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Suppression of adhesion and invasion of hepatoma cells in culture by tea compounds through antioxidative activity

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Abstract

To determine the actions of tea components on the invasion of a rat ascites hepatoma cell line of AH109A and to understand their modes of action, the cancer cells were co-cultured with a rat mesentery-derived mesothelial cell monolayer in the presence of tea components. The synergistic effects of (−)-epicatechin (EC) with (−)-epigallocatechin gallate (EGCG) on AH109A invasion were demonstrated. Further study showed that 10 μM of EGCG or theaflavins, or 2.5 μM of ethylenediaminetetraacetic (EDTA) entirely abolished the increase in AH109A adhesion and invasion stimulated by reactive oxygen species (ROS) from the hypoxanthine–xanthine oxidase system. Our results suggest that $^{\cdot}OH^{-}$- and other ROS-scavenging activity of EGCG and theaflavins may be responsible for the inhibition of $^{\cdot}OH^{-}$- and related ROS-potentiated AH109A adhesion and invasion to the cultured rat mesothelial cell monolayer.

Keywords: Adhesion; Invasion; Hepatoma; Reactive oxygen species; Theaflavins; Synergistic effects

1. Introduction

Tumour cells can produce a large amount of reactive oxygen species (ROS) [1]. ROS can also be generated under pathological conditions of tissue injury, inflammation, and during radiation therapies and chemotherapy treatments. These ROS are related to cancer invasion and metastasis [1,2]. The presence of metastasis is the main cause of mortality in patients with cancer. During the complicated multistep processes of cancer metastasis, adhesion and invasion of cancer cells to the host cell layer and extracellular matrix are the most critical characteristic steps [3–5]. Green tea has been shown to suppress the metastasis of mouse lung carcinoma cells [6]. We previously confirmed that higher concentrations of tea extracts and catechins suppress the proliferation and invasion of a rat ascites hepatoma cell line of AH109A [7,8]. However, it is unclear why tea can inhibit the invasion and metastasis of cancer cells. To address these questions, we investigated AH109A adhesion and invasion inhibition and the mode of action by characteristic tea components (−)-epigallocatechin gallate (EGCG) and (−)-epicatechin (EC) as well as theaflavins, the compounds commonly found in black tea.

2. Materials and methods

2.1. Materials

Ten milligrams of (−)-epigallocatechin gallate >98% pure, Funakoshi Co., Tokyo, Japan), and theaflavins (EC >98% pure, theaflavins >80% pure, Sigma Chemical Co., St. Louis, MO) were, respec-

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tively, dissolved in 10 ml phosphate-buffered saline (PBS(−)) for in vitro adhesion and/or invasion assays.

2.2. Culture of AH109A hepatoma cells

A rat ascites hepatoma cell line of AH109A was provided by the Institute of Development, Aging and Cancer, 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 Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co., Ltd., Tokyo) containing 10% calf serum (CS, from JRH, Lenexa, KS) for up to 2 months. These isolated AH109A cells were cultured for at least 1 week and then used for the assays.

2.3. In vitro invasion assay

The invasion assay was based on the method of Akedo et al. [9] with slight modifications as described previously [7,8]. 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-mmϕ culture dish with 2-mm grids. 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 50 μM EGCG, 100 μM EC, or 50 μM EGCG mixed with 100 μM EC (in the case of invasion assay for synergistic effect of EC with EGCG), or 10 μM EGCG or theaflavins (in the case of in vitro adhesion and invasion assay). After co-culturing for 48 h (invasion assay for the synergistic effect) or 24 h (invasion assay), the invaded AH109A cells and colonies were counted under a phase contrast microscope. Usually at least ten areas were counted and the invasive activity of the cells was indicated by the numbers of invaded cells and colonies/cm^2.

2.4. In vitro adhesion assay

Based on the invasion assay mentioned above, 2.4×10^5 AH109A cells were seeded on the M-cell monolayer in 10% CS/MEM medium in the absence or presence of each tea component. After co-culturing for 3 h, the unattached AH109A cells were aspirated with medium and then the dish was washed three times with PBS(−). The cells attached to the M-cell monolayer were counted under a phase contrast microscope. Usually at least ten areas were counted and the adhesive activity of the cells was indicated by the number of adhered cells/cm^2.

2.5. Pretreatment of AH109A cells with hypoxanthine and xanthine oxidase

AH109A cells were cultured for 1 h in the absence or presence of 2.5 μM EDTA (Sigma), 50 μM EGCG, 100 μM EC, or 50 μM EGCG mixed with 100 μM EC, or 10 μM EGCG or theaflavins, and/or 4 μg/ml hypoxanthine (HX, Sigma) with 7×10^{-4} U/ml xanthine oxidase (XO, Sigma) [10,11]. The treated AH109A cells were then washed with MEM medium and seeded on the M-cell monolayer in MEM containing 10% CS without tea samples. After co-culturing for 3 and 24 h, the adhesive and invasive capacities were assessed, respectively, as mentioned above.

2.6. Statistical analysis

Data were expressed as means ± SE of ten areas. Multigroup comparisons were conducted by a one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test.

3. Results

3.1. Synergistic inhibitory effects on AH109A invasion by EGCG and EC

The in vitro invasion assay indicated that 50 μM of EGCG could significantly suppress both exogenous ROS-potentiated and spontaneous (without HX and XO treatment) AH109A invasion underneath the M-cell monolayer. EC at 100 μM restrained only the exogenous ROS-potentiated AH109A invasion but failed to restrain the spontaneous invasion. In order to determine the possible synergistic effects of EC with EGCG on the AH109A invasion, we used the 100 μM of EC and 50 μM of EGCG to test their effects on AH109A invasion in the present study. Our results demonstrated that 100 μM of EC could exhibit synergistic inhibitory effects on both the exogenous ROS-potentiated and spontaneous AH109A invasion in the presence of 50 μM of EGCG (Fig. 1).

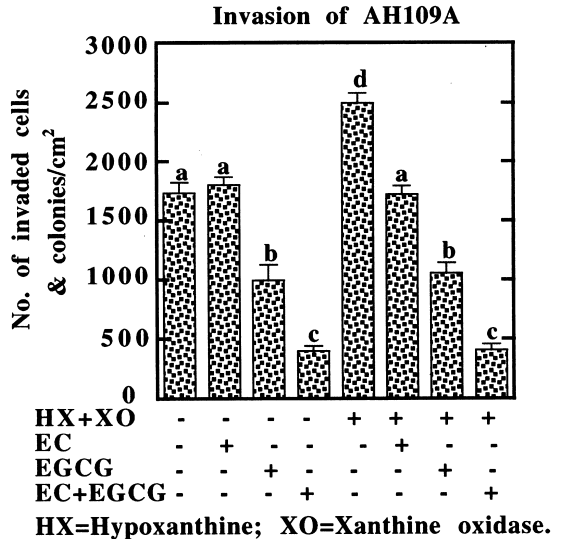

Fig. 1. Synergistic effects of EC with EGCG on ROS-enhanced AH109A invasion underneath the M-cell monolayer. AH109A cells were pretreated for 1 h in the absence or presence of 50 μM of EGCG, 100 μM of EC, or 100 μM of EC mixed with 50 μM of EGCG, and/or 4 μg/ml of hypoxanthine with 7×10^{-4} U/ml of xanthine oxidase. The treated AH109A cells were washed and then seeded on the M-cell monolayer. The synergistic effects of EC with EGCG on ROS-enhanced AH109A invasion underneath the monolayer was examined as described in Section 2. Each value and bar represents the mean and SEM for ten areas. All data were inspected at $P < 0.05$ by Tukey–Kramer multiple comparisons test. Values with different letters (a–d) differ significantly ($P < 0.05$). This figure is the representative of three similar experiments.

3.2. Inhibition of ROS-potentiated AH109A adhesion by low concentration EGCG, theaflavins and EDTA

The mode of AH109A adhesion inhibition by green and black tea components was determined by examining effects of 10 μM of EGCG and theaflavins, and 2.5 μM of EDTA on exogenous ROS-potentiated AH109A adhesion. At these concentrations, the three compounds did not significantly affect AH109A proliferation (data not shown). One hour pretreatment of AH109A cells with HX–XO producing ROS significantly enhanced the in vitro AH109A adhesion by 42% compared with the control group without pretreatment with HX–XO. The 10 μM of EGCG or theaflavins completely suppressed the enhancement of AH109A adhesion stimulated by the HX–XO pretreatment, although spontaneous AH109A adhesion on the M-cell monolayer was not eliminated. To suppress the Fenton reaction by chelating transition metals in HX–XO solution, 2.5 μM of EDTA was added during the 1 h pretreatment of AH109A with HX–XO. The enhancement of AH109A adhesion stimulated by this pretreatment was also suppressed completely by EDTA without significantly restraining AH109A proliferation (data not shown) and spontaneous adhesion was not significantly affected (Fig. 2).

3.3. Inhibition of ROS-potentiated AH109A invasion by low concentration EGCG, theaflavins and EDTA

To rule out the possible effects of a high concentration of EGCG, theaflavins or EDTA on AH109A proliferation, and also to further determine the mode of AH109A invasion inhibition by green and black tea components, we tested the effects of a low concentra-

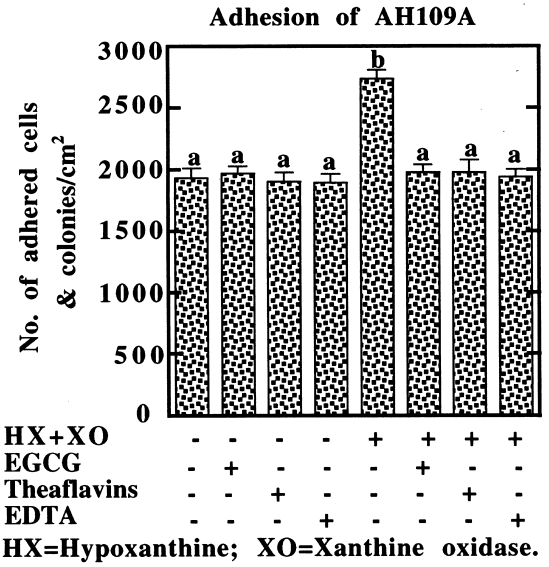

Fig. 2. Inhibitory effects of EGCG, theaflavins and EDTA on ROS-potentiated AH109A adhesion underneath the M-cell monolayer. AH109A cells were cultured for 1 h in the absence or presence of 10 μM of EGCG or theaflavins, or 2.5 μM of EDTA, and/or 4 μg/ml of hypoxanthine with 7×10^{-4} U/ml of xanthine oxidase. The treated AH109A cells were then seeded on the M-cell monolayer. The effects of EGCG, theaflavins and EDTA on adhesion of AH109A cells underneath the cultured M-cell monolayer were examined as described in Section 2. Each value and bar represents the mean and SEM for ten areas. All data were inspected at $P < 0.05$ by Tukey–Kramer multiple comparisons test. Values with different letters a and b differ significantly ($P < 0.05$). This figure is the representative of three similar experiments.

tion of EGCG, theaflavins, and EDTA on AH109A invasion and the mode of action. One hour pretreatment of AH109A cells with HX–XO producing ROS enhanced significantly the in vitro AH109A invasion by 36% compared with the control group which had not been pretreated with HX–XO. The in vitro assay indicated that 10 μM of EGCG or theaflavins could completely suppress the increase in AH109A invasion stimulated by the HX–XO pretreatment for 1 h without significantly changing AH109A proliferation. This exogenous ROS-induced AH109A invasion underneath the M-cell monolayer was also inhibited by 2.5 μM of EDTA (Fig. 3).

4. Discussion

In the present study, we confirmed that 1 h exposure to exogenous ROS from the HX–XO system increased AH109A invasion underneath the M-cell monolayer as previously observed in AH130 [11] and AH109A [12] treated with the system for 4 h. This increase in AH109A invasion was completely eliminated by EC and EGCG. EGCG also inhibited significantly spontaneous AH109A invasion at the concentration of 50 μM. More interestingly, EC displayed a synergistic inhibitory effect with EGCG against the invasion (Fig. 1). Further study showed that 10 μM of EGCG or theaflavins, or 2.5 μM of EDTA entirely suppressed ROS-potentiated AH109A adhesion on and invasion underneath the cultured M-cell monolayer (Figs. 2 and 3, respectively), without significantly changing AH109A proliferation (data not shown).

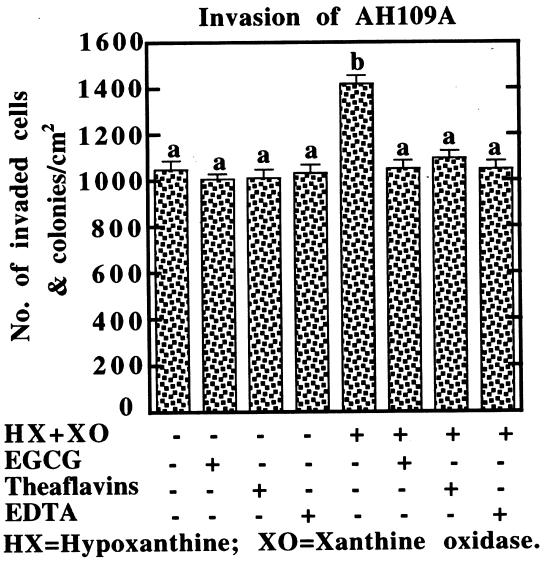

Fig. 3. Inhibitory effects of EGCG, theaflavins and EDTA on ROS-potentiated AH109A invasion underneath the M-cell monolayer. AH109A cells were cultured for 1 h in the absence or presence of 10 μM of EGCG or theaflavins, or 2.5 μM of EDTA, and/or 4 μg/ml of hypoxanthine with 7×10^{-4} U/ml of xanthine oxidase. The treated AH109A cells were then seeded on the M-cell monolayer. The effects of EGCG, theaflavins and EDTA on invasion of AH109A cells underneath the cultured monolayer were examined as described in Section 2. Each value and bar represents the mean and SEM for ten areas. All data were inspected at $P < 0.05$ by Tukey–Kramer multiple comparisons test. Values with different letters (a) and (b) differ significantly ($P < 0.05$). This figure is the representative of three similar experiments.

As mentioned above, tumour cells can produce a large amount of ROS [1]. If AH109A cells can also produce ROS, the spontaneous ROS would induce the spontaneous invasion of AH109A cells across the M-cell monolayer. In our present study, the HX–XO system produced $\dot{O}_2^-$ and H_2O_2 and finally $\dot{O}H^-$ via the Fenton reaction because transition metal ions are present in the solution [13]. EDTA inhibited both increase in AH109A adhesion and invasion possibly by chelating transition metal ions, and thus prevented the formation of $\dot{O}H^-$. Due to the presence of the 'catechol' structure, EC, EGCG and theaflavins are strong metal ion chelators [14]. They can bind the transition metal ions, prevent formation of $\dot{O}H^-$, and thus inhibit the enhancement of AH109A adhesion and invasion as EDTA did. Clearly, the antioxidative activity of EGCG and theaflavins may be primarily responsible for the exogenous ROS-potentiated AH109A adhesion to and invasion underneath the M-cell monolayer and might be partially responsible for spontaneous adhesion and invasion. EGCG has much stronger antioxidative capacity and higher affinity for the lipid bilayers of the cell membrane than EC [15], and can enter the nuclei of cancer cells [16]. This may partially explain why EGCG was able to effectively suppress both exogenous and spontaneous AH109A invasion while EC could only suppress exogenous ROS-increased AH109A invasion. The possible synergistic antioxidation of EC with EGCG may be responsible for the synergistic inhibitory effect against AH109A invasion. The arachidonic acid is metabolized to various products of cyclooxygenase and lipoxygenase. Some of the metabolites are

relevant to the invasion and metastasis of tumour cells [17,18]. Green tea polyphenols inhibited cyclooxygenase and lipoxygenase in vivo [19]. It is also possible that EGCG and theaflavins might inhibit AH109A invasion by suppressing the cyclooxygenase and lipoxygenase; however, this requires further study.

References

[1] T.P. Szatrowski, C.F. Nathan, Production of large amounts of hydrogen peroxide by human tumor cells, Cancer Res. 50 (1990) 2018–2021.

[2] Y. Nonaka, H. Iwagaki, T. Kimura, S. Fuchimoto, K. Orita, Effects of reactive oxygen intermediates on the in vitro invasive capacity of tumor cells and liver metastasis, Int. J. Cancer 54 (1993) 983–986.

[3] I.J. Fidler, Cancer metastasis, Br. Med. Bull. 47 (1991) 157–177.

[4] G.L. Nicolson, P.N. Belloni, R.J. Tressler, K. Dulski, T. Inoue, P.G. Cavanaugh, Adhesive, invasive, and growth properties of selected metastatic variants of a murine large-cell lymphoma, Invasion Metastasis 9 (1989) 102–116.

[5] L.A. Liotta, P.S. Steeg, W.G. Stetler-Stevenson, Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation, Cell 64 (1991) 327–336.

[6] M. Sazuka, S. Murakami, M. Isemura, K. Satoh, T. Nukiwa, Inhibitory effects of green tea infusion on in vivo invasion and in vivo metastasis of mouse lung carcinoma cells, Cancer Lett. 98 (1995) 27–31.

[7] Y. Miura, H. Shiomi, F. Sakai, K. Yagasaki, 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 (1997) 127–132.

[8] G.Y. Zhang, Y. Miura, K. Yagasaki, Effects of green, oolong and black teas and related components on the proliferation and invasion of hepatoma cells in culture, Cytotechnology 31 (1999) 37–44.

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

[10] M. Tanaka, K. Kogawa, Y. Nishihori, K. Kuribayashi, K. Nakamura, H. Muramatsu, K. Koike, S. Sakamaki, Y. Niitsu, Suppression of intracellular Cu–Zn SOD results in enhanced motility and metastasis of Meth A sarcoma cells, Int. J. Cancer 73 (1997) 187–192.

[11] K. Shinkai, M. Mukai, H. Akedo, Superoxide radical potentiates invasive capacity of rat ascites hepatoma cells in vitro, Cancer Lett. 32 (1986) 7–13.

[12] Y. Kozuki, Y. Miura, K. Yagasaki, Inhibitory effects of carotenoids on the invasion of rat ascites hepatoma cells in culture, Cancer Lett. 151 (2000) 111–115.

[13] C. Thomas, G.F. Vile, C.C. Winterbourn, The hydrolysis product of ICRF-187 promotes iron-catalyzed hydroxyl radical production via the Fenton reaction, Biochem. Pharmacol. 45 (1993) 1967–1972.

[14] Q. Guo, B. Zhao, M. Li, S. Shen, W. Xin, Studies on protective mechanisms of four components of green tea polyphenols against lipid peroxidation in synaptosomes, Biochim. Biophys. Acta 1304 (1996) 210–222.

[15] T. Hashimoto, S. Kumazawa, F. Nanjo, Y. Hara, T. Nakayama, Interaction of tea catechins with lipid bilayers investigated with liposome systems, Biosci. Biotechnol. Biochem. 63 (1999) 2252–2255.

[16] S. Okabe, M. Suganuma, M. Hayashi, E. Sueoka, A. Komori, H. Fujiki, Mechanisms of growth inhibition of human lung cancer cell line, PC-9, by tea polyphenols, Jpn. J. Cancer Res. 88 (1997) 639–643.

[17] X.H. Liu, J.M. Connolly, D.P. Rose, 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 (1996) 145–152.

[18] R. Reich, G.R. Martin, Identification of arachidonic acid pathways required for the invasive and metastatic activity of malignant tumor cells, Prostaglandins 51 (1996) 1–17.

[19] S. Katiyar, R. Agarwal, G.S. Wood, 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 (1992) 6890–6897.