lower than that reported to inhibit urokinase. Tumor cell invasion of a reconstituted 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 activator MT1-MMP, no increased gelatinase activation was found, and TIMP-1 and TIMP-2 inhibitors were up-regulated. Finally, concentrations of EGCG active in restraining proliferation 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 antimetastatic activity associated with green tea. ***Cancer* 2001;91:822–32.**
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KEYWORDS: green tea, epigallocatechin-3-gallate (EGCG), tumor invasion, MMP-2, MMP-9.

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 include inhibition of mutagenesis,[2] tumorigenesis,[1] tumor growth,[3] invasion,[4,5] metastasis,[6] and angiogenesis.[7]

Green tea contains polyphenols, most of which are flavanols, commonly known as catechins; these 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.[1] Urokinase (uPA), one of the hydrolases implicated in degradation of extracellular matrix and tumor invasion, is directly inhibited by epigallocatechin-3-gallate (EGCG),[8] the most prevalent flavanol of green tea. Taking this a step

further could suggest that EGCG inhibits the enzymatic cascade beginning with uPA and ending in activation of MMP-2 and MMP-9,[9] the two matrix metalloproteinases most frequently overexpressed in cancer and angiogenesis and instrumental in cutting through basement membrane barriers.[10] However, EGCG plasma concentrations (0.1–0.3 μM) after moderate green tea consumption (2–3 cups)[1] are much lower than those required for uPA inhibition (IC_{50} = 4 mM).

More recent in vivo experiments show that these plasma levels are instead sufficient to inhibit angiogenesis,[7] 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 studies.[11,12] However, the molecular mechanisms responsible for this activity remain to be elucidated.

Tumor invasion and angiogenesis are reduced by both tissue (TIMPs)[13] and synthetic inhibitors of MMPs. Some synthetic MMP inhibitors are currently in clinical trials for cancer treatment but carry undesirable side effects.[14] Understanding the molecular mechanisms by which EGCG interacts with and inhibits 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 anti-invasion drugs.

This study investigates the effects and interactions of EGCG with gelatinases MMP-2 and MMP-9, in relation 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 TIMPs[13] and is achieved at flavanol concentrations equivalent to those in the plasma of moderate green tea drinkers.

MATERIALS AND METHODS

Cell Lines

SK-N-BE human neuroblastoma,[15] HT1080 human fibrosarcoma, EAhy926 cells, derived from fusion of human umbilical vein endothelial cells with A431 human carcinoma,[16] and NIH-3T3 murine embryo fibroblasts were routinely grown in Dulbecco's modified Eagle's medium (Flow Laboratories, UK) supplemented with 10% heat-inactivated fetal calf serum (hi-FCS; Biochrom, Berlin, Germany), 100 U/mL penicillin, 100 μg/mL streptomycin (Labtek Eurobio, Corsio, Italy), in 5% CO_2 in air at 37 °C.

All experiments were initiated with cells in log phase of growth, at least at 25% confluence, and designed to be completed before 80% confluence. One-millimolar stock solutions of (-)epigallocatechin-3-gallate (EGCG) and of (-)epigallocatechin (EGC; Sigma, St. Louis, MO) were freshly prepared in culture medium and added to cultures at the reported concentration. At the end of the incubation, the conditioned media were clarified, aliquoted, and stored at −80 °C, and the cells were harvested for apoptosis evaluation, or counted for proliferation studies, or directly solubilized into the culture flask with 1 mL/25 cm^2 of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, for Western blot analysis.

FCS Treatment

Culture media were supplemented with either hi-FCS or gelatinase-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 100×*g* for 5 minutes, the supernatant and pellet were analyzed by gelatin zymography 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 mM) were mixed for 2 hours at 4 °C with 30 μL of gelatin-Sepharose. The suspension then was rinsed three times at 4 °C with chilled phosphate-buffered saline (PBS), and the pellet mixed gently for an additional 3 hours at 4 °C with 200 μL of HT1080 conditioned medium (10×). After centrifugation at 100×*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 conditioned medium (in this case no final rinsing), and then adding gelatin-Sepharose.

Aliquots of gelatin-Sepharose were preincubated with 100 μM EGCG, rinsed as above, and then incubated 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) spectrofluorimeter (excit. 330 nm, emiss. max 400 nM) and compared with positive and negative controls.

Cell Proliferation

After harvesting with trypsin-ethylenediamine tetraacetic acid solution (Biochrom), the cells were counted under a phase-contrast microscope by using a hemocytometer, and cell viability was determined by Trypan Blue exclusion. The results of two independent

observers were averaged and always showed standard deviation less than 10%.

Apoptosis Evaluation

After trypsinization, 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 Annexin V-FITC, and ApoAlert Propidium Iodide (Clontech Laboratories, Palo Alto, CA) following specific protocol and analyzed them by flow cytometry (excit. 488 nm, emiss. 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.[17] Matrigel (Collaborative Res., Beckton Dickinson, Bedford, MA) or gelatin (Bio-Rad, Hercules, CA) was used as the matrix for cells to migrate through, toward a chemoattractant represented by culture medium conditioned by NIH-3T3 cells. Polyvinylpyrrolidone-free polycarbonate filters (8-μm pore size) (Corning Inc., Acton, MA) were coated with gelatin (5 mg/mL) for chemotaxis or with Matrigel (15 μg/filter) for invasion. Cells were preincubated overnight in medium with and without EGCG 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 EGCG, nonmigrated cells on the upper surface of the filter were removed. The filter was rinsed in water and fixed with 100% ethanol for 30 seconds, stained with toluidine blue for 10 minutes, and scanned at 600 ppi. The cells that actively migrated to the under surface 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 chemoattractant.

Zymographic Analysis

Gelatinolytic activity in culture media was assayed as described.[18] Without heating the samples, zymography was performed by electrophoresing 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 mM NaCl, 10 mM $CaCl_2$, pH 7.4) or glycine-buffer (as above but 50 mM glycine), with and without 100 μM $ZnCl_2$.

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

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 HT1080 melanoma cells was coelectrophoresed as control to identify pro- and active gelatinolytic bands.[11] Digestion bands were quantitated by an image analyzer system with GelDoc 2000 and Quantity One software (Bio-Rad).

Activation of Gelatinases by Organomercurials

To increase the activated/zymogen form ratio of MMP-2 and MMP-9, we followed the method described by Stetler-Stevenson et al.[19] HT1080 conditioned medium was incubated at 37 °C with 1 mM 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 EGCG on gelatinases, we used a BIOTRAK MMP-2 activity assay system (Amersham Pharmacia Biotech), following the manufacturer's instructions, with slight modifications. The microtiter wells—coated with anti–MMP-2 antibodies—were saturated with the enzyme contained in 50 μL of medium conditioned by SK-N-BE human neuroblastoma cells; after overnight incubation at 4 °C, and exhaustive washing, the gelatinase was activated by treatment with 0.25 mM p-APMA. The wells were then incubated at 37 °C in presence of increasing amount of the peptide substrate, with (50 μM) or without EGCG, and the intensity of the color developed by the digested substrate was measured at 405 nm after 4 hours. A double-reciprocal plot of the results allowed the type and constant (K_i) of inhibition to be deduced.

Western Blotting

After SDS-PAGE, samples were electroblotted to a Hybond-C Extra nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was prehybridized at room temperature (RT) for 2 hours in PBS with 0.1% Tween 20 (PBS-T) containing 1% nonfat dried milk (Sigma) (solution1), and hybridized at RT for 1 hour in PBS-T containing 3% BSA and the first antibody. As first antibody, the following purified immunoglobulin (Ig) G were used: sheep anti-human

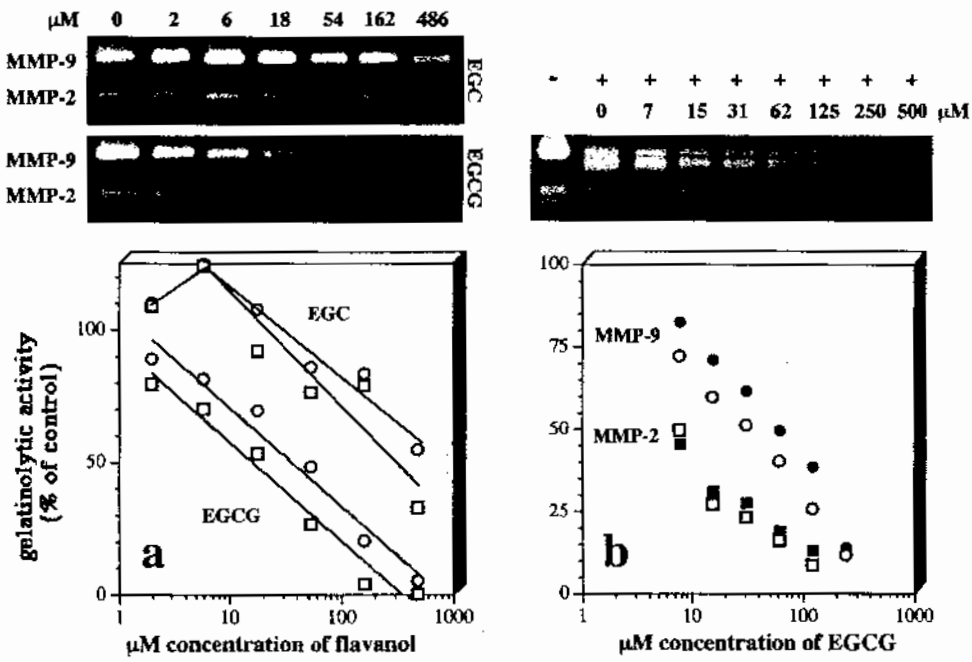

FIGURE 1. Inhibition of gelatinases by flavanols. (a) Dose–response suppression of MMP-2 (squares) and MMP-9 (circles) activity—as sum of zymogen and activated forms—by EGCG and EGC. (Top) relative gelatin zymograms of equal volumes of medium conditioned by HT1080 cells; the numbers give micromolar (μM) concentration of flavanol added to the zymography developing buffer. (b) Dose–response inhibition of separate zymogen (open circles and open squares) and activated (filled circles and filled squares) forms of MMP-2 and MMP-9 by EGCG. (Top) relative gelatin-zymogram. (−) and (+) lanes refer to p-APMA untreated or treated gelatinase-containing media, respectively. The numbers give μM concentration of EGCG added to the zymography developing buffer.

MMP-2 (1:200; The Binding Site, Birmingham, UK), rabbit anti-human uPA (1:1500, Monosan, Uden, The Netherlands), mouse monoclonal anti-human MMP-9 (1:200), MT1-MMP (1:300), TIMP-1 (1:200), and TIMP-2 (1:200) (the latter four purchased from Calbiochem, San Diego, CA). The membrane then was incubated at RT for 45 minutes in solution1 containing the second antibody. The second antibodies were horseradish peroxidase–labeled anti-sheep (1:2000; The Binding Site), anti-rabbit (1:4000; Sigma), anti-mouse (1:2000; Sigma) IgG. Each step was followed by extensive washing in PBS-T. Antigen detection was achieved by incubating the membrane for 1 minute at RT with 0.125 mL/cm^2 of ECL detection solution and exposing to Hyperfilm MP (both from Amersham Pharmacia Biotech) for 10 seconds up to 5 minutes.

Precipitation and Dot Blotting

Aliquots of gelatinase-containing HT1080 conditioned medium were incubated 1 hour at RT in presence of 100 μM EGCG or tannic acid (gallotannin; Sigma). After centrifugation 5 minutes at 15,000×*g*, equal aliquots of supernatant were directly absorbed to a Hybond-C Extra nitrocellulose membrane as above. The first antibody was the anti–MMP-9, and the developing procedure was as described for Western blotting.

RESULTS

Effect of EGCG and EGC Flavanols on MMP-2 and MMP-9

To compare the inhibition exerted on MMP-2 and MMP-9 gelatinases by the two most abundant flavanols in green tea, EGCG and EGC, HT1080 conditioned medium was analyzed by gelatin zymography in presence of increasing concentrations of flavanol. As shown in Figure 1a, in which the sum of zymogen and activated forms of each gelatinase is plotted, EGCG inhibited MMP-2 and MMP-9 in a dose-dependent manner (lowest registered values of IC_{50}: 8 and 13 μM, respectively). EGC inhibited gelatinase activity with an IC_{50} 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 organomercurial (to balance the zymogen/activated form ratio) and zymography in presence of EGCG, 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 flavanol than its activated counterpart (Fig. 1b).

The inhibition exerted by EGCG is noncompetitive, as determined by double-reciprocal plotting the results obtained from the MMP-2 capture assay (Fig. 2), in which the plots share a common $-1/Km$ on the abscissa. The calculated K_i 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 $ZnCl_2$. As shown in Figure 3, addition of 100 μM Zn^{2+} to the Tris buffer did not lower the inhibition exerted by EGCG on MMP-2 and MMP-9, but instead increased it several times. Addition of Zn^{2+} to the glycine buffer increased MMP-2

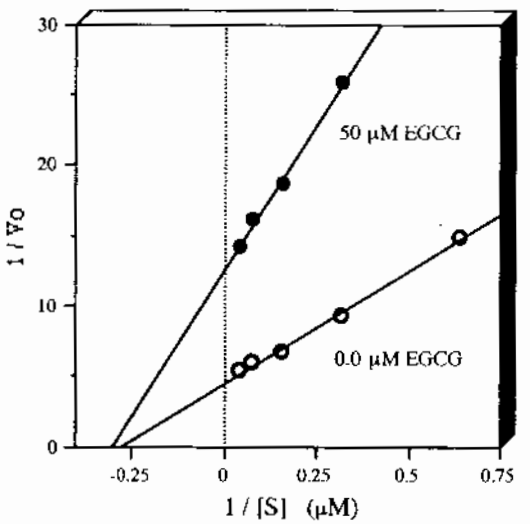

FIGURE 2. Characterization of the inhibition of MMP-2 activity by EGCG. The effect of increasing concentration of the substrate on the initial velocity V_0 of the enzymatic reaction was determined at 50 mM EGCG on antibody-captured MMP-2. Double-reciprocal plot showing that the inhibition exerted is noncompetitive. Each point represents the mean of triplicate determinations, with standard deviation less than 15%.

activity; however, EGCG inhibition remained effective. These data indicate that an excess of Zn^{2+} ions does not affect EGCG inhibition of MMP activity, suggesting the MMP inhibition by EGCG does not involve chelation of Zn^{2+} ions.

To determine whether EGCG binds to the enzyme, the substrate, or both, we first incubated the flavanol at 4 °C with gelatinase-containing medium and then with gelatin-Sepharose, or vice versa (in which case the unbound EGCG was rinsed off before adding the medium). The pellet then was analyzed by gelatin zymography. Although incubation of the enzyme with EGCG 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 EGCG, followed by washing, remained fairly constant at all EGCG concentrations used (Fig. 4a). This suggests a preferential binding of EGCG to gelatinases rather than to gelatin. Finally, incubation of the gelatin-Sepharose with 100 μM EGCG, washing, and analysis of the eluates after chemically induced dissociation (2 M NaCl, 2% SDS, or pH 11.0) failed to detect the flavanol in solution (not shown).

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

Effect of EGCG on Cells

Effects of EGCG 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 EGCG inhibited proliferation in a dose-dependent manner with an IC_{50} of 0.4 μM. At concentrations greater than 10 μM, the cells became round, detached from the plastic surface, and eventually shrank. Evaluation by specific antibody and cytofluo-

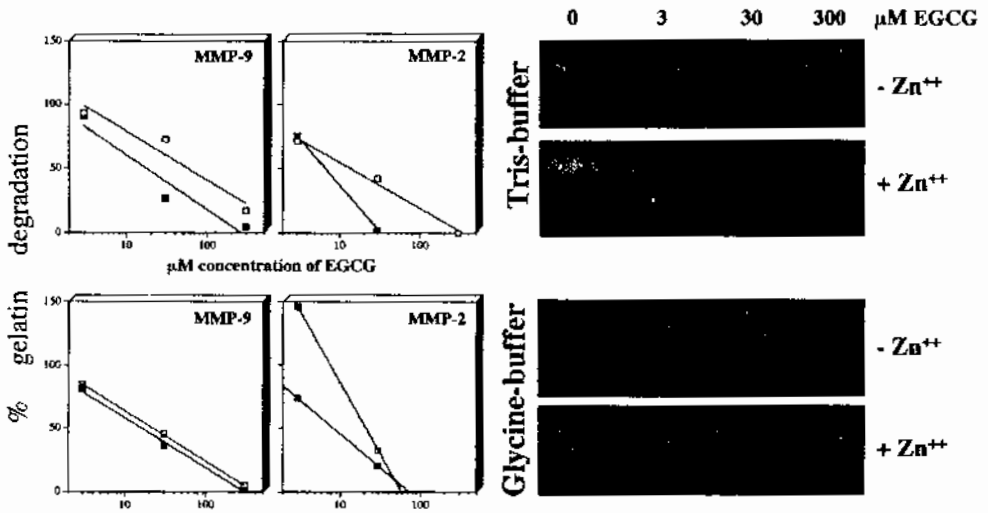

FIGURE 3. Zymography of MMP-2 and MMP-9 activities developed in Tris buffer or glycine buffer with (filled squares) or without (open squares) 100 μM Zn^{2+} to examine the role of Zn^{2+} chelation in MMP inhibition. Plotted values are taken from densitometric evaluation of digestion bands on the gelatin zymograms of equal volumes of medium conditioned by HT1080 cells. Example of a duplicate experiment.

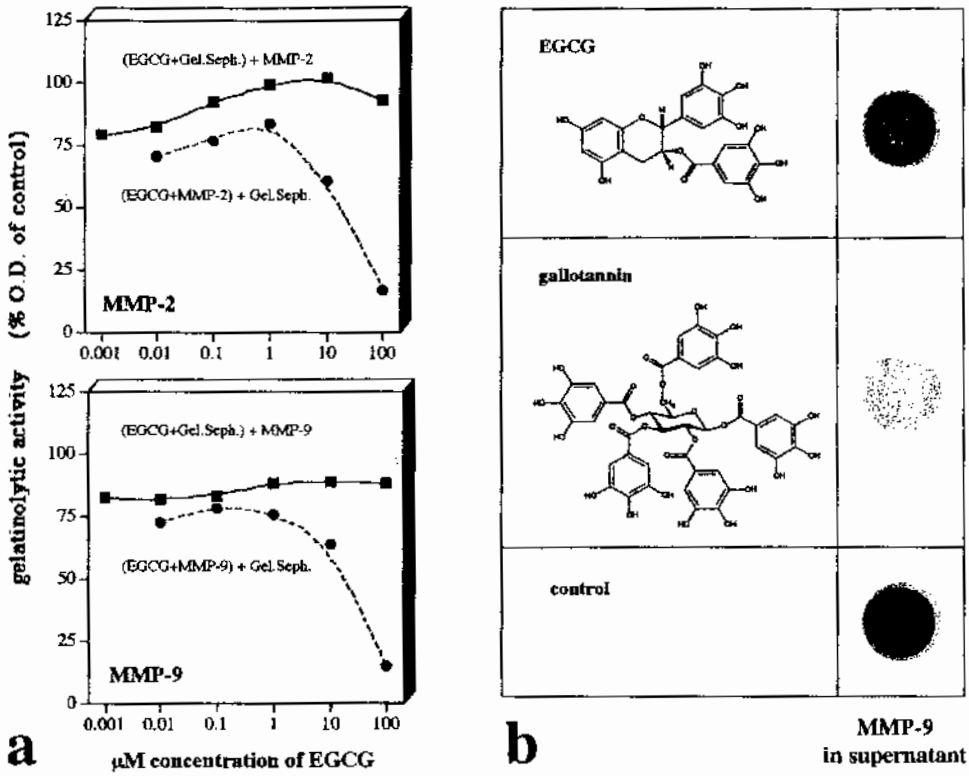

FIGURE 4. (a) Gelatinase-gelatin binding in presence of EGCG, after 3 hours incubation at 4 °C . (Top curve) EGCG was preincubated for 2 hours at 4 °C with gelatin-Sepharose, the unbound flavanol was rinsed off, and then HT1080-conditioned medium was added. (Bottom curve) EGCG was preincubated for 2 hours at 4 °C with HT1080-conditioned medium, and then gelatin-Sepharose was added. The pellets then were analyzed by gelatin-zymography and densitometry: at concentration greater than 1 μM, EGCG strongly reduces gelatinase binding to gelatin only in the second case. Example of a duplicate experiment. (b) Precipitation of MMP-9 by EGCG or tannic acid: anti–MMP-9 antibodies (dot blot) show that, after 1 hour at room temperature and clarification, the HT1080 gelatinase remains in solution in presence of 100 μM EGCG but completely precipitates with 100 μM tannic acid (example formula of a gallotannin).

rimetry of exposed phosphatidylserine indicated that most cells underwent apoptosis at these high concentrations (not shown). The effect of EGCG 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: 0.5 μM for HT1080 human fibrosarcoma cells and NIH-3T3 murine embryo fibroblasts and 0.8 μM for EAhy926 endothelial cells (not shown).

Zymography of SK-N-BE media, conditioned overnight with increasing concentrations of EGCG in absence of FCS, showed an unmodified level of MMP-2 up to 2 μM EGCG, 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 chemoattractant was dose–response restrained by EGCG in the very low dose range of 0.05–0.15 μM, with an IC_{50} of 0.08 μM (Fig. 5). No effect was registered at the same concentrations on chemotaxis (not shown).

Effects on the time course of gelatinase secretion were studied further on HT1080 cells, because these constitutively express a relatively balanced MMP-2/MMP-9 ratio as compared with SK-N-BE. To avoid starvation and overestimation of gelatinases, we cultured the cells in medium supplemented with gelatinase free FCS. The efficacy of serum gelatinase removal by gelatin-Sepharose was demonstrated in the control lanes of Figure 6 (panel c). 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 flavanol (Fig. 6, panel a), even though only 40% of the control cells were present after 32 hours with EGCG. 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 flavanol did not appreciably modify the zymogen/activated form ratio.

The levels of uPA and MT1-MMP then were investigated in HT1080 cells cultured 32 hours with 10% gelatinase free FCS with 1 μM EGCG. Western blotting of the cell extract showed that, as compared with the control, the presence of the flavanol 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 gelatinases by cells in presence of EGCG, we seeded HT1080 cells on Matrigel with and without the flavanol. Zymography of the medium supplemented with gelati-

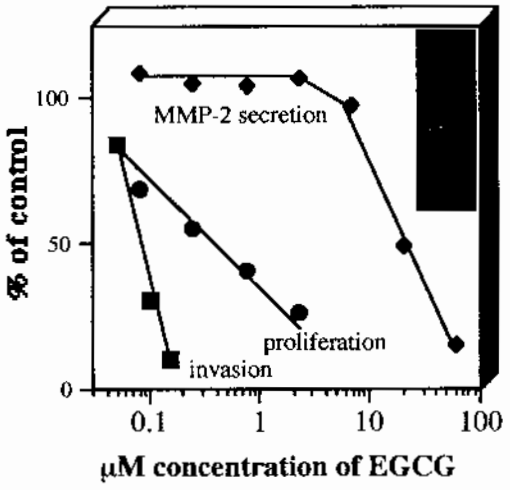

FIGURE 5. Dose-dependent effect of EGCG on cell proliferation, MMP-2 secretion and Matrigel invasion. After 14 hours of incubation of SK-N-BE cells in absence of serum, equal amounts of conditioned medium were analyzed by zymography for MMP-2 (example on insert). The incubation was continued for an additional 23 hours in the presence of 10% FCS for proliferation studies. The invasion assay on Matrigel was developed after 5 hours.

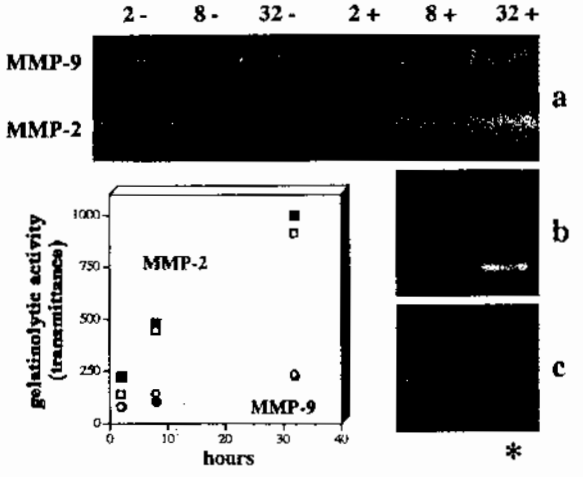

FIGURE 6. Zymograms of MMP-2 and MMP-9 gelatinases secreted by HT1080 cells. The cells were cultured 2, 8, and 32 hours with 1.0 (+) or 0.0 (−) μM EGCG in gelatinase-free hi-FCS–supplemented medium. In (a) volumes of medium conditioned by an equal number of seeded cells were loaded, while in (b) the two lanes refer to volumes conditioned by an equal number of cells at the end of the experiment (32 hours) in presence (right) or absence of EGCG. The plot refers to densitometric evaluation of gelatinolytic bands in (a): open and filled squares refer to MMP-2 −/+ EGCG, open and filled circles to MMP-9 −/+ EGCG, respectively. (c) Zymogram of 1 μL of FCS before and after (asterisk) incubation with gelatin-Sepharose, showing subtraction of both gelatinases.

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 substantially decreased and was superseded by that of MMP-9, independently of the flavanol (Fig. 8). 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 EGCG.

DISCUSSION

Micromolar concentrations of the major flavanol of green tea, EGCG, inhibit in a dose-dependent precipitation-independent manner the activity of both basement membrane collagen- and gelatin-degrading metalloproteinases, MMP-2 and MMP-9. In the experimental conditions used, the effect of this flavanol is exerted at an apparent lower concentration on MMP-2 than on MMP-9, with moderate differences for zymogen and activated forms in the latter. 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},[20] and the moderate differences may be attributable to the different methodologic approaches (capture ELISA, zymography, fluorometric assays) and substrates (gelatin, synthetic peptide). The noncompetitive nature of the inhibition, revealed by the double-reciprocal plot, is not surprising given the different structures of EGCG and the substrate of the gelatinases.

The inhibition exerted in vitro on tumor cell invasion occurred at concentrations of EGCG similar to those in the plasma of moderate green tea drinkers (0.1–0.3 μM),[1] which recently have been shown to inhibit angiogenesis in vivo.[7] EGCG thus is much more effective in vitro (two orders of magnitude) than either the natural tissue inhibitor TIMP-2[13] or even some synthetic MMP inhibitors currently in clinical trials[14] whose use must also come to terms with undesirable side effects.

Tumor cell proliferation was restrained at concentrations of EGCG approximately five times higher than values in which invasion is inhibited, thus indicating separate cellular targets of EGCG action. It has been suggested that EGCG exerts its growth-inhibitory effects either through modulation of the activities of several key G1 regulatory proteins such as cyclin-dependent kinases Cdk2 and Cdk4 or through induction of Cdk inhibitor p21 and p27.[21] In either case, compared with FGF-2–stimulated bovine capillary[7] 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 flavanol (IC_{50} = 100 and 0.4 μM, respectively). In addition, apoptosis was induced at much lower EGCG concentrations in cancer cells than

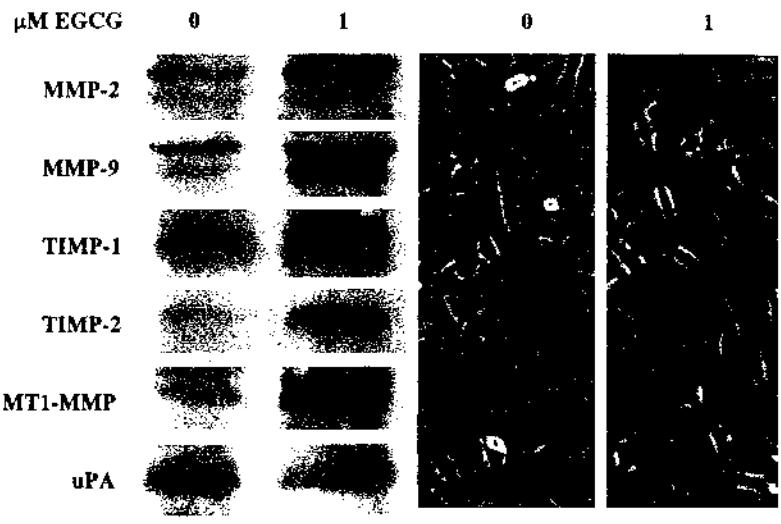

FIGURE 7. (Left) Western blotting of cell-associated antigens. HT1080 human fibrosarcoma cells were cultured for 32 hours in medium supplemented with gelatinase-free hi-FCS and 0.0 and 1.0 μM EGCG; samples correspond to equal numbers of cells at the end of incubation. (Right) Photographs of the cells at the end of incubation. Example of a duplicate experiment.

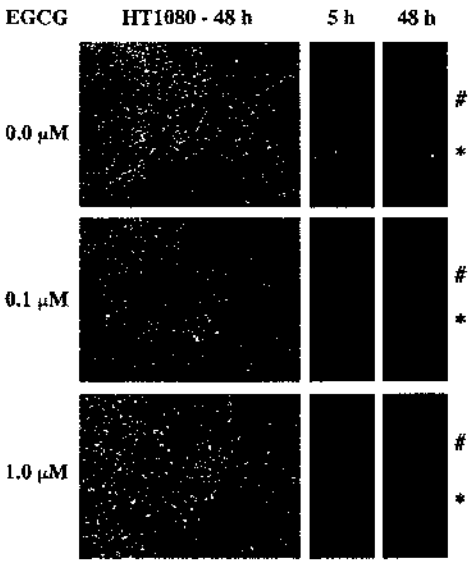

FIGURE 8. Morphology and gelatinase secretion by cells seeded on Matrigel. Example of HT1080 cells after 48 hours in gelatinase free medium on Matrigel, with different concentrations of EGCG as indicated. The invasive phenotype in absence of flavanol (elongated shape and centrifugal morphology) is lost with 0.1 μM, and proliferation restrained with 1 μM EGCG. Zymograms show the gelatinolytic activities (asterisk, MMP-2; pound sign, MMP-9) in the corresponding medium conditioned by 3000 seeded cells for 5 and 48 hours (h). No-cells control excluded passive release of active MMPs from Matrigel (not shown).

in normal cells, as already reported.[22] These marked differences in sensitivity may prove to be crucial in the clinical context. The extent to which the effect is due simply to their higher proliferation rate needs careful examination.

At EGCG concentrations in which proliferation is down-regulated, gelatinase secretion was strongly 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.[23] 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 reconstituted 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 over-expression of both secreted and membrane-bound gelatinases along with over-expression of the TIMPs and the membrane-bound activator MT1-MMP.[24] Then, direct inhibition of MT1-MMP by EGCG could explain why the level of activated form of gelatinase was found to be low, despite the up-regulation of MT1-MMP. This possibility is realized by other antitumor and anti-

angiogenic compounds that directly inhibit both the two gelatinases and MT1-MMP.[25]

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 contribute to the generation of angiostatin[26] by cleavage of plasminogen.[27] 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 linked 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 flavanol and angiostatin or similar angiogenesis inhibitors could result in a synergistic activity.

As mentioned, EGCG also is reported to block uPA.[8] However, this occurs only at concentrations approximately 500 times greater than the values reported here for gelatinases, far exceeding any reasonable tea-drinking level.[1] Down-regulation of cell-bound uPA by a much lower concentration of EGCG (1 μM) on HT1080 human fibrosarcoma cells now reveals that the flavanol exerts its effect on uPA upstream of direct inhibition of enzymatic activity. Nevertheless, because no EGCG-induced up-regulation was registered in the zymogen/activated form ratio of gelatinases, the uPA-plasminogen-plasmin activation cascade on MMP-2 and MMP-9[9] probably is not involved in this model system.

The registered effects on proteinases are not exclusive to EGCG. Green tea contains a family of flavanols, of which catechins EGCG, EGC, and ECG are the prevalent members, present in the ratio of approximately 10:5:2.[1] The latter two also exert inhibitory activity toward the two gelatinases, but whereas EGC is effective at concentrations 20–30 times higher than EGCG, ECG has been reported to block gelatinases at concentrations close to those registered here for EGCG.[11] This fits with the suggestion that both the flavanol 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 (gallotannin) shows an even stronger inhibition than EGCG; nevertheless, although the gallotannin–gelatinase interaction leads to enzyme precipitation, in presence of the flavanol the gelatinase remains in solution. This point therefore should be considered in designing new synthetic drugs with galloyl moieties on a flavanol skeleton.

In the same model system (HT1080 cells), ECG also inhibited the secretion of MMP-2 and MMP-9,[11] but only at cytotoxic concentrations (40–200 μM). Although EGCG gave parallel results within this range, invasion of reconstituted extracellular matrix (chemoinvasion) was inhibited at much lower concentrations (more than two orders of magnitude) than those reported for ECG in chemotaxis assays. This may indicate that the block of invasion is not due to cytoskeleton or cell motility impairment. Nevertheless, it has been suggested that the anti-invasive activity may be due to binding with laminin, a glycoprotein of basement membrane, which in turn leads to a lower cell adhesion to the extracellular matrix.[4] Interaction of EGCG with laminin cannot be excluded in the chemoinvasion 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 mechanisms by which EGCG blocks gelatinolytic activities. It has been suggested that the inhibition of gelatinases by EGCG is due to zinc chelation;[11] polyphenols do, in fact, have high complexation affinity to metal ions, and zinc is essential for enzymatic activity.[30] However, 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 glycine buffer confirms that a reduced Zn^{2+} availability may not be the explanation; although glycine is a good zinc chelator, addition of zinc does not lower the progressive inhibition exerted by EGCG.

Apart from possible chelation, direct EGCG–protein interaction has to be taken into account. The experimental results of tumor gelatinase binding to gelatin in presence of the flavanol (Fig. 4)—run at 4 °C to restrain enzymatic activities—can be described according 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 considers the formation of a ternary inactive complex. At high EGCG/MMP ratios, the lowering in gelatin-bound enzyme recovery indicates that the free flavanol 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. According 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 gelatin, after incubation with the flavanol and chemically induced dissociation (high ionic strength, denaturation, pH-induced ionization of phenolic groups).

Serum molarity of gelatinases less than 0.01 μM[31] is 10 times lower than that of EGCG in moderate green tea drinkers.[7] These gelatinases are most probably (reversibly) blocked by serum and/or tissue inhibitors, but even so, they may intercept EGCG. A close study of whether these interactions can take place in the plasma will reveal the extent to which the inhibition of the in-tissue tumor MMPs is influenced.

These data show: 1) that the EGCG concentration effective in inhibiting MMP-2 and MMP-9 is at least 500-fold lower than that reported for uPA; 2) that even lower concentrations (equivalent to that in the plasma of moderate green tea drinkers) are effective in reducing tumor cell invasion by 50%; 3) 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 trial synthetic inhibitors of MMPs; and 4) that the inhibitory activity is not due to metal-chelating properties of EGCG but correlates with direct binding of EGCG to gelatinases. In addition to having a preventive 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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