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Biphasic Effects of Kaempferol on the Estrogenicity in Human Breast Cancer Cells

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Dietary flavonoids have attracted a great deal of attention as agents for preventing estrogen-related diseases, such as postmenopausal symptoms, and for reducing the risk of estrogen-dependent cancer. Kaempferol is one of the most commonly found dietary phytoestrogen. The aim of this study was to investigate the estrogenic and/or antiestrogenic effect of kaempferol, which can confirm its potency as a preventive agent against estrogen-related diseases. Kaempferol has both estrogenic and antiestrogenic activity, which are biphasic response on estrogen receptor. The estrogenic activity of kaempferol induced *via* ER-mediated pathway depending on E_2 concentration ($\leq 10^{-12}$ M). Kaempferol (10^{-5} M) also caused antiproliferative effect on MCF-7 cell in the presence of E_2 (10^{-11} M) and restored to the addition of excess E_2 (10^{-7} M), which confirms that antiproliferation of kaempferol was induced via ER-dependent pathway. However, at 10^{-4} M, concentration higher than the concentrations at which the estrogenic effects of kaempferol are detected (10^{-5} M), kaempferol induced strong antiproliferative effect, but were unaffected by the addition of excess E_2 (10^{-7} M) indicating that kaempferol exerts antiproliferation via ER-independent pathway. In particular, kaempferol blocked the focus formation induced by E_2, which confirms that kaempferol might inhibit the malignant transformation caused by estrogens. Therefore, we suggested that kaempferol might regulate a suitable level of estrogenic activity in the body and is expected to have potential beneficial effects in preventing estrogen imbalance diseases (breast cancer, osteoporosis, cardiovascular disease and etc.).

Key words: Kaempferol, (Anti-)estrogenic activity, Chemopreventive effect

INTRODUCTION

The important biological roles of estrogen are reproduction and various physiological processes in a number of tissues, including the liver, bone, brain, blood vessels, adipose tissue etc. (Ciocca and Roig, 1995). Excess estrogen can cause cancer in various tissues such as the breast, ovaries, prostate, and colon (Henderson *et al.*, 1988; Yager and Liehr, 1996), and a deficiency can cause cardiovascular disease, menopausal symptoms and osteoporosis (Brzexinski and Debi, 1999; This *et al.*, 2001). Therefore, regulating the estrogen level is important for preventing estrogen-related disease.

Phytoestrogens are a group of polyphenolic chemicals from plants, and have attracted a great deal of attention as agents for preventing the abnormal effects caused by an estrogen imbalance. Dietary flavonoids are a group of natural phytoestrogens that are present in plants as glycoside conjugates. Recent epidemiological studies suggest that diets rich in flavonoids, particularly soy and unrefined grain products, may be associated with a low risk of breast and prostate cancer. It has also been suggested that dietary phytoestrogens can play a role in preventing other estrogen-related conditions, namely cardiovascular disease, menopausal symptoms and postmenopausal osteoporosis (Strauss *et al.*, 1998).

Kaempferol (Fig. 1) is one of the most commonly found dietary flavonols, which is a major subclass of flavonoid (De Vries *et al.*, 1998). The action of kaempferol was suggested to be mediated *via* an interaction with the estrogen receptors (Sathyamoorthy *et al.*, 1994; Wang and Kurzer, 1997; Oh and Chung, 2004) and the arylhydrocarbon receptor (Ciolino *et al.*, 1999). Wang and Kurzer

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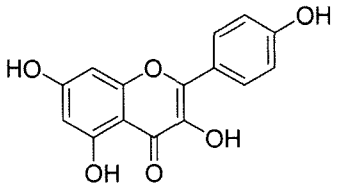

Fig. 1. Chemical structure of kaempferol

(1997) reported that kaempferol could function as either an estrogen agonist (low concentration; 1-10 μM) or a growth inhibitor (high concentration; 20-90 μM) depending on the concentrations used. It was reported that kaempferol (≤ 2.5 μM) did not exhibit antagonistic activity in the presence of 10^{-9} M E_2, but only showed agonist activity (Leung et al., 2004). In addition, Hung (2004) reported that kaempferol (17.5-70 μM) impaired the function of the ER-α and blocked estradiol-induced cell proliferation in ER-positive breast cancer cells. Therefore, it is possible that the estrogenic and antiestrogenic effects of kaempferol depend on estrogen and estrogen receptors. Accordingly, this study investigated the estrogenic and/or antiestrogenic activity of kaempferol in the presence of various concentration of the estradiol and estrogen receptor. The estrogenic/antiestrogenic activity of kaempferol was examined using a cell proliferation assay against the human breast cancer cell line, ER-positive MCF-7, in the presence (10^{-13}-10^{-7} M) or absence of E_2. In addition, the expression of the estrogen responsive-pS2 gene and a 2ERE (Estrogen Response Element)-luciferase reporter gene assay was examined. Finally, a MCF-7 focus assay was carried out in order to investigate the *in vitro* antitumor effect of kaempferol on breast cancer because the formation of the MCF-7 focus is an *in vitro* characteristic of a malignant transformation stimulated by E_2.

MATERIALS AND METHODS

Materials

17β-estradiol (E_2), tamoxifen (TM), and kaempferol (K) were purchased from the Sigma Chemical Co. The Dulbeccos Modified Eagles Medium (DMEM) was obtained from GIBCO BRL. The fetal bovine serum (FBS) was purchased from Hyclone. The estradiol and TM was stored as a 1 mM stock solution. The kaempferol solution was prepared in 100% DMSO as 100 mM. All chemical solutions were stored at -20°C until used.

Cell culture

MCF-7, human breast cancer, estrogen sensitive cells were kindly provided by Dr. Soto (Tufts University, Boston, USA). The cells were grown in Dulbeccos modified Eagle's medium (DMEM) supplemented with 5% Fetal bovine serum (FBS), penicillin and streptomycin in a humidified incubator at 37°C and 5% CO_2/95% air.

CDFBS preparation

The sex steroids were removed from the serum by treating the FBS with 5% charcoal-0.5% dextran (Olea *et al.*, 1996). Charcoal (acid washed, Sigma-Aldrich Co., MO, U.S.A.) was activated with cold sterile water immediately before use. A suspension of 5% charcoal with 0.5% dextran T70 (Pharmacia Biotech AB, Sweden) was prepared and centrifuged at 1,000×g for 10 min. The supernatants were aspirated, and a charcoal pellet was mixed with FBS and maintained in suspension by rolling at 6 cycles/min at 37 °C for 1 h. The suspension was centrifuged at 2,000×g for 50 min, and the supernatant was filtered through a 0.45 μm and 0.2 μm bottle top filter. The charcoal dextran-treated FBS (CDFBS) was stored at -20°C until needed.

Cell proliferation assay (E-SCREEN ASSAY)

The E-screen assay was based on the estrogen-receptor-binding induced proliferation of MCF-7 cells, a human breast cancer cell line. In the E-screen assay, maximum cell proliferative effect was observed with 10^{-10} M E_2. The proliferative effect of compounds relative to E_2 (10^{-10} M, 100%) is represented as RPE (Relative Proliferative Effect). The cell proliferation assay in the MCF-7 cells was carried out according to the methodology reported by Perez *et al.* (1998). The cells were harvested with 0.05% trypsin-0.53 mM EDTA·4Na and resuspended in 5% FBS DMEM. The cells were then seeded into 48-well plates at initial concentrations of 5,000 cells/well, and allowed to attach for 48 h. The seeding medium (5% FBS in DMEM) was replaced with the experimental medium (10% CDFBS supplemented with phenol red-free DMEM). MCF-7 cells were exposed to kaempferol (from 10^{-7} M to 10^{-4} M) in the presence (from 10^{-13} M to 10^{-9} M) or absence of E_2. The final concentration of the vehicle (dimethylsulfoxide, DMSO) in the medium never exceeded 0.1%. The assay was terminated after either 144 h by removing the media from the well, and SRB (Sulforhodamine B) assay was then carried out. The cell proliferative response is defined as an increase in the frequency of proliferating MCF-7 cells. The cell proliferation data (RPE, Relative Proliferation Effect) of the cells was calculated according to the following equation: RPE = [(S-1)/(E-1)]×100, Where S = the proliferation of the samples and E = the proliferation of the positive control (10^{-10} M, E_2).

Reporter gene assay

In reporter gene assay, the MCF-7 cell line was transfected with a 2ERE-luciferase reporter construct. This allows the expression of the firefly luciferase enzyme under the control of the estrogen regulatory element of the

Xenopus vitellogenin A2 gene in front of a SV40 promoter. The MCF-7 cells were seeded into 48-well plates at a density of 10^5 cells per well. The steroid level was depleted in the cells by incubating the cells with phenol red-free DMEM supplemented with 3% CDFBS and maintaining them in a 5% CO_2 incubator at 37°C for 24 h. After 24 h incubation, the cells were washed once with FBS-free OPTI-MEM I, and 0.5 mL FBS-free OPTI-MEM I medium was added to each well. 10 μg of pERE2-Luc and 20 μg of LipofectAMINE were mixed gently in 1 mL of FBS-free OPTI-MEM I medium and incubated at room temperature for 30 minutes prior to adding it to the MCF-7 cells. 0.2 mL of the resulting plasmid/LipofectAMINE solution to each well and incubated in 5% CO_2 incubator at 37°C for 15 h. The cells were washed with serum free DMEM-medium and 500 μL DMEM supplemented with 5% CDFBS was then added. The washed cells were treated with 0.1% (v/v) DMSO (control), 10^{-11} M 17β-E_2, kaempferol alone or in combination for 24 hr. In addition, tamoxifen (TM, 10^{-6} M) was added together with kaempferol at the estrogen response concentration in order to determine the estrogen receptor-mediated response of kaempferol. The final concentration of the vehicle in the assay medium was adjusted to 0.1%. After incubating the cells for 24 h, they were lysed with 100 μL of the reporter lysis buffer (Promega Biosciences, CA, U.S.A.). The luciferase activity in the cell lysate was assayed using a Luciferase Assay System kit (Promega Biosciences, CA, U.S.A.) according to the manufacturer's protocol, and the results were normalized to the protein concentration measured using the Bradford method.

pS2 mRNA expression assay (RT-PCR)

The MCF-7 cells were seeded into 6-well plates at a density of 5×10^5 cells per well. The steroid level in cells was depleted by incubating them with phenol red-free DMEM supplemented with 3% CDFBS and maintaining them in 5% CO_2 in air at 37°C for 24 h prior to use. After 24 h, the test compounds (E_2 and kaempferol) were added to the medium during 48 h. In order to determine the antiestrogenic activity, various concentrations of kaempferol were added together with 10^{-10} M 17β-E_2. Subsequently, the medium was removed and the cells were scraped from the dish to examine the amounts of pS2 mRNA. The total RNA was purified with TRIzol reagent (GIBCO BRL, NY, U.S.A.) according to the manufacturer protocol. RNA was stored at -80°C. The primers of human pS2 and α-actin used are as follows: (Knowlden *et al.*, 1997).

α-actin-up: 5'-GGAGCAATGATCTTGATCTT-3'
α-actin-down: 5'-CCTTCCTGGGCATGGAGTCCT-3'
pS2-up: 5'-CATGGAGAACAAGGTGATCTG-3'
pS2-down: 5'-CAGAAGCGTGTCTGAGGTGTC-3'

The PCR product was 204 bp for α-actin and 336 bp for pS2. Amplification was performed in a PCR thermocycler (MJ Research, Inc, MA, U.S.A.). According to the protocol of the Promega Access RT-PCR System (Promega A 1250), the thermal cycle profile of the first strand cDNA synthesis was 1 cycle at 48°C for 45 min, 94°C for 2 min. The thermal cycle profile of the second strand cDNA synthesis and PCR amplification for pS2 and α-actin was 25 cycles at 94°C for 30 seconds, 55°C for 1 min, 72°C for 2 min. The mRNA products were amplified with RT-PCR, and run in 1.5% agarose gel electrophoresis in a TBE buffer with ethidium bromide. The PCR products were detected and analyzed using a Gel documentation & analysis system (UVP, UK).

Focus formation

The MCF-7 focus assay, in which human breast cancer cells respond to E_2 by producing multicellular nodules or foci on a confluent monolayer background, was carried out as previously described (Gierthy *et al.*, 1991; Arcaro *et al.*, 1998). The cells were harvested with 0.05% trypsin-0.53 mM EDTA·4Na (Gibco), resuspended in 5% FBS DMEM, and plated onto 24-well plates. The cell density was 10^5 cells per well and the culture was maintained at 37°C in 5% CO_2 for 24 h. The steroid level in cells was depleted by incubating the cells with phenol red-free 3% CDFBS-DMEM for 3 days. The culture media was then changed with phenol red free 10% CDFBS-DMEM and treated with E_2 or kaempferol. The antiestrogenic activity of kaempferol was measured after treating the kaempferol was treated together with E_2. The final concentration of the vehicle (DMSO) in the medium never exceeded 0.1%. The cells were refed every 2 or 3 days with 2 mL of 10% CDFBS containing various concentrations of the test compounds. The cytotoxic effects were indicated by the changes in cell morphology, e.g. pycnosis, lysis or detachment. The cytostatic effects were indicated by a delay in reaching confluence compared with the control wells. After 14 days, the cells were washed with PBS, fixed with methanol, stained with Gimsa and photographed.

Data analysis

The data was analyzed using Sigma Plot software (Jandel Science Software, San Rafael, CA, U.S.A.) and Excel (Microsoft, NY, U.S.A.) with a Student's *t*-test for comparisons. A P-value < 0.05 was considered significant. Statistical differences between the groups were determined using the data expressed as a mean±standard deviation. At least three determinations were carried out for each data point.

RESULTS

ERE-Luciferase activity of kaempferol in the absence of E_2

A reporter gene assay, which was regulated by the 13 base pair vitellogenin A2 estrogen response element (ERE), was carried out to determine if kaempferol induces the estrogen agonistic activity in the absence of E_2. This reporter gene assay ensures that the induction or inhibition of the reporter gene (luciferase) occurs only through the ERE. As shown in Fig. 2A, kaempferol activated the transcription of the E_2-responsive reporter gene transfected into the MCF-7 in a dose-response manner. At 10^{-5} M, kaempferol showed the maximal effect (RLU = 15.25±1.18), and the effect of kaempferol (10^{-5} M) was blocked entirely by the addition of TM (10^{-6} M) (Fig. 2B). However, high concentrations (> 10^{-5} M) of kaempferol decreased the luciferase activity (Fig. 2A).

ERE-luciferase activity and pS2 gene expression of kaempferol in the presence of E_2

In order to investigate the estrogen antagonist properties of kaempferol, this study examined the effects of kaempferol on ERE-luciferase activity and pS2 gene expression in the presence of E_2. When the cells were treated with a combination of E_2 (10^{-11} M) and kaempferol in the presence of 5% phenol red/serum-free CDFBS DMEM, the luciferase activity, compared with the single treatment of 10^{-11} M E_2, was completely inhibited by kaempferol in a dose-response manner (Fig. 3A). At all the kaempferol concentrations tested, no cytotoxicity was observed in this cell line for incubation time of 24 h. As shown in Fig. 3B, the cells co-treated with an E_2 (10^{-10} M) and kaempferol (10^{-4} M) were significantly inhibited the pS2 mRNA induced by E_2 (10^{-10} M). This inhibition was similar to that of tamoxifen (Fig. 3B). However, the other kaempferol concentration did not show any inhibitory effect on pS2 gene expression (Data not shown).

Cell proliferation activity of kaempferol in the presence of E_2

In order to determine if kaempferol induces antiproliferation in the presence of E_2, the MCF-7 cells were treated with phenol red free 10% CDFBS medium supplemented with various concentrations of E_2 (10^{-13} M-10^{-9} M) and kaempferol (10^{-5} M or 10^{-4} M). As shown in Fig. 4A, a 10^{-5} M kaempferol, which showed the maximum estrogenic activity (Fig. 2A), induced cell proliferation compared with the unexposed control in the presence of low concentration of E_2 (10^{-13} M and 10^{-12} M), which did not induce cell proliferation in the MCF-7 cells. The cell proliferation of kaempferol at low concentrations of E_2 was similar to that (RPE = 74.40±1.27%, Oh and Chung, 2004) observed with kaempferol (10^{-5} M) in the absence of E_2. As shown in Fig. 4B, in the presence of 10^{-11} M E_2, which is generally used to investigate the antiestrogenic activity, kaempferol completely inhibited the cell proliferation caused by E_2 in a dose-dependent manner. However, the combination of high concentrations of E_2 (≥10^{-10} M) and kaempferol (10^{-5} M) did not inhibit the cell proliferation caused by E_2. In order to determine if the anti-proliferative effects of kaempferol was induced by competition with E_2 for binding to the ER, the MCF-7 cells were treated with kaempferol in the presence of excess E_2 (10^{-7} M). As shown in Fig. 4C,

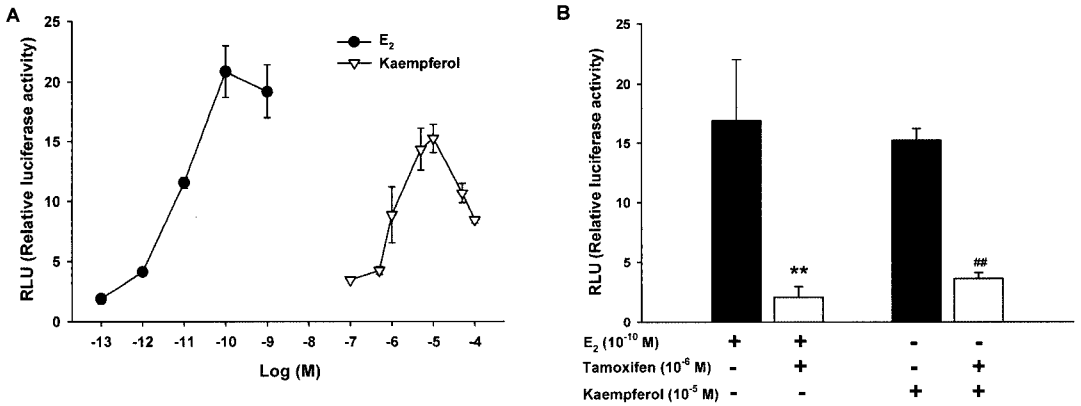

Fig. 2. Effects of kaempferol (A) and combination of kaempferol and tamoxifen (B) on luciferase activity in MCF-7 cells using reporter gene assay. The cells were plated in a 48-well plate at a cell density of 10,000 cells/well and allowed to attach for 24 h. The steroid levels in the cells were depleted by incubating the cells with phenol red-free 3% CDFBS-DMEM for 24 h. After 24 h incubations, the cells were transfected with the pERE2-luc construct using lipofectamine. The transfected cells were incubated in DMEM supplemented with 10% CDFBS with kaempferol (A) or a combination of kaempferol and tamoxifen (B) for 24 h. Incubation with DMSO alone was performed as a control and its final concentration in the medium never exceeded 0.1%. After the cells were incubated for 24 h, the total luciferase activity was measured using a Luciferase assay Reagent (Promega Biosciences CA, U.S.A.). The results are expressed as a mean ± standard deviation of three separate experiments for each data point. The comparisons were made using a Student's *t*-tests. Values are significantly different from 10^{-11} M E_2 (**p<0.01) or 10^{-5} M kaempferol (##p<0.01).

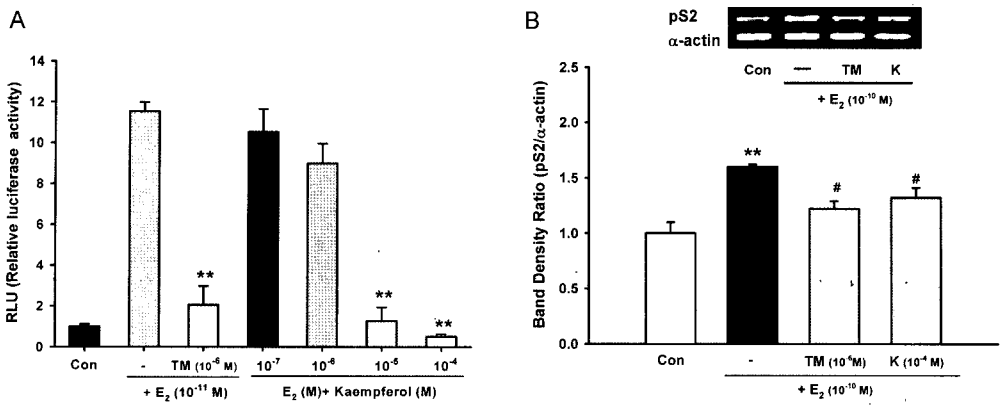

Fig. 3. Effects of kaempferol on luciferase activity (A) and pS2 gene expression (B) in MCF-7 cells. (A) The steroid-deprived MCF-7 cells were transiently transfected with the pERE-luc construct using lipofectamine. This cell was incubated in DMEM supplemented with CDFBS 10% with a combination of E_2 and kaempferol for 48 h. The total luciferase activity was measured using a Luciferase assay Reagent (Promega). The results are expressed as the mean ± standard deviation of three separate experiments for each data point. Comparisons were made using a Student's *t*-tests. Values are significantly different from E_2 (10^{-11} M) [**p<0.01]. (B) MCF-7 cells were incubated in DMEM supplemented with the steroid free CDFBS 10% with a combination of E_2 (10^{-10} M) and Kaempferol (K, 10^{-4} M) or Tamoxifen (T, 10^{-6} M) for 48 h. After the cells were incubated for 48 hr, the total RNA was extracted using a TRIzol reagent (Gibco BRL). The pS2 mRNA levels were measured by RT-PCR and normalized using α-actin mRNA as the internal standard. 1 μg of the total RNA was applied to the RT-PCR reaction for 25 cycles with the pS2 (336 bp) and a-actin (240 bp) primers. The RT-PCR products were run on an ethidium bromide stained 1.5% agarose gel, which was then scanned using a Gel documentation & Analysis system. Values are significantly different from the control (**p<0.01) or 10^{-10} M E_2 (#p<0.05). K: Kaempferol (10^{-4} M); TM: Tamoxifen (10^{-6} M).

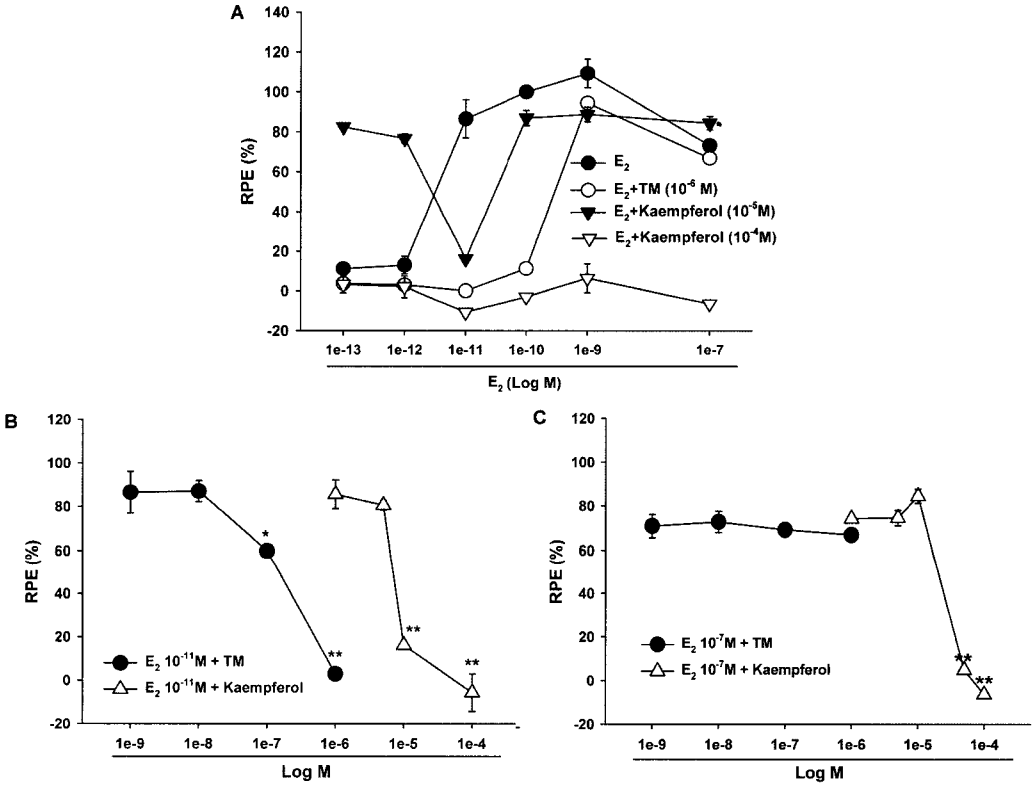

Fig. 4. Effects of kaempferol on estrogen-induced response in MCF-7 cells using E-screen assay. The cells were plated in 48-well plate at a cell density 5,000 cells/well and allowed to attach for 48 h. The cells were then incubated in DMEM supplemented with 10% CDFBS with E_2 or a combination of E_2 and kaempferol for 144 h. Incubation with DMSO alone was performed as a control and its final concentration in the medium never exceeded 0.1%. After the cells were incubated for 144 h, a SRB assay was conducted to measure the level of cell proliferation. The proliferative effect of compounds relative to E_2 (10^{-10} M, 100%) is represented as the RPE (Relative Proliferative Effect). The results are expressed as a mean±standard deviation of three separate experiments for each data point. Comparisons were made using a Student's *t*-tests. Values are significantly different from 10^{-11} M E_2 (B: *p<0.05, **p<0.01) or 10^{-7} M E_2 (C: **p<0.01).

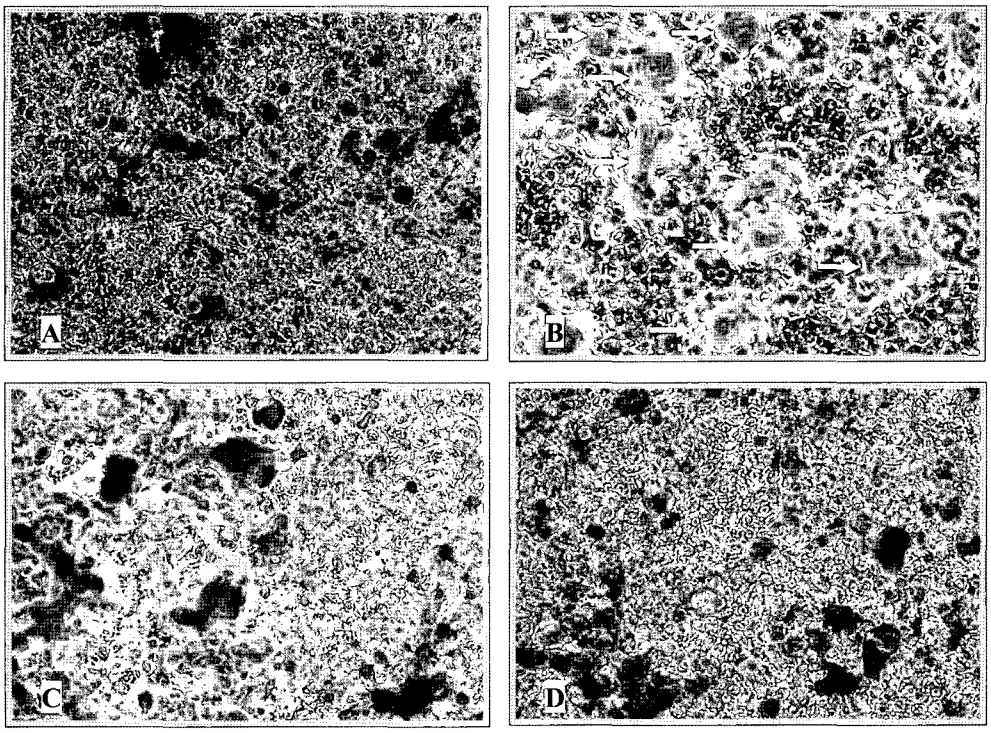

Fig. 5. Inhibitory effects of kaempferol on estrogen-dependent focus formation in MCF-7 cell cultures. The cells were plated in 24-well plate at a cell density 50,000 cells/well and allowed to attach for 24 h. The steroid levels in the cells were depleted by incubating the cells with phenol red-free 3% CDFBS-DMEM for 24 h. 24 h after seeding, the cells were incubated in phenol red-free 3% CDFBS-DMEM with combination of 10^{-9} M E_2 and kaempferol for 14 days. The cells were refed every 2 or 3 days with 2 mL of 3% CDFBS containing various concentrations of the test compounds. Incubation with DMSO alone was performed as a control and its final concentration in the medium never exceeded 0.1%. After the cells were incubated for 14 days, they were stained as described in Methods. Multi-cellular foci are evident (arrows) against a monolayer background (Original magnificent ×100). A, 0.1% DMSO; B, 10^{-9} M E_2; C, 10^{-9} M E_2+10^{-6} M TM; D, 10^{-9} M E_2+10^{-5} M Kaempferol.

when the MCF-7 cells were treated with a combination of E_2 (10^{-7} M) and TM (Tamoxifen), a weak estrogen, the level of cell proliferation was restored to almost 100% of that observed with E_2 (10^{-7} M). The cells treated with 10^{-5} M of kaempferol, which showed estrogenic activity, were restored to antiproliferation by the addition of excess E_2, indicating that the antiproliferation caused by kaempferol (10^{-5} M) occurs *via* an ER-dependent mechanism (Fig. 4A and 4C). However, high concentrations of kaempferol (>10^{-5} M) showed cytotoxicity did not restored to antiproliferation by the addition of excess E_2 (Fig. 4C), which suggests that the antiproliferation caused by high kaempferol concentrations occurred *via* an ER-independent mechanism.

Effect of kaempferol on the focus formation

The focus assay has been used to investigate the formation of a malignancy (Gierthy *et al.*, 1991) and this study examined the inhibitory effect of kaempferol on a malignant formation. In the presence of E_2 (10^{-9} M), kaempferol (10^{-5} M) completely inhibited the formation of cell foci induced by E_2 (Fig. 5). Because a 10^{-5} M kaempferol did not induce any cytotoxic effect in the serum-free CDFBS medium, we suggested that the observed activity of kaempferol was not due to a general decrease in growth rate or cytotoxicity. Therefore, this data suggested that kaempferol could inhibit the malignant transformation caused by the estrogen response. However, a 10^{-4} M kaempferol sharply decreased the cell density as a result of cell detachment compared with the control wells. So, the focus assay of a 10^{-4} M kaempferol could not be carried out for 14 days (Data not shown).

DISCUSSION

Dietary flavonoids have attracted a great deal of attention as agents for preventing estrogen-related diseases, such as postmenopausal symptoms, and for reducing the risk of estrogen-dependent cancer (Strauss *et al.*, 1998). Kaempferol is one of the most commonly found dietary phytoestrogen. Therefore, this study investigated the potency of kaempferol for the estrogenic and/or antiestrogenic effect in absence or presence of the estrogens and estrogen receptors, which can confirm the capacity as

preventive agents of the induced by estrogen imbalances.

The phytoestrogens such as genistein, biochanin A and resveratrol was reported to have a biphasic estrogenic activity depending on their concentration (Wang *et al.*, 1996; Hsu *et al.*, 1999; Basly *et al.*, 2000). A kaempferol also exerted a biphasic effect, stimulating luciferase activity at concentrations $\leq 10^{-5}$ M and decreasing luciferase activity at concentration $>10^{-5}$ M in the absence of estrogen. The luciferase activity of kaempferol was blocked by tamoxifen, which functions as an antiestrogen via competitive inhibition of the binding of E_2 to the ER (Cormier and Jordan, 1989; Macgregor and Jordan, 1998). Therefore, the results indicated that kaempferol ($\leq 10^{-5}$ M) could induce luciferase activity via the classical ER pathways. It was previously reported that kaempferol has the binding affinity for the ERs, α and β (Oh and Chung, 2004) and induced significantly MCF-7 cell proliferation and pS2 gene expression in a dose-dependent manner (Oh and Chung, 2004; Sathyamoorthy *et al.*, 1994). These studies indicated that kaempferol has an estrogen-like response in the absence of E_2 and the results of the present study appeared to be consistent with these findings.

The estrogenic activity of kaempferol (10^{-5} M) was induced at the presence of low E_2 (10^{-13} M and 10^{-12} M), which has little or no estrogenic activity. In addition, the same concentration of kaempferol (10^{-5} M) completely inhibited the cell proliferation induced by E_2 (10^{-11} M), which is generally used to investigate the antiestrogenic activity. The antiestrogenic effects of kaempferol (10^{-5} M) were also investigated in the luciferase reporter gene assay and pS2 gene expression assay. However, kaempferol (10^{-5} M) showed strong estrogenic activity rather than antiestrogenic activity by the addition to high or excess E_2 ($\geq 10^{-10}$ M). Le Bail *et al.* (1998) reported that kaempferol has additive estrogenic activities in the presence of 10^{-10} M E_2. It was reported that genistein reversed antiproliferation with the addition of excess (10^{-7} M), competing estrogen (So *et al.*, 1997). A weak estrogen receptor agonist, such as TM, did not inhibit the cell proliferation caused by excess estradiol (So *et al.*, 1997). Therefore, we suggested 10^{-5} M kaempferol can induce estrogenic and/or antiestrogenic activity depending on E_2 concentration and this effect was able to induce via classic ER pathway that competes with E_2 for binding to the estrogen receptor.

On the other hand, So *et al.* (1997) was reported that baicalein, galangin, hesperetin, naringenin and quercetin were unaffected by the addition of excess E_2 (10^{-7} M), so these flavonoids evidently exert their antiproliferative activity by ER-independent mechanism. In this study, a high concentration (10^{-4} M) of kaempferol induced antiproliferative activity did not restore by the addition of excess E_2 (10^{-7} M). In addition, in our previous studies, we confirmed that the high concentration (10^{-4} M) of kaempferol can induce cytotoxic effects in both MDA-MB-231 and MCF-7 cells in the serum medium at the incubation time of 72 h (Oh and Chung, 2004). Flavonoids generally induce antiproliferation or cytotoxicity at concentrations much higher than the concentrations at which the estrogenic effects are detected and the effects are observed in cells in the absence of ER expression, indicating that all of these effects are not ER mediated (Kurzer and Xu, 1997; Wang *et al.*, 1996; Peterson and Barnes, 1991). These ER-independent mechanisms of flavonoids, including the inhibition of protein tyrosine kinases, the suppression of angiogenesis, and the direct antioxidant effects, were proposed previously (Kurzer and Xu, 1997; Fotsis *et al.*, 1997). Furthermore, soy isoflavones can induce apoptosis in human breast cancer cells, specially ER-negative cell lines, which confirms that apoptosis occurs in a hormone-independent manner (Fioravanti *et al.*, 1998; Leung and Wang, 2000; Po *et al.*, 2002; Pozo-Guisado *et al.*, 2002; Upadhyay *et al.*, 2001; Nomoto *et al.*, 2002). Therefore, we suggested that the antiproliferative effect of a 10^{-4} M kaempferol might not have been induced by the competitive inhibition of E_2 binding to the ER, but were possibly induced by ER-independent cytotoxicity. However, the same concentration (10^{-4} M) of kaempferol inhibited the estrogen-responsive pS2 gene expression and the luciferase activity induced by E_2 without showing any cytotoxicity over a 48 h incubation period in the absence of serum. The expression of the pS2 gene product was directly induced by estrogen at the transcriptional level and has been used as an ideal model for studying the mechanism of estrogenic and antiestrogenic activity (Masiakowski *et al.*, 1982; Brown *et al.*, 1984). In addition, in the transactivation assay using pGL3-ERE (estrogen response element)-promoter construct, the induction of the luciferase reporter gene was stimulate only *via* the ERE. Therefore, these results suggested that the antiproliferation induced by high concentration of kaempferol can also occur *via* the ER-dependent pathways.

Many mechanisms of anticancer effect have been identified for phytoestrogens prevention of cancer, including estrogenic/antiestrogenic activity, antiproliferation, induction of cell cycle arrest and apoptosis, induction of detoxification enzymes, regulation of the host immune system, changes in cellular signaling, inhibit angiogenesis, and aromatase enzyme inhibition, stimulation of sex hormone binding globulin (SHBG) synthesis, and antioxidant properties (Birt *et al.*, 2001). The MCF-7 foci formation was stimulated by E_2 and suppressed by tamoxifen (TM). It was reported that estrogen-dependent focus development may represent the basic characteristics of an estrogenic response i.e. the induction of concerted gene expression via an ER-mediated process, which

results in tissue restructuring through the enhanced postconfluent cell proliferation (Girthy *et al.*, 1991). In this study, kaempferol (10^{-5} M) significantly inhibited the formation of cell foci induced by E_2 (10^{-9} M). Because the observed effect of kaempferol was not induced by a general decrease in the growth rate or cytotoxicity, which confirms that kaempferol can inhibit the malignant transformation caused by estrogens. Recently, a lot of epidemiological studies have demonstrated that high consumption of phytoestrogen, which has similar responses to that induced by kaempferol, may be associated with a low incidence of breast cancer, although this could not be completely confirmed (Ingram et al., 1997; Messina et al., 1997; Strauss *et al.*, 1998; Peeters *et al.*, 2003). Therefore, we suggested that kaempferol can be considered as a candidate of chemotherapeutic agents on estrogen-dependent cancer.

In conclusion, we confirmed that kaempferol has a biphasic response on the estrogen activity depending on concentration of E_2 or kaempferol via ER-dependent and/or independent pathway. In particular, kaempferol blocked the focus formation induced by E_2, indicating that kaempferol might inhibit the malignant transformation caused by estrogens. Therefore, kaempferol might regulate a suitable level of estrogenic activity in the body and have potential beneficial effects in preventing estrogen imbalance diseases (breast cancer, osteoporosis, cardiovascular disease and etc.).

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