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Isoflavone inhibits cell proliferation and the expression of signal transducer and activator of transcription 3 in human endometrial cancer cells

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Abstract

The active signal transducer and activator of transcription 3 (STAT3), which is known to play a role in oncogenesis, is a novel target for cancer therapy. Isoflavone, a phytoestrogen, has long been studied for its anticarcinogenic effects. In this study, we investigated the inhibitory effects of isoflavone on cell proliferation and the expression of STAT3 in human endometrial cancer cells. Two established endometrial cancer cell lines [Ishikawa, which expresses both estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) and HEC-1, which expresses only ERβ] and endometrial epithelial cells obtained from endometrial cancer tissue of 12 patients were treated with isoflavone (22 μM) and estrogen 17β-estradiol (E2 ; 10 nM). Cell proliferation assay and protein expression of STAT3 and p-STAT3 were analyzed in these cancer cell lines. Isoflavone treatment significantly suppressed the cell growth (76.36%) and protein expression levels of both STAT3 (23%) and p-STAT3 (42%) in Ishikawa cells compared with control. In HEC-1 cells isoflavone treatment showed no effect. E2 significantly increased cell growth (56%) and protein expression of p-STAT3 (38%) in Ishikawa cells. These results suggest that isoflavone may inhibit cell growth in Ishikawa cells. As Ishikawa cells express ERα whereas HEC-1 cells do not, the ERα pathway was thought to play a role in the inhibitory effects of isoflavone, and hence ERα knockdown (KD) was carried out. Interestingly, isoflavone or E2 treatment did not affect the cell growth in ERαKD Ishikawa cells. Furthermore, mRNA expression of STAT3 was analyzed in endometrial epithelial cells derived from endometrial cancer patients. Human endometrial epithelial cancer cells treated with isoflavone showed a significant decrease in mRNA expression of STAT3. In conclusion, the findings of this study suggest that isoflavone suppress cell growth by modulating STAT3 expression through ERα in endometrial cancer cells.

Introduction

Endometrial cancer, which arises from the uterine endometrium, is the sixth most common cancer and the third most common cause of death among women worldwide[1]. The overall 5-year survival rate of endometrial cancer is 18% when diagnosed in advanced stages with distant metastasis[2]. The main risk factor for endometrial cancer is an excess of endogenous or exogenous estrogen, such as from postmenopausal estrogen therapy, without adequate counteraction by progestin[3]. Effective therapies are currently lacking for advanced, aggressive, and recurrent cases of endometrial cancer, and hence there is an urgent need for cost-effective treatments with maximal effects.

Isoflavone, which is one of a group of organic compounds called phytoestrogens, is found mainly in soybean, and was shown to have anticarcinogenic effects[4-5]. Isoflavone is known to inhibit the growth of breast, prostate, colon, and ovarian cancer cell lines[6-10]. Isoflavone is known to bind to both estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), and was demonstrated to have inhibitory effects on estrogen-associated endometrial carcinogenesis[11-12].

Signal transducer and activator of transcription 3 (STAT3) is a cytoplasmic transcription factor that acts as

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a molecular hub for various signaling pathways. Activated STAT3 mediates cell proliferation, invasion, and metastasis in malignant tumors. STAT3 expression is increased in chemotherapy-resistant cancer patients and aggressive cancer cell lines[13]. Therefore, the inhibition of active STAT3 may lead to the suppression of cancer cell growth and apoptosis, indicating the possibility that STAT3 signaling is required for cell survival and growth[14]. Estrogen 17β-estradiol (E2) is well known for its proliferative effects in cancer cells. Thus, E2 was used in comparison to the effects of isoflavone in endometrial cancer cells.

In the present study, we aimed to clarify the inhibitory effects of isoflavone on cell proliferation and the expression of STAT3 in human endometrial cancer cells. We also investigated whether the inhibitory effects are associated with ERα.

Materials and Methods

Cell culture and treatment

Two endometrial cancer cell lines, namely, Ishikawa cells and HEC-1 cells were used in this study[15-16]. Cells were cultured in phenol red-free Minimum Essential Medium-α (Life Technologies, Camarillo, CA, USA) supplemented with 10% charcoal-stripped fetal bovine serum (Life Technologies), 2 mM L-glutamine (Life Technologies) and an antibiotic combination of 100 μg/mL streptomycin sulfate and 100 U/mL penicillin G sodium (Life Technologies) at 37°C in humidified air with 5% CO_2. Cells were cultured for 24 hours to allow them to attach to the culture dish before treatment with isoflavone [22 μM, AglyMax-60 (daidzein : genistein : glycitein at a ratio of 7 : 1 : 2) ; Nichimo, Shinagawa, Tokyo, Japan] and E2 (Sigma-Aldrich, MO, USA). A preliminary study concluded that isoflavone was cytostatic at 22 μM and E2 promoted adequate cell proliferation at 10nM, at 96 hours.

Transient transfection of ERα knockdown (ERαKD)

To achieve ERαKD, a shRNA sequence targeting the ERα gene was subcloned into the pLKO.1 TRC cloning vector (Addgene, Cambridge, MA, USA). At 80% confluence, Ishikawa cells were transfected with the ERαKD shRNA vector using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, MA, USA, Fig. 4D) according to the manufacturer's protocol.

Cell proliferation [Methyl thiazoyldiphenyl tetrazolium bromide (MTT)] assay

Aliquots of cell suspensions (1×10^4 cells/well) were added to each well of a 96-well micro titer plate in triplicate and incubated for 96 hours. A premixed, optimized dye solution (15 μL) (Promega, Madison, WI, USA) was added to each well and then incubated for 4 hours. Then, 100 μL of solubilization/stop solution was added to the culture wells to solubilize the formazan product, and the absorbance was recorded at 570 nm using a 96-well plate reader (SpectraMax 190 Absorbance Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). SoftMax Pro 7 software (Molecular Devices) was used to calibrate the data.

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA (1×10^4 cells/well) was isolated using Isogen (1mL, Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA (1-μg) was transcribed to complementary DNAs. Target primers are STAT3, ERα, and ERβ (Life Technologies). All results were normalized to those obtained using a human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mix (20x, Life Technologies). The 96-well reaction plate was run on an Applied Biosystems StepOnePlus™ Real-Time PCR System (Life Technologies). Data were analyzed using StepOnePlus™ Real-Time PCR System Software (Life Technologies).

Western blot analysis

Cells were lysed with RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA), containing, Halt™ protease inhibitor cocktail (Thermo Fisher Scientific), and ethylenediaminetetraacetic acid solution (Thermo Fisher Scientific). Protein concentrations were measured using Quick Start™ bovine serum albumin standard set (BIO-RAD, Hercules, CA, USA). A 40-μg protein sample was loaded onto a NuPAGE 4%-12% Bis-Tris gel (Life Technologies) and transferred to a polyvinylidene fluoride membrane. The membrane was blocked using ECL Prime blocking reagent (Amersham Biosciences, Piscataway, NJ, USA) for 2 hours, and then treated with primary antibodies overnight at 4 °C and secondary antibodies for 2 hours at room temperature using the concentrations recommended by the manufacturer. Antibodies against total STAT3 (1 : 1,000, Cell Signaling, Beverly, MA, USA), phosphorylated STAT3 [p-STAT3, (Tyr705, 1 : 1,000, Cell Signaling)], ERβ (1 : 1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and ERα (1 : 1,000, Santa Cruz Biotechnology) were used to detect corresponding proteins on the membrane. Band intensities were normalized to those of actin (Millipore, Billerica, MA, USA). Band densities were quantitatively analyzed using NIH image J software (NIH, Bethesda, MD, USA).

Human endometrial cancer tissue

Samples of endometrial cancer tissues from 12 patients diagnosed with endometrial cancer were collected after obtaining written consent. Ethical approval was obtained from Tokyo Medical University Hospital. The endometrial tissue was dissociated in Hank's balanced salt solution (Thermo Fisher Scientific) with collagenase type I (Worthington Biochemical Corporation, Freehold, NJ, USA) and incubated for 2 hours at 37°C. Tissue

samples were then filtered through a 100-μm sieve to remove cell debris. The cell suspension was filtered through a 40-μm sieve to separate epithelial cells from stromal cells. The epithelial cells were recovered by back washing the 40-μm sieve. The epithelial cells were cultured in a collagen-coated 6-well plate for 24 hours. RT-qPCR was performed after 48 hours of treatment with isoflavone and E2.

Statistical analysis

All experiments were repeated three times. All data were analyzed using Student *t*-test. A *p*-value of less than 0.05 was considered to indicate a statically significant difference between two groups.

Results

Anti-proliferative effects of isoflavone on Ishikawa and HEC-1 cells

To elucidate the anti-proliferative effects of isoflavone on endometrial cancer cell lines, Ishikawa cells (expressing both ERα and ERβ) and HEC-1 cells (expressing only ERβ) were treated for 96 hours with isoflavone or E2 (Fig. 1A). Growth rates of Ishikawa cells treated with isoflavone were lower than that of untreated control cells ; Ishikawa cells treated with isoflavone had a 76.36% decrease in cell growth compared with the control ($p < 0.05$). Growth rates of Ishikawa cells treated with E2 had 56% increase in cell growth compared with control ($p < 0.05$). In contrast to Ishikawa cells, isoflavone did not suppress cell growth of HEC-1 cells. HEC-1 cells treated with E2 increased cell growth by 19.8%, but statistical significance was not found.

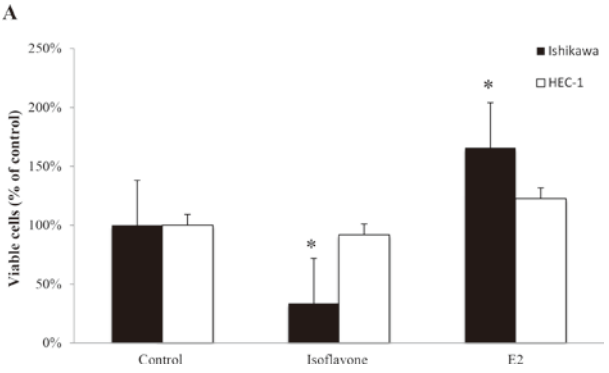

Fig. 1 Effect of isoflavone or E2 on endometrial cancer cell lines (A) Ishikawa and HEC-1 cells (1×10^4 cells/well, 96-well plate) treated with isoflavone (22 μM) or E2 (10 nM) in comparison to control cells are shown at 96 hours. Control was set to 100% viability. The average absorbance values were graphed in percentage. Isoflavone treatment suppressed cell growth in Ishikawa cells. E2 promoted cell growth in Ishikawa cells. Results are representative of three independent experiments. *$p < 0.05$ compared with control.

Isoflavone decreased protein expression of STAT-3 and p-STAT3 in Ishikawa cells

Western blot analysis demonstrated that untreated Ishikawa cells expressed both ERα and ERβ proteins, whereas untreated HEC-1 cells expressed ERβ but not ERα (Fig. 2A). Treatment of Ishikawa cells with isoflavone for 48 hours resulted in a decrease in the protein expression of STAT3 by 23% and p-STAT3 by 42% ($p < 0.05$) compared to control untreated cells (Fig. 2B, D, and E). Unlike in Ishikawa cells, protein expression levels of STAT3 remained consistent in isoflavone-treated HEC-1 cells. E2 treatment of Ishikawa cells resulted in a significant increase in p-STAT3 protein expression levels (38%) (Fig. 2C, D, and E).

ERαKD *desensitized Ishikawa cells to isoflavone*

Ishikawa cells treated with isoflavone had decrease cell growth and protein expression levels of STAT3 and p-STAT3 (Fig. 1 and 2). We further aimed to clarify whether isoflavone would continue to decrease cell proliferation in Ishikawa cells upon the KD (knockdown) of ERα. We decided to analyze the effects of Isoflavone or E2 treatment in Ishikawa cells with ERαKD. A shRNA vector was designed targeting the ERα (5′-GCAGGATT GTTGTGGCTACTA-3′). Ishikawa cells treated with irrelevant shRNA (5′-CCTAAGGTTAAGTCGCCCTC G-3′) were control KD cells. Western blot analysis confirmed the effective KD of ERα after 48 hours of transfection (Fig. 3A). ERαKD and control KD cells were used to perform cell proliferation assays. Isoflavone or E2 treatments did not affect the cell growth in ERαKD Ishikawa cells (Fig. 3B).

Isoflavone decreased STAT3 mRNA expression in human endometrial cancer cells

We next aimed to investigate the effect of isoflavone on endometrial epithelial cells obtained from 12 patients. The mRNA expression levels of STAT3 were significantly decreased in endometrial cancer cells upon treatment with isoflavone compared with control untreated cells ($p < 0.05$) (Fig. 4). The endometrial cancer cells treated with E2 showed a significant increase in mRNA expression levels of STAT3 ($p < 0.05$) (Fig. 4).

Discussion

Janus protein tyrosine kinase (JAK) binds to cytokine receptors and growth factor receptors, activates the STAT3 cascade, and thereby mediates cell proliferation[17]. The JAK-STAT3 signaling pathway is transient in normal cells but is constantly active in various cancer cells. Therefore, studies on human ovarian and breast cancers have shown that the suppression of overexpressed STAT3 is a promising therapeutic strategy for the inhibition of cancer growth, invasion, and metastasis[18-19]. Isoflavone, which is a natural product obtained from plants, has been suggested to exert inhibitory effects

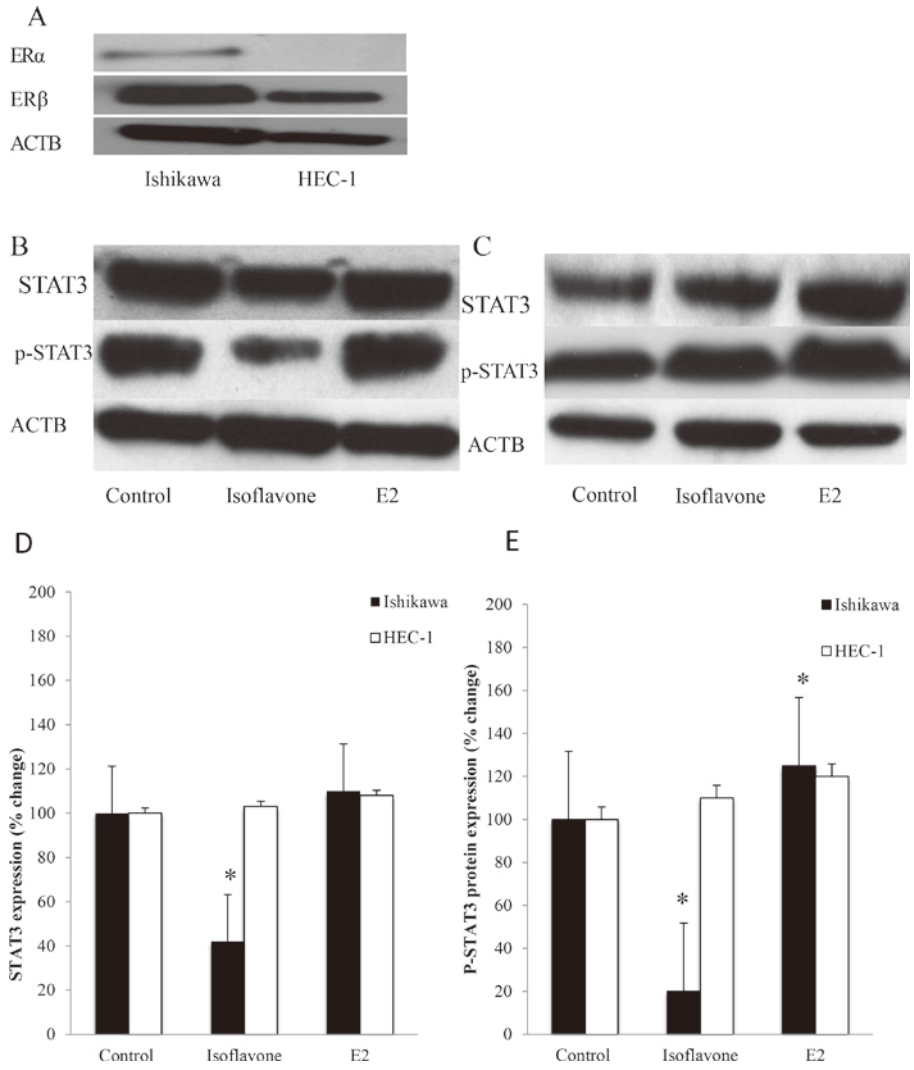

Fig. 2 STAT3 and p-STAT3 protein expression in isoflavone-treated endometrial cancer cell lines
(A) Western blot analysis of ERα and ERβ protein levels in Ishikawa and HEC-1 cells. The figure shows protein levels of STAT3 and p-STAT3 in (B) Ishikawa cells and (C) HEC-1 cells treated with isoflavone (22 μM) and E2 (10 nM) for 48 hours. Actin was used as an internal control. The relative protein expression of STAT3 and p-STAT3 were quantified in the arbitrary unit and converted to the percentage. Intensities of western blot bands were quantified by densitometric analysis and results were graphed in percentage ; (D) STAT3 and (E) p-STAT3. Ishikawa cells treated with isoflavone showed statistical significance. Data are shown as means ± SD, n=3. *$p < 0.05$ compared with internal control.

on endometrial carcinogenesis in mice, possibly by suppressing the expression of the estrogen-associated genes c-fos and c-jun, as well as cytokines[11]. Isoflavone has been studied for its chemopreventive and therapeutic effects in various cancer cells. However, its effect on endometrial cancer cells, which express the classical ERs (ERα and ERβ), had not been studied extensively. Therefore, in the present study, we aimed to understand the effects of isoflavone on endometrial cancer cells.

The treatment of endometrial cancer cell lines with isoflavone inhibited cell proliferation. In particular, anti-proliferative effects of isoflavone were observed in Ishikawa cells. On the other hand, isoflavone did not exert similar effects on HEC-1 cells. ERα is the primary receptor involved in the proliferation of Ishikawa cells in response to E2[20]. In the present study, we found that E2 treatment significantly increased cell proliferation in Ishikawa cells compared with control cells.

Consistent with other studies, Ishikawa cells expressed ERα and ERβ, whereas HEC-1 cells only expressed ERβ, which was verified by protein expression analyses. In experiments using prostate and ovarian cancer cells, pretreatment with isoflavone inhibited STAT3 activation[21-22]. Our study confirmed the downregulation of STAT3 expression by isoflavone in endometrial cancer cells. In the present study, isoflavone decreased the mRNA expression levels of STAT3 in endometrial epithelial cells. Ishikawa cells treated with isoflavone showed decreased protein level of STAT3 and p-STAT3. In contrast, HEC-1 cells treated with isoflavone did not

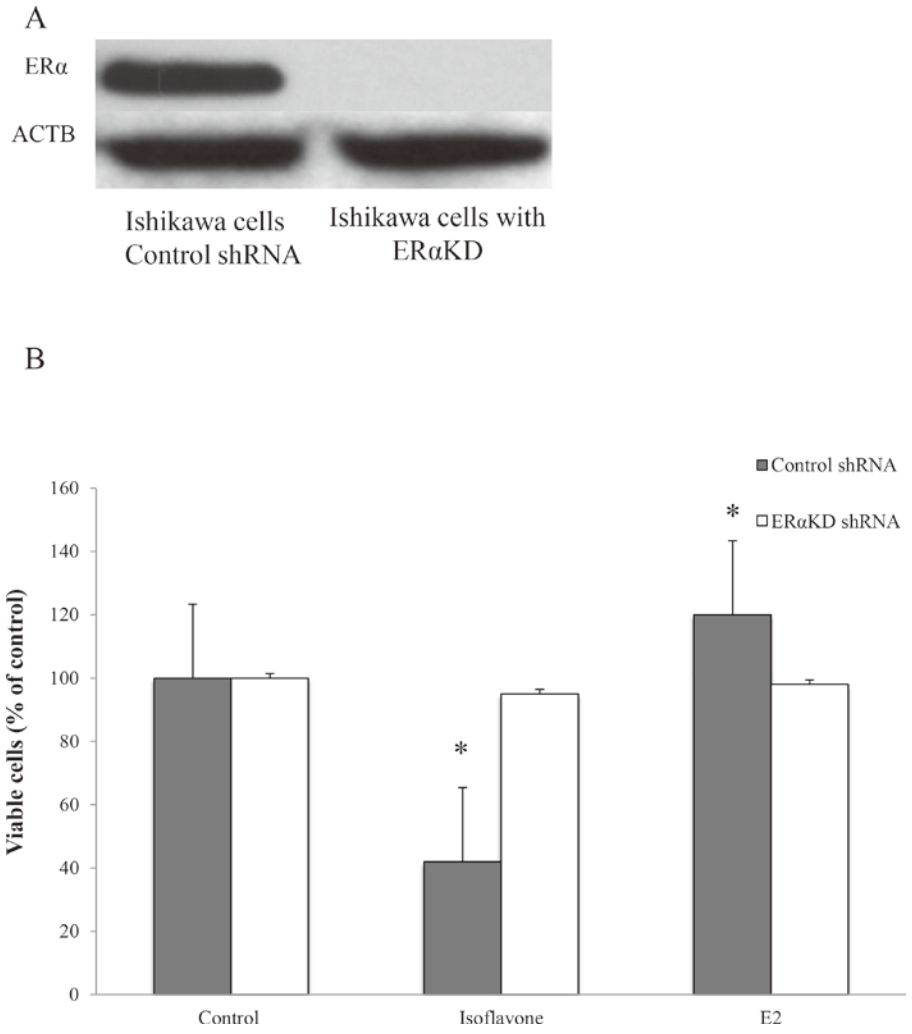

Fig. 3 Ishikawa cells with ERαKD
(A) Western blot analysis of ERα protein levels in ERαKD shRNA cells compared to control shRNA cells. (B) ERαKD shRNA and control shRNA cells were treated with Isoflavone (22 μM) and E2 (10 nM) for 96 hours. Isoflavone or E2 treated ERαKD shRNA cells showed statistical significance compared to control shRNA cells. Results are representative of three independent experiments. *$p < 0.05$ compared with control.

result in a significant suppression of STAT3 expression. MCF-7 cells treated with isoflavone were found to show a significant decrease in ERα protein expression[23]. In this study, Ishikawa cells treated with E2 showed an increase in cell growth, protein and mRNA expression of STAT3 respectively.

Previous studies have shown that E2 treatment leads to an increase in the expression levels of p-STAT3 in ERα-positive MCF-7 cells[24]. In this study, Ishikawa and endometrial epithelial cells treated with E2 showed a significant increase in the expression of STAT3 and p-STAT3.

Isoflavone binds to ERs to maintain basal levels of estrogen in the human body and inhibits hormone-associated cancers[25]. Recent studies have targeted the pleiotropic effects of isoflavone on cancer cells through multiple cellular signaling pathways. The results of our study demonstrated the anti-proliferative effects of isoflavone on ERα positive Ishikawa cells. Consistent with our findings, other studies have emphasized that ERα is expressed by 60%-70% of endometrial cancers with a favorable prognosis compared with ERα negative endometrial cancers[26]. ERα is predominantly expressed in both normal and malignant endometrial cancer cells.

In the present study, to clarify whether ERα is essential for the isoflavone-induced anti-proliferative effects of ERα positive cancer cells, as well as to re-analyze the effects of E2 on Ishikawa cells, ERαKD experiments were carried out on Ishikawa cells. We found that anti-proliferative effects of isoflavone and the proliferative effects of E2 were inhibited by ERαKD.

Isoflavone has gained a large amount of attention and is one of the most frequently studied organic compounds. Studies have demonstrated that a high intake of isoflavone is associated with a reduced risk of endometrial cancer[27]. A study published in March 2017

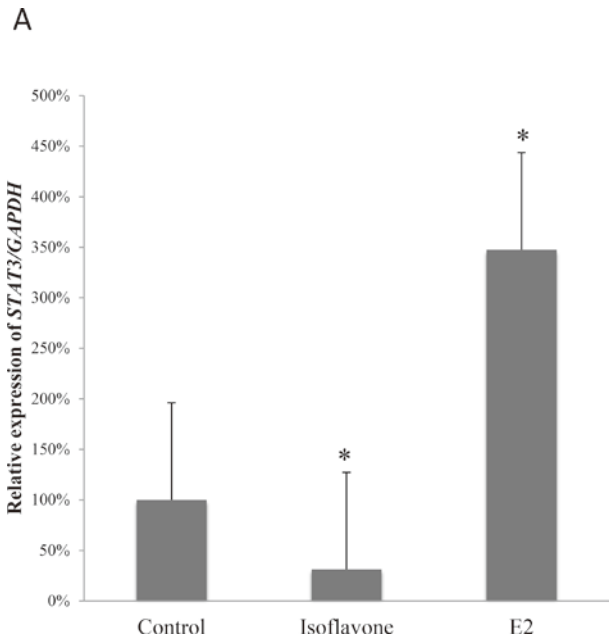

Fig. 4 Effect of isoflavone and E2 on STAT3 mRNA expression levels in endometrial cancer cells derived from human tissues

(A) RT-qPCR analysis of mRNA STAT3 expression levels in endometrial cancer cells derived from human tissues. Cells were treated with isoflavone or E2 for 48 hours. GAPDH was used as an internal control. Endometrial cancer cells treated with isoflavone suppressed while E2 increased the mRNA STAT3 expression levels. Relative gene expression was quantified by StepOnePlus™ Real-Time PCR System Software and results were graphed in percentage. Data are shown as means ± SD, n=3.

*$p < 0.05$ compared with control.

concluded that high dietary intake of isoflavone among breast cancer survivor's reduced the mortality rate[28]. Previous studies have identified STAT3 and ERα as new target oncogenes for cancer therapy[29]. ER directly interacts with stat proteins, and has been suggested to promote the progression of endometrial cancer[30]. Therefore, inhibiting ER may have an inhibitory effect on STAT3 expression. Chemotherapeutic agents that inhibit ERα may suppress activated STAT3 and hence prevent carcinogenesis. Many studies have shown that estrogen promotes cancer cell proliferation[31]. The overexpression of ER was found in breast cancer, and as E2 binds to ER, this results in the proliferation of malignant mammary cells[32]. Similarly, estrogen acts as an agonist in endometrial cancer cell lines. Estrogen stimulates ER to activate the JAK/STAT3 pathway and express mutated cancer genes[31]. Thus, if isoflavone competes with E2 at ER binding sites, this should inhibit the JAK/STAT3 pathway. Therefore, it is possible that isoflavone suppresses STAT3 expression by its interaction with ERα.

This study has several limitations. As primary epithelial cells fall into senescence within a week, and epithelial cell samples were limited because of the small numbers of patients, we were unable to perform cell proliferation and protein studies on primary epithelial cells.

In conclusion, the findings of our study confirm that isoflavone inhibits STAT3 expression in human endometrial cancer cells. The use of safe dietary agents, such as isoflavone, to inhibit endometrial cancer cells is a promising novel approach to design alternative clinical strategies for endometrial cancer treatment.

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イソフラボンは子宮内膜癌細胞においてSTAT3の発現を抑制し、細胞増殖を阻害する

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【要旨】 活性型 signal transducer and activator of transcription 3（STAT3）はがん原因子として知られ、がん治療のための有力な標的である。植物性エストロゲンの一つイソフラボンは、これまで長きにわたりその抗がん効果が研究されてきた。そこで本研究では、子宮内膜がん細胞の増殖および STAT3 発現に対するイソフラボンの抑制効果を検討した。Estrogen receptor alpha（ERα）と ERβ の両方を発現する Ishikawa 細胞株と ERβ のみ発現する HEC-1 細胞株の二つの樹立された子宮内膜がん細胞株、および 12 人の子宮内膜がん患者より採取した子宮内膜上皮細胞をイソフラボン（22 μM）および 17βestradiol（E2、10 nM）で処理し、細胞増殖および STAT3、ERα、ERβ の発現を解析した。Ishikawa 細胞株において、イソフラボン処理は細胞増殖（76.36%）および STAT3（23%）とリン酸化 STAT3（42%）の発現を有意に減少させた。一方、HEC-1 細胞株ではイソフラボンによる抑制効果は認められなかった。E2 処理は Ishikawa 細胞の細胞増殖とリン酸化 STAT3 の発現を有意に増加させた。Ishikawa 細胞株は HEC-1 細胞で発現していない ERα を発現していることから、ERα シグナル経路がイソフラボンの抑制効果に貢献していると考えられ、そこで ERα のノックダウン実験を行った。ERα ノックダウン Ishikawa 細胞ではイソフラボンおよび E2 処理による細胞増殖が認められなかった。加えて、ヒトより採取した子宮内膜上皮細胞においてもイソフラボン処理による STAT3 の mRNA 発現の減少が認められた。結論として、イソフラボンは子宮内膜がん細胞において STAT3 の発現を抑制し、細胞増殖を阻害することが本研究より明らかとなった。

〈キーワード〉子宮内膜癌細胞、STAT3、イソフラボン、ERα