Inhibition of Collagenases from Mouse Lung Carcinoma Cells by Green Tea Catechins and Black Tea Theaflavins

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Theaflavin and theaflavin digallate, which are components of black tea were examined by in vitro invasion assay with mouse Lewis lung carcinoma LL2-Lu3 cells, which are highly metastatic. The compounds inhibited invasion by the tumor cells. Gelatinzymography showed that the cells secreted matrix metalloproteinases (MMPs), probably including MMP-2 and MMP-9, which may be involved in tumor cell invasion and metastasis. Theaflavin and theaflavin digallate also inhibited MMPs from the culture medium of these tumor cells, as did (−)-epigallocatechin gallate. These results suggest that theaflavin, theaflavin digallate, and (−)-epigallocatechin gallate inhibit tumor cell invasion by inhibiting type IV collagenases of the LL2-Lu3 cells.

Key words: (−)-epigallocatechin gallate; theaflavin; theaflavin digallate; matrix metalloproteinase; invasion

Orally administered green tea and (−)-epigallocatechin gallate (EGCG), a major constituent of green tea, have antitumor effects.1–4 Green tea inhibits in vivo metastasis and in vitro invasion of mouse Lewis lung carcinoma LL2-Lu3 cells, which are highly metastatic.5 This inhibition may be related to the inhibition of matrix metalloproteinases (MMPs) from these cells6 because this family of matrix-degrading zinc-enzymes seem to be important in tumor cell invasion and metastasis.6–9

Theaflavins are constituents of black tea and are derived from catechins by fermentation (Fig. 1).10 Theaflavins may have effect on tumor cells similar to that of catechins. Here, we examined the effect of theaflavins on invasion by LL2-Lu3 cells.

Materials and Methods

Materials. Catechins were obtained from Funakoshi Co., Ltd., Tokyo. EGCG, (±)-catechin, theaflavin, and theaflavin digallate each were coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech Inc., Tokyo) at the concentration of 5 mg/ml wet gel as described previously.3,11 Theaflavin and theaflavin digallate were prepared as described earlier.12,13 Gelatin (Sigma Chemical Co., St. Louis, MO) was coupled to CNBr-activated Sepharose 4B at the concentration of 10 mg/ml wet gel. Matrigel was purchased from Collaborative Biochemical Products, Bedford, MA. Chemotaxis cell chambers with polycarbonate filters (pore size, 8 μm) were obtained from Kurabo Co., Ltd., Osaka.

Cells. Highly metastatic LL2-Lu3 cells (LL2-Lu3 cells) were obtained as described before,5 and maintained in a serum-free culture medium, Cosmedium-001 (Cosmo Bio Co., Ltd., Tokyo).

In vitro Matrigel invasion assay. This assay was done as reported previously.5 In brief, the upper surface of the chemotaxis cell chamber filter was coated with 10 μg of Matrigel in a volume of 100 μl of Dulbecco's modified Eagle medium (DMEM) and dried. Filters were washed three times with 0.1% bovine serum albumin in DMEM just before use. LL2-Lu3 cells were suspended at 4 × 10^6 cells/ml in DMEM with 0.2% bovine serum albumin. Chemotaxis cell chambers were hung on a 24-well microplate. Cell suspensions (100 μl) and 100 μl of DMEM or a test solution were put into upper compartments of the chemotaxis cell chambers and the cell culture medium (500 μl) was put into wells of the microplate. After incubation at 37°C in a CO2 incubator for 6–8 h, the LL2-Lu3 cells on the upper surface of the filters were wiped away with a cotton swab. Filters were fixed with methanol and stained with hematoxylin and eosin. The numbers of LL2-Lu3 cells that had penetrated to the lower surface of the

Fig. 1. Structures of EGCG (1), Theaflavin (2), and Theaflavin Digallate (3).
filters were counted under a microscope at the magnification of × 200. Solutions of theaflavins at various concentrations in DMEM were filtered to stabilize them and used as test solutions.

MMPs from LL2-Lu3 cells. LL2-Lu3 cells were cultured in Cosmedium-001 and the conditioned medium (500 ml) was put on a gelatin-agarose column and passed through a gel bed of 10 ml. The column was washed with 0.01 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, and 0.01% (v/v) Brij-35. The bound fraction was eluted with 0.05 M Tris-HCl buffer, pH 7.5, containing 1 M NaCl, 5% dimethyl sulfoxide (Me2SO), and 0.01% (v/v) Brij-35, and fractions of 1 ml were collected. Fractions were monitored by gelatin zymography as described elsewhere.[14]

Type IV collagenase assay. Collagenases from LL2-Lu3 cells separated by the gelatin-agarose column as described above were dialyzed against 0.05 M Tris-HCl buffer (pH 7.5) containing 10 mM CaCl2. The dialyzed collagenase solution was concentrated 10-fold in a Centricon-10 microconcentrator (Amicon, Danvers, MA) and collagenases were activated with p-aminophenylmercuric acetate. The activity of type IV collagenases was measured with a type IV collagenase activity test kit (Cosmo Bio Co., Ltd., Tokyo). Catechins and theaflavins dissolved in 0.05 M Tris-HCl buffer (pH 7.5) containing 1 mM CaCl2 were used as collagenase inhibitor solutions. A 25 μl portion of a solution of type IV collagen labeled with fluorescein isothiocyanate was added to a mixture of 25 μl of neutral buffer, 40 μl of activated collagenases from LL2-Lu3 cells, and 10 μl of the collagenase inhibitor solution at 1000, 500, 250, 125, 62.5, or 0 μM. The mixture was then incubated at 37°C for 4 h. To the digest was added 300 μl of ethanol and the mixture was kept at 4°C for 30 min. Finally, 2.7 ml of phosphate-buffered saline, pH 7.5, was added to 300 μl of the supernatant of the reaction mixture obtained by centrifugation at 8000 × g for 10 min. Fluorescence was measured with excitation at 490 nm and emission at 520 nm.

Interaction between MMPs and EGCg. (+)-catechin, theaflavin, or theaflavin digallate. Conditioned culture medium (300 ml) of LL2-Lu3 cells was put on an EGCg-agarose column with a gel bed of 5 ml. The column was washed with 0.01 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, and 0.01% (v/v) Brij-35. The bound fraction was eluted with 0.05 M Tris-HCl buffer, pH 7.5, containing 1 M NaCl, 5% Me2SO, and 0.01% (v/v) Brij-35, and fractions of 1 ml were collected. Fractions were monitored by gelatin zymography. Similar experiments were done with affinity columns of (+)-catechin, theaflavin, and theaflavin digallate immobilized on agarose.

Results

In vitro Matrigel invasion assay

When LL2-Lu3 cells were cultured in the presence of theaflavin or theaflavin digallate, their Matrigel invasion was inhibited in a concentration-dependent way (Fig. 2).

Collegenases from LL2-Lu3 cells

When serum-free conditioned medium of cultured LL2-Lu3 cells was studied by affinity chromatography with gelatin-agarose, zymography on gels containing gelatin showed strong gelatinolytic activities of substances with molecular weights of approximately 66,000 and 92,000 together with other positions (Fig. 3). Estimation of molecular mass suggested the presence of 66-kDa gelatinase A (matrix metalloproteinase-2; MMP-2) and 92-kDa gelatinase B (MMP-9).[7]

Effects of catechins and theaflavins on type IV collagenase from LL2-Lu3 cells

EGCg, theaflavin, and theaflavin digallate inhibited type IV collagenase activity in a concentration-dependent way (Fig. 4). (+)-Catechin and (−)-epicatechin at concentrations up to 100 μM did not inhibit type IV collagenases.

Fig. 3. Zymography of MMPs from LL2-Lu3 Cells.

Standard proteins (lane 1): rabbit muscle myosin (205,000), Escherichia coli β-galactosidase (116,000), rabbit muscle phosphorylase b (97,000), bovine serum albumin (66,000), chicken ovalbumin (45,000), and bovine carbonic anhydrase (29,000) from top to bottom. Lane 2, conditioned culture medium of LL2-Lu3 cells. Lane 3, gelatin-agarose bound fraction obtained by elution with 1 M NaCl. Lane 4, EGCg-agarose bound fraction obtained by elution with 1 M NaCl. Lane 5, theaflavin-agarose bound fraction obtained by elution with 1 M NaCl. Lane 6, theaflavin digallate-agarose bound fraction obtained by elution with 1 M NaCl. Bands with collagenase activity are still unstained after staining with Coomassie brilliant blue R-250.
Interaction between type IV collagenases and EGCg, (+)-catechin, theaflavin, or theaflavin digallate

When serum-free conditioned medium of cultured LL2-Lu3 cells was studied by affinity chromatography with EGCg-agarose, zymography showed that collagenases were bound to and eluted from the column (Fig. 3). The collagenases seemed to include MMP-2 and MMP-9. When similar experiments were done with theaflavin-agarose and theaflavin digallate-agarose, bands of M MPs were also detected from the bound fraction (Fig. 3). When plain (unsubstituted) Sepharose 4B was used, MMP bands were not detected from the bound fraction. MMP bands were not detected in the fraction bound by (+)-catechin-agarose, either.

Discussion

Here, we found that invasion by lung carcinoma LL2-Lu3 cells was inhibited by theaflavin and theaflavin digallate. Two catechins that contain gallate, (-)-epicatechin gallate and EGCg, inhibit such invasion, but (+)-catechin and (-)-epicatechin do not; we have proposed that the mechanism of inhibition includes the inhibition of collagenase by these catechins. 5

LL2-Lu3 cells produced M MPs, probably including MMP-2 and MMP-9. That the type IV collagenase activity of these matrix metalloproteinases was inhibited by EGCg, theaflavin, and theaflavin digallate, but was not inhibited by (+)-catechin which did not inhibit invasion by cells either, suggests that inhibition of such invasion and inhibition of these collagenases are related. In other words, EGCg, theaflavin, and theaflavin digallate inhibited the Matrigel invasion of LL2-Lu3 cells by inhibiting their matrix metalloproteinases.

Inhibition of cell invasion and collagenase activities by theaflavin, which lacks a gallate group, is interesting. Perhaps EGCg and theaflavin have similar steric configurations of phenolic hydroxyl groups.

Affinity chromatography showed that LL2-Lu3 cell collagenases, presumably including MMP-2 and MMP-9, were bound to EGCg, theaflavin, and theaflavin digallate, although the MMP-2-like collagenase seems to have less affinity than MMP-9 (Fig. 3). This finding, together with the finding that the collagenases were not bound by (+)-catechin-agarose, suggests that the mechanism of the inhibition of collagenases by EGCg and these theaflavins involves direct binding between them.

Wang et al. found that orally administered black tea could inhibit the formation of ultraviolet-light-induced skin tumors, and decreased tumor size in SKH-1 mice treated with 7,12-dimethylbenz[a]anthracene. 6,7 Their results may be explained, at least in part, by our finding that theaflavins, components of black tea, inhibit collagenases thought to be involved in tumor cell invasion and endothelial growth. 6 It is possible that oral administration of black tea might also prevent cancer metastasis.

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References