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Estrogenicity of Isoflavones on Human Endometrial Stromal and Glandular Cells

UMIT A. KAYISLI, CINAR AHMET H. AKSU, MURAT BERKKANOGLU, AND AYDIN ARICI

Section of Reproductive Endocrinology (U.A.K., C.A.H.A., M.B., A.A.), Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, Connecticut 06520; and Department of Histology and Embryology (U.A.K.), Akdeniz University School of Medicine, Antalya, Turkey 07070

Endometrium consists of different cell populations such as epithelial and stromal cells and is mainly regulated by sex steroids. Isoflavones are plant-derived estrogenic compounds that have estrogenic and antiestrogenic properties in a cell-specific manner. We hypothesized that one of the potential health benefits of isoflavones may be their ability to regulate endometrial cell function. The present study was conducted to assess estrogenic and/or antiestrogenic effects of isoflavones (genistein, genistin, daidzein, and daidzin) in cultured human endometrial stromal and glandular (Ishikawa) cells by MTT colorimetric cell proliferation assay, proliferating cell nuclear antigen expression, and alkaline phosphatase activity assays. Experiments were performed in a time- (24–96 h) and concentration-dependent (10^{-12} to 10^{-5} M) manner. All isoflavones used in the present study induced endometrial stromal and Ishikawa cell proliferation when compared with control (vehicle) group in a time- (at 48 h and afterward) and concentration-dependent manner (at 10^{-8} M and above) ($P < 0.05$). However, isoflavones (at 10^{-8} and above concentrations) were also antiestrogenic when combined with estradiol (E_2) ($P < 0.05$). The isoflavones revealed a weak estrogenic activity (39–67% less than E_2) as assessed by alkaline phosphatase activity ($P < 0.05$), but when administered together with E_2, they antagonized estrogen induced alkaline phosphatase activity by 36–89% ($P < 0.05$). We conclude that, although isoflavones alone have weak estrogenic effects on endometrial stromal and glandular cells, in the presence of E_2 they act as antiestrogens. (*J Clin Endocrinol Metab* 87: 5539–5544, 2002)

PHYTOESTROGENS ARE A FAMILY of chemically diverse compounds found in plants and are known to have estrogenic effects on human tissues. Isoflavones, derived from soybeans, are such subclass of phytoestrogens. Epidemiological studies suggest that isoflavones may reduce the risk of cancer, osteoporosis, heart disease, and atherosclerosis (1, 2).

In many studies, isoflavones have been shown to behave as estrogenic compounds, stimulating plasma prolactin levels, mammary gland proliferation, and uterine weight, and altering vaginal cytology in ovariectomized rats (3, 4). However, in another study of surgically induced menopausal macaques, isoflavones demonstrated no significant estrogenic effect (5).

Soybean phytoestrogens comprise three main isoflavones, genistin, daidzin, and glycitin, found as glycosylated structures (glucosyl-7-genistein, glucosyl-7-daidzein and glucosyl-7-glycitein) in the plant and first two are metabolized to bioactive aglycons in the gut as genistein and daidzein, respectively (6). The effects of different isoflavones may be quite variable. It has been shown that daidzin inhibits human mitochondrial aldehyde dehydrogenase (7). Moreover, orally administered daidzin and genistin have shown to be protective against bone loss in ovariectomized rats (8). Recently, genistin, daidzin, and their aglycosylated forms (genistein and daidzein) have been reported to have some neuroprotective effect by decreasing LDH release (9). Genistein acts as an estrogen agonist *in vivo* and *in vitro*, resulting in the proliferation of cultured human MCF-7 breast cancer cells (10). On the other hand, although genistein was reported to inhibit MDA-MB-231 breast cancer cell growth *in vitro*, at the same concentrations in plasma, genistein did not inhibit the same cell's growth *in vivo* (11). Duncan *et al.* (12) have studied the effect of three soy extracts, each containing different concentrations of isoflavones in postmenopausal women. They found that neither isoflavones nor soy produce an estrogenic effect on vaginal epithelium or endometrium. In contrast, Foth and Cline (13) have shown that isoflavones, when given simultaneously with exogenous estradiol (E_2), have antiproliferative effect on the endometrium and mammary gland of postmenopausal macaques.

These contradictory studies illustrate that the effect of isoflavones on endometrial cell function and proliferation is not clear. Although isoflavones alone may have a weak estrogenic effect on endometrial stromal and glandular cells in culture, they may also antagonize estrogenic activity in the presence of estrogen. In this study, we evaluated the effect of four major isoflavones (genistein, genistin, daidzein, and daidzin) on endometrial stromal and glandular cells using a colorimetric cell proliferation assay based on a water-soluble tetrazolium salt, cellular ELISA for proliferating cell antigen (PCNA), and alkaline phosphatase estrogenicity assay.

Materials and Methods

Tissue collection

Endometrial tissues were collected from human uteri after hysterectomy conducted for reasons other than endometrial disease. Informed consent was obtained from each woman before the surgery, and the study was approved by the Human Investigation Committee of Yale

Abbreviations: DMSO, Dimethylsulfoxide; E_2, estradiol; MTT, 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCNA, proliferating cell nuclear antigen.

University. Tissue samples were transported from the operating room to the laboratory in Hanks' balanced salt solution for separation and culture of endometrial stromal cell cultures.

Ishikawa cell (a well-differentiated endometrial adenocarcinoma cell line) was provided to us by Dr. R. Hochberg (Department of Obstetrics and Gynecology, Yale University, New Haven, CT) from a frozen stock. Thawed cells were maintained in T75 flasks (BD Biosciences, Franklin Lakes, NJ) until passage.

Isolation and culture of human endometrial cells and culture of Ishikawa cells

Endometrial stromal cells were separated and maintained in primary cell culture as described previously (14). Briefly, endometrial tissue was digested by incubation of the tissue minces in Hanks' balanced salt solution that contained HEPES (25 mM), penicillin (200 U/ml), streptomycin (200 mg/ml), collagenase (1 mg/ml), and deoxyribonuclease (0.1 mg/ml, 1500 U/mg) for 30 min at 37 C with agitation. The dispersed endometrial cells were separated by filtration through a wire sieve (73-μm diameter pore). The endometrial stromal cells were maintained in Ham's F12/DMEM (1:1, vol/vol) containing antibiotics-antimycotics (1% vol/vol) and FBS (10%, vol/vol). Endometrial stromal cells were plated in T75 plastic flasks, maintained at 37 C in a humidified atmosphere (5% CO_2 in air), and allowed to replicate to confluence. After first passage, only stromal cells are retained. Other cell types such as epithelial cells, endothelial cells and macrophage/monocytes are depleted. Stromal cells were passed by standard methods of trypsinization and plated in 96-well plates (1.5×10^4 cells/well) and allowed to proliferate to 60–70% confluence before commencement of each experiment. At the beginning of each experiment the cells were maintained in serum- and phenol red-free F12/DMEM for 24 h before the application of treatments. Thereafter cells were incubated with the isoflavones (genistin, genistein, daidzin, and daidzein) in a time- (24–96 h) and concentration- (10^{-12} to 10^{-5} M) dependent manner. All the isoflavones used in this study were obtained as 99% pure powder form from INDOFINE Chemical Co. (Somerville, NJ).

Ishikawa cells, after being thawed from their frozen stocks, were maintained to 80% confluence in T75 flasks with F12/DMEM and passed to 96-well plates for the experiments. Experiments were performed in a manner similar to the endometrial stromal cells described above.

MTT cell proliferation assay

Cell proliferation was assessed by a colorimetric assay using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. MTT assay that detects the formation of dark blue formazan product from MTT in active mitochondria was performed as described previously (15). Four hours before the end of each experiment, 10 μl MTT solution are added onto each well of 96-well plates. The optical absorbance at 570 nm is read within 30 min with a microplate reader (Thermomax, Molecular Devices Corp., Menlo Park, CA). Data are expressed in OD units. The last column of each 96-well plate did not contain cells and was used as a blank.

Alkaline phosphatase assay

To bioassay estrogenic potency, we used a well-developed technique using induction of alkaline phosphatase activity in Ishikawa cells (16). This technique is based on a colorimetric assay of alkaline phosphatase enzyme activity in 96-well microtiter plates. Previous studies have shown that estrogens stimulate and antiestrogens (tamoxifen and 4-hydroxytamoxifen) inhibit estrogen-stimulated alkaline phosphatase activity in Ishikawa cells (16). Alkaline phosphatase activity was assayed exactly as described (16).

Indirect cellular ELISA for PCNA

We have devised a cellular ELISA method to quantitate the regulation of PCNA expression by estrogens. Primary endometrial stromal cells grown in 96-well plates and treated with E_2 and/or the isoflavones were fixed with 100% methanol for 20 min at –20 C. After washing three times with PBS at room temperature, the endogenous cellular peroxidase activity was quenched by 20 min incubation in 3% H_2O_2 methanol at room temperature. Each well was incubated with 2% BSA in PBS for 20 min to reduce nonspecific binding and thereafter mouse monoclonal IgG2a anti-PCNA (Sigma, St. Louis, MO) was applied into each well for 30 min at room temperature by gently shaking. After washing three times with PBS, biotinylated antimouse IgG (Vector Laboratories, Burlingame, CA) was applied with 1% horse serum for 30 min. Following several rinses with PBS, horseradish peroxidase-avidin complex was added for 30 min. After washing with PBS, 3,3′,5,5′-tetramethylbenzidine Substrate Kit solution (Vector Laboratories) was added for 10 min, and the reaction was stopped with the addition of 50 μl of 1 N sulfuric acid per well. One of the columns of 96-well plate was free from cell and used as blank, and another column of the plate was incubated with the isotype of the primary antibody (mouse IgG2a) and used as negative control. The colorimetric evaluation was done at 450 nm with the microplate reader (Thermomax).

Statistical analyses

Data from the ELISA, MTT cell proliferation, and alkaline phosphatase activity assays were normally distributed as tested by Kolmogorov-Smirnov test. Thus, ANOVA and *post hoc* Tukey test for pairwise multiple comparisons were used for statistical analysis. $P < 0.05$ was considered to be significant. Statistical calculations were performed using Sigmastat for Windows, version 2.0 (Jandel Scientific Corp., San Rafael, CA).

Results

Effect of isoflavones on endometrial cell proliferation

Concentration-dependent (10^{-9} to 10^{-6} M) proliferative effects of genistein, genistin (glucosyl-7-genistein), daidzein (7–4′-dihydroxyisoflavone), and daidzin (7-glucosyl-4′-hydroxyisoflavone) alone or in the presence of E_2 (10^{-9} to 10^{-8} M) on endometrial stromal and Ishikawa cells were assessed using the MTT colorimetric assay. Endometrial stromal and Ishikawa cells were treated with isoflavones for 24–96 h. All isoflavones tested induced endometrial stromal cell proliferation in a time- and concentration-dependent manner starting at 48 h. The proliferative effect was significant at 10^{-7} M and higher concentrations ($P < 0.05$; Fig. 1, A and B). The proliferative effect of isoflavones (10^{-8} to 10^{-6} M) was 8–15% lower than that induced by E_2 (10^{-8} M) ($P < 0.05$; Fig. 1, A and B). There were no significant differences among the isoflavones tested. When isoflavones were combined with 10^{-9} M E_2, rather than increasing the proliferative effect, they antagonized the proliferative effect of E_2 by 10–20% ($P < 0.05$; Fig. 2).

Regulation of PCNA expression by isoflavones in endometrial stromal cells

We assessed the regulation of PCNA in endometrial stromal cells by E_2 and four isoflavones (genistein, genistin, daidzein, and daidzin) using a cellular ELISA for PCNA. Following 96 h of treatment, isoflavones alone (10^{-7} M) increased PCNA up to 15% in endometrial stromal cells ($P < 0.05$; Fig. 3A). Similar to cell proliferation assay results, when combined with E_2, isoflavones (10^{-6} M) decreased the E_2-induced PCNA expression ($P < 0.05$, Fig. 3B).

Estrogenicity of isoflavones on Ishikawa cells

We used the effect of estrogens to induce alkaline phosphatase activity in Ishikawa cells to compare the estrogenic potencies of the four isoflavones (genistein, genistin, daidzein and daidzin) (12). Ishikawa cells were treated with E_2

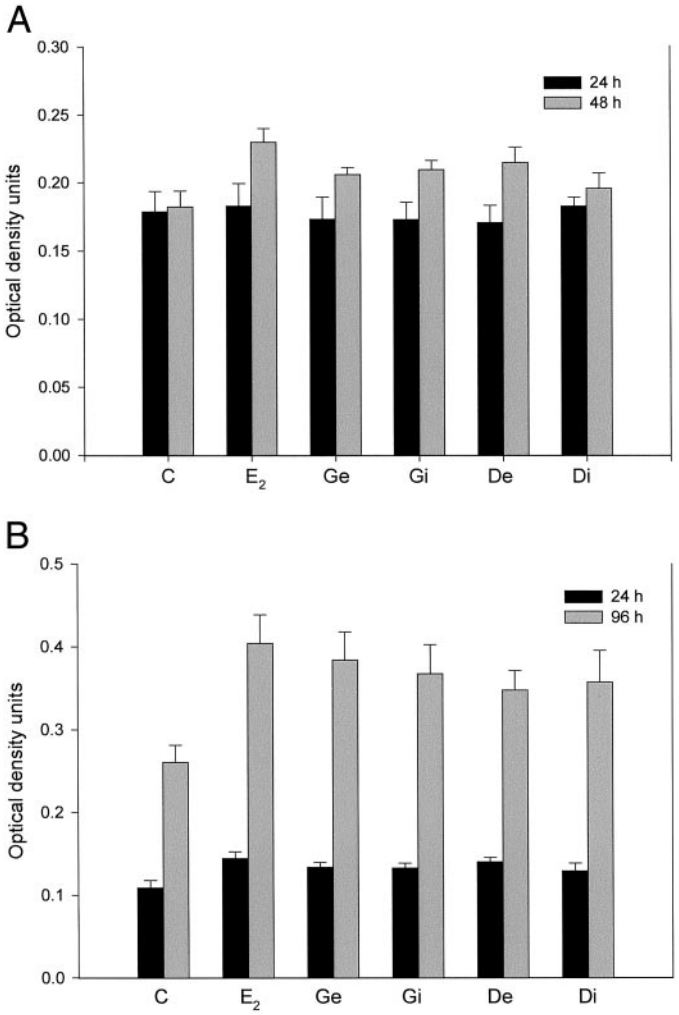

FIG. 1. Effect of isoflavones on endometrial stromal cell (A) and gland (Ishikawa) cell (B) proliferation. Cells were treated with E_2 (10^{-9} M), genistein (Ge), genistin (Gi), daidzein (De), and daidzin (Di) (all 10^{-7} M) or with dimethylsulfoxide (DMSO, vehicle) as control (C) for 24–96 h. Cell proliferation was analyzed in 96-well microplate by MTT colorimetric assay. Values are expressed as mean ± SEM of eight wells for each group. Following 24 h treatment, there were no significant differences between the groups. However, at 48 h and thereafter (not shown), both E_2 and isoflavone-treated groups had significantly increased proliferation compared with the control group ($P < 0.05$) (A). Ishikawa cells treated with isoflavones or E_2 showed significant increase in cell proliferation when compared with control group ($P < 0.05$) at 96 h of treatment (B).

(10^{-12} to 10^{-5} M) and with isoflavones (10^{-12} to 10^{-5} M) for 24–96 h. All four isoflavones (at 10^{-8} M and above concentrations) stimulated alkaline phosphatase activity in a time-dependent manner, but their potency was 39–67% less than E_2 ($P < 0.05$; Figs. 4 and 5A).

Ishikawa cells were treated with E_2 (10^{-8} M) alone and in combination with isoflavones at similar or 100-fold higher concentrations for up to 96 h. Cells treated with E_2 and 10^{-8} M isoflavones revealed no significant differences in their alkaline phosphatase activity (Fig 5B). Ishikawa cells were treated with 10^{-6} M isoflavones 30 min before E_2 treatment, and cells were incubated for 48 h, there was a significant decrease by the addition of isoflavones, in a time-dependent manner

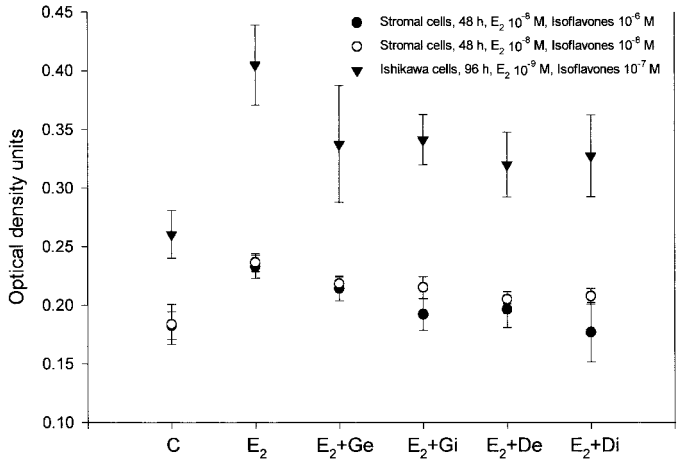

FIG. 2. Effect of isoflavones on E_2-induced endometrial stromal cell and gland (Ishikawa) cell proliferation. Cells were treated with E_2, E_2 + genistein (E_2 + Ge); E_2 + genistin (E_2 + Gi); E_2 + daidzein (E_2 + De); and E_2 + daidzin (E_2 + Di) or with DMSO as vehicle (C) at different concentrations for 48 or 96 h. Values are expressed as mean ± SEM of eight wells for each group. Isoflavones significantly decreased the cell proliferation induced by E_2 ($P < 0.05$).

(36–89%, $P < 0.05$, Fig 5B). When the cells were treated with isoflavones (10^{-6} M) and estradiol (10^{-8} M) simultaneously, isoflavones were less effective in decreasing the E_2-induced alkaline phosphatase activity (data not shown).

Discussion

Soy products that contain compounds with estrogenic and antiestrogenic properties are becoming increasingly popular in human and infant foods. Phytoestrogens are naturally produced plant estrogens that are reputed to have beneficial effects. They are phenolic compounds that are known to bind to ERs (6, 17).

Isoflavones are a major subclass of the phytoestrogen family. They may have roles in ameliorating hormone-related diseases, osteoporosis, menopausal symptoms, and cancer (18). Two main isoflavones, genistin and daidzin, are found as glycosylated structure in the plant and are metabolized to bioactive aglycons in the gut as genistein and daidzein, respectively (6). However, our results show that genistin and daidzin could be bioactive in *in vitro* conditions. Supporting these hypotheses, previous studies have shown that daidzin and genistin are selective inhibitors of human mitochondrial aldehyde dehydrogenase and could behave neuroprotective *in vitro* (7, 9). In the present study, we observed that isoflavones produce an estrogenic effect on endometrial cells. However, relatively high concentrations of isoflavones (10^{-8} to 10^{-6} M) were required, and thus, they were only weakly estrogenic compared with E_2. On the other hand, they acted as antiestrogens in the presence of E_2.

PCNA is involved in many aspects of DNA replication and processing, forming a sliding platform that can mediate the interaction of proteins with DNA. PCNA was originally discovered as an antigen found only in the nuclei of dividing cells. Many proteins, including those that are involved in cell cycle control, bind to this stabilizing molecule. This molecule is accepted as a surrogate index for cellular proliferation (19,

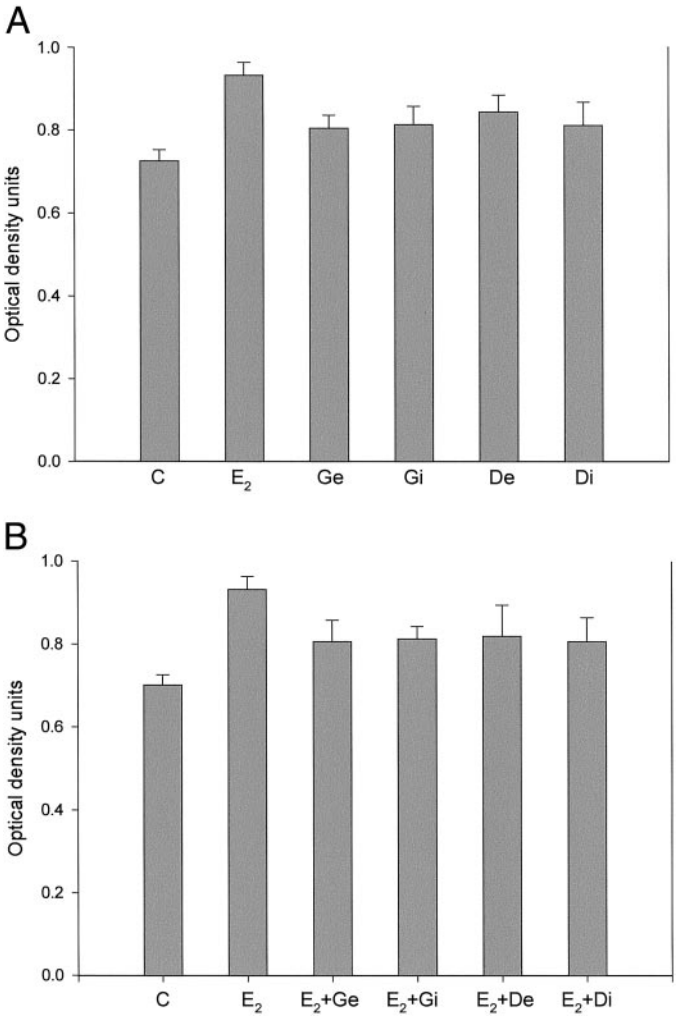

FIG. 3. Effect of E_2 and isoflavones on PCNA in endometrial stromal cells. Regulation of PCNA was assessed by a cellular ELISA. Effect of isoflavones alone (A) or in combination with E_2 (B) on endometrial stromal cells was assessed. Cells were treated with genistein (Ge), genistin (Gi), daidzein (De), and daidzin (Di) 10^{-7} M when treated alone, 10^{-6} M when treated with E_2 (10^{-8} M) for 96 h DMSO (vehicle) as control (C) alone or with E_2. Values are expressed as mean ± SEM of eight wells for each group. The increase of PCNA in cells treated with isoflavones alone compared with control group (A), and the decrease of PCNA in cells treated with isoflavones and E_2 combined compared with cells treated with E_2 alone (B) were significant ($P < 0.05$).

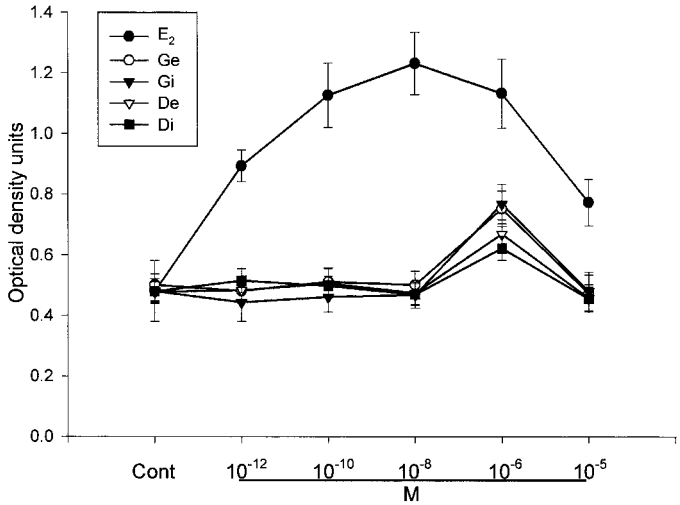

FIG. 4. Concentration-dependent stimulation of alkaline phosphatase activity in Ishikawa cells by genistein (Ge), genistin (Gi), daidzein (De), daidzin (Di), and E_2 for 48 h. E_2-treated cells have a significant increase in alkaline phosphatase activity ($P < 0.001$), which peaked at 10^{-8} M. Isoflavones also stimulated alkaline phosphatase activity and induced the peak activity at 10^{-6} M ($P < 0.05$). At 10^{-5} M and above concentrations isoflavones and E_2 seem to be toxic in endometrial cells.

20). Our results showed that isoflavones could regulate cell proliferation by affecting PCNA expression.

Previous studies also have shown that isoflavones exhibit weak estrogenic activity in other cell types such as mammary gland and hypothalamic/pituitary cells (4, 21). However, these compounds were also reported to be normally present at 10- to 100-fold higher concentrations in serum compared with that of E_2 (22, 23). Recently Lu *et al.* (24) have shown that isoflavones when taken at 5 mg/d dosage decrease circulating ovarian steroids in women without affecting gonadotropin levels. Thus, this is probably not an estrogenic effect and probably originates from the known inhibition of flavonoids on aromatase, 3β-hydroxysteroid dehydrogenase Δ5/Δ4 isomerase, and 17β-hydroxysteroid dehydrogenase activities (25, 26). In the present study, we showed that assessment of alkaline phosphatase activity in Ishikawa cells to evaluate estrogenicity of naturally produced plant estrogens could be used and is easily applicable.

To the contrary, Duncan *et al.* (27) found that a low dose isoflavone diet decreases LH and FSH levels during periovulatory phase, and high dose isoflavone diet decreases estrone levels during the midfolicular phase. Likewise, women with high phytoestrogen concentrations in their blood are reported to have a lower rate of menopausal symptoms such as hot flushes, compared with women with low concentrations of phytoestrogens (28). Estrogens are known to elevate bone mineralization in both female and male (29, 30). Recently, another *in vivo* study (31) has shown that postmenopausal women with the lowest level of isoflavone intake, compared with postmenopausal women with high levels of isoflavone intake, have a significantly lower lumbar spine (L2–4) bone mineral density and Ward's triangle bone mineral density. Moreover, women with the highest-level intake of isoflavone had significantly lower levels of serum PTH, osteocalcin, and urinary *N*-telopeptide compared with those with low intake of isoflavone. On the other hand, there was no relationship between the phytoestrogen intake and bone mineral density in premenopausal women with high endogenous estrogen level (31). Thus, it appears from this study that both estrogen and isoflavones are important for the estrogenic or antiestrogenic behavior of isoflavones. Diel *et al.* (32) have shown that genistein increased the uterine weight and stimulated uterine estrogen-dependent gene expression. They concluded that this isoflavone is as a weak ER agonist in ovariectomized rats. Our findings that isoflavones are weak estrogens in agreement with those *in vivo* studies because at high concentrations (10^{-8} to 10^{-6} M), they increase the endometrial cell proliferation and have estrogenic potency *in vitro*. Our results further indicate that neither

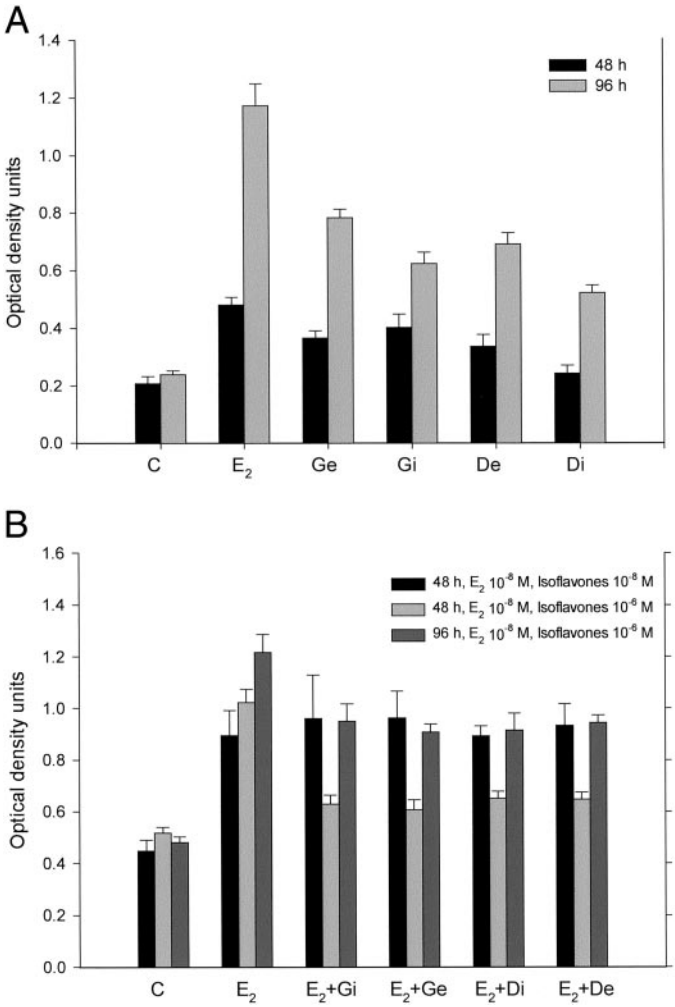

FIG. 5. Time-dependent stimulation of alkaline phosphatase activity in Ishikawa cells in culture by genistein (Ge), genistin (Gi), daidzein (De), daidzin (Di) (all at 10^{-6} M), and E_2 (10^{-8} M). E_2- and isoflavone-treated groups showed a significant increase in alkaline phosphatase activity in a time-dependent manner ($P<0.05$) (A). At the same concentration with E_2, isoflavones (10^{-8} M) did not reveal significant changes on E_2-induced alkaline phosphatase activity (B). E_2-induced alkaline phosphatase activity was down-regulated significantly when isoflavones were pretreated 30 min before E_2 and the concentration of isoflavones was 100-fold higher (10^{-6} M) than that of E_2 (10^{-8} M) for 48 h and 96 h ($P < 0.05$) (B).

endometrial gland nor stromal cell growth is inhibited by isoflavones alone *in vitro*.

Most of the proliferative and estrogenic effects are achieved via ERs in endometrial cells. Mammary glands are one of the most sensitive tissues for estrogen. In previous studies, it has been shown that the effects of weak estrogenic or antiestrogenic compounds are carried through ERs (33, 34). Makela *et al.* (35) have also shown that genistein, with a 20-fold higher binding affinity to ERβ than to ERα, provides a dose-dependent vasculoprotective effect in an *in vivo* rat carotid artery injury model, and it was also shown that ERβ is found at relatively higher level in blood vessel cells. Our results showed that isoflavones used in this study have antiestrogenic and antiproliferative effects in the presence of E_2. These effects were observed mostly in the presence of high concentrations of isoflavones (10^{-8} to 10^{-6} M) combined with physiologic concentrations of E_2 (10^{-10} to 10^{-8} M) following 48 h incubation and afterward. Phytoestrogens have been identified in many physiological fluids in humans consuming ordinary diets. Many studies have reported that dietary-related phytoestrogen concentrations in human plasma show considerable variations related to geographic localization, sex, and daily diet consumption. Adlercreutz *et al.* (36) have shown that various populations consuming diets without or with soy have different level of isoflavones in human plasma and urine excretion. Urinary isoflavones in Finish women, American women, and Asian immigrant women in Hawaii and Japanese women and men vary between 67.5 nmol/d and 8770 nmol/d. On the other hand, plasma level of isoflavones has been shown to vary from 4.2–1204 nM (36, 37), compatible with the concentration range that we have used in our study.

Because the effect of E_2 on alkaline phosphatase activity and on cell proliferation is carried out by genomic mechanisms, antiproliferative and antiestrogenic effects of isoflavones are likely to be explained by a competition between isoflavones and E_2 for ERs. Moreover, more efficient decrease on alkaline phosphatase activity in Ishikawa cells treated with isoflavones 30 min before E_2 treatment compared with cells treated with isoflavones and E_2 simultaneously also supports this hypothesis.

Animal studies have shown that genistein treatment could have a protective effect against cancer development, especially in estrogen-dependent diseases (5, 38). Most of the previous studies have shown isoflavones with antiproliferative and antiestrogenic actions depending on their experimental design, such as presence or absence of endogenous estrogen or the concentration of the isoflavones (17, 39). A clinical study performed among postmenopausal women showed no estrogenic effect, even at high quantities of isoflavones (40). Moreover, Phipps *et al.* (41) have shown no significant increase in menstrual cycle length, but an increase in sex steroid hormone binding globulin (an estrogen affected protein) level in women who consumed dietary isoflavones.

In conclusion, isoflavones alone behave as weak estrogenic factors by increasing proliferation and alkaline phosphatase activity in endometrial stromal and glandular (Ishikawa) cells. On the other hand, E_2-induced alkaline phosphatase activity or cell proliferation is down-regulated by isoflavones. Further studies are required to understand the interaction between isoflavones and their molecular targets such as ERs and other transcription factors in endometrial cells.

Acknowledgments

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Address all correspondence and requests for reprints to: Aydin Arici, M.D., Section of Reproductive Endocrinology, Department of Obstetrics and Gynecology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06520-8063. E-mail: aydin.arici@yale.edu.

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