Short communication

Inhibition of hepatoma cell invasion beneath mesothelial-cell monolayer by sera from tea- and related component-treated rats and their modes of action

Guoying Zhang, Yutaka Miura & Kazumi Yagasaki*

Department of Applied Biological Science, Tokyo Noko University, Saiwaicho 3-5-8, Fuchu, Tokyo, 183-8509, Japan

(* Author for correspondence; E-mail: yagasaki@cc.tuat.ac.jp; Fax: +81 42 367 5713)

Received 28 November 2000; accepted 2 February 2001

Key words: hepatoma, invasion, reactive oxygen species, sera from tea-treated rats

Abstract

The bioavailability and action of teas on the invasion of a rat ascites hepatoma cell line, AH109A, were determined and their modes of action were by co-culturing the cancer cells with a rat mesentery-derived mesothelial-cell (M-cell) monolayer in the presence of sera from rats orally given teas and their component, (-)-epigallocatechin gallate (EGCG). The rat sera obtained 2 and 5 hr after oral intubation of a low concentration of green, oolong, or black tea, or EGCG significantly inhibited AH109A invasion underneath the M-cell monolayer. These sera showed a time-dependent and significant inhibitory effect on the AH109A invasion. The 2-hr sera and 2.5 μ M EDTA in the medium completely eliminated the enhancement of AH109A invasion induced by a reactive oxygen species (ROS)-generating system. These results show that the inhibition of relevant ROS-potentiased invasion of AH109A cells across the M-cell monolayer may be due to the antioxidative action of EGCG, the *in vivo* metabolites, and tea-induced changes in the endogenous substances. The results suggest that the drinking of tea in daily life may have certain preventive and therapeutic effects against cancer cell invasion.

Abbreviations: PGT, powdered green tea; POT, powdered oolong tea; PBT, powdered black tea; EGCG, (-)-epigallocatechin gallate; ROS, reactive oxygen species; HX-XO, hypoxanthine and xanthine oxidase; RS, rat serum; EDTA, ethylenediaminetetraacetic acid; M-cells, mesothelial-cells.

Introduction

The metastatic capacity of cancer cells is one of the critical factors affecting the survival of cancer patients. Invasion across the host cell layers and extracellular matrix are the most characteristic and key steps in the metastatic cascade (Nicolson et al., 1989; Fidler, 1991; Liotta et al., 1991; Al-Mehdi et al., 2000). Clearly, an agent which could efficiently inhibit the invasion of cancer cells would be a hopeful candidate to suppress cancer metastasis. Epidemiological and experimental studies have suggested the potential of teas in the prevention of some cancers (Taniguchi et al., 1992; Hirose et al., 1993; Nishida et al., 1994;

Sazuka et al., 1995; Blot et al., 1996; Katiyar and Mukhter, 1996; Okabe et al., 1997). We have previously demonstrated that higher concentrations of tea extracts and tea polyphenolic compounds inhibit the proliferation and invasion of a rat ascites hepatoma cell line, AH109A cells (Miura et al., 1997; Zhang et al., 1999). However, whether drinking tea in our daily life really has certain preventive and therapeutic effects against the invasion of tumor cells is unclear. The mechanisms of the inhibition of invasion of cancer cells by the sera from tea- or (-)-epigallocatechin gallate (EGCG)-treated rats remain unknown. To answer these questions, we employed in this study *ex vivo* models of the invasion of rat hepatoma cells across

Figure 1. Inhibition of the invasion of hepatoma cells across the M-cell monolayer by sera from rats treated with a low concentration of PGT, POT, or PBT. (A) Confluent rat M-cell monolayer after culturing for 7 days. (B) Growing AH109A cells (a rat ascites hepatoma cell line). (C) The invasion of AH109A cells underneath the cultured M-cell monolayer after co-culturing for 24 hr. (D, E, and F) Inhibition of AH109A invasion by rat sera obtained 2 hr after oral intubation of a low concentration of PGT, POT, or PBT, respectively.

the rat mesothelial cell (M-cell) monolayer in the co-culture, and systematically investigated the effects and mechanisms of action of the sera from rats treated with a low concentration of tea and EGCG against the invasion of these cells.

Materials and methods

Preparation of sera from PGT-, POT-, PBT- and EGCG-treated rats

Two grams of powdered green tea (PGT), powdered oolong tea (POT) and powdered black tea (PBT) (Yamato Tea Co., Ltd., Nara, Japan) were respectively extracted by 100 ml of boiling water for 3 min. (-)-epigallocatechin gallate (EGCG) (>95% pure, Sigma Chemical Co. St. Louis, MO, U.S.A.) was dissolved in water at a concentration of 4 mg ml⁻¹. Each solution of PGT, POT, PBT, and EGCG was, respectively, intubated at 1 ml 100 g⁻¹ body weight to 5-week-old male Donryu rats (NRC Haruna, Gunma, Japan). This treatment is respectively equivalent to drinking about 2–3 cups of green, oolong, or black tea, and 2-fold EGCG in 2–3 cups of green tea liquor in daily life (Lee et al., 1995; Yang et al., 1996, 1998). Before oral intubation of each tea solution, these rats were deprived of their diet but allowed free access to water for 16 hr. Blood was collected at 2 and 5 hr after oral intubation. The sera were prepared by centrifugation and sterilized by filtration for *ex vivo* invasion assays.

Stock culture of AH109A hepatoma cells

A rat ascites hepatoma cell line, AH109A was provided by the Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan. AH109A cells were maintained in the peritoneal cavities of the male Donryu rat, prepared from accumulated ascites and cultured *in vitro* in Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% calf serum (CS, JRH, Lenexa, KS, U.S.A.) for up to 2 months. These isolated AH109A cells were cultured for at least one week to eliminate macrophages and neutrophils and then used for the assays.

Ex vivo invasion assay

The invasion assay was based on the method of Akedo et al. (Akedo et al., 1986; Horai et al., 1992) with slight modifications as described previously (Miura et

Table 1. *Ex vivo* effects of sera obtained at different times after oral intubation of PGT, POT, PBT, and EGCG against AH109A invasion across M-cell monolayer^a

Treatment	No. of invaded cells and colonies cm ⁻²
Control (RS)	1408 ± 51 a ^b
PGT-RS (2 hr-sera)	848 ± 45 b
PGT-RS (5 hr-sera)	1130 ± 31 c
POT-RS (2 hr-sera)	843 ± 45 b
POT-RS (5 hr-sera)	1150 ± 32 c
PBT-RS (2 hr-sera)	838 ± 32 b
PBT-RS (5 hr-sera)	1133 ± 22 c
EGCG-RS (2 hr-sera)	1080 ± 34 c
EGCG-RS (5 hr-sera)	1162 ± 53 c

^a Each rat serum was obtained 2 and 5 hr after oral intubation of a low concentration of PGT, POT, PBT, or EGCG. The number of invaded AH109A cells and colonies across the cultured M-cell monolayer was examined 48 hr later.

^b a, b, c: Values not sharing a common letter are significantly different at $P < 0.05$ by Tukey-Kramer multiple comparisons test. Values are expressed as means ± SEM of 10 areas.

This table represents 2 similar experiments.

al., 1997; Zhang et al., 1999). Briefly, the M-cells were isolated from the male Donryu rat mesentery. After digestion by trypsin, 1.2×10^5 cells were plated in a 60 mmΦ culture dish (Nalge Nunc International Co., Tokyo) with 2 mm grids. After M-cells had been cultured in MEM with 10% CS to a confluent state in 7–10 days, 2.4×10^5 AH109A cells were seeded on the M-cell monolayer in MEM in the presence of 10% rat sera (RS) obtained 2 or 5 hr after oral intubation of PGT, POT, PBT, or EGCG. The method of preparing the sera was mentioned above. After being co-cultured for 48-hr or 12, 24, 36, and 48 hr, respectively, the invaded AH109A cells and colonies were counted under a phase contrast microscope. At least 10 areas were counted and the invasive activity of the cells was indicated by the number of invaded cells and colonies cm⁻².

Pretreatment of AH109A cells with hypoxanthine and xanthine oxidase

AH109A cells were cultured for 1 hr in the absence or presence of 2.5 μM EDTA (Sigma, MO, U.S.A.) in MEM containing 10% RS, the 10% RS obtained 2 hr after oral intubation of PGT, POT, PBT, or EGCG, and 2.4 μg ml⁻¹ of hypoxanthine (HX, Sigma, U.S.A.) with 6.9×10^{-4} U ml⁻¹ of xanthine oxidase (XO,

Figure 2. Time-course of *ex vivo* inhibitory effects of sera from PGT-, POT-, and PBT-treated rats against AH109A invasion across the M-cell monolayer. Each rat serum was obtained 2 hr after oral intubation of a low concentration of PGT, POT, or PBT. Time-course of inhibitory effects of these rat sera on AH109A invasion beneath the cultured M-cell monolayer was examined at 12, 24, 36, and 48 hr after co-culture as described in the section 'Materials and Methods'. Each point and vertical bar represents the mean and SEM of 10 areas. Values not sharing a common letter a or b are significantly different at $P < 0.05$ by Tukey-Kramer multiple comparisons test. This figure represents 3 similar experiments.

Sigma, MO, U.S.A.) (Shinkai et al., 1986; Tanaka et al., 1997; Kozuki et al., 2000; Zhang et al., 2000). Then the treated AH109A cells were washed with MEM and seeded on the M-cell monolayer in MEM containing 10% RS without tea samples. After co-culturing for 24 hr, the invasive capacities were assessed as described above.

Statistical analysis

Data were expressed as means $\pm$ standard errors of ten areas. Multigroup comparisons were conducted by a one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test.

Results

Inhibition of AH109A invasion by sera from PGT-, POT-, PBT-, or EGCG-treated rats

Figure 1 shows, respectively, the confluent M-cell

monolayer (A), growing AH109A cells (B), AH109A cells invading beneath the cultured M-cell monolayer (C) and the inhibition of AH109A invasion by sera from PGT-, POT-, or PBT-treated rats (D, E, and F, respectively). The control AH109A cells invaded abundantly and time-dependently underneath the M-cell monolayer. As shown in Figure 2, compared with the control, the sera obtained 2 hr after oral intubation of the low concentration of PGT, POT, or PBT could significantly and time-dependently suppress AH109A invasion across this monolayer. The inhibitory rate was about 52–55%, 43–52%, 36–47%, and 39–40%, respectively, at 12, 24, 36, and 48 hr after being co-cultured. In another experiment (Table 1), the sera obtained 2 and 5 hr after oral intubation of PGT, POT, PBT, or EGCG also significantly inhibited AH109A invasion. The 2-hr sera showed much stronger inhibition than 5-hr sera from PGT-, POT-, or PBT-treated rats, but there was no significant difference between the 2- and 5-hr sera from EGCG-treated

Figure 3. Inhibitory effects of EDTA and the sera from PGT-, POT-, PBT-, or EGCG-treated rats against ROS-potentiated AH109A invasion across the M-cell monolayer. Each rat serum was obtained 2 hr after oral intubation of a low concentration of PGT, POT, PBT, or EGCG. AH109A cells were cultured for 1 hr in the absence or presence of $2.5 \mu\text{M}$ of EDTA or these rat sera, and $2.4 \mu\text{g ml}^{-1}$ hypoxanthine with $6.9 \times 10^{-4} \text{U ml}^{-1}$ of xanthine oxidase. The treated AH109A cells were then seeded on the M-cell monolayer. The effects of these rat sera on invasion of AH109A cells underneath the cultured M-cell monolayer were examined as described in the section 'Materials and Methods'. Each value and bar represents the mean and SEM of 10 areas. Values not sharing a common letter a or b are significantly different at $P < 0.05$ by Tukey-Kramer multiple comparisons test. This figure represents 3 similar experiments.

rats, or between the 2-hr EGCG sera and 5-hr PGT, POT, or PBT sera.

Inhibition of ROS-potentiated AH109A invasion by EDTA and sera from PGT-, POT-, PBT-, or EGCG-treated rats

To understand the mechanism of the inhibition of AH109A invasion by the sera from these low concentration tea-treated rats, we examined the effects of reactive oxygen species (ROS) on AH109A invasion across the M-cell monolayer. As shown in Figure 3, 1 hr pretreatment of AH109A cells with HX-XO pro-

ducing ROS increased significantly the invasion by 39% over the control group without such pretreatment, when AH109A proliferation was not significantly affected by the pretreatment with EDTA, these sera or HX-XO (data not shown). EDTA ($2.5 \mu\text{M}$) in MEM containing 10% RS and the sera obtained 2 hr after oral intubation of PGT, POT, PBT, or EGCG suppressed completely to the basal value the enhancement of AH109A invasion induced by the HX-XO pretreatment (Figure 3).

Discussion

Our present results confirmed that the sera obtained after intubation of a low concentration of PGT, POT, PBT, or EGCG could completely eliminate the enhancement of AH109A invasion caused by exogenous ROS produced from the HX-XO system (Figure 3). Even in the absence of a ROS-generating system, these sera also suppressed significantly the spontaneous AH109A invasion within 48 hr in a time-dependent manner (Figures 1 and 2, and Table 1). Compared with rats, the absorption of tea components including catechins is more efficient in humans (Yang et al., 1996). These findings mean that drinking tea might have a certain inhibitory effect against the invasion of cancer cells. EGCG, the *in vivo* metabolites, and tea-induced changes in the endogenous substances may produce these anticancer activities. Indeed, EGCG has been detected in rat and human plasmas after the drinking of decaffeinated tea (Lee et al., 1995; Yang et al., 1998). The 2-hr EGCG sera showed weaker inhibition than 2-hr PGT, POT, and PBT sera against AH109A invasion (Table 1). This suggests that not only the one component, EGCG, but other tea components may also possess certain anticancer activities. Another tea component (-)-epicatechin has displayed the synergistic inhibitory effects with EGCG against both spontaneous and exogenous ROS-potentiated AH109A invasion underneath the M-cell monolayer (Zhang et al., 2000).

The HX-XO solution generates both superoxide anion ($\cdot\text{O}_2^-$) and hydrogen peroxide (H_2O_2), and finally hydroxyl radical ($\cdot\text{OH}$) via the Fenton reaction if trace amounts of transition metal ions are present (Thomas et al., 1993). EDTA in MEM containing 10% RS inhibits the enhancement of AH109A invasion, possibly by chelating the transition metal ions and thus preventing the formation of $\cdot\text{OH}$ (Sandstrom et al., 1997; Kachur et al., 1998). Since EGCG possesses the chelating ability (Guo et al., 1996), EGCG and certain tea components in the sera from tea-treated rats also may suppress the enhancement of AH109A invasion, possibly by preventing the formation of $\cdot\text{OH}$ as EDTA did. The present study further confirms our previous results that EDTA in MEM containing 10% calf serum suppressed the increase of invasion of AH109A in the HX-XO system (Zhang et al., 2000). Our previous *in vitro* study demonstrated that 10 μM of EGCG or theaflavins inhibited the $\cdot\text{OH}$ - and ROS-potentiated AH109A invasion in the HX-XO system as the chelator EDTA did by restraining $\cdot\text{OH}$ and ROS

effects (Zhang et al., 2000). Tumor cells can produce a large amount of ROS, which is thought to be involved in cancer invasion and metastasis (Szatrowski and Nathan, 1990). The $\cdot\text{O}_2^-$ and H_2O_2 may promote AH109A invasion and also be scavenged by the sera from the PGT-, POT-, PBT-, or EGCG-treated rats. As an antioxidant, tea has the ability to scavenge not only $\cdot\text{OH}$, but also $\cdot\text{O}_2^-$ and H_2O_2 . This ROS-scavenging activity may be another explanation for the suppressive actions of tea- or EGCG-treated rat sera. Indeed, the sera from PGT-, POT-, PBT-, or EGCG-treated rats have suppressed the spontaneous invasion of AH109A cells (Figures 1 and 2, and Table 1). It is reported that diets rich in linoleic acid stimulate the metastasis of human breast cancer cells. This omega-6-fatty acid is metabolized to various lipoxygenase and cyclooxygenase products, several of which are associated with tumor cell invasion and metastasis (Liu et al., 1996). Green tea polyphenols inhibited 12-O-tetradecanoyl-phorbol-13-acetate-induced epidermal lipoxygenase and cyclooxygenase in mice (Katiyar et al., 1992). It is also likely that the polyphenolic components in these sera from the tea-treated rats might suppress the lipoxygenase and cyclooxygenase and thus the AH109A invasion underneath the M-cell monolayer. Actually, EGCG and green tea have shown inhibitory effects against lung metastasis of mouse B16 melanoma cells (Taniguchi et al., 1992) and mouse Lewis lung carcinoma cells (Sazuka et al., 1995), respectively. However, whether the antioxidative activity of EGCG and green tea is the major mechanism of *in vivo* inhibition of invasion and metastasis of cancer cells requires further confirmation.

References

- Akeda H, Shinkai K, Mukai M, Mori Y, Tateishi R, Tanaka K, Yamamoto R & Morishita T (1986) Interaction of rat ascites hepatoma cells with cultured mesothelial cell layers: A model for tumor invasion. *Cancer Res* 46: 2416–2422.
- AL-Mehdi AB, Tozawa K, Fisher AB, Shientag L, Lee A & Muschel RJ (2000) Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: A new model for metastasis. *Nature Medicine* 6(1): 100–102.
- Blot WJ, Chow WH & McLaughlin JK (1996) Tea and cancer: A review of the epidemiological evidence. *Eur J Cancer Prev* 5: 425–438.
- Fidler IJ (1991) Cancer metastasis. *Br Med Bull* 47: 157–177.
- Guo Q, Zhao B, Li M, Shen S & Xin W (1996) Studies on protective mechanisms of four components of green tea polyphenols against lipid peroxidation in synaptosomes. *Biochim Biophys Acta* 1304: 210–222.

- Hirose M, Hoshiya T, Akagi K, Takahashi S, Hara Y & Ito N (1993) Effects of green tea catechins in a rat multi-organ carcinogenesis model. *Carcinogenesis* 14, 1549–1533.
- Horai T, Imamura F, Nakae T, Nakamura S, Mukai M, Shinkai K, Higashino K & Akedo H (1992) Interactions of lung cancer cells with the human mesothelial cell monolayer: An *in vitro* model for cancer invasion. *Jpn J Clin Oncol* 22: 67–72.
- Kachur AV, Tuttle SW & Biaglow JE (1998) Autoxidation of ferrous ion complexes: A method for the generation of hydroxyl radicals. *Radiat Res* 150: 475–482.
- Katiyar S, Agarwal R & Wood GS (1992) Inhibition of 12-O-tetradecanoylphorbol-13-acetate-caused tumor promotion in 7,12-dimethylbenz(a)anthracene-initiated SENCAR mouse skin by a polyphenolic fraction isolated from green tea. *Cancer Res* 52: 6890–6897.
- Katiyar SK & Mukhter H (1996) Tea in chemoprevention of cancer: Biological and experimental studies. *Int J Oncol* 8: 221–238.
- Kozuki Y, Miura Y & Yagasaki K (2000) Inhibitory effects of carotenoids on the invasion of rat ascites hepatoma cells in culture. *Cancer Lett* 151: 111–115.
- Lee M-J, Wang ZY, Li H, Chen L, Sun Y, Gobbo S, Balentine D & Yang CS (1995) Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer Epidemiol Biomarkers Prev* 4: 393–399.
- Liotta LA, Steeg PS & Stetler-Stevenson WG (1991) Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. *Cell* 64: 327–336.
- Liu XH, Connolly JM & Rose DP (1996) Eicosanoids as mediators of linoleic acid-stimulated invasion and type IV collagenase production by a metastatic human breast cancer cell line. *Clin Exp Metastasis* 14: 145–152.
- Miura Y, Shiomi H, Sakai F & Yagasaki K (1997) Assay system for screening food components that have anti-proliferative and anti-invasive activity to rat ascites hepatoma cells: *In vitro* and *ex vivo* effects of green tea extract. *Cytotechnology* 23: 127–132.
- Nicolson GL, Belloni PN, Tressler RJ, Dulski K, Inoue T & Cavanaugh PG (1989) Adhesive, invasive, and growth properties of selected metastatic variants of a murine large-cell lymphoma. *Invasion Metastasis* 9: 102–116.
- Nishida H, Omori M, Fukutomi Y, Ninomiya M, Nishiwaki S, Suganuma M, Moriwaki H & Muto Y (1994) Inhibitory effects of (-)-epigallocatechin gallate on spontaneous hepatoma in C3H/NeNCrj mice and human hepatoma-derived PLC/PRF/5 cells. *Jpn J Cancer Res* 85, 221–225.
- Okabe S, Suganuma M, Hayashi M, Sueoka E, Komori A & Fujiki H (1997) Mechanisms of growth inhibition of human lung cancer cell line, PC-9, by tea polyphenols. *Jpn J Cancer Res* 88: 639–643.
- Sandstrom BE, Svoboda P, Granstrom M, Harms-Ringdahl M & Candeias LP (1997) H₂O₂-driven reduction of the Fe³⁺-quin2 chelate and the subsequent formation of oxidizing species. *Free Radic Biol Med* 23: 744–753.
- Sazuka M, Murakami S, Isemura M, Satoh K & Nukiwa T (1995) Inhibitory effects of green tea infusion on *in vitro* invasion and *in vivo* metastasis of mouse lung carcinoma cells. *Cancer Lett* 98: 27–31.
- Shinkai K, Mukai M & Akedo H (1986) Superoxide radical potentiates invasive capacity of rat ascites hepatoma cells *in vitro*. *Cancer Lett* 32: 7–13.
- Szatrowski TP & Nathan CF (1990) Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res* 50: 2018–2021.
- Tanaka M, Kogawa K, Nishihori Y, Kuribayashi K, Nakamura K, Muramatsu H, Koike K, Sakamaki S & Niitsu Y (1997) Suppression of intracellular Cu-Zn SOD results in enhanced motility and metastasis of Meth A sarcoma cells. *Int J Cancer* 73: 187–192.
- Taniguchi S, Fujiki H, Kobayashi H, Go H, Miyado K, Sadano H & Shimokawa R (1992) Effect of (-)-epigallocatechin gallate, the main constituent of green tea, on lung metastasis with mouse B16 melanoma cell lines. *Cancer Lett* 65: 51–54.
- Thomas C, Vile GF & Winterbourn CC (1993) The hydrolysis product of ICRF-187 promotes iron-catalysed hydroxyl radical production via the Fenton reaction. *Biochem Pharmacol* 45: 1967–1972.
- Yang CS, Chen L, Lee M-J & Landau JM (1996) Effects of teas on carcinogenesis in animal models and humans. In: *Dietary Phytochemicals in Cancer Prevention and Treatment* (Am. Inst. Cancer Res.) (pp. 51–56), New York, Plenum Press.
- Yang CS, Chen L, Lee MJ, Balentine D, Kuo MC & Schantz SP (1998) Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers. *Cancer Epidemiol Biomarkers Prev* 7: 351–354.
- Zhang GY, Miura Y & Yagasaki K (1999) Effects of green, oolong and black teas and related components on the proliferation and invasion of hepatoma cells in culture. *Cytotechnology* 31: 37–44.
- Zhang GY, Miura Y & Yagasaki K (2000) Suppression of adhesion and invasion of hepatoma cells in culture by tea compounds through antioxidative activity. *Cancer Lett* 152(2): 169–173.