Inhibitory effect of serum from rats administered with coffee on the proliferation and invasion of rat ascites hepatoma cells

Yutaka Miura, Tamio Furuse & Kazumi Yagasaki

Department of Applied Biological Science, Tokyo Noko University, Fuchu, Tokyo 183, Japan

Received 24 February 1997; accepted 13 May 1997

Key words: coffee, hepatoma, invasion, oral intubation, proliferation

Abstract

The action of coffee on the proliferation and invasion of a rat ascites hepatoma cell line of AH109A was investigated using *in vitro* and *ex vivo* assay systems. When rats were given oral intubation of instant coffee powder solution, the sera of those rats had the potent inhibitory activity on both the proliferation and invasion of AH109A. The activity of rat serum was both time- and dose-dependent. The instant coffee powder also inhibited the proliferation and invasion of AH109A *in vitro*. These results indicate that coffee has anti-proliferative and anti-invasive activity both *in vitro* and *ex vivo*. They also suggest that some anti-proliferative and anti-invasive material(s), which may be the ingredient(s) of coffee or their metabolites, appear in rat serum when rats are given oral intubation of coffee, although a possibility that host defense systems may be activated by the oral intubation of coffee cannot be ruled out.

Introduction

It is well known that tumor cells have two biological characteristics, that is, endless proliferation and metastasis. Especially metastasis, which is achieved by a series of complex sequential steps (Fidler *et al.*, 1978), is the major cause that a tumor becomes fatal. The efforts to find the effective materials or methods which inhibit tumor cell proliferation and metastasis have been conducted by many researchers. For this purpose, some *in vitro* assay systems have been often used. For tumor cell metastasis, many assay systems to study tumor cell invasion, the most important step in tumor metastasis (Liotta *et al.*, 1983, Mareel, 1980), were reported (Armstrong *et al.*, 1982, Albini *et al.*, 1987, Welch *et al.*, 1989). However, the effective ways to prevent tumor invasion are not still found because of two reasons. One is that tumor invasiveness in such systems and the invasive behavior of tumor cell in its 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 rat ascites hepatoma cell line, AH130, could invade underneath the monolayer of cultured mesentery-derived mesothe-

lial cells and they showed that the *in vitro* invasiveness of AH130 in their culture model correlate well to *in vivo* invasive behavior of AH130. The other is that some materials which show the inhibitory potential on tumor cell invasion *in vitro* are often ineffective *in vivo* tumor cell invasion. In the previous report, we described the assay method which overcomes the discrepancy between the *in vitro* and *in vivo* effects using *in vitro* and *ex vivo* assay systems in combination (Miura *et al.*, 1997). We also reported the effectiveness of green tea extract on tumor cell proliferation and invasion with the assay system.

In this study, we describe that coffee, another popular beverage as teas, has both anti-proliferative and anti-invasive activity to rat ascites hepatoma cells *in vitro* and *ex vivo*. Especially, the serum from rats administered with coffee has the potent inhibitory effects on both the proliferation and invasion of rat ascites hepatoma cells.

Materials and methods

Materials

Instant coffee powder (ICP) was a generous gift from Nestlé Japan Ltd. (Kobe, Japan). The components of ICP, that is, caffeic acid, chlorogenic acid and quinic acid were purchased from Sigma (St. Louis, MO, USA) and caffeine was from Wako Pure Chemicals Co. Ltd. (Osaka, Japan). These components were added to the medium at the concentrations indicated in figures. All other reagents were of the best grade commercially available.

Cultures of AH109A hepatoma cells

AH109A cells, a rat ascites hepatoma cell line, were provided by SRL (Tokyo, Japan). The hepatoma cells were maintained in peritoneal cavities of male Donryu rats (NRC Haruna, Gunma, Japan). AH109A cells prepared from accumulated ascites were able to be cultured *in vitro* in DM-160 medium (Kyokuto Pharmaceutical Co., Tokyo, Japan) supplemented with 10% calf serum (CS, obtained from JRH, Lenexa, KS, USA), streptomycin (100 µg/ml, Meiji-seika Kaisha, Tokyo, Japan) and penicillin (100 U/ml, Ban-yu Pharmaceutical Co., Tokyo, Japan) (10% CS/DM160) for up to 2 months. AH109A cells cultured at least for 1 week after preparing from rat ascites were used for assays described below in order to eliminate contaminated macrophages and neutrophils.

In vitro proliferation assay

Effects of ICP and coffee components on AH109A proliferation were examined either by the incorporation of [methyl-³H] thymidine (20 Ci/mmol, New England Nuclear, Boston, USA) into acid-insoluble fraction of cells as described previously (Yagasaki *et al.*, 1992) or MTT assay (Mosmann, 1983) as described previously (Miura *et al.* 1997).

In vitro invasion assay

In vitro invasion assay was performed as described previously (Miura *et al.*, 1997). Briefly, mesentery-derived mesothelial cells of Donryu rats (4–6 weeks old) were isolated by trypsin digestion and were cultured for 1 week to the confluent state in 10% CS/DM160. Then, 2.4×10^5 cells of AH109A were overlaid on the confluent cultured M-cell mono-

layer in 10% CS/DM160 in the absence or presence of ICP or ICP components and cultured for 48 hr. The invasive activity of AH109A was measured by counting invaded AH109A cells and colonies under phase contrast microscopy. Since M-cells were cultured in a 60 mm culture dish with a 2 mm grid (Coster, Cambridge, MA, USA), the number of invaded AH109A cells and colonies in the area of 4 mm² were able to 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/cm².

Ex vivo proliferation and invasion assay

ICP was intubated to male Donryu rats at a dose indicated in figures. Blood was collected at the indicated time after the intubation. Sera were prepared by centrifugation, sterilized by filtration, and added to culture media at a concentration of 10% instead of calf serum in *in vitro* proliferation and invasion assay of AH109A described above. The proliferative and invasive activity of AH109A in the presence of sera of rats intubated with ICP was measured as described above.

Statistical analysis

Data were analysed 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

We first investigated the *in vitro* effect of ICP on the proliferation and invasion of AH109A. In this case, the effect of ICP on AH109A proliferation was determined by the incorporated radioactivity of [methyl-³H] thymidine because ICP solution has the significant absorbance at 570 nm and 630 nm, the wave lengths that are used for MTT assay. As shown in Fig. 1, ICP has the strong anti-proliferative (Fig. 1A) and anti-invasive (Fig. 1B) activity *in vitro*. Both inhibitory actions were dose-dependent, but anti-proliferative activity is stronger than anti-invasive activity at all concentrations examined in this study.

We next examined the *ex vivo* effect of ICP on the proliferation and invasion of AH109A, that is, whether or not the serum from a rat given oral intubation of ICP had the inhibitory effects on the proliferation and invasion of AH109A. For this purpose, rats were given oral

Figure 1. *In vitro* effect of instant coffee powder (ICP) on the proliferation and invasion of AH109A. ICP was directly dissolved in culture medium at the concentrations indicated in figures. The proliferative activity of AH109A (A) was determined by the incorporation of [methyl- ^3H] thymidine and the invasive activity (B) by invasion assay as described in Methods section. 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.

intubation of ICP solution at a dose of 100 mg/ml/100 g body weight and blood was withdrawn 0, 0.5, 1, 2, 3 and 5 hr thereafter. Sera were prepared and the effects of those ICP-loaded rat sera on the proliferation and invasion of AH109A were investigated *in vitro*. The serum commenced to significantly inhibit both the proliferation and invasion of AH109A 1 hr after oral intubation and this inhibitory action lasted for up to 5 hr after oral intubation (Figs. 2A and B). These results showed that ICP had the potent *ex vivo* inhibitory activity on both the proliferation and invasion of AH109A when orally given. Moreover, we also confirmed that these *ex vivo* inhibitory effects of ICP were dose-dependent when sera were prepared 2 hr after oral intubation of ICP solution (Figs. 3A and B).

Discussion

In this report, we found that ICP-loaded rat serum could inhibit AH109A proliferation and invasion *in vitro*. Its inhibitory effect was observed 1 hr after oral intubation of ICP for both the proliferation and invasion, which lasted for another 4 hr. Moreover, it was dose-dependent on the loaded amount of ICP. These results suggest the existence in ICP-loaded rat serum of some effective compound(s) that inhibit the proliferation and invasion of AH109A. As shown in Fig. 1, ICP, when

added directly to culture medium, inhibited the proliferation and invasion of AH109A in a dose-dependent manner. There is therefore a possibility that some effective component(s) in ICP are absorbed across the gastrointestinal tract and emerge in rat serum. In fact, we have already confirmed that caffeic acid, one of the major components in ICP, can inhibit the *in vitro* invasion of AH109A and that caffeic acid-loaded rat serum can inhibit the invasion of AH109A in dose- and time-dependent manners (Furuse *et al.* unpublished observations). However, some abundant components in ICP, that is, caffeic acid, caffeine, quinic acid and chlorogenic acid had no inhibitory effect on the proliferation of AH109A *in vitro* (data not shown). Some trace components are likely to be responsible for the inhibition of AH109A proliferation. However, other possibilities that some metabolites of ICP's components are responsible for the inhibitory effects of ICP-loaded rat serum and that host defense systems are stimulated by the intubation of ICP cannot be excluded. In the previous report, we described the *in vitro* inhibitory activity of green tea extract on the proliferation and invasion of AH109A. The inhibitory effect of green tea-loaded rat serum on AH109A invasion was observed, but the inhibitory effect on proliferation disappeared when the tea extract was given by oral intubation (Miura *et al.*, 1997). Unlike green tea extract, the inhibitory effect of ICP on AH109A proliferation was observed both *in*

Figure 2. *Ex vivo* effect of ICP on the proliferation and invasion of AH109A. ICP-loaded rat serum was obtained at the indicated times after oral intubation. Its effect on the proliferation (A) and invasion (B) was examined *in vitro*. The proliferative activity was determined by MTT assay and the invasive activity by invasion assay as described in Methods section. 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.

Figure 3. Dose-dependent *ex vivo* effect of ICP on the proliferation and invasion of AH109A. ICP was dissolved in water and given by oral intubation to Donryu rats at doses indicated in figures. Sera were prepared at 2 hr after oral intubation. The effect of ICP-loaded rat serum on the proliferation and invasion of AH109A was determined *in vitro*. The proliferative activity of AH109A (A) was determined by MTT assay and the invasive activity (B) by invasion assay as described in Methods section. 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.

vitro and *ex vivo*, suggesting that effective components suppressing the proliferation of AH109A cells in ICP may be different from those in green tea.

ICP's inhibitory effect on AH109A invasion may be attributable to caffeic acid. As to green tea extract,

some tea catechins were considered to have anti-invasive activity. Both caffeic acid and catechins have a radical scavenging activity. This property of these compounds may therefore be involved in their anti-invasive action. In fact, it was reported that superoxide

radicals potentiate the invasive activity of AH130 and that superoxide dismutase and catalase inhibit AH130 invasion *in vitro* (Mukai *et al.*, 1987, Shinkai *et al.*, 1986). However, the involvement of arachidonic acid metabolites in tumor cell invasion and metastasis was recently reported (Reich and Martin, 1996, Liu *et al.*, 1996). Caffeic acid is known as a lipoxygenase inhibitor (Koshihara *et al.*, 1983). A possibility that caffeic acid inhibits AH109A invasion by suppressing lipoxygenase activity cannot be ruled out.

In conclusion, we have demonstrated the *in vitro* and *ex vivo* inhibitory activity of ICP on both the proliferation and invasion of AH109A. These results again demonstrate the usefulness of our assay system for the screening of anti-proliferative and anti-invasive compounds in foods and natural sources. Further investigations are needed to clarify the chemical entity of effective components and the precise mechanism(s) for anti-proliferative and anti-invasive activity of ICP, and are now in progress in our laboratory.

Acknowledgements

This work was supported in part by grants from the All Japan Coffee Association and the Nestlé Science Promotion Committee.

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.
- Fidler IJ, Gersten DM and Hart IR (1978) The biology of cancer invasion and metastasis. *Adv. Cancer Res* 28: 149–250.
- Koshihara Y, Neichi T, Murota S, Lao A, Fujimoto Y and Tatsuno T (1983) Selective inhibition of 5-lipoxygenase by natural compounds isolated from chinese plants, *Artemisia rubripes* Nakai. *FEBS Lett.* 158: 41–44.
- Liotta L, Rao CN and Barsky SH (1983) Tumor invasion and the extracellular matrix. *Lab Invest* 49: 636–649.
- Liu X-H, Connolly JM and 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.
- Mareel MM (1980) Recent aspects of tumor invasiveness. *Int Rev Exp Pathol* 22: 65–129.
- Miura Y, Shiomi H, Sakai F and Yagasaki K (1997) 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. *Cytotechnology* 23: 127–132.
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immuno Methods* 65: 55–63.
- Mukai M, Shinkai K, Tateishi R, Mori Y and Akedo H (1987) Macrophage potentiation of invasive capacity of rat ascites hepatoma cells. *Cancer Res* 47: 2167–2171.
- Noel A, Calle A, Emonard HP, Nussgens BV, Simar L, Foidart J, Lapiereb 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.
- Reich R and Martin GR (1996) Identification of arachidonic acid pathways required for the invasive and metastatic activity of malignant tumor cells. *Prostaglandins* 51: 1–17.
- Shinkai K, Mukai M and Akedo H (1986) Superoxide radical potentiates invasive capacity of rat ascites hepatoma cells *in vitro*. *Cancer Lett* 32: 7–13.
- 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: Kazumi Yagasaki, Department of Applied Biological Science, Tokyo Noko University, Fuchu, Tokyo 183, Japan.