Effect of isoflavones on breast cancer cell development and their impact on breast cancer treatments

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Abstract

Purpose Epidemiological studies have suggested that intake of soy isoflavones is associated with a reduced risk of development of breast cancer and an improved prognosis in patients with breast cancer. In addition, basic research has demonstrated the antitumor effects of these compounds on breast cancer cell lines. However, the detailed effects of the intake of equol, which is one of the metabolites of the soy isoflavones, are yet to be clarified on the risk of development and recurrence of breast cancer and its interactions with drugs used for treating breast cancer. This study aimed to determine the antitumor effects of equol and investigate the impact of adding equol to therapeutic agents for breast cancer using breast cancer cell lines.

Methods We examined the antitumor effect of equol on breast cancer cell lines using MTS assay. We also studied the combined effect of equol and the existing hormonal or chemotherapeutic agents using combination index. We evaluated the expressions of the related proteins by western blot analysis and correlated the findings with the antitumor effect.

Results Equol showed bi-phasic protumor and antitumor effects; at a low concentration, it promoted the tumor growth in hormone receptor-positive cell lines, whereas antitumor effects were generally observed when an excessive amount of dose unexpected in the blood and the tissue was administered. When used with tamoxifen, equol might have some antagonistic effect, although it depends on equol concentration and the type of cancer cells.

Conclusions We confirmed that equol has dual action, specifically a tumor growth-promoting effect and an antitumor effect. Although the results suggested that equol might exert an antagonistic effect against tamoxifen depending on the concentration, equol did not exert an antagonistic effect on other therapeutic agents.

Keywords: isoflavones, equol, tamoxifen, breast cancer

Introduction

Soy isoflavones have been suggested to be effective in preventing osteoporosis, reducing cardiovascular events, and ameliorating menopausal symptoms such as hot flushes by exerting estrogenic actions [1], and they are currently used as a supplement to relieve these symptoms.

Several meta-analyses of epidemiological studies have suggested that an increased soybean intake is associated with a reduced risk of developing breast cancer [2,3]. A prospective cohort study of Japanese subjects showed that frequent miso soup and isoflavone consumption was associated with a reduced risk of breast cancer [4]. The intake of soy isoflavones may also improve the prognosis of patients with breast cancer [2,5-12].

Isoflavones are hydrolyzed to yield biologically active compounds such as daidzein, genistein, and glycysteine. Among these compounds, daidzein is metabolized to equol by intestinal bacteria, which in turn is absorbed into the body and exerts a biological effect on target organs [13]. However, only 30%-50% of Japanese individuals are reported to harbor equol-producing bacteria [14,15]. The binding strength of soy isoflavone components to the estrogen receptor (ER) ranges from 1/1000 to 1/10,000 compared to that of estradiol, and equol has the highest ER-binding strength among these components [16].

Even though it should be noted that the concentration of equol is pharmacologically higher than that reached in the human body, Charalambous et al. have shown that equol exerts antitumor effects on

hormone receptor (HR)-positive breast cancer cell lines in basic *in vitro* research [17]. Because of its anti-estrogenic activity, equol is expected to act as a selective estrogen receptor modulator (SERM), that is, equol is similar to tamoxifen, which is an existing anti-estrogenic agent used in treating breast cancer. In addition, Shi et al. showed that equol exerted antitumor effects even on HR-negative breast cancer cell lines and that it induced apoptosis by inhibiting the expression of nuclear factor-kappaB [18]. However, the effects of equol and the underlying mechanisms are yet to be clarified in detail.

Some individuals, including patients receiving breast cancer treatments, may take equol to achieve estrogenic effects; nevertheless, the safety of equol and its associated risk of breast cancer recurrence remain unclear. Because tamoxifen and chemotherapy antagonize each other's effects [19], they are not used concurrently in clinical practice. If equol is also a SERM, it may antagonize the effects of breast cancer drugs. Therefore, it is of importance to investigate the effects and safety of combining the intake of equol with existing treatments for breast cancer because no such detailed studies have been conducted to date.

In the current study, we investigated the antitumor effects of equol on breast cancer cell lines and examined the effects of combining equol with existing hormonal or chemotherapeutic agents used in treating breast cancer.

Materials and Methods

Cell culture and agents

MCF-7, T-47D, ZR-75-1, and MDA-MB-231 cell lines were obtained from ATCC (Rockville, MD, USA), and Drs. Koki Tsuboi and Shin-ichi Hayashi (Tohoku University, Sendai, Miyagi, Japan) kindly provided the aromatase-overexpressing MCF-7 cell line (E10arom) for this study [20]. All of the cell lines were maintained in RPMI-1640 (Thermo Fisher Scientific, Waltham, MA, USA) medium supplemented with 10% fetal calf serum (FCS), and cultured in a humidified incubator with 5% CO_2 at 37°C. (S)-Equol (Cayman Chemical, Ann Arbor, MI, USA), 17-β-estradiol (E2), testosterone, 4-hydroxytamoxifen, fulvestrant, letrozole, paclitaxel, doxorubicin and 5-fluorouracil (Sigma-Aldrich, St. Louis, MO, USA) were obtained from commercial sources. In experiments to verify the effect of equol alone, MCF-7, T-47D, ZR-75-1, and E10arom cells were cultured in a phenol red-free medium, which was estrogen-deprived medium, containing 10% dextran-coated, charcoal-treated FCS (DCC-FCS) (Hyclone, Logan, UT, USA).

Cell viability assay

The cell proliferative ability was determined by a modified MTS assay. Cells were seeded in 96-well plates at cell density of 3×10^3-5×10^3 cells/well. After 24-hours' incubation, the cells were cultivated in the presence of various concentrations of equol alone, an anti-estrogenic drug (4-hydroxytamoxifen or fulvestrant) alone, a chemotherapeutic agent (doxorubicin, paclitaxel, or 5-fluorouracil) alone, or of these agents in various combinations, for 72 hours at 37°C in a 5% CO_2 atmosphere. The MTS assay was

performed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Fitchburg, WI, USA), in accordance with the manufacturer's protocol. The anti-proliferative effects of the agents were expressed in terms of the 50% inhibitory concentration (IC_{50}). The assays were carried out independently in duplicate to quadruplicate, and the mean IC_{50} values were determined.

Cell proliferation assays, using equol at low concentrations, were used to determine the effects of equol as a growth-promoting agent. MCF-7, T-47D, and ZR-75-1 cells were cultured for three days in a phenol red-free medium containing 10% DCC-FCS and transferred to 6-well plates (1×10^4-2×10^4 cells/well) with equol, 4-hydroxytamoxifen or fulvestrant, with or without E2 10 nM. E10arom cells were also cultured and transferred to 6-well plates with medium containing 10% DCC-FCS and equol, 4-hydroxytamoxifen, fulvestrant or letrozole with testosterone at a concentration of 10 nM. Cells were harvested on day 5 after addition of the test compounds and were counted using a micro cell counter (Sysmex, Hyogo, Japan). All assays were carried out in duplicate to quadruplicate.

Western blot analysis

Samples for western blot analysis were prepared from the cultured cells (MCF-7, T-47D, ZR-75-1, and MDA-MB-231) [21,22]. The primary antibodies were used as follows: antibodies to ERα, and CyclinD1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), progesterone receptor (PgR) (Abnova), and β-actin (used as the loading control) (Merck Millipore, Billerica, MA, USA). Anti-mouse immunoglobulin G (IgG)-conjugated horseradish peroxidase (Santa Cruz Biotechnology) was used as the secondary antibody.

To detect specific signals, the membranes were examined using ECL Prime Western Blotting Detection Reagent (GE Healthcare, Amersham, UK) and by scanning them in the LAS-3000 imager (Fujifilm, Tokyo, Japan).

Effects of drug combinations

The combined effect of two drugs was evaluated based on the combination index (CI), as described previously [19]. It was calculated using the Calcusyn software (Biosoft, Cambridge, UK), as follows:

CI=(D)1/(Dx)1+(D)2/(Dx)2+(D)1(D)2/(Dx)1(Dx)2,

where: (Dx)1 is the dose of Drug 1 alone required to produce an X% effect, (D)1 is the dose of Drug 1 required to produce the same X% effect in combination with Drug 2, (Dx)2 is the dose of Drug 2 alone required to produce an X% effect, and (D)2 is the dose of Drug 2 required to produce the same X% effect in combination with Drug 1. The effects of a drug combination were defined as follows: CI < 1, synergistic effect; CI = 1, additive effect; CI > 1, antagonistic effect.

The MTS assay was used to examine the effects of equol combined with each of the existing drugs, at a fixed concentration, using MCF-7, T-47D, and ZR-75-1 cells. Cells were seeded on to 96-well plates at a cell density of 3×10^3-5×10^3 cells/well. After 24-hours' incubation, the cells were treated for 72 hours with equol (0-10 μM) plus a hormone drug (4-hydroxytamoxifen or fulvestrant) or a chemotherapeutic agent (doxorubicin, paclitaxel, or 5-fluorouracil), at one of two concentrations (0.1 μM or 1 μM).

Statistical analysis

All experiments were performed in duplicate to quadruplicate. Values are presented as the means ± SEM. Statistical significance was evaluated using student's t-test for paired comparisons. $P < 0.05$ was considered as indicative of a statistically significant difference. In the cell proliferation assays with a single-agent, control was set as the state in which equol, 4-hydroxytamoxifen or fulvestrant were not administered, and the cells exposed to each agent at the respective concentrations were compared to control. In the combination experiments, the state of each drug without equol (control) was compared to the state with respective concentrations of equol.

Results

Effects of equol on breast cancer cell lines

The expression status of ER/PgR in HR-positive cell lines (MCF-7, T-47D, and ZR-75-1) and HR-negative cell line (MDA-MB-231) used in this study is shown in Fig. 1a. The antitumor effect of equol in each cell line was examined by cell viability assay (Fig. 1b) and the IC_{50} values are shown in Table 1, ranging from 12 to 22 μM, which are unable to achieve in blood or in tissue pharmacologically. Regardless of the expression of HR, antitumor effect was observed at 40 μM of equol in any cell lines. In contrast, low concentration of equol (0.625 μM) promote cell proliferation in T-47D cell (Fig. 1b). Therefore, equol concentration-dependent changes in estrogenic and anti-estrogenic activities were examined in detail. HR-positive cell lines were treated with estrogen-deprived medium that was or was

not supplemented with 17β estradiol (E2) plus equol, 4-hydroxytamoxifen (4-OH-TAM), or fulvestrant. The target concentration was determined based on the IC_{50} value of equol and drugs. Although 4-OH-TAM and fulvestrant inhibited the growth of MCF-7, T-47D, and ZR-75-1 cells (Supplementary Fig. 1a-c), equol induced cell proliferation at low concentrations, under estrogen-deprived conditions in the MCF-7 and ZR-75-1 cell lines. The T-47D cell line produced similar results, although there was no significant difference (Fig. 1c-e).

We performed western blot analyses to investigate changes in the ER signaling pathway (mediated by PgR and CyclinD1 proteins) resulting from differences in equol concentration. At a low concentration of equol, these proteins were expressed at a rate similar to proteins induced by E2, and estrogenic activity was observed. Conversely, at high concentrations, expression levels of proteins of the ER pathway were suppressed (Fig. 2a-c).

Effects of combining equol with hormone drugs

The combination index (CI) was calculated and evaluated to determine the effects of equol combined with therapeutic agents. The molar ratios for combining equol with the drugs were determined based on the individual IC_{50} values (Table 2a). The CI at 50% and 75% effective doses showed an antagonistic effect on cell lines treated with the combination of equol and 4-OH-TAM. However, either the antagonistic or synergistic effect was observed when equol was combined with fulvestrant depending on the effective dose and the cell lines used (Table 2b). Furthermore, to simulate clinical conditions, the concentrations of

4-OH-TAM or fulvestrant were kept constant, while the concentration of equol added was changed (Fig. 3a-d and Supplementary Fig. 2a-b). The target concentration was determined based on the IC_{50} value. Under these conditions, these drugs exerted tumor growth-inhibitory effects as single agents. Therefore, the highest concentration was set as 1 μM, which corresponded to about 1/10 of IC_{50}, and the lowest concentration was set at 0.1 μM (1/10 of the highest concentration) for this investigation. In MCF-7 and ZR-75-1, the tumor growth effect was observed at a low concentration of 4-OH-TAM (0.1 μM) and at a low concentration of equol compared with the treatment with 4-OH-TAM alone. In contrast, the addition of 10 μM equol on 4-OH-TAM significantly suppressed the tumor growth (Fig. 3a and 3b). Regarding the interaction with fulvestrant, no significant antagonistic effect of equol was observed with this combination at either low or high concentrations, and when the addition of 10 μM equol on fulvestrant, the tumor suppressive effect was significantly greater than that obtained with either drug alone (Fig. 3c and 3d). Similar examinations were performed with T-47D cell lines. T-47D showed no antagonism by the combined equol with 4-OH-TAM (Supplementary Fig. 2a), and fulvestrant (Supplementary Fig. 2b). The western blot analyses showed that when equol was used in combination with 4-OH-TAM, the expression of ER decreased in 3 ER-positive cell lines, as did the expression of PgR in MCF-7(Fig. 4a) and ZR-75-1 (Fig.4b) cell lines. CyclinD1 was down-regulated in MCF-7 (Fig.4a). However, these changes were not observed in T-47D cells (Supplementary Fig. 3a). Fulvestrant itself caused ER down-regulation, and when equol was combined with fulvestrant, additional down-regulation was

achieved in MCF-7, ZR-75-1, and T-47D (Fig. 4c and 4d, Supplementary Fig. 3b).

Effects of combining equol with an aromatase inhibitor

To investigate the effects of equol in the postmenopausal hormonal environment, aromatase-expressing cell lines were used. Table 3a shows the molar ratios at which equol and letrozole were combined. When equol was combined with letrozole, the CI at 50% and 75% effective doses showed a synergistic effect in E10arom, which is an aromatase-overexpressing MCF-7 cell line (Table 3b). Furthermore, when the combined effects of equol and letrozole at constant concentrations were examined, no antagonism of the antitumor effect of letrozole was observed, regardless of the equol concentration (Fig. 5).

Effects of combining equol with chemotherapeutic agents

Among chemotherapeutic agents, evaluations based on the CI suggest that these drugs and equol may have inhibitory effect, depending on the effective dose and cell lines used (Table 4b). Equol did not inhibit chemotherapeutic agents when it was combined with drugs at fixed concentrations; in fact, an enhancing effect of adding equol was noted, especially when chemotherapeutic agents were used at a high concentration (Fig. 6a-c, Supplementary Fig. a-f).

Discussion

In some patients with breast cancer who are receiving postoperative therapy, the quality of life may deteriorate and the patients may not be able to continue with these therapy because of side effects such as

hot flashes, and joint and muscle pain caused by the anti-estrogenic effects of the drugs. Equol improves menopausal symptoms and may be able to prevent treatment interruption and maximize the benefits of treatment by controlling side effects associated with insufficient estrogen. Nevertheless, the effects of equol on the risk of development and recurrence of breast cancer, as well as its effect on breast cancer treatment outcomes, are yet to be elucidated to date. The current study examined the effects of equol on breast cancer and its treatment.

In the current study, although the results were somewhat different depending on the cell lines used, low concentrations of equol were generally associated with the cell proliferation of breast cancer, which may have occurred because of the estrogenic effects through ERα, similar to E2. Concurrently, as its concentration increased, equol showed an antitumor effect by downregulating ERα, a mechanism similar to the one underlying the effect of fulvestrant. Moreover, equol has been previously reported to exert ERβ-mediated antitumor effects [23-27]. ERβ inhibits cell proliferation, and patients with breast cancer and high ERβ expression have better survival than patients without ERβ expression [28]. These observations suggest that equol exerts antitumor effects through ERα and other targets in HR-positive and -negative breast cancer cell lines.

At high equol concentrations, ERα was down-regulated even in combination with tamoxifen, and an antitumor effect was observed. It is also reported that equol enhanced the apoptotic effect of tamoxifen [17]. Whereas at low concentrations, our data indicated that equol competed with tamoxifen for binding

to ERα, resulting in the inhibition of the antitumor effects of tamoxifen. Equol-tamoxifen interactions varied depending on equol concentration, with low equol concentration appearing as a cause for concern. Overall, these results indicate that the use of equol during hormone therapy for breast cancer requires caution, and further studies are needed. In contrast, equol did not show any antagonistic effect on fulvestrant at any concentration; moreover, the actions of fulvestrant are mediated by ERβ, and equol may enhance the phosphorylation of ERβ [29]. In the presence of high levels of aromatase expression, equol exerted no inhibitory effect, even when combined with an aromatase inhibitor, suggesting that equol is safe for patients with breast cancer and menopausal symptoms. In addition, our findings suggest that equol does not inhibit the activity of chemotherapeutic drugs, despite an expectation of antagonistic effects with equol used in combination with these drugs.

The blood concentration of equol was reported to peak at 1 μM within 1 hour after ingesting 10 mg of equol-containing food, indicating that some patients possibly are exposed to low concentrations of equol [30-32]. Furthermore, it is difficult to maintain a constant daily concentration of equol in the body because equol concentration depends on isoflavone intake and the state of intestinal bacteria, which is vulnerable to the host's physical condition and the use of antibiotics and other drugs. Equol might have different effects at different concentration, suggesting that variations in equol body concentration should be considered in clinical practice. It should be noted that the antitumor effect observed in the current study resulted from overdose of equol in the human body. In addition, drugs may have an off-target effect,

which is a non-specific effect caused by an action on a site other than the original target site. Thus, it is necessary to carefully verify whether the antitumor effect observed *in vitro* at high concentration of equol shows the same result *in vivo* and in the human body.

In conclusion, we demonstrated that equol might show bi-phasic protumor and antitumor effects. Moreover, equol might exert an antagonistic effect against tamoxifen at its specific concentrations and depending on the cell lines used. Notwithstanding these effects, equol might to be able to be used in breast cancer treatment, provided it is used at suitable concentrations and in combinations with the drugs with which it does not exert antagonistic effects. Further preclinical and clinical research is needed to establish how best to use equol in clinical situations.

Acknowledgements This work was supported by a Management Expenses Grants for National University Corporations in Japan. We thank Dr. Koki Tsuboi and Dr. Shin-ichi Hayashi (Department of Molecular and Functional Dynamics, Graduate School of Medicine, Tohoku University, Sendai, Miyagi, Japan) for providing us with the aromatase-overexpressing MCF-7 cell line (E10arom) for this study.

Compliance with Ethical Standards

Conflicts of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with

human participants or animals performed by any of the authors.

Informed consent Informed consent was not obtained because this study included only data of preclinical studies using cell lines and no personal data or information.

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Figure legends

Fig. 1 Effects of equol on breast cancer cell lines

(a) The expressions of ER and PgR in hormone receptor (HR)-positive cell lines (MCF-7, T-47D, and ZR-75-1) and HR-negative cell line (MDA-MB-231) were evaluated by western blot analysis. (b) Cell viability was evaluated using the MTS assay in the MCF-7, T-47D, ZR-75-1, and MDA-MB-231 cell lines maintained in RPMI-1640. Each cell line (3×10^3-5×10^3 cells/well) was seeded in 96-well plates and treated with different concentrations of equol. After 72 h, the cell viability was evaluated using the MTS assay. Bars correspond to the standard error of the mean (SEM). (c-e) ER-positive MCF-7, T-47D, and ZR-75-1 cells (1×10^4-2×10^4 cells/well) were plated in 6-well plates and treated with estrogen-deprived medium that was or was not supplemented with 10 nM of 17β estradiol (E2) plus 0-20 μM equol (Eq) for 5 days. Cell viability was determined using a micro cell counter. Each dataset was compared to the state without drugs. $*p < 0.05$ for comparison between experimental (with equol) and control (without equol) conditions with uncontained E2, and $**p < 0.05$ for comparison between experimental (with equol) and control (without equol) conditions with contained E2. Error bars correspond to SEM.

Fig. 2 Effects of different equol concentrations on ERα and signal transduction downstream of ERα

MCF-7, T-47D, and ZR-75-1 cells were treated with equol (0.1-20 μM) for 48 h in the presence or absence of E2 at 10 nM. Protein expressions were analyzed by western blot analysis.

Fig. 3 Effects of combining equol with hormone therapeutic agents

The effects of combining equol with each of the drugs at a fixed concentration were evaluated using the MTS assay in MCF-7 (a,c) and ZR-75-1(b,d) cells. Cells were seeded in 96-well plates at a cell density of 3×10^3-5×10^3 cells/well. After incubation for 24 h, the cells were treated with equol (0-10 μM) and a hormone drug [4-hydroxytamoxifen (TAM) (a,b)] or fulvestrant (FUL (c,d)] at one of the two fixed concentrations (0.1 μM or 1 μM) for 72 h. *$p < 0.05$ for comparison between experimental (TAM or FUL of 0.1 μM with equol) and control (TAM or FUL of 0.1 μM only) conditions, and **$p < 0.05$ for comparison between experimental (TAM or FUL of 1 μM) and control (TAM or FUL of 1 μM only) conditions with contained E2. Error bars correspond to SEM**.**

Fig. 4 Effect of equol and 4-hydroxytamoxifen and fulvestrant on ERα and signal transduction downstream of ERα

MCF-7 and ZR-75-1 cells were treated for 48 h with equol (0.05-20 μM) and a hormone drug (4-hydroxytamoxifen [TAM] [a,b] or fulvestrant [FUL] [b,c]) in the presence of E2 at 10 nM. Protein expressions were then analyzed using western blots.

Fig. 5 Effects of combining equol with an aromatase inhibitor

The effects of combining equol (0-10 μM) with letrozole (LET) at a fixed concentration (0.1 μM or 1 μM) were evaluated using the MTS assay and E10arom. Error bars correspond to the SEM.

Fig. 6 Effects of combining equol with chemotherapeutic agents

The effects of combining equol with each of the chemotherapeutic drugs at a fixed concentration were evaluated using the MTS assay and MCF-7 cells. The cells were treated with equol (0-10 μM) plus a chemotherapeutic agent (doxorubicin [DXR] [a], paclitaxel [PTX] [b], or 5-fluorouracil [5-FU] [c]) at one of the two fixed concentrations (0.1 μM or 1 μM). *$p < 0.05$ for comparison between experimental (DXR, PTX, or 5-FU of 0.1 μM with equol) and control (DXR, PTX, or 5-FU of 0.1 μM only) conditions, and **$p < 0.05$ for comparison between experimental (DXR, PTX, or 5-FU of 1 μM) and control (DXR, PTX, or 5-FU of 1 μM only) conditions with contained E2. Error bars correspond to SEM.