*Cytotechnology* **23:** 127–132, 1997.



*Special Issue*

# Assay systems 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

Yutaka Miura, Hiroshi Shiomi, Fumihiko Sakai & Kazumi Yagasaki
*Department of Applied Biological Science, Tokyo Noko University, Fuchu, Tokyo 183, Japan*

*Key words:* green tea extract, hepatoma cells, invasion, proliferation

**Abstract**

We have developed *in vitro* and *ex vivo* assay systems for screening food components and natural substances that suppress the proliferation and/or invasion of a rat ascites hepatoma cell line of AH109A and have used them to study the effect of green tea extract. AH109A cells were found to penetrate underneath the monolayer of primary cultured mesothelial cells isolated from Donryu rat mesentery in the presence of 10% newborn bovine serum. Green tea extract inhibited this AH109A penetration in a dose dependent manner and also inhibited AH109A proliferation *in vitro* dose-dependently. Green tea tannin, the major polyphenolic substances in green tea extract, also inhibited the proliferation and invasion of AH109A *in vitro* in a dose dependent manner. When rat serum obtained 0.5 h after oral intubation of green tea extract was added to the culture media instead of newborn bovine serum at a concentration of 10%, the invasion of AH109A was significantly inhibited as compared to control rat serum (before green tea extract intubation); the inhibitory effect lasted for 1 h and disappeared 3 h after oral intubation of green tea extract, but those rat sera showed no inhibition of AH109A proliferation. These results suggest that green tea extract has an inhibitory effect on the invasion of AH109A both *in vitro* and *ex vivo*, but on the proliferation of AH109A only *in vitro*, and that these assay systems are effective for the screening of food components which inhibit tumor cell proliferation and invasion.

## Introduction

Tumor cells have two biological characteristics: endless proliferation and metastasis. Several *in vitro* assay systems have been used to screen materials which inhibit these properties. Particularly for tumor cell metastasis which is achieved by a series of complex and sequential steps (Fidler *et al*., 1978), many assay systems to study tumor cell invasion, the most important step in tumor metastasis (Liotta *et al*., 1983; Mareel, 1980), were reported. Systems using chick chorioallantoic membranes (Armstrong *et al*., 1982, Chambers *et al*., 1982; Easty and Easty, 1974) and the Boyden chamber with the membrane coated with extracellular matrices (Albini *et al*., 1987; Welch *et al*., 1989) are often used. However, two factors have hindered the finding of an effective way to prevent tumor invasion. One is that tumor invasiveness in such systems and the invasive behavior of tumor cells in their original host were reported to be poorly correlated (Poste *et al*., 1980; Noel *et al*., 1991). Akedo *et al*. (1986) developed a model culture system in which a rat ascites hepatoma cell line, AH130, could invade underneath the monolayer of cultured mesentery-derived mesothelial cells; they showed that the *in vitro* invasiveness of AH130 in their culture model correlated well with the *in vivo* invasive behavior of this cell line. The other factor is that some materials which show the inhibitory potential on tumor cell invasion *in vitro* are often ineffective in *in vivo* tumor cell invasion. Assay systems which overcome the discrepancy between the *in vitro* and *in vivo* effects are thus needed.

In this study, using mesentery-derived mesothelial cells and rat ascites hepatoma cells (AH109A), we describe novel *in vitro* and *ex vivo* culture systems to find some food materials which have the potential to inhibit tumor cell proliferation and invasion. Like AH130 cells, AH109A cells grow in the rat pertioneal cavity and invade the mesentery during the course of growth. AH109A cells are also capable of proliferating in an *in vitro* culture system. Our system allows us to assess the ability of a tested material to inhibit AH109A proliferation and invasion *in vitro*; materials which are found to have this inhibition capability are further studied by an *ex vivo* system to determine whether they retain this effect on AH109A invasion when given orally to rats. These culture systems thus partially overcome the above described discrepancy between the *in vitro* and *in vivo* effects on tumor cell proliferation and invasion.

Using these systems, we found that green tea extract could inhibit AH109A invasion both *in vitro* and *ex vivo*, but that it inhibited the proliferation of AH109A only *in vitro*.

## Materials and methods

### *Preparation of green tea extract*

2.4 g of green tea leaves (Itoen Co., Ltd., Tokyo, Japan) were extracted by 100 ml of hot water (80 °C–90 °C) for 1 min. After filtrating through a filter paper (No. 2, Advantec Toyo Co., Ltd., Tokyo, Japan), the extract was evaporated to dryness. The dried materials were reconstituted by 10 ml of phosphate buffered saline (PBS) (that is, concentrated 10 fold), then sterilized by filtration and added to culture media at the concentrations indicated in the figures.

### *Primary cultures of mesentery-derived mesothelial cells*

Mesentery-derived mesothelial cells (M-cells) were prepared and cultured according to Akedo *et al*. (1986) with slight modifications. Briefly, the abdominal cavity of a male Donryu rat (4–6 weeks old, obtained from NRC Haruna, Gunma, Japan) was opened and the mesentery was cut off by scissors. About 15 mesenteries were usually obtained from one rat. The mesenteries were then incubated in 0.25% trypsin (Sigma, St. Louis, MO, USA) in PBS at 37 °C for 20 min with gentle stirring. Trypsin digestion was stopped by adding equal volume of DM-160 medium (Kyokuto Pharmacutical Co., Tokyo, Japan) supplemented with 10% newborn bovine serum (NBS, JRH, Lenexa, KS, USA), streptomycin (100 $\mu$g/ml, Meiji-Seika Kaisha, Tokyo, Japan) and penicillin (100 U/ml, Ban-yu Pharmaceutical Co., Tokyo, Japan) (10% NBS/DM-160) and the cell suspension was filtrated through a mesh sieve. The M-cells were pelleted by centrifugation at 1500 rpm and 4 °C for 10 min, washed once with 10% NBS/DM-160, and seeded at a density of 1.5 x $10^5$ cells/60 mm$\phi$ culture dish with 2 mm grids (Coster, Cambridge, MA, USA) in 3 ml of 10% NBS/DM-160. The cells were cultured in an atmosphere of 5% $CO_2$-95% humidified air at 37 °C to the confluent state by replacing culture media every other day and were used for the *in vitro* and *ex vivo* invasion assays described below.

### *Cultures of AH109A hepatoma cells*

AH109A cells were provided by SRL (Tokyo, Japan), and were maintained in the peritoneal cavities of male Donryu rats. AH109A cells prepared from accumulated ascites were able to be cultured *in vitro* in 10% NBS/DM-160 for up to 2 months. AH109A cultured at least for 1 week after preparing from rat ascites were used for the assays described below in order to eliminate contaminated macrophages and neutrophils.

### *In vitro proliferation assay*

Effect of green tea extract on AH109A proliferation was examined by the incorporation of [methyl-$^3$H] thymidine (20 Ci/mmol, New England Nuclear, Boston, USA) as described previously (Yagasaki *et al*., 1992) and MTT assay (Mosmann, 1983). For MTT assay, 0.5 $\times$ $10^5$ cells of AH109A were cultured for 44 h in a well of 24-well-plate containing 10% NBS/DM-160 in the absence or presence of green tea extract. MTT solution was then added and the cells were cultured for another 4 h. Viable cells can cleave MTT and produce dark brown formazan in the cells. The formazan was extracted by 0.04 N HCl/isopropanol and absorbance at 570 nm and 630 nm was measured. The relative proliferative activity of AH109A was calculated from the difference between $A_{570}$ and $A_{630}$.

*In vitro invasion assay*

*In vitro* invasion assay was performed accoding to Akedo *et al*. (1986) with slight modifications. $2.4 \times 10^5$ cells of AH109A were overlayed on the confluently cultured M-cell monolayer in 10% NBS/DM-160 in the absence or presence of green tea extract and cultured for 48 h. After washing nonadherent AH109A with PBS, the cells were fixed with 0.25% glutaraldehyde in PBS. The invasive activity of AH109A was measured by counting the invaded AH109A cells and colonies under a phase contrast microscope. Since M-cells were cultured in 60 mm$\phi$ culture dishes with 2 mm grid, the number of invaded AH109A cells and colonies in a 4 $mm^2$ area could be counted. Usually at least 10 areas were counted and the invasive activity of AH109A was indicated by the number of invaded cells and colonies/$cm^2$.

*Ex vivo proliferation and invasion assay*

Green tea extract was intubated to male Donryu rats at a dose of 1.0 ml/100 g body weight and blood was collected at the times indicated. Sera were prepared, sterilized by filtration, and added to culture media at a concentration of 10% in place of NBS in the *in vitro* proliferation and invasion assays of AH109A as described above. The proliferative and invasive activities of AH109A in the presence of these sera were then measured as described above.

*Effect of tannin on the in vitro proliferation and invasion of AH109A*

Tannin, the major polyphenolic substances in green tea extract, was obtained from Mitsui Norin Co., Ltd. (Shizuoka, Japan). Tannin was added to culture media at concentrations indicated in Figure 3 and the effect of tannin on the proliferation and invasion of AH109A was assessed by the *in vitro* proliferation (in this case, only MTT assay was done) and invasion assays as described above.

*Statistical analysis*

Data were analyzed by a one-way analysis of variance (ANOVA). When F values were significant ($P<0.05$), differences among the data were inspected at $P<0.05$ by Tukey's Q test.

**Results and discussion**

Effect of green tea extract on the *in vitro* proliferation and invasion of AH109A is illustrated in Figure 1. This extract which is 10 times concentrated inhibited the *in vitro* proliferation of AH109A as determined both by the incorporated amount of [methyl-$^3$H]-thymidine (Figure 1A) and by MTT assay (Figure 1B) in a dose-dependent manner. Green tea extract also inhibited dose-dependently the *in vitro* invasion of AH109A underneath the cultured M-cell monolayer (Figure 1C). The inhibitory effect of the extract on this invasion was more potent than that on AH109A proliferation at all concentrations examined in this study. These results suggest that different component(s) in green tea extract are responsible for the inhibition of the *in vitro* proliferation and invasion of AH109A.

Rats were given oral intubation of green tea extract, blood was withdrawn 0, 0.5, 1.0, 2.0 and 3.0 h thereafter and the effect of green tea-loaded rat serum on the proliferation and invasion of AH109A were investigated *in vitro*. The serum obtained 0.5 h after oral intubation significantly inhibited the invasion of AH109A and its inhibitory effect was also observed at 1.0 h, but at 2–3 h it had disappeared (Figure 2B). The green tea-loaded rat serum, however had no effect on the proliferation of AH109A at any time points investigated (Figure 2A). These results clearly showed that the effective components in green tea extract on AH109A invasion was absorbed from the gastrointestinal tract and that those on AH109A proliferation was not. These findings are in accordance with the *in vitro* results described above.

How does green tea extract inhibit AH109A invasion both *in vitro* and *ex vivo*? Tannin, the major polyphenolic substances in green tea extract, inhibited the *in vitro* invasion of AH109A in a dose dependent manner (Figure 3B). The concentrations of tannin used in Figure 3 are equivalent to the amounts of the component contained in the experimental media shown in Figure 1C. Therefore, tannin is possibly responsible for the inhibitory effect of green tea extract on the *in vitro* invasion of AH109A. It is well known that tannin contains some polyphenolic substrances, e.g., catechin, epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate, which have potent antioxidative activity (Ruch *et al*., 1989). Shinkai *et al*. (1986) reported that superoxide radical potentiates the invasive activity of AH109A cells *in vitro*, using the same *in vitro* invasion system as that used in this study. Furthermore epigallocatechin gallate, the main polyphe-

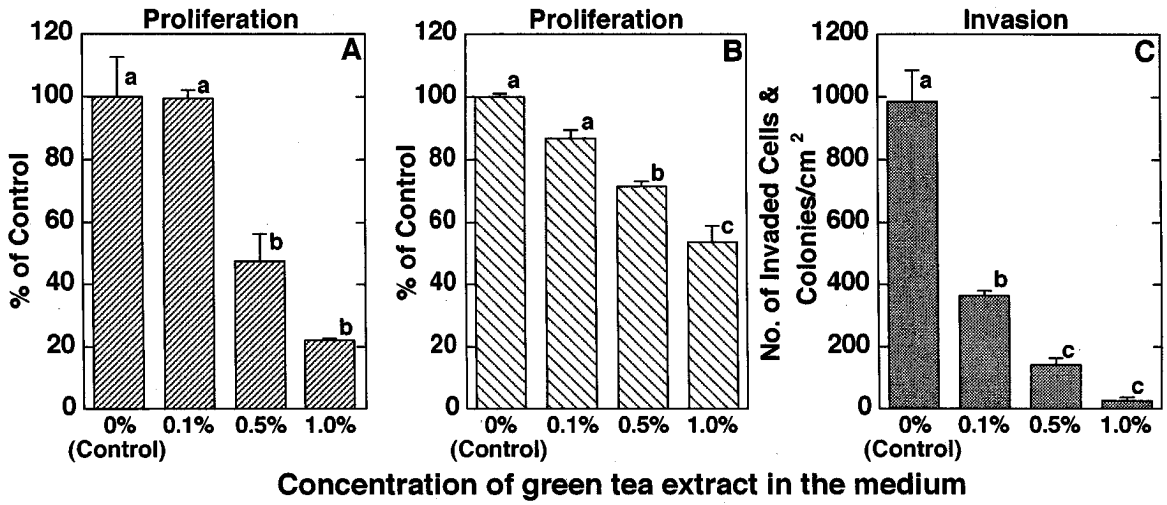


*Figure 1. In vitro* effect of green tea extract on the proliferation and invasion of AH109A. Green tea extract was added at the concentrations of 0.1%, 0.5% and 1.0% to culture media. Its effect on the *in vitro* proliferation of AH109A by measuring the incorporated amount of [methyl-$^3$H] thymidine for 2 hr (A), by MTT assay (B), and on the *in vitro* invasion of AH109A underneath the cultured M-cell monolayer (C) was examined as described in Materials and methods. PBS instead of green tea extract was added to control medium (0%). Each column and vertical bar represents the mean and SEM for 4 wells (A,B) or 10 areas (C). Values not sharing a common letter are significantly different at $P$ <0.05 by Tukey's Q test. This figure is representative of at least 3 similar experiments.

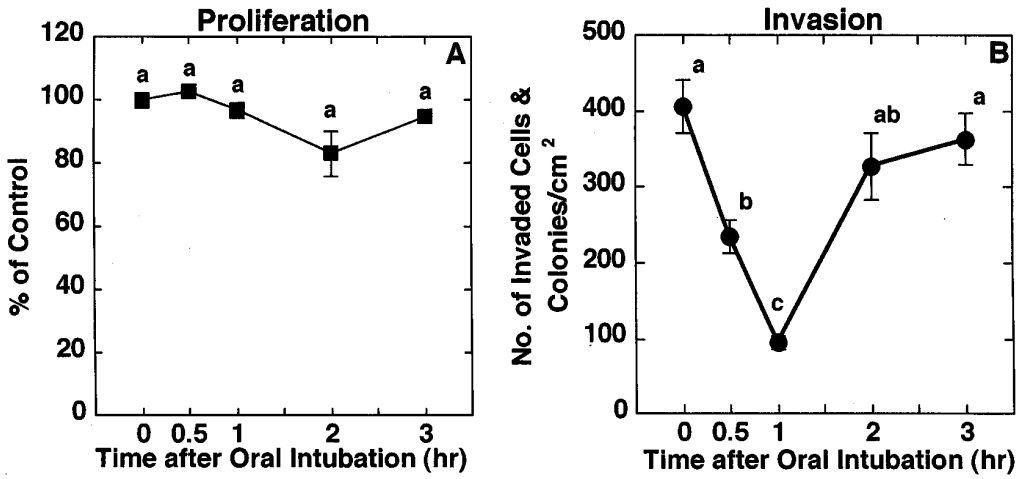


*Figure 2. Ex vivo* effect of green tea extract on the proliferation and invasion of AH109A. Green tea extract-loaded rat serum was obtained at the indicated times after oral intubations. Its effect on the proliferation (A) and invasion (B) was examined *in vitro*. Each point and vertical bar represents the mean and SEM for 4 wells (A) or 10 areas (B). Values not sharing a common letter are significantly different at $P$ <0.05 by Tukey's Q test. This figure is representative of at least 3 similar experiments.

nolic constituent of green tea, was reported to have a suprressive effect on lung metastasis of mouse B16 melanoma cell lines in both experimental and spontaneous systems (Taniguchi *et al*., 1992). It is also known to inhibit mouse lung carcinoma cell adhesion to the endothelial cells (Isemura *et all*., 1993). It is therefore possible that epigallocatechin gallate is responsible for the inhibitory effect of green tea extract observed in this study on the invasion of AH109A both *in vitro* and *ex vivo*. However, this substance also reportedly has some cytotoxic activity on the endothelial cells in culture (Isemura *et al*., 1993). In this study, we found no shrinking or detachment of M-cells throughout all experiments. There is thus a possibility that some sub-

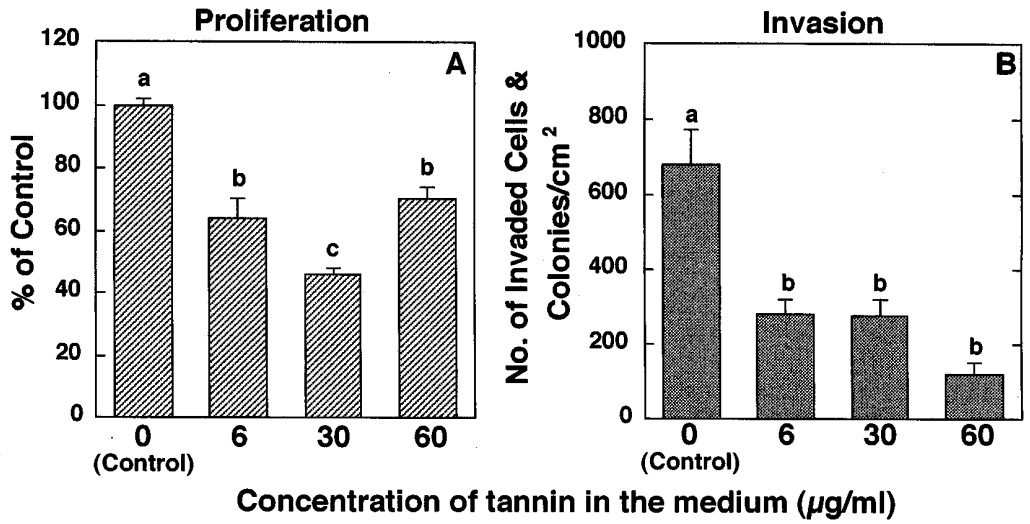


*Figure 3.* Effect of tannin on the *in vitro* proliferation and invasion of AH109A. Tannin was added to culture media at concentrations indicated in figure and its effect on the proliferation (A) and invasion (B) was examined *in vitro*. Each column and vertical bar represents the mean and SEM for 4 wells (A) or 10 areas (B). Values not sharing a common letter are significantly different at $P < 0.05$ by Tukey's Q test.

stances other than epigallocatechin gallate are responsible for the inhibitory effect of green tea extract on AH109A invasion both *in vitro* and *ex vivo*.

Green tea extract did inhibit AH109A proliferation *in vitro*, but not *ex vivo*. Tannin also inhibited the proliferation of AH109A *in vitro* at concentrations equivalent to the amounts of the component contained in the experimental media in Figure 1A and 1B (Figure 3A). It is reported that epigallocatechin (not epigallocatechin gallate) shows a considerable cytotoxicity on mouse 3LL mouse lung carcinoma cells (Isemura *et al.*, 1993); it is also known to be much less well absorbed from the gastrointestinal tract as compared with epigallocatechin gallate. We therefore conclude that the inhibitory effect of green tea extract on AH109A proliferation *in vitro* and its ineffectiveness *ex vivo* are due to epigallocatechin, although the possibility that other substances may be responsible cannot be excluded.

In summary, we showed using our assay systems that green tea extract significantly inhibits the invasion of AH109A both *in vitro* and *ex vivo* and that it also inhibits the proliferation *in vitro*, but not *ex vivo*. We are now testing the *in vivo* effect of the extract on AH109A proliferation and metastasis, using a spontaneously metastatic system recently developed in our laboratory (Miura *et al.*, manuscript in preparation). The described *in vitro* and *ex vivo* systems are thought to be useful for the primary stage of screening antitumor or antimetastatic substances, and we have already found some food components which inhibit the proliferation and/or invasion of AH109A; characterization of the effective substance(s) is now in progress.

## Acknowledgement

This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan (No. 05258208).

## References

Akedo H, Shinkai K, Mukai M, Mori Y, Tateishi R, Tanaka K, Yamamoto R and Morishita T (1986) Interaction of rat ascites hepatoma cells with cultured mesothelial cell layers: A model for tumor invasion. Cancer Res 46: 2416–2422.

Albini A, Iwamoto Y, Kleinman HK, Matrin G, Aaronson SA, Kozlowski JM and McEwan RN (1987) A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. Cancer Res 47: 3239–3245.

Armstrong PB, Quigley JP and Sidebottom E (1982) Transepithelial invasion and intramesenchymal infiltration of chick embryo chorioallantois by tumor cell lines. Cancer Res 42: 1826–1837.

Chambers AF, Shafir R and Ling V (1982) A model system for studying metastasis using the embryonic chick. Cancer Res 42: 4018–4025.

Easty DM and Easty GC (1974) Measurement of the ability of cells to infiltrate normal tissues *in vitro*. Br J Cancer 29: 36–49.

Fidler IJ, Gersten DM and Hart IR (1978) The biology of cancer invasion and metastasis. Adv Cancer Res 28: 149–250.

Isemura M, Suzuki Y, Satoh K, Narumi K and Motomiya M (1993) Effect of catechins on the mouse lung carcinoma cell adhesion to the endothelial cells. Cell Biol Int 17: 559–564.

Liotta L, Rao CN and Barsky SH (1983) Tumor invasion and the extracellular matrix. Lab Invest 49: 636–649.

Mareel MM (1980) Recent aspects of tumor invasiveness. Int Rev Exp Pathol 22: 65–129.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immuno Methods 65: 55–63.

Noel A, Calle A, Emonard HP, Nusgens BV, Simar L, Foidart J, Lapiere CM and Foidart J-M (1991) Invasion of reconstituted basement membrane matrix is not correlated with the malignant metastatic cell phenotype. Cancer Res 51: 405–414.

Poste G, Doll J, Hart IR and Fidler IJ (1980) *In vitro* selection of murine B16 melanoma variants with enhanced tissue-invasive properties. Cancer Res 40: 1636–1644.

Ruch RJ, Cheng S and Klaunig JE (1989) Prevention of cytotoxicity and inhibition of intercellular communication by anti-oxidant catechins isolated from chinese green tea. Carcinogenesis 10: 1003–1008.

Shinkai K, Mukai M and Akedoo H (1986) Superoxide radical potentiates invasive capacity of rat ascites hepatoma cells *in vitro*. Cancer Lett 32: 7–13.

Taniguchi S, Fujiki H, Kobayashi H, Go H, Miyado K, Sadano H and 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.

Welch DR, Lobl TJ, Seftor EA, Wack PJ, Aeed PA, Yohem KH, Seftor REB and Hendrix MJC (1989) Use of the membrane invasion culture system (MICS) as a screen for anti-invasive agents. Int J Cancer 43: 449–457.

Yagasaki K, Tanabe T, Ishihara K and Funabiki R (1992) Modulation of the proliferation of cultured hepatoma cells by urea cycle-related amino acids. In: Murakami H *et al*. (eds.) Animal cell technology: Basic and Applied Aspects, Vol. 4 (pp. 257–263) Kluwer Academic Publishers, The Netherlands.

*Address for Correspondence:* K. Yagasaki, Ph.D., Department of Applied Biological Science, Tokyo Noko University, Fuchu, Tokyo 183, Japan.