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Effects of Soy-isoflavonoid on Molecular Markers Related to Apoptosis in Mature and Ovariectomized Female Rats, and Mammalian Tumor Cell Lines

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Abstract Alteration of molecular markers related to apoptosis of *in vivo* normal system and *in vitro* cancerous system by soy-isoflavonoid with estrogen was investigated. Down-regulation of Bcl-2 was accompanied by decreased expression of COX-2 (cyclooxygenase-2) in mature female rats treated with soy-isoflavonoid and estrogen. In ovariectomized rat system, Bax was regulated by higher concentration of soy treatment. Bax up-regulation by soy-isoflavonoid genistein treatment was observed in MCF-7 mammary cancer cell system. Estrogen without soy induced similar pattern of Bax expression as soy-isoflavonoid *in vivo*, but exhibited opposite trend *in vitro*. These findings suggest soy-isoflavonoid may have potential to induce apoptosis at higher concentrations through up-regulation of Bax or down-regulation of Bcl-2 expressions depending on normal or cancerous state, and physiological status of rats.

Keywords: soy-isoflavonoids, molecular markers related to apoptosis, mature estrogen-supplemented rats, ovariectomized rats, MCF-7 cells

Introduction

Genistein (4,5,7-trihydroxyisoflavone), the major phyto-estrogen in soybeans and legumes, is structurally similar to the estrogens. The phytoestrogens reported in human and animal studies appeared to have estrogenic activity as well as antagonize the estrogenic effects of the endogenous estrogen (1, 2). Population studies have suggested that consumption of a phytoestrogen-rich diet could prevent degenerative diseases such as atherosclerosis and relieve some estrogen-deficiency symptoms in postmenopausal women (3-5). In addition, epidemiological evidence indicates positive associations between chemoprevention and dietary soy consumption. A cross-national study involving 50 countries identified soy products as having a highly significant protective effect against prostate cancer (6). Asian women with high soy intake have low incidences of breast cancer (7, 8); however, in second generation US immigrants, the protection by soy intake is lost following adoption of the Western-based diet (9). Furthermore, urinary levels of phytoestrogens were lower in breast cancer cases compared with case-controls (10, 11). Despite the positive association between soy product intake and risk of breast cancer in many epidemiological studies, the chemopreventive effect appeared to be determined by other complex factors such as the concentration and type of genistein (12, 13). At physiological concentrations (1 nM to 10 μM) genistein stimulates the growth of MCF-7 cells, whereas, at high concentrations, inhibits the proliferation of the cells (14). This kind of biphasic effect has been attributed to genistein exerting estrogen-like effects at lower concentrations, whereas, at higher levels, genistein acts as an estrogen-antagonist. Furthermore, the circulating/surrounding level of estrogen could play an important role in the function of genistein (15, 16). Biochemical role of genistein for understanding and recommending its use as a chemopreventive agent has not yet been clarified. One of the speculated modes of action has been suggested to be cell cycle arrest; Davis *et al.* (17) showed that induction of apoptosis by genistein is accompanied by G_2/M arrest.

The maintenance of normal tissues involves apoptosis, a regulated cell death process. Abnormal regulation of apoptosis is related to many disorders including tumor promotion, autoimmune and immunodeficiency diseases, and neurodegenerative pathologies (18). Bcl-2 family proteins play a role in regulating apoptosis (19). Overexpression of Bcl-2 enhances the survival of several cell types and prevents apoptosis induced by various chemotherapeutic drugs (20). Bax represents a pre-apoptotic member of the Bcl-2 family, which controls important checkpoints during the apoptotic process. Overexpression of Bax has been shown to accelerate cell death (21). A high ratio of Bcl-2-to-Bax protein expression is closely associated with early recurrence of a certain type of cancer (22). Li *et al.* suggested that the up-regulation of Bax may be the molecular mechanism by which genistein induces apoptosis (23). On the other hand, estrogen has been reported to possess anti-apoptotic activity in a breast cancer cell line such as MCF-7 (24).

A number of cellular and animal model experiments indicate that COX-2 may play a role in apoptosis. Overexpression of COX-2 was shown to be related to the inhibition of apoptosis, thereby inducing tumorigenesis. Studies also revealed overexpression of COX-2 in many tumors, and this up-regulation of COX-2 has been shown to promote cancer progression and recurrence (25-27).

The purpose of this study was to examine the modulation of the expressions of Bcl-2 and Bax proteins, whose gene products are known to be involved in the

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regulation of apoptosis, by soy-isoflavonoid in comparison with estrogen in mature and ovariectomized female rats, as well as in a mammalian breast tumor cell line. In addition, the interaction between apoptosis-related protein expression and COX-2 expression was examined in mature female rats fed soy-isoflavonoid.

Materials and Methods

Chemicals Genistein, 17β-estradiol, cholesterol, L-cysteine, α-cellulose, choline bitartrate, and tert-butylhydroquinone were purchased from Sigma Chemical (St. Louis, MO, USA). Soy-isoflavonoids used in animal experiments were in the form of Genistein Combined Polysaccharides (GCP; Amino Up Co., Sapporo, Japan). One gram of GCP contains 116 ± 8.4 mg genistein, 28.5 ± 5.4 mg daidzein, 13.5 ± 2.6 mg glycitein, and 3% insoluble polysaccharides from basiodiomycetes. Other reagents were of chemical grade and purchased from commercial reagent suppliers. Corn starch was supplied by Miwon Co. (Seoul, Korea), casein was a product of The New Zealand Dairy Board (Wellington, New Zealand), and soybean oil and lard were commercial brands.

Animals and feeding regimens Female Sprague-Dawley rats, forty-eight weeks old, were fed a standard laboratory diet (Cheil Feed Co., Seoul, Korea) for one week. Using a randomized complete block design, rats were divided by initial body weight into three groups of nine. Rats were housed individually in an environmentally controlled animal laboratory with a 12-h light:dark cycle. For four weeks, rats were fed one of the three diet regimens (Table 1) and water *ad libitum*. The basal diet was the high fat and high cholesterol diet to provide dietary environment more susceptible to carcinogenesis. Diets were stored at -40°C before use. To observe the effect of ovariectomy, female Sprague-Dawley rats, eight-week-old, were fed a standard laboratory diet (Cheil Feed Co.) for one week. To obtain full response of ovariectomy (i.e. post-menopausal state), rats were ovariectomized or sham-operated prior to feeding a laboratory diet for three weeks. They were housed individually in an environmentally controlled animal laboratory with a 12-h light:dark cycle. For eight weeks, rats were fed one of the six diet regimens (Table 2) and water *ad libitum*. Diets were stored at -40°C before use.

Tissue collection After the designated feeding periods, the rats were anesthetized with ethylether, and 4-8 mammary buds were collected, frozen in liquid nitrogen, and stored at -80°C before use.

Cell culture preparation The MCF-7 cells were incubated with phenol red free Dulbeccos Eagle medium containing 10 *u*g/ml insulin (bovine), 10% fetal bovine serum, 1% antibiotic-antimycotics, 3.7 g $NaHCO_3$ at 37°C in a 5% CO_2 incubator for 48 hr. Ten different plates were prepared, added with DMSO (control), and 25, 50, and 100 *u*M genistein (Sigma Co.), 12.5, 25, and 50 nM 17β-estradiol (Sigma Co.) or combinations of 25 *u*M genistein and 12.5 nM 17β-estradiol, 50 *u*M genistein and 25 nM 17β-estradiol or 100 *u*M genistein and 50 nM 17β-estradiol, and incubated for additional 48 hr before cell harvest.

Western Blotting Collected tissues or cells were lysed in ice-cold 120 ml lysis buffer [150 mM NaCl, 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.4, 20 mM EGTA, 1 mM DTT, 1 mM Na3VO4, protease inhibitor cocktail tablet (Boehringer Mannheim, Mannheim, Germany)] for 40 min. Lysates were centrifuged at 14,800 g for 30 min,

Table 1. The composition of the diet in experiment using mature female rats

Groups	
1. Control	No supplementation
2. Estrogen supplemented	17β-estradiol (600 *u*g/kg)*
3. Estrogen + Soy-isoflavonoid supplemented	17β-estradiol (200 *u*g/kg) soy-isoflavonoids (10 g/kg)

The basal diet was high fat (120 g lard/kg diet) and high cholesterol (1 g/kg diet).[1)]
[1)]High-fat and high-cholesterol diet contains corn starch 438 g; sucrose 100 g; soybean oil 41 g; lard 120g; cholesterol 1g;casein, 200; L-cysteine, 3.0; α-cellulose, 50; choline bitartrate, 2.5; tert-butylhydroquinone, 0.014; AIN 93G salt mix[2)], 35.0; AIN 93G vitamin mix[3)] 10.0 g/kg.
[2)]AIN 93G salt mix (g/kg): calcium carbonate, 357.0; potassium phosphate monobasic, 196.0; potassium citrate, 70.78; sodium chloride, 74.0; potassium sulfate, 46.6; magnesium oxide, 24.4; ferric citrate, 6.08; zinc carbonate, 1.65; manganous carbonate, 0.63; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenate, 0.01025; ammonium paramolybdate, 0.00795; chromium potassium sulfate, 0.275; sodium meta-silicate, 1.45; powdered sucrose, 221.2268
[3)]AIN 93G vitamin mix (g/kg): nicotinic acid, 3.0; calcium pantothenate, 1.6; pyridoxine hydrochloride, 0.7; thiamin hydrochloride, 0.6; riboflavin, 0.6; biotin, 0.02; folic acid, 0.2; vitamin B_{12}, 0.025; α-tocopherol acetate, 15.0; retinyl acetate, 0.8; vitamin D_3, 0.25; vitamin K_1, 0.075; powdered sucrose, 974.655
*The level of estradiol used in this study was based on the conventional concentrations adapted by researchers such as Sahlin et al. J. Steroid Biochem. Mole. Biol. 80:457-467 (2002).

Table 2. The composition of the diet in experiment using ovariectomized rats

Groups	
1. Sham-operated Control	No supplementation
2. Ovariectomized Control	No supplementation
3. Ovariectomized Estrogen	17β-estradiol (200*u*g/kg)
4. Sham-operated Low soy-isoflavonoid	soy-isoflavonoids (0.8 g/kg)
5. Ovariectomized Low soy-isoflavonoid	soy-isoflavonoids (0.8 g/kg)
6. Ovariectomized High soy-isoflavonoid	soy-isoflavonoids (4.0 g/kg)
7. Ovariectomized Genistein	genistein (0.25 g/kg)

The basal diet was high fat (120 g lard/kg diet) and high cholesterol (1 g/kg diet).[1)]
[1)]High-fat and high-cholesterol diet contains corn starch 438 g; sucrose 100 g; soybean oil 41g; lard 120g; cholesterol 1g;casein, 200; L-cysteine, 3.0; α-cellulose, 50; choline bitartrate, 2.5; tert-butylhydroquinone, 0.014; AIN 93G salt mix[2)], 35.0; AIN 93G vitamin mix[3)] 10.0 g/kg.
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and aliqouts of supernatant containing 30 mg protein were boiled in SDS sample loading buffer for 5 min before electrophoresis on 12% SDS-polyacrylamide gel. After 3 hr transfer of SDS-polyacrylamide gel to PVDF membrane (Amersham Life Sciences, Arlington Heights, IL, USA), the blots were blocked with 5% fat-free dry milk-PBST buffer [Phosphate-buffered saline (PBS) containing 0.1% Tween-20] for 2 hr at room temperature and washed in PBST buffer. The membranes were incubated for 1 hr at room temperature with 1:1000 dilution of goat Bcl-2, Bax or COX-2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 hr. Blots were rinsed with PBST, incubated with 1:5000 dilution of anti-gout-horseradish peroxidase conjugated-secondary antibody and washed again three times in PBST buffer for 5 min. The transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Life Sciences) according to the manufacture's procedure.

Results and Discussion

The effects of soy-isoflavonoid and estrogen or the combination of soy-isoflavonoid and estrogen on the expressions of molecular markers related to apoptosis were monitored in the systems of estrogen-deficient and estrogen-supplemented rats as well as in cultured mammalian tumor cells.

In the system of mature estrogen-supplemented rats, soy-isoflavonoid induced higher down-regulation of Bcl-2 protein (Fig. 1) compared to the estrogen-supplemented rats. Bcl-2/Bax ratio was lowest in soy-isoflavonoid supplemented group. COX-2 expression showed similar down-regulation patterns as observed in Bcl-2 with the soy-isoflavonoid-supplemented group (Fig. 2).

In the system of ovariectomized estrogen-deficient female rats, Bcl-2 expression was slightly up-regulated at low concentration of soy-isoflavonoid in genistein-treated animals compared to the sham or ovariectomized control rats (Fig. 3). Up-regulation of Bax was observed with estrogen, and low and high concentrations of soy-isoflavonoid. Bcl-2-Bax-ratio decreased in estrogen- and high concentration soy-isoflavonoid-treated groups. In sham-operated or ovariectomized control rats (lanes 1 and 2, Fig. 3), down-regulation of both Bcl-2 and Bax resulted in decreased Bcl-2/Bax ratio. However, in the estrogen-treated ovariectomized group, decrease in Bcl-2/Bax ratio was due to the higher level of Bax compared to Bcl-2. Treatment of low concentration soy-isoflavonoids to sham and ovariectomized rats resulted in overexpression of Bcl-2, and slightly lower expression of Bax resulted in higher ratio of Bcl-2 and Bax. Finally, lower Bcl-2/Bax ratio, almost comparable to that of the control groups, was observed with treatment of high concentration soy-isoflavonoid (lane 6, Fig. 3). The highest ratio of Bcl-2 and Bax was observed with genistein treatment. COX-2 expression in ovariectomized estrogen-deficient female rats appeared to be independent of Bcl-2 expression (Fig. 4). The highest expression of COX-2 was observed with the estrogen-supplemented group and high soy-isoflavonoid-supplemented groups.

In MCF-7 cell line, genistein, one of the soy-isoflavonoids, was tested for its effect on Bcl-2 and Bax

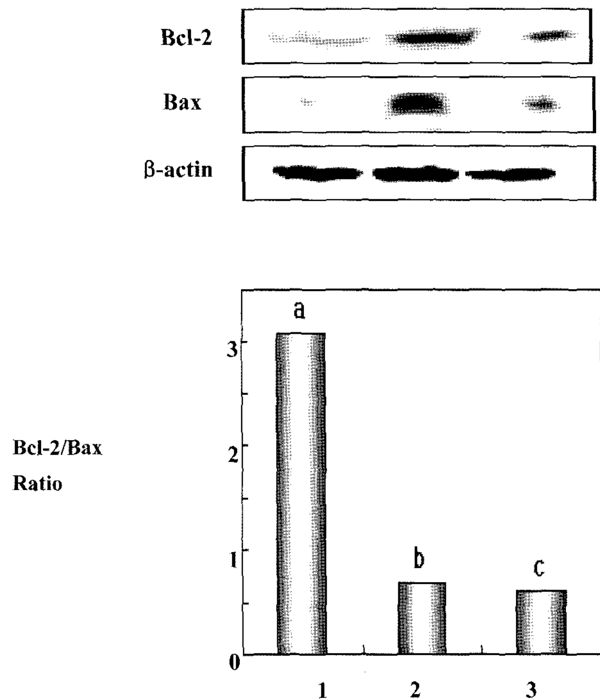

Fig. 1. Expression of Bcl-2 and Bax proteins (top) and Bcl-2/Bax ratio (bottom) after estrogen or estrogen plus soy-isoflavonoid treatments in mature female rats. Rats were treated with soy-isoflavonoids and estradiol for four weeks. Tissues were collected and lysates were prepared, subjected to electrophoresis on 7% SDS-PAGE, Western- blotted, and visualized with ECL detection kit described in Materials and Methods. Lane 1. Control; lane 2. 17 β-estradiol treated and lane 3. 17 β-estradiol + soy-isoflavonoid-treated respectively as described in Materials and Methods.

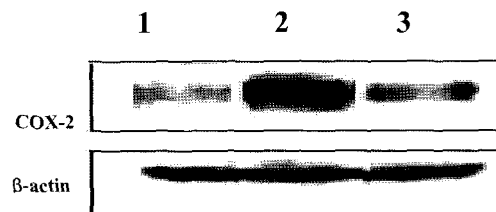

Fig. 2. Expression of COX-2 after estrogen or estrogen plus soy-isoflavonoid treatments in mature female rats. Rats were treated with soy-isoflavonoids and estradiol for four weeks. Tissues were collected and lysates were prepared, subjected to electrophoresis on 7% SDS-PAGE, Western- blotted, and visualized with ECL detection kit described in Materials and Methods. Lane 1. Control; lane 2. 17 β-estradiol treated and lane 3. 17 β-estradiol + soy-isoflavonoid-treated respectively as described in Materials and Methods.

when treated alone or with estrogen at various concentrations and combinations (Fig. 5). The medium and highest concentrations of genistein and the lowest concentration of estrogen treatment resulted in the stimulation of Bax expression, whereas Bcl-2 expression was not significantly changed by either genistein or estrogen treatments. Genistein treatment decreased, whereas estrogen treatment increased, Bcl-2/Bax ratio in a concentration-dependent manner. The combined treatment showed higher ratio, with maximum response observed from the combination of the lowest concentrations of

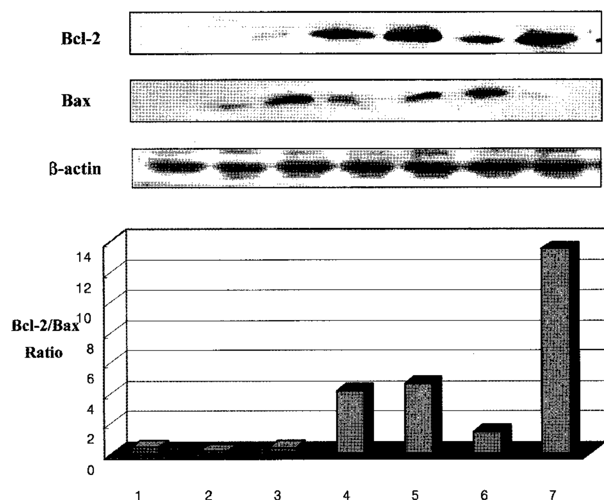

Fig. 3. Expression of Bcl-2 and Bax proteins (top) and Bcl-2/ Bax ratio (bottom) after soy-isoflavonoids. estrogen or genistein treatments in ovariectomized female rats. Rats were treated with soy-isoflavonoids, estrogen or genistein for eight weeks. Tissues were collected and lysates were prepared, subjected to electrophoresis on 7% SDS-PAGE, Western- blotted, and visualized with ECL detection kit described in Materials and Methods. Lane 1. Untreated control (sham); lane 2. Control (ovariectomized); lane 3. 17 β-estradiol treated (ovariectomized); lane 4. Low soy-isoflavonoid treated (sham); lane 5. Low soy-isoflavonoid treated (ovariectomized); lane 6. High soy-isoflavonoid treated (ovariectomized) and lane 7. Genistein treated (ovariectomized) respectively as described in Materials and Methods.

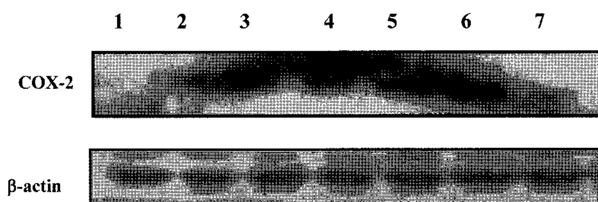

Fig. 4. Expression of COX-2 after soy-isoflavonoids. estrogen or genistein treatments in ovariectomized female rats. Rats were treated with soy-isoflavonoids, estrogen or genistein for eight weeks. Tissues were collected and lysates were prepared, subjected to electrophoresis on 7% SDS-PAGE, Western- blotted, and visualized with ECL detection kit as described in Materials and Methods. Lane 1. Untreated control (sham); lane 2. Control (ovariectomized); lane 3. 17 β-estradiol treated (ovariectomized); lane 4. Low soy-isoflavonoid treated (sham); lane 5. Low soy-isoflavonoid treated (ovariectomized); lane 6. High soy-isoflavonoid treated (ovariectomized) and lane 7. Genistein treated (ovariectomized) respectively as described in Materials and Methods.

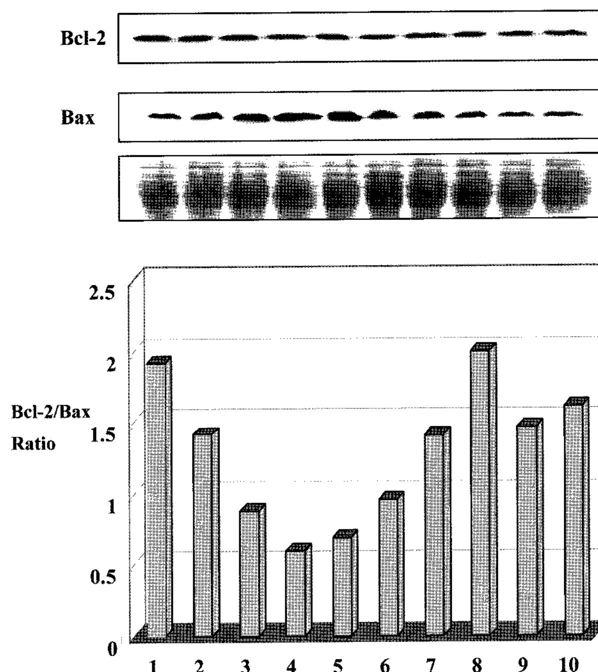

Fig. 5. Expression of Bcl-2 and Bax (top), and Bcl-2/Bax ratio (bottom) after genistein and/or estradiol treatments. MCF-7 cells treated with genistein and/or estradiol for 48 hr at 37°C. Whole cells were harvested and lysateds were prepared, subjected to electrophoresis on 7% SDS-PAGE, Western- blotted, and visualized with ECL detection kit described in Materials and Methods. Lane 1. Untreated control (DMSO); lanes 2-4, cells incubated with 25, 50 or 100 uM genistein, lanes 5-7, cells incubated with 12.5 nM 17 β-estradiol, 25 nM 17 β-estradiol, and 50 nM 17 β-estradiol, and lanes 8-10, cells incubated with 25 uM genistein and 12.5 nM 17 β-estradiol, 50 uM genistein and 25 nM 17 β-estradiol, or 100 uM genistein and 50 nM 17 β-estradiol, respectively.

genistein and estrogen.

Genistein, the active component of soy-isoflavonoid, is a naturally occurring plant-derived compound. This biologically active type originates from soybean products through conversion with intestinal bacteria, and is a hormone-like compound with antioxidative and estrogen receptor-binding activities (28). This plant-derived estrogen as well as other products such as daidzein and glycetin appear to exert both estrogenic and antiestrogenic effects on metabolism, depending on their biological concentration, endogenous estrogen levels, and individual traits such as sex and menopausal status (16, 29). Initially, the interference at the level of estrogen receptor was suggested as the major role of genistein in the inhibition of tumor-promoting effect of estrogens (30, 31). At concentrations lower than 10 micromol/L, the growth of MCF-7 cell, an estrogen receptor-positive cancer cell line, was stimulated by genistein; however, genistein does not stimulate the growth of estrogen receptor-negative breast cancer cell. The antiestrogenic activity may be partially explained by competition with the endogenous 17β-estradiol for estrogen receptors (31). Depending upon the concentration, phytoestrogen shows estrogen agonistic or antagonistic action (32, 33). Moreover, interaction with two kinds of estrogen receptors (alpha and beta) appears to play a crucial role in exhibiting the complex responses of phytoestrogens (34-37). The mechanism of genistein on chemopreventive action has recently been reported in the field of cell cycle regulation including p21 and cdks (38, 39).

To investigate the effect of soy-isoflavonoid in the presence of estrogen on COX-2 expression and apoptosis markers, 48-month-old female rats were supplemented with estrogen or combination of estrogen and soy-isoflavonoid. Up-regulation was observed in antiapoptotic protein Bcl-2 expression with estrogen, and this up-regulation could be reversed by co-treatment with soy-isoflavonoid. In the experiment using ovariectomized rats, the effect of soy-isoflavonoid on Bcl-2 expression was

dose-dependent, showing stimulated expression at low concentration and decreased expression with higher level of soy-isoflavonoid. In MCF-7 cells, the regulation of Bax appeared to be dependent on the concentration of genistein, showing stimulated expression at the medium and highest concentrations. Considering that estrogens are potent mitogens in the mammary gland, playing a pivotal role in the development and progression of mammary carcinoma (40), proapoptotic potential of soy-isoflavonoid by the down-regulation of Bcl-2, up-regulation of Bax or both may have an implication on the chemopreventive activity of soy products. Nevertheless, the concentration of soy supplementation as well as that of estrogen should be taken into consideration. Defects in homeostasis of apoptotic mechanisms play a role in the pathogenesis of various cancers, and the chemopreventive measures to stimulate apoptosis can provide the basis of anti-cancer effects of various phytochemicals. The possible regulation of *bcl-2* and/or *bax* has been suggested as the key element of proapoptotic mechanism of genistein in several reports, contrary to other published results (41-44). In genistein-treated MCF-7 cells, Bcl-2 phosphorylation increased at 24 to 48 hr, while Bax expression was not elevated during the same period (41). Li *et al.* (42) reported that genistein induced up-regulation of Bax and down-regulation of Bcl-2. The concentration dependency of Bcl-2 expression has been observed in genistein-treated MCF-7 cells (43). At the threshold concentration of genistein capable of inducing apoptosis (25 mol/L), Bcl-2 expression was not elevated. However, at higher concentrations, both Bcl-2 and Bax were up-regulated. In the study of Xu and Loo (44), the ratio of Bax to Bcl-2 increased up to 48 hr in MCF-7 cells and decreased thereafter. In the present study, on the other hand, Bcl-2/Bax ratio decreased in a concentration-dependent manner after 48-hr treatment with genistein.

In the present study of MCF-7 cells, up-regulation of Bax was observed at medium and higher concentrations of genistein, and the addition of estrogen overrode this effect. However, estrogen blocking of the proapoptotic potential of genistein by hindering the up-regulation of Bax, thereby increasing Bcl-2/Bax ratio, has not yet been clearly elucidated. Furthermore, the difference observed in the response of Bcl-2 to genistein and estrogen between *in vivo* systems and cancerous cell culture system requires explanation. This could be simply explained by the observation of the differential effects of anti-tumor compounds on cell proliferation between normal or neoplastic cell lines (45). However, the difference of signaling protein regulation between these two systems might have played a significant role, although the complexity of signaling protein regulation in normal system and tumor cell lines has not been resolved completely.

A possible mechanism by which COX-2 affects cancer survival is by increasing cell resistance to apoptosis (46). It appears that COX-2 inhibitors induce apoptosis by inactivating the Akt-dependent pathway, which mediates cell survival through the phosphorylation of Bcl-2 (47).

This study has shown the concentration dependency of antiapoptotic protein Bcl-2 and proapoptotic protein Bax regulations by soy-isoflavonoid and estrogen in both *in vivo* and *in vitro* systems. In normal female rats, Bcl-2, rather than Bax, was regulated by soy-isoflavonoid and estrogen treatments, whereas, in a mammary tumor cell line, the modulation by Bax was more evident. Proapoptotic potential of estrogen in the presence of genistein was observed at low concentrations *in vivo*, whereas at all concentrations *in vitro*. The observed agonistic and antagonistic activities of genistein to estrogen at a concentration-dependent manner and the altered pattern of the genistein activity in the presence of estrogen require further investigation. Because dose of genistein and physiological status such as estrogen sufficiency and deficiency were important factors in this study, practical application of soy-isoflavonoids in the prevention of degenerative diseases and post-menopausal syndromes warrants in-depth study on these factors.

Acknowledgments

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