

Anti-invasive activity of α -tocopherol against hepatoma cells in culture via protein kinase C inhibition

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Effects of α -, β -, γ - and δ -tocopherols on the proliferation and invasion of AH109A hepatoma cells and their modes of action were investigated. Four tocopherols inhibited the invasion as well as the proliferation of AH109A cells. Their inhibitory effects were more prominent on the invasion than on the proliferation. At 1 μ M, α -tocopherol showed most potent anti-invasive activity without any influence on the proliferation. We have previously demonstrated that reactive oxygen species increase the invasion of AH109A cells. α -Tocopherol suppressed the reactive oxygen species-induced invasion but failed to suppress the reactive oxygen species-induced rises in intracellular peroxide level. GF 109203X, a protein kinase C inhibitor, decreased the invasive activity of AH109A cells. In contrast, phorbol-12-myristate-13-acetate, a protein kinase C activator, increased the invasive capacity of AH109A cells. α -Tocopherol suppressed the phorbol-12-myristate-13-acetate-induced increase in the invasion, and canceled the phorbol-12-myristate-13-acetate-induced rises in protein kinase C activity and phosphorylation of extracellular signal-regulated kinase. These results suggest that tocopherols, especially α -tocopherol, possess inhibitory effect more strongly on the invasion of AH109A cells than on the proliferation. They also suggest that the anti-invasive activity of α -tocopherol is raised through suppression of PKC/ERK signaling.

Key Words: tocopherol, hepatoma, invasion, protein kinase C, reactive oxygen species

Cancer invasion is a critical step of metastasis, which is the most important property of cancer cells.⁽¹⁾ The blockade or prevention of this process by drugs and food factors will lead to prolongation of the life span of host. In our previous study, some food factors such as L-ascorbic acid, carotenoids and resveratrol have anti-invasive effect against a rat ascites hepatoma cell line of AH109A.⁽²⁻⁴⁾ These compounds possessing antioxidative activity led us to assume that their inhibitory effect on invasion is mediated through scavenging reactive oxygen species (ROS). Tumor cells are known to produce a large amount of ROS than do normal cells.⁽⁵⁾ ROS may be a key mediator for tumor metastasis including migration and invasion.^(6,7) In fact, ROS increased invasive activity of AH109A cells.⁽⁸⁾ Molecular mechanisms for metastasis promotion by ROS were reportedly related to activation of mitogen activated protein kinase (MAPK) signaling through various tyrosine kinases and protein kinase C (PKC).⁽⁹⁾ PKC is a family of serine/threonine kinases and is classified into three groups, such as classical PKC (α , β , γ), novel PKC (δ , ϵ , η , θ), and atypical PKC (ζ , ι/λ). PKC is known to regulate a variety of cell functions including proliferation, cytoskeletal organization, and cell migration.^(9,10)

Tocopherols differ in the number of methyl groups present on the chroman head and are known as one of lipid-soluble vitamins.

PKC is known as a main intracellular target of α -tocopherol.^(11,12) Moreover, tocopherols have antioxidative activity and anti-proliferative action on some tumor cells.^(13,14) However, little is known about the effects of tocopherols on the invasion of cancer cells and their modes of action. The aim of this study was to investigate the effects of tocopherols on the invasion as well as the proliferation of AH109A hepatoma cells and to clarify their modes of action.

Materials and Methods

Materials. α -, β -, γ - or δ -Tocopherol (CN Biosciences, Inc., La Jolla, CA) was dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO) or ethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Each tocopherol solution was added to the medium at the final DMSO concentration of 0.5% or the final ethanol concentration of 0.1%. The control medium contained 0.5% DMSO or 0.1% ethanol alone. GF 109203X (GF, Sigma Chemical Co.) and phorbol-12-myristate-13-acetate (PMA, Calbiochem, La Jolla, CA) were dissolved in DMSO and added to the medium at the final DMSO concentrations of 0.5% (GF) and 0.1% (PMA), respectively. Anti- γ -tubulin antibody was purchased from Sigma Chemical Co., and anti-phospho-ERK1/2 (anti-p-ERK1/2) and anti-ERK1/2 (anti-ERK1/2) antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Culture of AH109A hepatoma cells. The animal experiments in this study were conducted in accordance with guidelines established by the Animal Care and Use Committee of Tokyo University of Agriculture and Technology. Male Donryu rats (3 weeks of age) were purchased from Japan SLC, Inc. (Shizuoka, Japan), and were used for invasion assay as described below.

A rat ascites hepatoma cell line of AH109A cells was provided by the Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan. AH109A cells were maintained and cultured in Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% calf serum (CS, obtained from GIBCO BRL, Grand Island, NY) (10% CS/MEM) as described previously.⁽¹⁵⁾

In vitro proliferation and invasion assays. The effects of tocopherols on AH109A proliferation were examined by measuring the incorporation of [methyl-³H]thymidine (0.15 μ Ci/well, specific radioactivity; 20 Ci/mmol, New England Nuclear, Boston, MA) into DNA as described previously.^(4,16) The proliferative activity of AH109A cells treated with PMA was determined using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). The effects of tocopherols on AH109A invasion were estimated

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by co-culturing AH109A cells with rat mesentery-derived mesothelial cell (M-cell) monolayers, which were isolated from male Donryu rats as described previously⁽¹⁷⁾ with slight modifications.⁽¹⁵⁾

Treatment of AH109A cells with hypoxanthine (HX) and xanthine oxidase (XO). AH109A cells were cultured for 4 h in the absence or presence of 1 μ M of tocopherols and/or 4 μ g/ml of HX (Sigma) with 7×10^{-4} U/ml of XO (Sigma).^(18,19) After treatment, the hepatoma cells were washed once with the medium and seeded onto the M-cell monolayer in 10% CS/MEM with or without 1 μ M of tocopherols, and cultured for another 24 h.

Flow cytometric analysis. Intracellular peroxide level in AH109A cells was investigated by flow cytometric analysis using a peroxide-dependent fluorescent dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Inc., Eugene, OR) according to Bass *et al.*⁽²⁰⁾ by flow cytometry with an EPICS ELITE EPS (Beckman-Coulter, Hialeah, FL) as described previously.⁽²⁾

Measurement of PKC activity. AH109A cells were pre-cultured with or without α -tocopherol for 10 min. At the end of the incubation, PMA was added to the medium to activate PKC. After stimulation by PMA for 3 min, the cells were washed and dissolved with lysis buffer [20 mM MOPS (Wako Pure Chemical Industries), 50 mM glycerol 2-phosphate disodium salt *n*-hydrate (Wako Pure Chemical Industries), 50 mM sodium fluoride (Wako Pure Chemical Industries), 1 mM sodium orthovanadate (Sigma Chemical Co.), 5 mM EGTA (ICN Biomedicals Inc., Aurora, OH), 2 mM EDTA (Wako Pure Chemical Industries), 1% IGEPAL CA-630 (Sigma Chemical Co.), 1 mM 1,4-dithiothreitol (DTT, MERCK, Darmstadt, Germany), 1 mM benzamide (Sigma Chemical Co.), 1 mM phenylmethanesulphonylfluoride (PMSF, Sigma Chemical Co.), 10 μ l/ml protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan)]. Protein concentrations were measured by Micro BCA protein assay kit (Pierce Chemical Co., Rockford, IL). Aliquots of the lysates (2 μ g of total protein) were prepared for PKC activity assay by using a commercial kit (StressXpress[®] PKC Kinase Activity Assay Kit, Assay Designs, Ann Arbor, MI).

Western blotting analysis. After treatment of AH109A cells with and without tocopherols in the presence or absence of PMA for 10 min, these cells were lysed with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate), 10 μ l/ml protease inhibitor cocktail (Nacalai Tesque, Inc.) and 10 mM sodium orthovanadate (Sigma Chemical Co.). Samples were placed on ice for 30 min and then centrifuged at $18,000 \times g$ and 4°C for 20 min. Protein concentrations were measured by Micro BCA protein assay kit (Pierce Chemical Co.). Aliquots of the lysates (43 μ g of total protein) were boiled for 5 min and electrophoresed on 10% SDS-polyacrylamide gel. After transferring, the membranes were blocked with 5% BSA (Sigma Chemical Co.) in tris-HCl buffered saline (TBS) at 4°C overnight. P-ERK1/2, total ERK1/2 and γ -tubulin were detected by specific monoclonal antibodies at 4°C overnight and then horse radish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare UK, Buckinghamshire, UK). Finally, protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Pierce Chemical Co.). After the detection of the bands, intensities of those bands were measured by NIH Image J software.

Statistical analysis. Data are expressed as means \pm SEM. Multiple comparison was performed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test, and $p < 0.05$ was considered statistically significant.

Results

Effects of α -, β -, γ - and δ -tocopherols on the proliferation and invasion of AH109A cells. We first examined the effects of α -, β -, γ - and δ -tocopherols on the proliferation and invasion of

hepatoma (AH109A) cells at concentrations of 0, 1, 5, 10 and 50 μ M, using *in vitro* assay systems. All tocopherols dose-dependently inhibited the proliferation of AH109A cells to 10 μ M and maintained the significant suppressive effect up to 50 μ M by *ca.* 20%. At 1 μ M, all tocopherols exerted no significant influence on the proliferation (Fig. 1 A–D), and α -tocopherol alone significantly inhibited the proliferation at 5 μ M (Fig. 1A). On the other hand, all tocopherols noticeably and significantly inhibited the invasion of hepatoma cells at all the concentrations (1–50 μ M) employed (Fig. 1 E–H). Their inhibitory effects were more prominent on the invasion than on the proliferation. In particular, all tocopherols showed the significant anti-invasive activity on AH109A cells even at 1 μ M (Fig. 1 E–H) without affecting the proliferation at the concentration (Fig. 1 A–D). Thus, 1 μ M was employed as a proper concentration of tocopherols in the following experiments.

We next conducted the invasion assay at 1 μ M of each tocopherol to clarify which tocopherol would be most effective. As shown in Fig. 2, the invasive activity of AH109A cells treated with α -tocopherol significantly decreased as compared with β -, γ -tocopherols and tended to decrease as compared with δ -tocopherol. From this result, α -tocopherol was demonstrated to possess most potent anti-invasive activity against AH109A cells. Thus, we thereafter focused our attention on the effect of α -tocopherol on the invasion of AH109A cells.

Effect of α -tocopherol on the invasion and intracellular peroxide level of AH109A cells pre-treated with HX and XO, a ROS-generating system. To examine whether or not α -tocopherol would inhibit the invasion of hepatoma cells by its antioxidative activity, an invasion assay was designed with AH109A cells pre-cultured in ROS-generating system. As shown in Fig. 3A, the invasive activity of AH109A cells pre-cultured in HX-XO-containing medium was significantly increased in comparison with that of AH109A cells pre-cultured in the control (HX-XO-free) medium. On the other hand, α -tocopherol at 1 μ M completely canceled the ROS-induced invasive activity of cancer cells to the control level. To clarify the suppressive effect of α -tocopherol on the ROS-induced invasion, we next measured the intracellular peroxide level in AH109A cells by flow cytometric analysis using DCFH-DA as an indicator. As shown in Fig. 3B, this level was elevated by treatment of AH109A cells with HX-XO system. However, α -tocopherol failed to suppress the ROS-elevated intracellular peroxide level (Fig. 3B), despite that it canceled the ROS-potentiated invasion of AH109A cells treated with HX-XO as seen in Fig. 3A. These results suggested that anti-invasive effect of α -tocopherol was not due to its ROS-scavenging property.

Effects of PKC inhibitor and activator (PMA) on the proliferation and invasion of AH109A cells. Activation of PKC by ROS was known to cause amplification of signal transduction toward cell migration.⁽⁹⁾ To investigate whether or not PKC would play an important role in the invasive activity of AH109A cells, we examined the proliferation and invasion of AH109A cells that were exposed to a PKC inhibitor GF 109203X (0.5–4 μ M), which is a highly specific inhibitor of cPKC and partial nPKC, for 1 h followed by proliferation and invasion assay. Pre-treatment of AH109A with GF 109203X exerted no significant influence on the proliferation up to 1 μ M but significantly inhibited the proliferation at 2 and 4 μ M (Fig. 4A). Under the similar condition, GF 109203X commenced to significantly decrease the invasive activity from 1 μ M and kept the inhibitory effect up to 4 μ M (Fig. 4B).

To investigate whether or not PMA, a PKC activator, would increase the invasive activity of AH109A cells, we examined the proliferation and invasion of AH109A cells that were exposed to PMA which is known as a specific activator of cPKC and nPKC in the PKC family.⁽²¹⁾ While PMA slightly inhibited the proliferation of AH109A cells pre-cultured with PMA (0.1–10 nM)

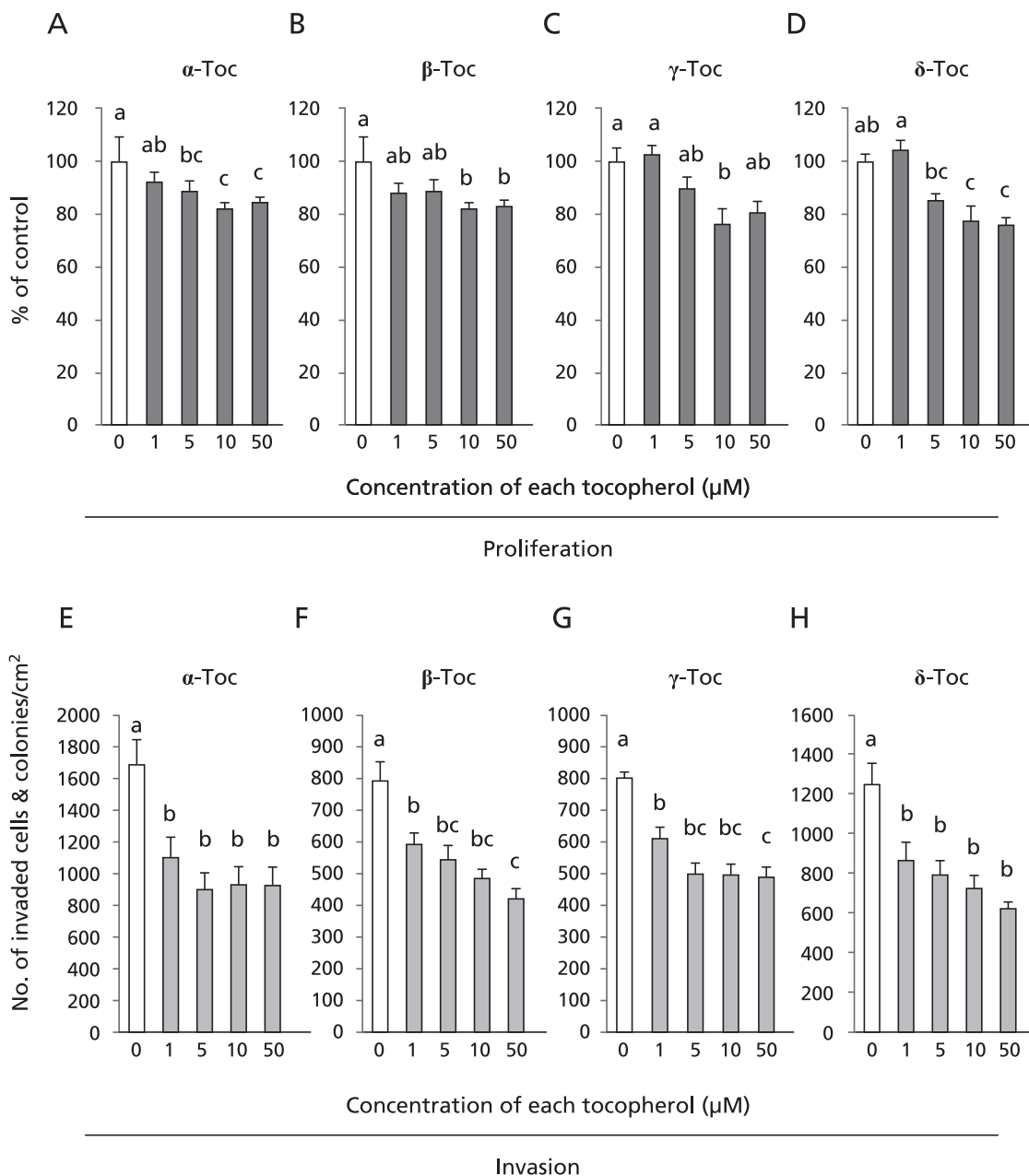


Fig. 1. Effects of α -, β -, γ - and δ -tocopherols on the proliferation and invasion of AH109A cells. Tocopherols were dissolved in DMSO. Each tocopherol solution was added to culture medium at a final DMSO concentration of 0.5%. All tocopherols were added to experimental media at final concentrations of 0, 1, 5, 10 and 50 μ M. The proliferation of AH109A cells was evaluated by measuring the incorporation of [methyl- 3 H]thymidine into the DNA fraction. Each value represents the mean \pm SEM of six wells (Fig. 1 A–D). The invasive activity of AH109A cells was determined by invasion assay. Each value represents the mean \pm SEM of ten areas (Fig. 1 E–H). Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey-Kramer multiple comparisons test. Toc indicates tocopherol.

for 4 h (Fig. 4C), the PKC activator dose-dependently elevated the invasive activity of AH109A cells (Fig. 4D). These results suggested an involvement of PKC in the invasive activity of AH109A cells.

Effect of α -tocopherol on the PMA-induced invasion of AH109A cells. α -Tocopherol has been reported to inhibit activity of PKC in smooth muscle cells.⁽¹¹⁾ Thus, we examined the effect of α -tocopherol on the PMA-induced invasive activity of AH109A cells (Fig. 5A). PMA (10 nM) treatment significantly increased the hepatoma invasion as compared with the control. α -Tocopherol canceled the PMA-induced rise in the invasion of hepatoma cells at 1 μ M, the concentration where α -tocopherol

exerted no influence on the invasion of PMA-untreated AH109A cells.

Effects of PMA and α -tocopherol on PKC activity and its down stream. The fact that α -tocopherol at 1 μ M suppressed the PMA-induced invasion in the present study (Fig. 5A) prompted us to directly measure the PKC activity in AH109A cells. AH109A cells were pre-cultured in the absence or presence of α -tocopherol at 1 μ M, followed by treatment with or without PMA at 10 nM (Fig. 5B). PKC activity of AH109A cells pre-cultured with α -tocopherol alone was equal to that of the control level. On the other hand, PKC activity was significantly stimulated by PMA as compared with the control (PMA-free, α -

tocopherol-free). However, PMA-induced PKC activity in the AH109A cells pre-cultured with α -tocopherol was significantly suppressed to the control level.

PKC is an upstream regulator of the Raf-MEK1/2-p44/p42 MAPK cascade.⁽⁹⁾ To elucidate whether or not α -tocopherol would be involved in phosphorylation of PKC signaling, we examined the effect of α -tocopherol on the ERK1/2 phosphorylation in

AH109A cells, which is the downstream of PKC signaling. As shown in Fig. 5 C and D, the ERK1/2 phosphorylation in AH109A cells treated with PMA (10 nM) for 10 min was 1.4-fold as high as the basal ERK1/2 phosphorylation in AH109A cells without PMA treatment. However, α -tocopherol (1 μ M) almost completely suppressed the PMA-stimulated phosphorylation of ERK1/2, indicating that α -tocopherol inhibited the downstream of PKC signaling.

Discussion

In the present study, we examined *in vitro* effects of α -, β -, γ - and δ -tocopherols on the proliferation and invasion of AH109A cells and found that four tocopherols all inhibited both the proliferation and invasion of hepatoma cells. Higher concentrations (10–50 μ M) of α -, β -, γ - and δ -tocopherols significantly inhibited the proliferation by 20% (Fig. 1 A–D) and the invasion by 47% (Fig. 1 E–H) as compared with the corresponding controls. Previous studies also demonstrated that tocopherols at higher concentrations in the range of 10–50 μ M inhibited proliferative activities of different cancer cells such as prostate cancer, breast cancer, colorectal adenocarcinoma, osteosarcoma and glioma cells.^(14,22,23) In case of AH109A hepatoma cells, anti-proliferative activities of lower concentrations (1–5 μ M) of tocopherols, except for α -tocopherol (Fig. 1A), were not statistically significant as compared with untreated AH109A cells (Fig. 1 B–D), but four tocopherols at 1–5 μ M significantly suppressed the invasion (Fig. 1 E–H). At the lower concentrations, their inhibitory effects were more prominent on the invasion than on the proliferation. In the present study, α -tocopherol was demonstrated for the first time to most strongly prevent the invasion of AH109A cells among four tocopherols (Fig. 2). Moreover, present study showed that the order of anti-invasive activity of the four tocopherols was α -tocopherol > δ -tocopherol \geq β -tocopherol = γ -tocopherol. In our previous studies, carotenoids, fish oil and theanine, for instance, did not show any anti-proliferative activity but did anti-invasive activity against AH109A cells at lower concentrations,^(3,24,25)

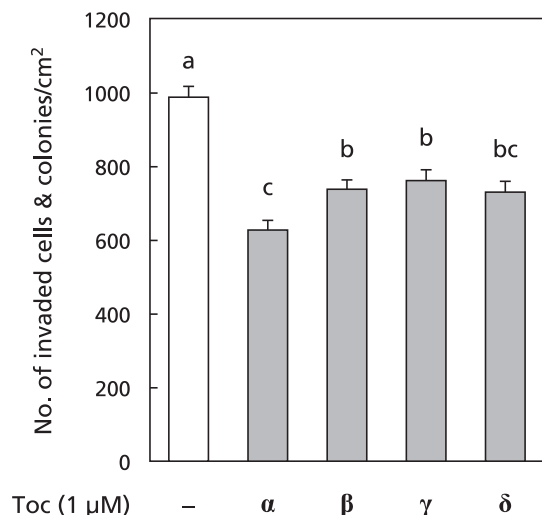


Fig. 2. Comparative effects of α -, β -, γ - and δ -tocopherols on the invasion of AH109A cells. Tocopherols were dissolved in ethanol. Each tocopherol solution was added to culture medium at a final ethanol concentration of 0.1%. The invasive activity of AH109A cells in the absence or presence of 1 μ M of each tocopherol was determined by invasion assay. Each value represents the mean \pm SEM of ten areas. Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey-Kramer multiple comparisons test. Toc indicates tocopherol.

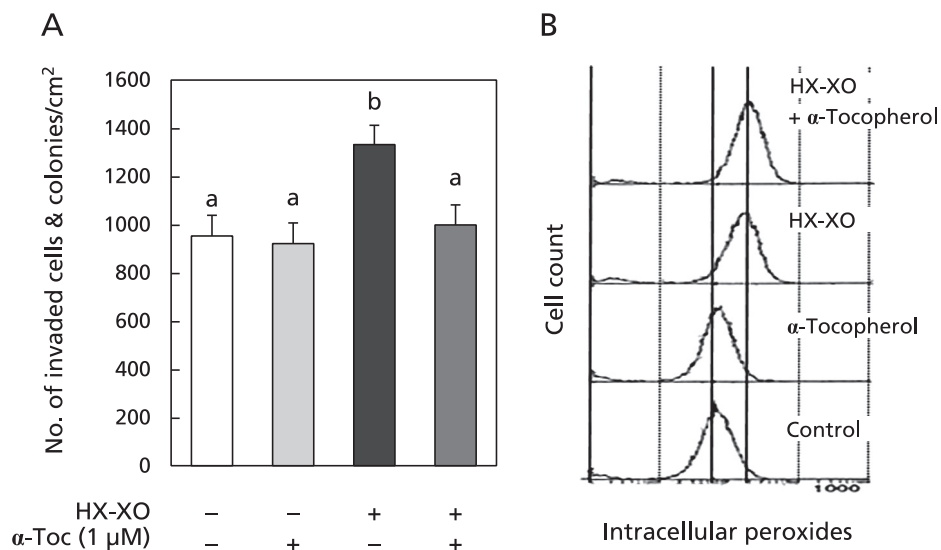


Fig. 3. Effect of α -tocopherol on the invasion and intracellular peroxide level of AH109A cells pre-cultured with ROS generated by HX-XO system. α -Tocopherol was dissolved in DMSO. The α -tocopherol solution was added to culture medium at a final DMSO concentration of 0.5%. (A) AH109A cells were cultured for 4 h in the presence or absence of 1 μ M of α -tocopherol and/or HX (4 μ g/ml) with XO (7×10^{-4} U/ml). After treatment, the cells were washed and applied onto M-cell monolayers in 10% CS/MEM without α -tocopherol and HX-XO. The invasive activity was determined by invasion assay 24 h later. Each value represents the mean \pm SEM of ten areas. Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey-Kramer multiple comparisons test. (B) After treatment of cells with α -tocopherol and/or ROS for 4 h, DCFH-DA was added, and cells were incubated for another 20 min and analyzed with a flow cytometer. Basal and ROS-increased intracellular peroxide levels are indicated as solid lines. A representative result is shown. Toc indicates tocopherol.

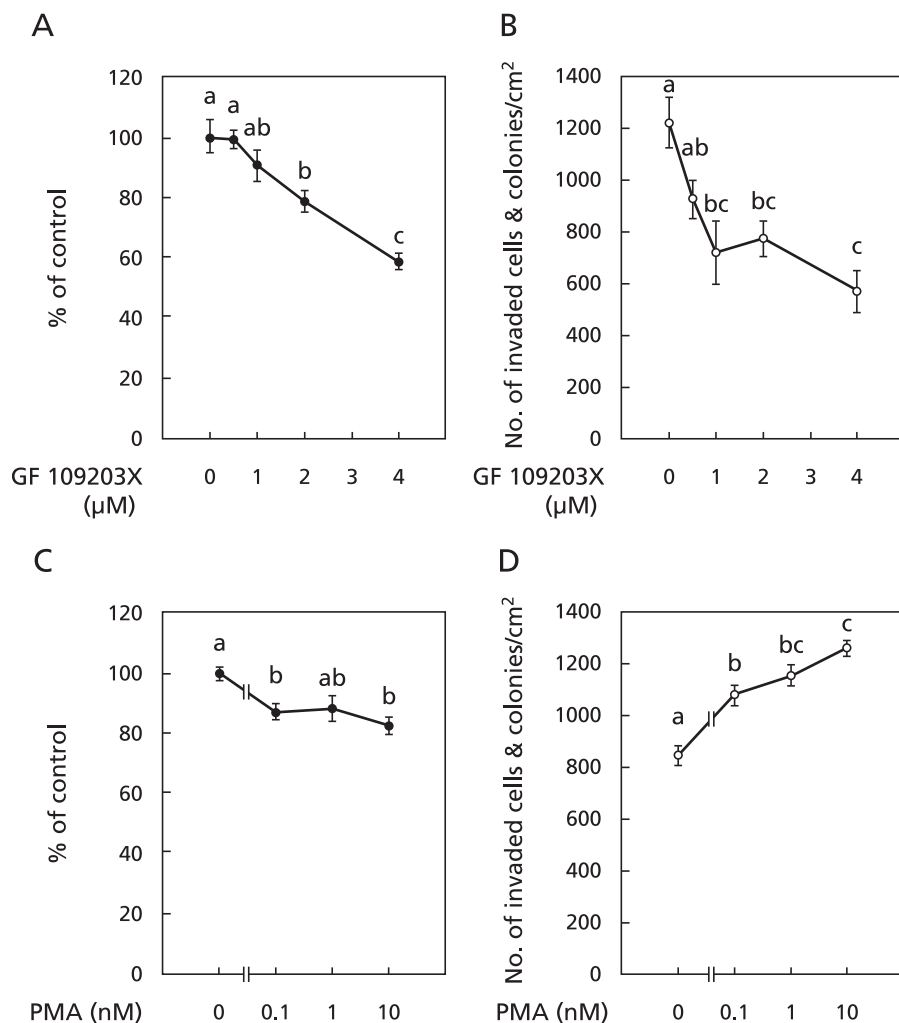


Fig. 4. Effects of PKC inhibitor (GF 109203X) and PKC activator (PMA) on the proliferative and invasive activities of AH109A cells. (A) GF 109203X was dissolved in DMSO. The GF 109203X solution was added to culture medium at a final DMSO concentration of 0.5%. After treatment of AH109A cells with 0, 0.5, 1, 2 and 4 μM GF 109203X for 1 h, the cells were washed and the proliferation of AH109A cells was evaluated by measuring the incorporation of [methyl-³H]thymidine into the DNA fraction. Each value represents the mean ± SEM of six wells. (B) After treatment of AH109A cells with 0, 0.5, 1, 2 and 4 μM GF 109203X for 1 h, the cells were washed and overlaid onto M-cell monolayers. The invasive activity was determined by invasion assay. Each value represents the mean ± SEM of ten areas. (C) Phorbol-12-myristate-13-acetate (PMA) was dissolved in DMSO. The PMA solution was added to culture medium at a final DMSO concentration of 0.1%. PMA was added to the medium at the concentrations of 0, 0.1, 1 and 10 nM. The proliferative activity of AH109A cells was determined with a cell counting kit. Each value represents the mean ± SEM of six wells. (D) The invasion of AH109A cells was determined by invasion assay. Each value represents the mean ± SEM of ten areas. Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey-Kramer multiple comparisons test.

indicating that the effects on the proliferation and invasion of AH109A cells depend on the properties of food components.

As tocopherols are known to have antioxidative activity, we have employed a ROS-stimulated invasion assay system. α -Tocopherol, which showed the most potent anti-invasive activity, suppressed the invasion of AH109A cells cultured in a ROS-generating system with HX-XO (Fig. 3A). The HX-XO system is reported to generate both superoxide anion ($O_2^{\cdot-}$) and H_2O_2 , and finally hydroxyl radical ($\cdot OH$) via the Fenton reaction.⁽²⁶⁾ Interestingly, α -tocopherol (1 μM) did not decrease the intracellular peroxide level irrespectively of the presence of the ROS-generating system (Fig. 3B). In the similar system, other antioxidants such as ascorbic acid (62.5 μM) and resveratorol (50 μM) were reported to distinctly decrease the intracellular peroxide level in AH109A hepatoma cells treated with exogenous ROS.^(2,27) Although α -tocopherol was reported to have most potent antioxidative reaction rate *in vitro*,⁽¹³⁾ it did not affect the intracellular peroxide level in

cytoplasm (Fig. 3B). Thus, the effect of α -tocopherol against the invasion might not be mediated by antioxidative function under the present experimental conditions.

ROS-induced oxidative stress may be implicated in adhesion, migration and invasion of tumor cells, and also intracellular ROS play critical roles in mediating cross talk of PKC with integrin.⁽²⁸⁾ Multiple cysteine residues within PKC can be oxidatively activated by ROS.⁽²⁸⁾ As activation of PKC by ROS was thought to be involved in ROS-stimulated invasion, we examined the contribution of PKC in invasion of AH109A cells using PKC inhibitor and PKC activator. GF, an ATP competitive inhibitor of PKC, decreased the invasive activity of AH109A cells at the concentration (1 μM) where GF exerted no influence on the proliferation (Fig. 4 A and B). PMA, a PKC activator like corresponding action to diacylglycerol (DAG), increased the invasion of AH109A cells (Fig. 4D), although it weakly reduced the proliferation (Fig. 4C). Thus, PKC is confirmed to be important in the AH109A invasion.

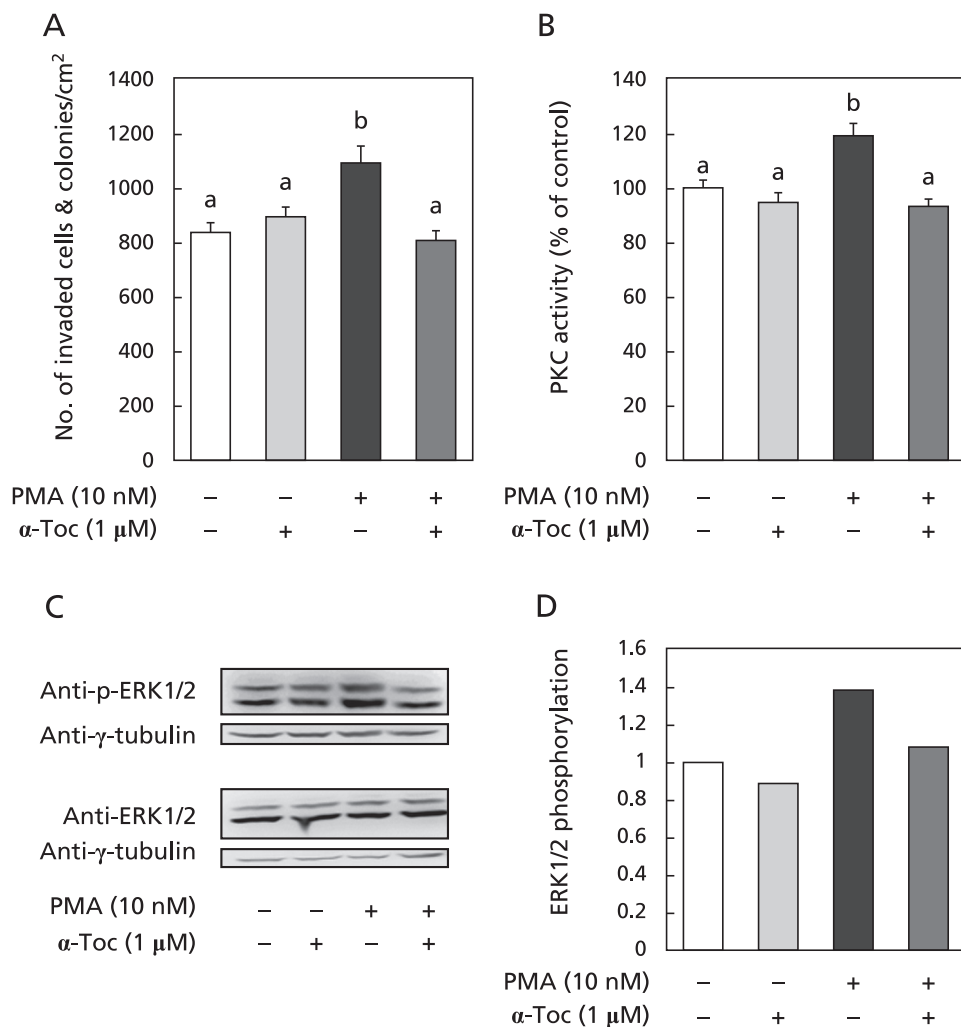


Fig. 5. Effect of α -tocopherol on PMA-induced invasion, PKC activity and ERK phosphorylation of AH109A cells. α -Tocopherol and PMA were dissolved in ethanol and DMSO, respectively. The α -tocopherol and PMA solutions were added to culture medium at a final concentration of 0.1% ethanol and DMSO. (A) Effect of α -tocopherol on the PMA-induced invasion of AH109A cells. AH109A cells were cultured for 4 h in the presence or absence of 1 μ M of α -tocopherol and/or PMA (10 nM). After treatment, the cells were washed and applied onto M-cell monolayers in 10% CS/MEM without α -tocopherol and PMA. The invasive activity was determined by invasion assay 24 h later. Each value represents the mean \pm SEM of ten areas. Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey-Kramer multiple comparisons test. (B) Effect of α -tocopherol on the PKC activity stimulated by PMA. AH109A cells were pre-cultured with α -tocopherol (1 μ M) for 10 min and thereafter exposed to PMA (10 nM) for 3 min. After treated with α -tocopherol and PMA, PKC activity in the cells was determined by ELISA. Each value represents the mean \pm SEM of three samples. Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey-Kramer multiple comparisons test. (C) Effect of α -tocopherol on the PMA-induced phosphorylation of ERK. AH109A cells were treated with PMA (10 nM) and/or α -tocopherol (1 μ M) for 10 min. Western blot analyses were performed using antibodies to phospho-ERK1/2 (anti-p-ERK1/2), ERK1/2 (anti-ERK1/2) and γ -tubulin (anti- γ -tubulin). γ -Tubulin was used as internal control. A representative result is shown. (D) Relative amount of ERK1/2 phosphorylation (p-ERK1/2/ERK1/2) was calculated from the result of Fig. 5C after each band was divided by each anti- γ -tubulin band. Toc indicates tocopherol.

In addition, cPKC and nPKC including DAG- and PMA-binding domain would play an important mediation role in the invasion process. Using the invasion assay with PMA, α -tocopherol (1 μ M) was found to suppress the PMA-induced invasive activity of AH109A cells (Fig. 5A). From these results, the inhibitory effect of α -tocopherols on the ROS-induced invasion of AH109A cells was suggested to be attributable to the inhibition of PKC activity. Activated PKC is reported to translocate from cytoplasm to membrane by PMA,⁽²⁹⁾ suggesting that activated PKC is most attractive target of α -tocopherol.

We therefore tried to directly measure PKC activity. From an experiment on time-dependency of PKC activation by PMA (data not shown), we decided to expose cells to PMA for 3 min. As shown in Fig. 5B, α -tocopherol inhibited PMA-stimulated PKC activity. Furthermore, the rise in phosphorylation of ERK1/2 in

AH109A cells treated with PMA was downregulated by α -tocopherol (Fig. 5 C and D). PMA and growth factor activate the PKC-Raf-MEK-ERK signal pathway.⁽³⁰⁾ Our present results are in consistent with the previous findings.

In addition, downstream effectors of PKC activations on the invasion of tumor cells resulted in increasing hepatocyte growth factor (HGF) secretion.⁽³¹⁾ We have already reported that the HGF-c-Met system is strongly implicated in the motility and invasion of AH109A cells.⁽⁸⁾ Treatment of AH109A cells with ROS seems to accelerate the secretion of HGF and invasive activity in an autocrine fashion.⁽⁸⁾ Thus, ROS-PKC-HGF-c-Met system is thought to be involved in the invasive activity of AH109A cells. In the present study, α -tocopherol was demonstrated to suppress ROS- and hence activated PKC-induced invasion of AH109A cells. These results suggest that the suppressive action of α -

tocopherol on the ROS-induced invasion is due to its inhibitory property against PKC activity rather than its ROS-scavenging property. As β -, γ - and δ -tocopherols at 1 μ M also canceled the PMA-induced elevation in the invasive activity (data not shown), effects of β -, γ - and δ -tocopherols on the rise in the ROS-induced invasion, the PMA-induced PKC activity and ERK1/2 phosphorylation remain to be elucidated.

In summary, α -, β -, γ - and δ -tocopherols showed more notable inhibitory effect on the invasion of AH109A cells than that on the proliferation. α -Tocopherol (1) showed the strongest anti-invasive activity among four tocopherols at a low concentration of 1 μ M, (2) suppressed the rise in ROS-induced invasion of AH109A without scavenging intracellular peroxides, (3) suppressed the PMA-induced rise in the invasive activity of AH109A, (4) suppressed the rise in the PMA-induced PKC activation in AH109A and (5) suppressed the rise in the PMA-induced ERK1/2 phosphorylation of PKC downstream in AH109A cells.

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Abbreviations

CS	calf serum
DCFH-DA	2',7'-dichlorofluorescein diacetate
DG	diacylglycerol
DMSO	dimethyl sulfoxide
DTT	1,4-dithiothreitol
ERK	extracellular signal-regulated kinase
GF	GF 109203X
HGF	hepatocyte growth factor
HX	hypoxanthine
MAPK	mitogen activated protein kinase
MEM	Eagle's minimum essential medium
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethanesulphonyl fluoride
ROS	reactive oxygen species
Toc	tocopherol
XO	xanthine oxidase

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