Cytotechnology **36:** 195–200, 2001.

Short communication

Inhibitory effects of theanine and sera from theanine-fed rats on receptor-mediated cancer cell invasion beneath mesothelial-cell monolayers

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 2 July 2001; accepted 15 August 2001

Key words: AP-5, hepatoma, invasion, NMDA receptor, theanine

Abstract

To investigate the bioavailability and mode of action of theanine against cancer, we examined *in vitro* and *ex vivo* effects of theanine on invasion of a rat ascites hepatoma cell line of AH109A. Theanine dose-dependently inhibited the invasion of AH109A cells across rat mesentery-derived mesothelial-cell (M-cell) monolayers without restraining AH109A cell proliferation *in vitro*. Rat sera obtained after oral intubation of theanine also inhibited the invasion. A competitive N-methyl-D-aspartate (NMDA) type glutamate receptor antagonist, (±) 2-amino-5-phosphonopentanoic acid (AP-5), dose-dependently counteracted the theanine-mediated *in vitro* and *ex vivo* inhibition of AH109A invasion. A competitive non-NMDA type glutamate receptor antagonist, 6,7-dinitroquinoxaline 2,3-dione (DNQX), did not affect this inhibition by theanine *in vitro*. These results suggest that the inhibition of AH109A invasion by theanine may be mediated by the NMDA receptor of AH109A.

Abbreviations: AP-5, (±) 2-amino-5-phosphonopentanoic acid; DNQX, 6,7-dinitroquinoxaline 2,3-dione; NMDA, N-methyl-D-aspartate; M-cells, mesothelial cells.

Introduction

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 of AH109A cells (Zhang et al., 1999) and induce apoptosis and cell cycle arrest in these cells (Zhang et al., 2000a). The sera from low concentration tea- or (-)-epigallocatechin gallate (EGCG)-fed rats also suppress AH109A invasion and the mode of action is related to tea's antioxidative activity (Zhang et al., 2000b) like carotenoids (Kozuki et al., 2000), curcumin (Kozuki et al., 2001a) and resveratrol (Kozuki et al., 2001b). In order to examine effects of other effective tea components against cancer cell invasion and further understand their mechanisms of action, in the present study we investigated the effect of theanine on the invasion of AH109A cells.

Theanine, γ-glutamylethylamide, is one of the major amino acids in Japanese green tea. The amino acid is transported through the intestinal brush-border membrane (Kitaoka et al., 1996), incorporated into the serum, liver and brain (Terashima et al., 1999), and excreted into urine as itself or after being degraded into glutamic acid and ethylamine (Terashima et al., 1999). In the brain of rats, theanine is reported to act through N-methyl-D-aspartate (NMDA) type glutamate receptor (Yokogoshi et al., 1998) which is one of three types of glutamate receptor; NMDA, non-NMDA and metabotropic types of glutamate receptor. Our results show that theanine significantly inhibits AH109A invasion across the normal rat mesothelial-cell (M-cell) monolayers *in vitro* and *ex vivo*. This inhibition is suggested to be mediated by the NMDA type glutamate receptor of AH109A.

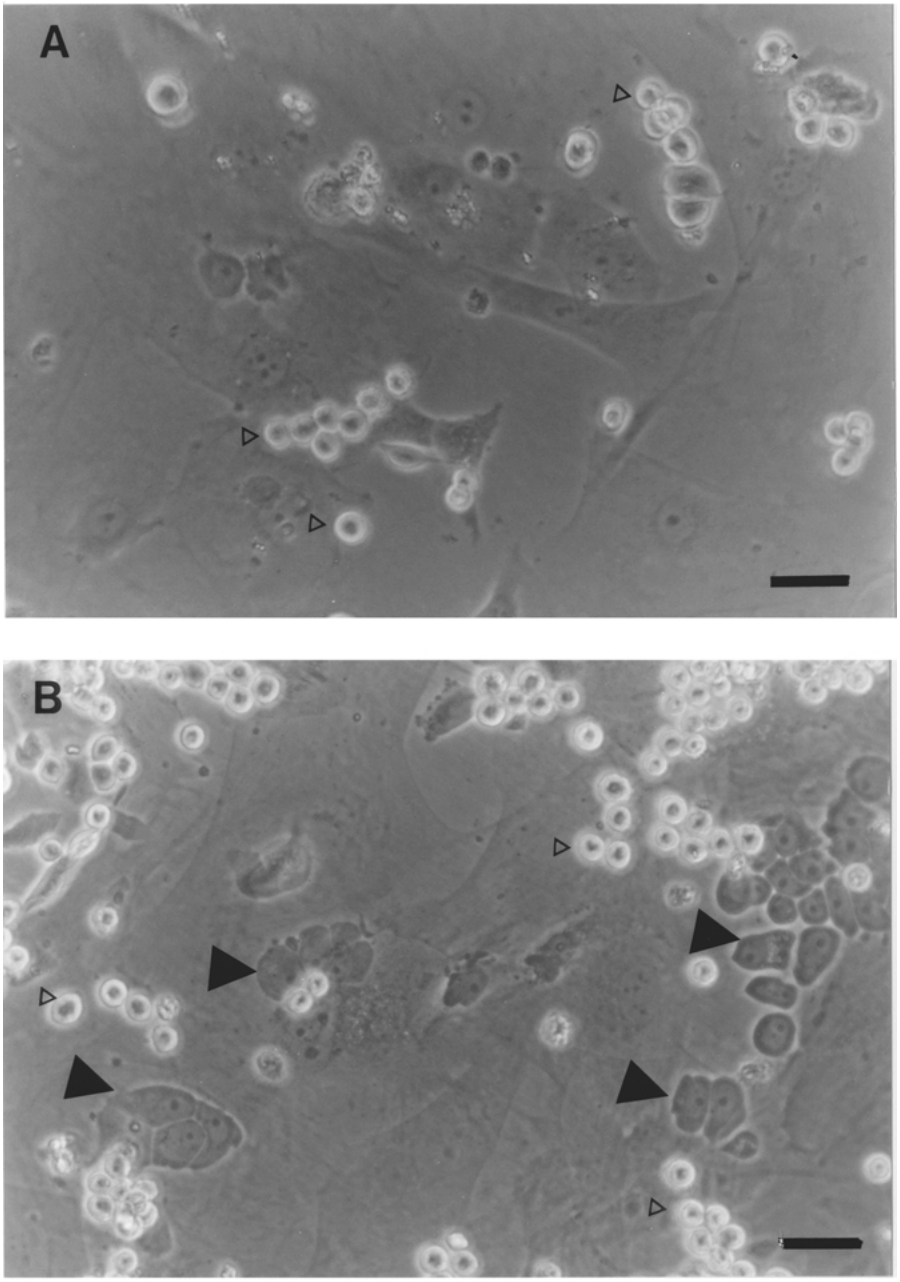

Figure 1. AH109A cells on and beneath M-cell monolayer. Phase-contrast micrographs were taken at 0 hr (A) and 48 hr (B) after seeding the hepatoma cells. ▲, hepatoma cells penetrating M-cell monolayer; △, hepatoma cells on M-cell monolayer. Scale: 30 μm.

Materials and methods

Preparation of the solutions of theanine, AP-5 and DNQX

Theanine (>98% pure, Tokyo Chemical Industry Co., Tokyo, Japan) and (±) 2-amino-5-phosphonopentanoic acid (AP-5, Funakoshi Co., Tokyo, Japan) were, respectively, dissolved in Ca-Mg-free phosphate-buffered saline (PBS (-)) for *in vitro* invasion assay. 6, 7-Dinitroquinoxaline 2, 3-dione (DNQX, Funakoshi Co.) were first dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO, U.S.A.) and then diluted with PBS (-) for *in vitro* assay. In an

experiment on DNQX, DMSO was contained in all experimental media at a final concentration of 0.1%. No cytotoxic effect on M-cells and AH109A was observed at this DMSO concetration.

Animal experiment and preparation of the sera from theanine-fed rats

Male Donryu rats (4 weeks old) were purchased from NRC Haruna, Gunma, Japan. Animals were treated in accordance with guidelines established by the Animal Care and Use Committee at Tokyo Noko University. The animals were given water and a stock pellet diet (CE-2, CLEA JAPAN, Tokyo, Japan) *ad libitum*, and kept in an air-conditioned room with an 8:00 a.m. to 8:00 p.m. light cycle. They were deprived of their diet at 6:00 p.m. but allowed free access to water until oral administration of theanine which was conducted at 10:00 a.m. the next day. Theanine was dissolved in water at the concentration of 40 mg ml^{-1} (Terashima et al., 1999). The theanine solution was orally intubated to the rats at a dose of 1 ml 100 g^{-1} body weight. Blood was, respectively, collected at 0, 0.5, 1, 2, 3, 6, and 12 hr after oral intubation of the solution. The sera were prepared by centrifugation and sterilized by filtration. The prepared sera were added to Eagle's minimun essential medium (MEM, Nissui Pharmaceutical Co., Tokyo, Japan) at a concentration of 10% for *ex vivo* invasion assay.

Culture of AH109A hepatoma cells

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

In vitro proliferation assay

Effect of theanine on AH109A proliferation was examined by WST-1 method (Ishiyama et al., 1996; Zhang et al., 1999). For WST-1 assay, 5×10^3 AH109A cells were cultured for 44 hr in each well of a 96-well-plate containing 10% CS/MEM in the absence or presence of different concentrations of theanine. WST-1 solution was then added and the cells were cultured for another 4 hr. Viable cells can make WST-1 produce highly colored formazan dyes and the absorbance at 415 and 595 nm was measured with a microplate reader (BIO-RAD, Model 450, California, U.S.A.). The relative proliferation rate of AH109A was calculated between the control group and test groups treated with different concentrations of theanine.

In vitro and ex vivo invasion assays

The invasion assay was based on the method described previously (Akedo et al., 1986) with slight modifications as described previously (Miura et al., 1997; Zhang et al., 1999, see Figure 1). Briefly, mesothelial cells (M-cells) were isolated from the male Donryu rat mesentery. After digestion by trypsin, 1.2×10^5 cells were plated in a 60 mm$\varnothing$ culture dish with 2 mm grids (Nalge Nunc International Co., Tokyo). When M-cells were cultured to a confluent state within 7–10 days, 2.4×10^5 AH109A cells were seeded on the M-cell monolayer in MEM in the presence of 10% CS containing different concentrations of theanine, AP-5, or DNQX (in the case of *in vitro* invasion assay) or 10% rat serum (RS) containing different concentrations of AP-5 or the 10% RS obtained at different times after oral intubation of theanine (in the case of *ex vivo* invasion assay). After co-culture for 48 hr, the invaded AH109A cells and colonies underneath the rat M-cell monolayers were counted under a phase contrast microscope. Usually at least 10 areas were counted and the invasive activity of the cells was indicated by the numbers of invaded cells and colonies/cm^2.

Statistical analysis

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

Results

Effect of theanine on AH109A proliferation and invasion

The *in vitro* proliferation assay indicated that theanine at the concentrations of 25 to 1600 μM did not restrain the proliferation of AH109A cells (Figure 2A). At the same concentrations, theanine displayed dose-dependent and significant effects against AH109A cell

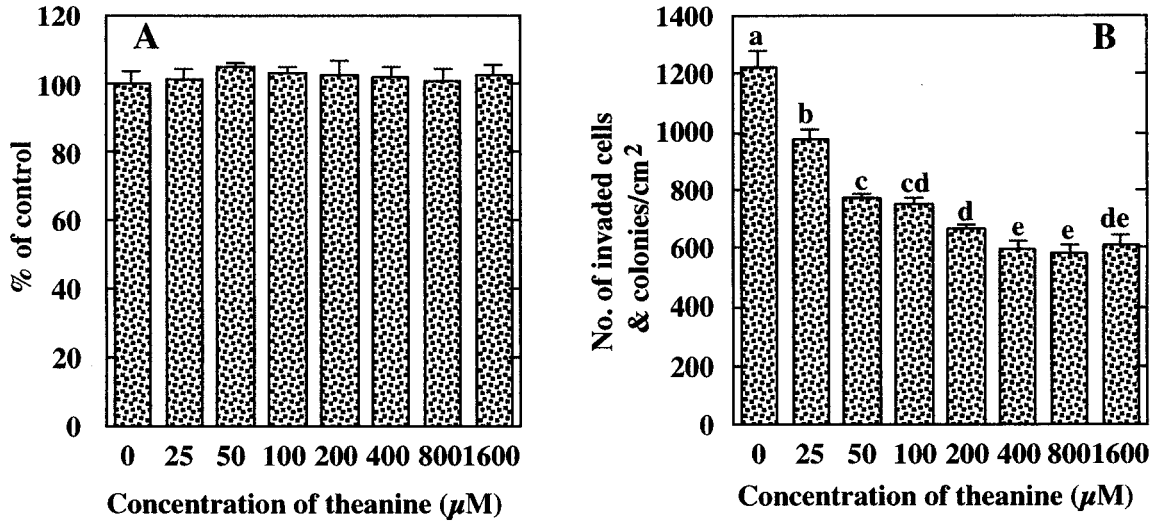

Figure 2. Effect of theanine on *in vitro* proliferation (A) and invasion (B) of AH109A cells. Theanine was added at the concentrations indicated and the effects on the proliferation (A) and invasion (B) were, respectively, examined by WST-1 assay and invasion assay *in vitro* as described in the section 'Materials and Methods'. Each column and bar represents the mean and SEM of 8 wells (A) or 10 areas (B). All data were inspected at $P < 0.05$ by Tukey-Kramer multiple comparisons test. Values with different letters (a–e) differ significantly ($P < 0.05$). This figure is representative of 3 similar experiments.

invasion across the rat M-cell monolayers. Theanine at the concentrations of 25 and 400 μM could significantly suppress about 20 and 50% of such invasion. Increase in theanine concentration to above 400 μM did not significantly enhance the inhibition (Figure 2B).

Effects of AP-5 and DNQX on inhibition of AH109A cell invasion across M-cell monolayers by theanine in vitro

Theanine is a glutamate derivative. To investigate whether its activity is related to the NMDA receptor in rats, the effect of theanine on the AH109A cell invasion across the M-cell monolayers was examined in the presence or absence of AP-5, a competitive NMDA type glutamate receptor antagonist (Baunez and Amalric, 1996; Erecinska et al., 1987) and DNQX, a competitive non-NMDA type glutamate receptor antagonist in rats (Honore et al., 1988). As shown in Figure 3A, compared with the control without theanine or AP-5 treatment, 100 μM theanine significantly suppressed AH109A invasion across the M-cell monolayers. AP-5 at the concentrations of 100 and 200 μM did not significantly affect the invasion. However, 100 and 200 μM of AP-5 significantly and dose-dependently counteracted the inhibitory effect of 100 μM theanine on the AH109A cell invasion. Figure 3B indicates that DNQX did not affect theanine-mediated inhibition of this invasion across the M-cell monolayers at the concentrations of 50 and 100 μM.

Ex vivo time course effects of sera from theanine-fed rats on AH109A invasion and effect of AP-5 on theanine-mediated ex vivo inhibition of AH109A invasion

To further confirm the bioavailability of theanine and *in vivo* possibility of its inhibitory effect on AH109A invasion, we examined the effects on the invasion across the M-cell monolayers of AP-5 as well as the rat sera obtained at different times after oral intubation of theanine. The 0.5, 1, 2, and 3 hr sera from theanine-fed rats showed significant inhibitory effects on AH109A cell invasion across the M-cell monolayers, while the 6 and 12 hr sera had no significant inhibition. The 1 hr serum showed the strongest inhibition but this inhibition was completely counteracted by 200 μM of AP-5. The rat serum containing 200 μM AP-5 did not significantly affect AH109A cell invasion (Figure 4).

Discussion

Our present results demonstrated that theanine dose-dependently and significantly inhibited the AH109A cell invasion across the rat M-cell monolayers at concentrations where the amino acid did not restrain the

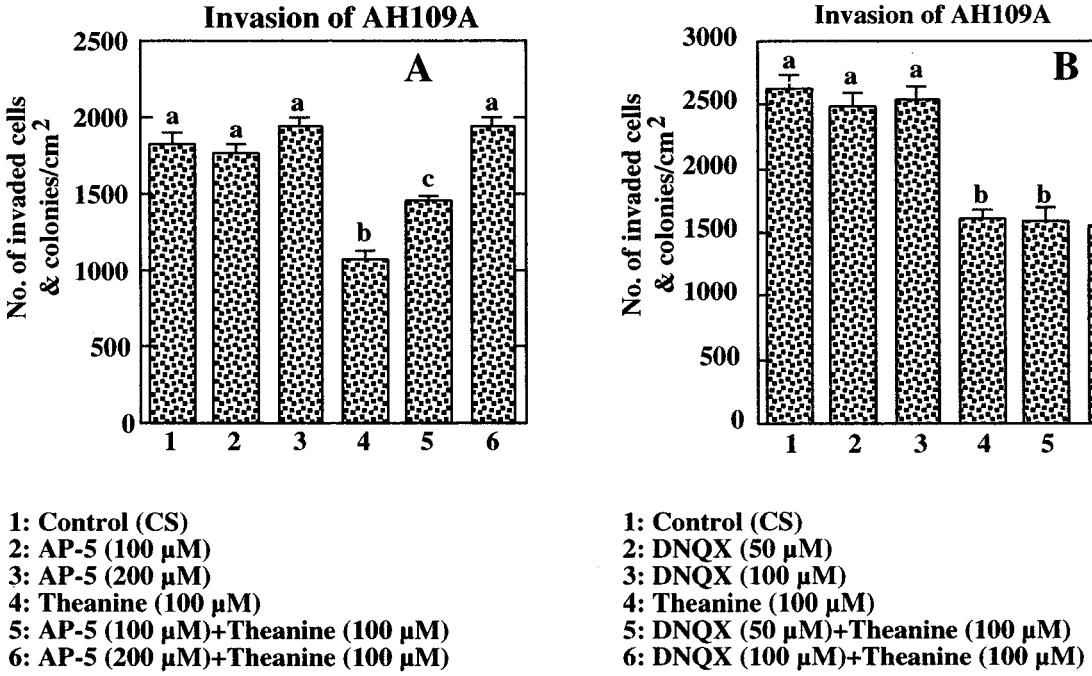

Figure 3. Effects of AP-5 and DNQX on theanine-mediated *in vitro* inhibition of AH109A cell invasion across the M-cell monolayers. (±) 2-Amino-5-phosphonopentanoic acid (AP-5) (A) or 6,7-dinitroquinoxaline 2,3-dione (DNQX) (B) was added at the concentrations indicated and their effects on the invasion of AH109A across the M-cell monolayers were examined by invasion assay *in vitro* as described in the section 'Materials and Methods'. Each column and bar represents the mean and SEM of 10 areas. All data were inspected at $P < 0.05$ by Tukey-Kramer multiple comparisons test. Values with different letters (a–c) differ significantly ($P < 0.05$). This figure is representative of at least 2 similar experiments.

AH109A cell proliferation *in vitro* (Figure 2). The *ex vivo* study indicated that the serum obtained 30 min after intubation of theanine commenced to significantly suppress the AH109A cell invasion beneath the M-cell monolayers. The 1 hr serum from theanine-fed rats showed the strongest inhibition of the invasion (Figure 4). This finding is consistent with results of previous work in rats (Terashima et al., 1999) in which a peak concentration of theanine in serum was obtained 1 hr after intragastrical administration of theanine. The inhibitory effects of theanine-treated rat sera were still significant but gradually weakened 2–3 hr after its administration, and completely diminished 6 hr later and thereafter. These results confirmed the bioavailability of theanine and the *in vivo* possibility of suppressing the invasion and thus metastasis of cancer cells. Glutamate acts as an intercellular messenger and affects intracellular calcium by binding its receptors (Maechler and Wollheim, 1999). The NMDA receptor in rats is a glutamate receptor (Baunez and Amalric, 1996; Erecinska et al., 1987; Honore et al., 1988). Theanine is reported to act through NMDA type glutamate receptor in the brain of rats (Yokogoshi et al., 1998). Thus, we tried to learn whether or not theanine inhibit AH109A cell invasion via its interaction with NMDA receptor using receptor antagonists. As a competitive NMDA and a competitive non-NMDA type glutamate receptor antagonist, AP-5 (Baunez and Amalric, 1996; Erecinska et al., 1987) and DNQX (Honore et al., 1988) were, respectively, employed to test their effects on theanine-induced inhibition of AH109A cell invasion. Our present results have shown that AP-5 counteracts the inhibitory effects of theanine and serum from theanine-fed rats on AH109A cell invasion across the M-cell monolayers *in vitro* and *ex vivo* (Figures 3A and 4). DNQX did not affect the inhibition of AH109A cell invasion by theanine (Figure 3B). These results suggest that an NMDA receptor-mediated signaling pathway may be involved in AH109A invasion and that theanine probably affect this pathway. We have recently confirmed gene expression of NMDA receptor subtypes even in a hepatoma cell line of AH109A (unpublished observation).

As an important antitumor component in tea, theanine has been reported to enhance antitumor activ-

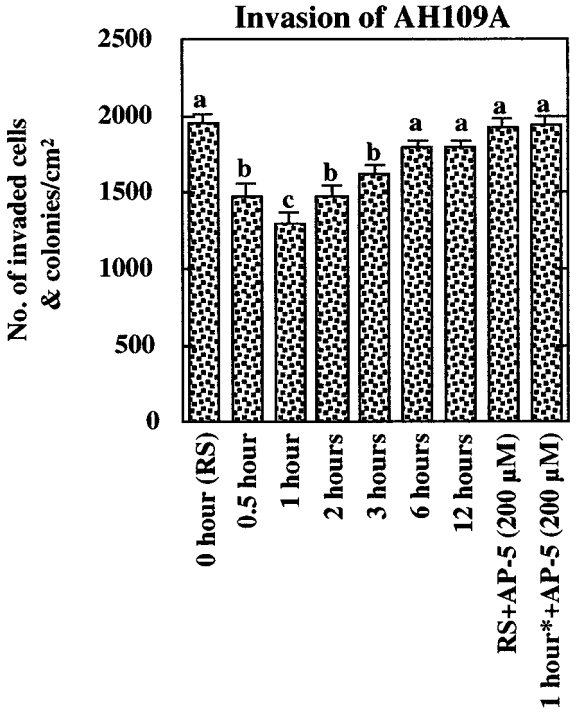

Figure 4. Ex vivo time course effects of sera from theanine-fed rats on AH109A invasion and effect of AP-5 on theanine-mediated *ex vivo* inhibition of AH109A invasion. After rats received oral intubation of theanine solution, blood was individually collected at the indicated times. Serum was pooled with an identical volume from at least two rats at each indicated time, and pooled sera were subjected to the invasion assay. Their *ex vivo* effects on the AH109A cell invasion across the M-cell monolayers and the effect of AP-5 on the inhibition of the invasion by the sera from theanine-fed rats were examined by invasion assay *ex vivo* as described in the section 'Materials and Methods'. Each column and bar represents the mean and SEM of 10 areas. All data were inspected at $P < 0.05$ by Tukey-Kramer multiple comparisons test. Values with different letters (a–c) differ significantly ($P < 0.05$). This figure is representative of 3 similar experiments. ∗AP-5 (200 μM) mixed with rat serum obtained 1 hr after oral intubation of theanine.

ity by inhibiting the anticancer drugs pirarubicin and adriamycin efflux from M5076 ovarian sarcoma cells (Sugiyama and Sadzuka, 1998; Sugiyama et al., 1999). Our present results are the first report that theanine directly suppressed invasion of cancer cells *in vitro* and *ex vivo* and that the mechanisms of its action might be related to the NMDA receptor. Further intensive studies are needed to clarify exact mechanisms.

References

Akedo A, 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.

Baunez C & Amalric M (1996) Evidence for functional differences between entopeduncular nucleus and substantia nigra: Effects of APV (DL-2-amino-5-phosphonovaleric acid) microinfusion on reaction time performance in the rat. Eur J Neurosci 8: 1972–1982.

Erecinska M, Pastuszko A, Wilson DF & Nelson D (1987) Ammonia-induced release of neurotransmitters from rat brain synaptosomes: Differences between the effects on amines and amino acids. J Neurochem 49: 1258–1265.

Honore T, Davies SN, Drejer J, Fletcher EJ, Jacobsen P, Lodge D & Nielsen FE (1988) Quinoxalinediones: Potent competitive non-NMDA glutamate receptor antagonists. Science 241: 701–703.

Ishiyama M, Tominaga H, Shiga M, Sasamoto K, Ohkura Y & Ueno K (1996) A combined assay of cell viability and *in vitro* cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. Biol Pharm Bull 19: 1518–1520.

Kitaoka S, Hayashi H, Yokogoshi H & Suzuki Y (1996) Transmural potential changes associated with the *in vitro* absorption of theanine in the guinea pig intestine. Biosci Biotechnol Biochem 60: 1768–1771.

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.

Kozuki Y, Miura Y & Yagasaki K (2001a) Inhibitory effect of curcumin on the invasion of rat ascites hepatoma cells *in vitro* and *ex vivo*. Cytotechnology 35: 57–63.

Kozuki Y, Miura Y & Yagasaki K (2001b) Resveratrol suppresses hepatoma cell invasion independently of its anti-proliferative activity. Cancer Lett 167: 151–156.

Maechler P & Wollheim CB (1999) Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. Nature 402: 685–689.

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.

Sugiyama T & Sadzuka Y (1998) Enhancing effects of green tea components on the antitumor activity of adriamycin against M5076 ovarian sarcoma. Cancer Lett 133: 19–26.

Sugiyama T, Sadzuka Y, Nagasawa K, Ohnishi N, Yokoyama T & Sonobe T (1999) Membrane transport and antitumor activity of pirarubicin, and comparison with those of doxorubicin. Jpn J Cancer Res 90: 775–780.

Terashima T, Takido T & Yokogoshi H (1999) Time-dependent changes of amino acids in the serum, liver, brain and urine of rats administered with theanine. Biosci Biotechnol Biochem 63: 615–618.

Yokogoshi H, Kobayashi M, Mochizuki M & Terashima T (1998) Effect of theanine, γ-glutamylethylamide, on brain monoamines and striatal dopamine release in conscious rats. Neurochem Res 23: 667–673.

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 (2000a) Induction of apoptosis and cell cycle arrest in cancer cells by *in vivo* metabolites of teas. Nutr Cancer 38: 265–273.

Zhang GY, Miura Y & Yagasaki K (2000b) Suppression of adhesion and invasion of hepatoma cells in culture by tea compounds through antioxidative activity. Cancer Lett 159: 169–173.