RESEARCH COMMUNICATION

Prevention of Mammary Carcinogenesis in C3H/OuJ Mice by Green Tea and Tamoxifen

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Abstract

Background: Tamoxifen (TAM) is useful in the chemoprevention of breast cancer, and green tea catechins, including (-)-epigallocatechin gallate (EGCG), may have similar actions. In this study, we investigated their effects, alone or in combination, on mammary carcinogenesis using breast cancer cells and preneoplastic lesions inC3H/OuJ mice. Methods: Growth inhibitory effects of EGCG and TAM on MCF-7 cells were evaluated with the anchorage-independent colony forming assay. The effects on mammary tumor carcinogenesis and preneoplastic lesions were assessed *in vivo* using animals treated with GTE in drinking water (1%, 0.1%), or a tamoxifen pellet (10 mg/ animal, subcutaneously inoculated) or both agents in combination (1%GTE + 10 mg TAM). The number and size of mammary tumors were measured weekly during treatment. At 48 weeks of age, mice were sacrificed for the examination of hyperplastic alveolar nodules (HAN) and argyrophilic nucleolar organizer regions (AgNOR). Results: In the anchorage-independent growth assay, EGCG and TAM exhibited dose-dependent antiproliferative effects on MCF-7 cells. In the tumor formation assay, tumor incidences were decreased in the GTE, TAM, and GTE+TAM groups, particularly in the latter, in which no tumors developed. AgNOR counts were also significantly lower in the 1%GTE+TAM compared with the 1%GTE group, suggesting an additional anticarcinogenic effect. Conclusion: These data suggest that GTE and TAM, individually and in combination, have potential for chemoprevention of breast cancer.

Keywords: Chemoprevention - epigallocatechin gallate - tamoxifen - alveolar nodules - nucleolar organizer regions

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Introduction

Approximately 1,200,000 new cases of breast cancer occur globally each year (Cuzick, 2008). Unfortunately, although our understanding of the biologic behavior of these tumors has improved, the incidence of breast cancer continues to rise throughout the world (Patel et al., 2007). Asian countries are no exception; in Japan, more than 40 000 women were diagnosed with breast cancer in 2004 (Matsuda et al., 2008). Diagnosis at earlier, more curable stages due to improved screening modalities is considered responsible for some of this increase. Nevertheless, large numbers of breast cancer cases and deaths continue to be observed, denoting an ongoing problem of significant proportions. A high priority should therefore be placed on research aimed at prevention of breast cancer (Jemal et al., 2009).

While nonrandomized studies have reported that prophylactic mastectomy or oophorectomy can significantly reduce the risk of breast cancer, these approaches are unacceptable to the majority of women (Bao et al., 2006). Chemoprevention, which is defined as the prevention of cancer by pharmacological agents that inhibit or reverse the process of carcinogenesis, has thus increasingly become the focus of breast cancer prevention efforts (Powles et al., 2007; Cuzick et al., 2007). The first-generation selective estrogen receptor modulator (SERM) tamoxifen (TAM) is the only drug approved by the US Food and Drug Administration for breast cancer prevention (Vogel et al., 2006; Jenkins et al., 2008). In the NSABP P-01 trial, more than 13 000 women were randomly assigned to receive placebo or TAM for 5 years. After 7 years of follow-up, the cumulative rate of invasive breast cancer was reduced from 42.5 per 1000 women in the placebo group to 24.8 per 1000 in the TAM group (risk ratio (RR) = 0.57) and the cumulative rate of noninvasive breast cancer was reduced from 15.8 per 1000 women in the placebo group to 10.2 per 1000 in the TAM group (RR = 0.63) (Fisher et al., 2005).

The current incidence rate for premenopausal breast cancer is approximately fourfold higher in Western countries than in Far East Asian nations. Migrants from Asia to the USA typically acquire a breast cancer risk associated with their host nation by the second generation, suggesting a direct influence of environmental rather than genetic factors (Limer and Speirs, 2004). The recent

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adoption of a more westernized diet correlates with an increased breast cancer incidence in urban areas of Japan, Singapore and China (Dai et al., 2001).

A comparison of Asian and Western diets indicates that, among many differences, green tea consumption is higher in Asian countries. Importantly, increasing evidence suggests that components in green tea exert chemopreventive effects (Wu et al., 2003). Epidemiological studies suggest that green tea consumption may reduce the risk of cancers including those of the lung, skin, prostate, and breast.

Green-tea polyphenols have various anticarcinogenic effects, including strong antioxidant activity, inhibition of nitrosation and cell proliferation, and induction of apoptosis among carcinoma cells (Tsubono et al., 2001). Several studies demonstrate that administration of green tea extract or its most abundant component, (-)-epigallocatechin gallate (EGCG), suppresses tumor development in various organs including the lung, liver, intestine, bladder, prostate, and breast in a variety of rodent models (Yang et al., 1997).

In this study we investigated the chemopreventive potential of green tea extract (GTE) and TAM, alone and in combination. We examined the in vitro antiproliferative effect of these agents with anchorage-independent growth assay of MCF-7 cells. The preventive effects were investigated with an in vivo tumor formation study in C3H/OuJ mice. To evaluate the mechanism of the preventive effects, we examined hyperplastic alveolar nodule (HAN) count as a measure of preneoplastic lesions and argyrophylic nucleolar organizer region (AgNOR) count as an indicator of proliferative activity of neoplasms.

Materials and Methods

Agents

GTE was kindly provided by Dr. Hirota Fujiki from Saitama Cancer Institute (Saitama, Japan). GTE consists of freeze-dried solids containing approximately 11.2% epigallocatechin gallate, 10.3% epigallocatechin, 6.6% caffeine, 2.5% epicatechin, and 2.3% epicatechin gallate as analyzed by U.S. Tea Association. (-)-Epigallocatechin gallate (EGCG), TAM, and TAM pellets were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell line

The estrogen receptor (ER) positive human breast cancer cell line MCF-7 was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Cells were maintained in Dulbecco's Modified Eagles Medium (D-MEM) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich Japan, Tokyo, Japan), and incubated at 37°C in a humidified atmosphere consisting of 95% air and 5% CO2. All experiments were performed using exponentially growing cells.

Anchorage-independent growth assay

Six-well plates were precoated with 0.3% soft agar in MEM with 10%FBS. We added EGCG (0.01-1 μM) or TAM (0.001-1 μM), or both, followed by MCF-7 cells (1.0 x 10^4 cells/ well). The plates were incubated at 37 ˚C for 14 days, after which the number of colonies greater than 400 μm in diameter was counted macroscopically.

Tumor formation study

C3H/OuJ transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and were kept in a pathogen-free facility. All procedures were approved by the Animal Laboratory Center of the Keio University School of Medicine. Ten-week-old mice (7 per group) were randomly assigned to treatment groups consisting of GTE (0.1 or 1% in drinking water), TAM (10 mg pellet), the combination of 1% GTE and 10 mg tamoxifen, and control. A freshly prepared solution of 0.1% or 1% GTE in tap water was supplied everyday beginning at 10 weeks of age as the sole source of drinking water in the GTE groups. Control animals were similarly handled and supplied with tap water for the duration of the experiment. TAM pellets were subcutaneously administered at 10 weeks of age. Mammary tumor development was monitored weekly by palpation and tumor size was measured with calipers. After 38 weeks of treatment, the mice were sacrificed by cervical dislocation and tumors and mammary pads were excised. Tumors were fixed in 10% phosphate-buffered formalin and embedded in paraffin for histological examination with hematoxylin and eosin (H&E) staining.

Hyperplastic alveolar nodule (HAN) count

Fat pad tissues containing normal mammary gland derived from C3H/OuJ mice were fixed in 10% neutral buffered formalin at room temperature for 18 to 24 hours, dehydrated in ethanols, embedded in paraffin, sectioned (6 μm), and stained with H&E. For mammary whole-mount preparations, isolated glands were flattened between glass microscope slides and immersed in acetone immediately after collection or after 1 hour of fixation in 10% neutral buffered formalin. Glands were defatted by three to four changes of acetone (24 hours each), rehydrated through graded alcohols, and stained with a solution of 50 mg methylene blue in 100 ml formalin for 3 minutes. Specimens were destained in graded ethanols and samples were stored in methyl salicylate for further examination. HANs, which appear as darkly stained, variably sized collections of alveolar-like structures, were counted macroscopically. The number of discrete HANs was recorded for each gland.

Argyrophilic nucleolar organizer region (AgNOR) count

Paraffin embedded fat pad tissues were deparaffinized in xylene and descending concentrations of ethanol, each for 5 minutes. The sections were washed four times in de-ionized double distilled water for 3 minutes each time. The tissue was then incubated in a dark chamber for 40 minutes with a 50% aqueous solution of silver nitrate in 2% gelatin and 1% formic acid solution. The sections were again washed four times in distilled water and then incubated for 5 minutes with 5% sodium thiosulfate solution to arrest the staining reaction. The sections were then washed twice, dehydrated in ascending concentrations of ethanol and xylene and coverslipped without counterstaining. The AgNOR content of at least 100 epithelial cell nuclei per area (at 400x magnification) was evaluated in one focal

plane. AgNORs were observed as black dots in the nuclei and the number of AgNORs per nucleus was measured.

Statistical analysis

Statistical analysis was carried out using statistical software (GraphPad Prism version 5.0, GraphPad Software Inc., CA and SPSS Statistics 17.0, SPSS Japan Inc., Tokyo, Japan). The two-sided Student's t test was used to determine significant differences between the various groups analyzed. Statistical significance was defined as $p < 0.05$. Proliferation data were analyzed by the methods established by Chou and Talalay (1984). Multiple drug effect analysis was performed using CalcuSyn software (Biosoft, Cambridge, United Kingdom), which quantitatively describes the interaction between two or more drugs (Junttila et al., 2009). This method assigns combination index (CI) values to each drug combination and defines drug synergy as CI <1 and drug antagonism as CI ≥ 1.

Results

Anchorage-independent growth assay

The anchorage-independent growth assay showed that individually, EGCG (0.01–10 μM) and TAM (0.001–1 μM) exhibited dose-dependent antiproliferative activity on ER-positive MCF-7 breast cancer cells (Figure 1). The IC50 of EGCG was 0.07 μM and that of TAM was 0.18 μM. The combination of EGCG and TAM was more effective than either agent given alone. The CI calculations for 0.01–10 μM EGCG and 0.001–1 μM TAM combinations consistently showed CI values <1, indicating synergism.

Tumor formation study

The cumulative incidence of palpable tumors in mice treated with GTE, TAM, or GTE + TAM is shown in Figure 2. The mammary tumors were morphologically designated as mixed solid and glandular adenocarcinomas and resembled what has been called human invasive ductal carcinoma. GTE in drinking water and subcutaneously inoculated TAM reduced the incidence of tumor compared with the control group. No tumors appeared in the combination group. While all C3H control mice developed tumors by 38 weeks of age, mice treated with EGCG or TAM exhibited tumors after 40 weeks of age or later. Hence both agents slowed tumor formation.

The average number of tumors was smaller in the 1% GTE group (0.25) and the TAM group (0.14) than in the control group (1.10) (Figure 3). Both GTE and TAM therefore appear to reduce the number of tumors per mouse.

HAN count

Average HAN count was lower in the 1% GTE group (6.13) than in the control group (9.20) ($p < 0.001$) (Figure 4). HAN count was also reduced in the TAM group (6.00) compared with the control group (9.20) ($p = 0.028$). Both 1% GTE and TAM appear to reduce preneoplastic HAN formation. There was no significant difference in HAN

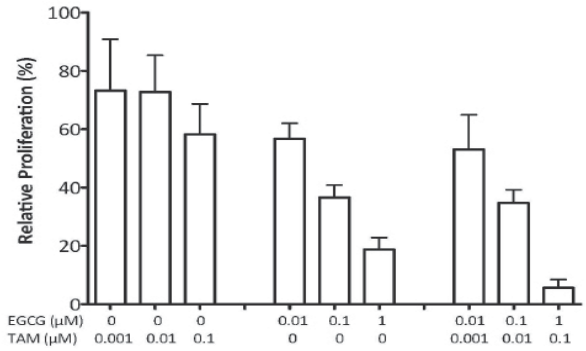

Figure 1. Anchorage-independent Cell Growth of MCF-7 Treated with EGCG, TAM, or EGCG+TAM. Six-well plates were precoated with 0.3% soft agar in MEM with 10%FBS. We added EGCG (0.01-1 μM) or TAM (0.001-1 μM), or both, followed by MCF-7 cells (1.0 x 104 cells/well). Anchorage-independent cell growth of MCF-7 was counted in each condition. Synergism between EGCG and TAM was demonstrated. EGCG,(-)-epigallocatechin gallate; TAM, tamoxifen

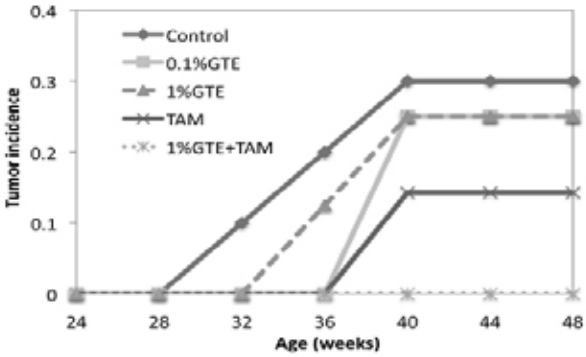

Figure 2. Mammary Tumor Incidence in C3H/OuJ Mice. Ten-week old mice were assigned to treatment groups consisting of GTE (0.1 or 1% in drinking water), TAM (10 mg pellet, subcutaneously inoculated), or the combination of 1% GTE and TAM. Mammary tumor development was monitored weekly by palpation and tumor size was measured with calipers. GTE, green tea extract; TAM, tamoxifen

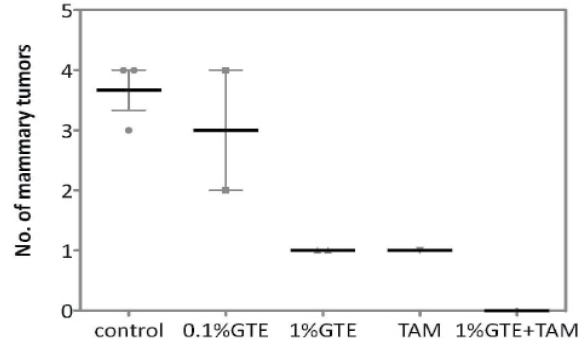

Figure 3. Average Number of Mammary Tumors in C3H/OuJ Mice. Results are expressed as mean with standard error (SEM) for each treatment group. GTE, green tea extract; TAM, tamoxifen

count between the combination group (5.83) and the 1% GTE group (6.13). HAN counts were also similar in the combination group (5.83) and TAM group (6.0).

AgNOR count

Average AgNOR count was lower in both the 1% GTE group (1.82) ($p = 0.008$) and the TAM group (1.70) ($p < 0.001$) than in the control group (2.15) (Figure 5).

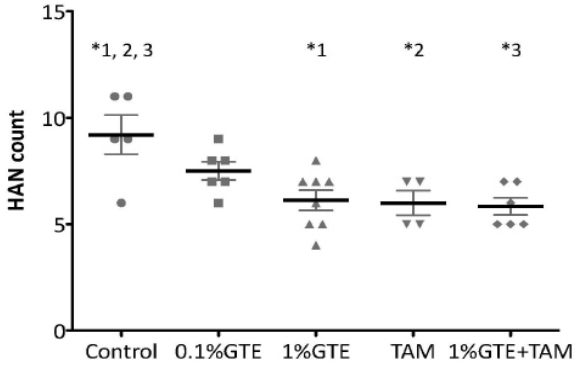

Figure 4. HAN Count in Mammary Glands of C3H/OuJ Mice. Mammary glands were removed, fixed in formalin, and stained with methylene blue. HANs were then counted. Results are expressed as mean HAN count with standard error for each treatment group. HAN, hyperplastic alveolar nodule, GTE: green tea extract, TAM: tamoxifen, *1, $p < 0.001$; *2, $p = 0.028$; *3; $p = 0.006$

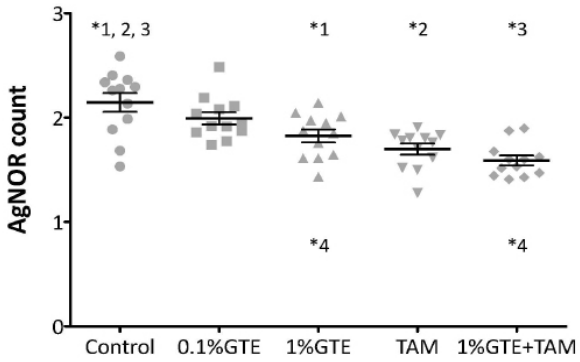

Figure 5. AgNOR Count in Mammary Glands of C3H/OuJ Mice. Tissues were fixed in formalin and processed with a silver nitrate staining procedure. The number of AgNORs (observed as black dots) per nucleus was counted. Results are expressed as mean AgNOR count with standard error for each treatment group. AgNOR, argyrophilic nucleolar organizer region; GTE, green tea extract; TAM, tamoxifen; *1, $p = 0.008$; *2, $p < 0.001$; *3, $p < 0.001$; *4, $p = 0.006$

Accordingly, both 1% GTE and TAM seem to reduce AgNOR formation.

AgNOR count was significantly lower in the combination group (1.59) than in the 1% GTE group (1.82) ($p = 0.006$), but there was no difference between the combination group and the TAM group (1.70).

Discussion

In the anchorage-independent growth assay, CI values were <1 over most of the effective range of the agents, demonstrating that the combination of EGCG and TAM synergistically inhibits proliferation of MCF-7 cells.

The C3H mouse is one of the most popular animal models of mammary tumor formation. This strain carries the C3H mammary tumor virus type S and breeding females typically show a high incidence of mammary tumors by 11 months of age. Our study shows that GTE and TAM, both individually and in combination, can reduce the incidence of mammary tumors in these mice. It was notable that no tumors developed in the combination group of 1% GTE and 10 mg TAM.

The principal preneoplastic lesion in the murine mammary gland is the HAN, which is a focus of hyperplastic lobuloalveolar development in an area of nonstimulated mammary gland (Leong et al., 2008). HANs can be induced in mice by mammary tumor viruses (MMTVs), chemical carcinogens, X-irradiation, and prolonged hormone stimulation (Medina, 2008). They are considered preneoplastic lesions as they exhibit enhanced tumorigenic potential when transplanted into syngeneic mice (Tsubono et al., 2001; Sartippour et al., 2006). In this study, HAN count was significantly reduced in the 1% GTE group, TAM group, and combination group compared with the control group. This finding suggests that both GTE and TAM, or their combination, could potentially have a chemopreventive effect against breast cancer.

AgNOR count correlates with the proliferative activity of neoplasms (Mourad et al., 1994). Some studies have demonstrated that quantitative analysis of AgNORs has prognostic value in breast cancer. The combination of MIB-1 immunostaining and AgNOR measurements in MIB-1 positive nuclei improves prognostication (Biesterfeld et al., 2001). Metaphase identification of AgNORs as brown-black granules has been made possible by a simple silver staining technique. In the present study, the formation of AgNORs was significantly reduced in the 1% GTE group, TAM group, and combination group compared with the control group. In addition, AgNOR was also significantly reduced in the combination group compared with the 1% GTE group. This suggests that GTE and TAM have synergistic inhibitory effects on cell proliferation in the mammary gland.

EGCG has also been observed to act synergistically with sulindac to prevent colon carcinogenesis in rats, and with tamoxifen on the lung cancer cell line PC-9 (Suganuma et al., 1999; Fujiki et al., 2003). A recent study reported synergistic in vitro cytotoxicity of 4-hydroxytamoxifen (4-OHT) and EGCG in ER-negative breast cancer MDA-MB231 cells (Chisholm et al., 2004). Since 4-OHT and EGCG are both extensively glucuronidated, 4-OHT might inhibit the conjugation of EGCG and thus increase its cytotoxicity. In a pharmacokinetic analysis, Shin and Choi reported that EGCG significantly increased the AUC0–∞ and Cmax of oral tamoxifen in rats. Because orally administered tamoxifen is a substrate for CYP3A-mediated metabolism and P-gp-mediated efflux in the intestine and liver, the presence of EGCG might effectively obstruct this metabolic pathway (Shin and Choi, 2009). Sartippour et al. suggested that GTE inhibits breast cancer growth via a direct anti-proliferative effect on tumor cells, as well as by indirect effects on endothelial cells (Lu et al., 1998). They found that GTE reduces levels of three important angiogenic factors in breast cancer: VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor), and aFGF (acidic fibroblast growth factor). Other researchers have shown that green tea can upregulate tumor suppressor genes. Green tea may also inhibit HER-2/neu signaling in breast cancer cells (Pianetti et al., 2002).

In our in vitro study, EGCG and TAM synergistically inhibited the proliferation of MCF-7 cells. From the results of our in vivo study, it is notable that no tumors appeared

in the combination group treated with 1% GTE and TAM. HAN formation was reduced with GTE and TAM given either individually or in combination, hence these agents could have potential in the chemoprevention of breast cancer. AgNOR evaluation showed that GTE and TAM act synergistically in their antiproliferative effects on the mammary gland. In conclusion, our study suggests that GTE and TAM, particularly in combination, have potential in the chemoprevention of breast cancer.

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