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Retinoic acid dramatically enhances cytotoxicity of equol against human monoblastic U937 cells, but not against human peripheral neutrophils

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ABSTRACT — Since phytoestrogens (e.g. biochanin A, coumestrol, daidzein, genistein and glycitein) are structurally similar to estrogen, they mimic estrogen function. Phytoestrogens consist in certain edible plants, especially in soybeans. Among them, interestingly, equol that has the greatest estrogenic activity is generated from daidzein by gut microbes. Therefore, the relationship between phytoestrogens and gut (including intestinal cells and bacteria) is being watched with strong interest. In this paper, we revealed that all-trans retinoic acid (RA) dramatically enhances cytotoxicity of several phytoestrogens against U937 cells. While β-estradiol and phytoestrogens tested showed no effect on the viability of U937 cells in the absence of RA, 10 μM coumestrol, (±)-equol and genistein brought about remarkably reduced viability of U937 cells at 24 h (to ~15%, ~7% and ~35%, respectively) in the presence of 1 μM RA. In particular, the cytotoxicity of (±)-equol was drastically enhanced in the presence of RA. Moreover, very interestingly, (±)-equol showed no effect on the viability of human peripheral neutrophils even in the presence of RA. As is well known, human monoblastic leukemia U937 cells have been used as an in vitro model for macrophage that exists in intestine and plays significant roles to maintain intestinal homeostasis. These data suggest that equol not only can serve as an effective modifier in therapy for leukemia in combination with RA but also may affect the maintenance of intestinal homeostasis.

Key words: Phytoestrogen, Equol, Cytotoxicity, Macrophage, Neutrophil, U937

INTRODUCTION

Polyphenol compounds are secondary metabolites in plants, and protect them from various harms such as ultraviolet irradiation and numerous infections. They are contained in usual human diet and show various biological activities including nutritional effects. Therefore, polyphenols are increasingly gaining considerable attention for maintaining human health. Phytoestrogens belonging to plants-derived polyphenol compounds not only are structurally similar to β-estradiol (a typical estrogen) but also mimic its biological function (Schmitt et al., 2003; Desmawati and Sulastri, 2019; Kiyama, 2019; Hasan et al., 2020). As is well known, phytoestrogens (e.g. biochanin A, coumestrol, daidzein, genistein and glycitein) consist in certain edible plants, above all abundantly in...
soy (Jargin, 2014). In addition, equol is generated from daidzein by gut microbes (Rafii, 2015; Mayo et al., 2019). They show agonist or antagonist actions on estrogen receptor-mediated processes dependent on the cells or tissues. For example, phytoestrogens are known to modulate multiple target molecules in breast cancer cells (Basu and Maier, 2018; Molina et al., 2018). In addition, phytoestrogens have also effects on anti-aging (Liu et al., 2020), immunomodulation (Soria-Jasso et al., 2019), developing brain (Rosenfeld, 2019), colorectal neoproliferative lesions (Viggiani et al., 2019), vaginal health and dyspareunia (Dizavandi et al., 2019), intestinal microbiome (Kolatorova et al., 2018; Rosenfeld, 2019), urinary incontinence, pelvic organ prolapse and fecal incontinence (Cardenas-Towers et al., 2018). While genistein, one of phytoestrogens, induces apoptotic cell death in various types of leukemia (Yamasaki et al., 2010, 2013; Li et al., 2011; Hsiao et al., 2019), it also can induce infant leukemia cell death (Azarova et al., 2010).

As mentioned above, intestinal bacteria converts daidzein to equol (Rafii, 2015; Mayo et al., 2019). Although equol is the phytoestrogen with the greatest estrogenic activity (Mayo et al., 2019), only about 30 ~ 50% of human can produce equol in the intestine (Rafii, 2015). Equol has great influences on atherosclerosis, arterial stiffness, decline/dementia (Sekikawa et al., 2019), developing brain, gut microbiota, neurobehavioral disorders (Rosenfeld, 2019). However, the understanding of the effects of equol against leukemia cells is still poor.

In this paper, we investigated the effects of β-estradiol and various phytoestrogens on viability of human monoblastic U937 cells, and revealed that all-trans retinoic acid (RA) dramatically enhances cytotoxicity of several phytoestrogens against the cells.

MATERIALS AND METHODS

Materials

Biochanin A, (±)-equol and genistein (Tokyo Chemical Industry, Tokyo, Japan), daidzein, β-estradiol and glycitein (Fujifilm Wako, Osaka, Japan), coumestrol (Cayman Chemical, Ann Arbor, MI, USA), RPMI-1640 culture medium and trypan blue solution (Gibco Laboratories, Gaithersburg, MD, USA), RA (Sigma, St Louis, MO, USA), fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA) and plasmocin (InvivoGene, San Diego, CA, USA) were obtained from companies indicated respectively.

Cell culture and treatment with various reagents

Human monoblastic leukemia U937 cells (RCB0435) were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Cells were grown in RPMI-1640 culture medium containing 10% FBS and 5 μg/mL plasmocin as described (Kikuchi and Imajoh-Ohmi, 1995; Kikuchi et al., 1996, 2011, 2018, 2019; Akiyoshi et al., 2019). Cells (1.0 x 10⁶) in 5 mL of culture medium were incubated in the presence of 10 μM various reagents (biochanin A, coumestrol, daidzein, (±)-equol, β-estradiol, genistein or glycitein) and 1 μM RA for 24 hr. Concerning (±)-equol, cells (1.0 x 10⁶) in 5 mL of culture medium were also incubated with 1 ~ 5 μM (±)-equol in the presence of 1 μM RA at 37°C for up to 48 hr. A hemocytometer was used to count total cells under the microscope. Viable cells were counted by the trypan blue dye exclusion method (Kikuchi et al., 2016).

Peripheral neutrophils

Human neutrophils were prepared from peripheral blood of a healthy volunteer using Lymphoprep (Stemcell Technologies, Vancouver, Canada) followed by hypotonic treatment. Human neutrophils (1.0 x 10⁶ cells/mL) were suspended in PBS containing 10% fetal calf serum (FCS) and 1 mM EDTA. In the absence or presence of 1 μM RA, human neutrophils (1.0 x 10⁶ cells/mL) were cultured with 5 μM daidzein or 5 μM (±)-equol in the same buffer (PBS/10% FCS/1 mM EDTA) at 37°C for 24 hr. A hemocytometer was used to count total cells under the microscope. Viable cells were counted by the trypan blue dye exclusion method (Kikuchi et al., 2016).

Statistical analysis

Data obtained with cell viability assays are presented as averages of four (U937 cells) or eight (neutrophils) separate experiments. Error bars indicate standard deviation. Statistical differences were calculated with Student’s t test.

Ethical considerations

This experiment complies with the Declaration of Helsinki (adopted in 1964 and amended in 2013) and ethical approval for this study was obtained from the Research Ethics Committee of Kawasaki Medical School and Hospital (Approval Number: 3615).

RESULTS AND DISCUSSION

First, in order to study the effects of β-estradiol and various phytoestrogens (biochanin A, coumestrol, daidzein, (±)-equol, genistein and glycitein Fig. 1) on viability of U937 cells, the cells were treated with 10 μM
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**Fig. 1.** Chemical structures of estrogen and phytoestrogens.

**Fig. 2.** Effects of RA on cytotoxicity of estrogen and phytoestrogens against U937 cells. (A) Cytotoxicity of β-estradiol and phytoestrogens in the absence of RA. Cells (1.0 x 10⁶) in 5 mL of culture medium were incubated with 10 μM of each reagent at 37°C for 24 hr. Viable cells were counted by the trypan blue dye exclusion method. Data represent the average of four separate experiments. Statistical differences were calculated using Student’s t test. Error bars indicate standard deviation. (B) Cytotoxicity of β-estradiol and phytoestrogens in the presence of RA. Cells (1.0 x 10⁶) in 5 mL of culture medium were incubated with 10 μM of each reagent in the presence of 1 μM RA at 37°C for 24 hr. Viable cells were counted by the trypan blue dye exclusion method. Data represent the average of four separate experiments. Statistical differences were calculated using Student’s t test. Error bars indicate standard deviation. **, p < 0.01 compared with the data without β-estradiol or phytoestrogens (referred to as “None”).

of each reagent. β-Estradiol and phytoestrogens tested showed no effect on the viability of U937 cells in the absence of RA at 24 hr (Fig. 2A). On the other hand, coumestrol, (±)-equol and genistein brought about remarkably reduced viability of U937 cells (to ~15%, ~7% and ~35%, respectively) in the presence of RA (Fig. 2B). In contrast, β-estradiol and other phytoestrogens used showed no effect on the viability of U937 cells even in the presence of RA (Fig. 2B).

Second, to investigate the influences of these three
phytoestrogens on cytotoxicity in the presence of RA in more detail, we examined the dose-dependency of them on viability. As shown in Fig. 3, in the presence of RA, three phytoestrogen tested showed remarkable cytotoxicity in a dose-dependent manner. In particular, the cytotoxicity of (±)-equol was drastically enhanced in the presence of RA (Fig. 3B). While daidzein showed little cytotoxicity with or without RA, interestingly, (±)-equol generated from daidzein displayed very strong cytotoxicity in the presence of RA. As is well known, (±)-equol is generated from daidzein by intestinal bacteria. These findings suggested that conversion from daidzein to (±)-equol by intestinal bacteria brings about drastic strong cytotoxicity against monoblastic U937 cells that have been used as an in vitro model for macrophage in the presence of RA.

In other words, intestinal bacteria can affect the intestinal cells including macrophages through conversion from daidzein to (±)-equol.

Finally, to examine the effects of equol on cytotoxicity against normal cells (not leukemia cells) in the presence of RA, human peripheral neutrophils were incubated with 5 μM daidzein or 5 μM (±)-equol in the presence of 1 μM RA for 24 hr. Viable cells were counted by the trypan blue dye exclusion method. Data represent the average of eight separate experiments. Statistical differences were calculated using Student’s t test. Error bars indicate standard deviation. Either with or without RA, there were no significant differences between without daidzein or (±)-equol (referred to as “None”) and with daidzein or (±)-equol.

Our results revealed that RA dramatically enhances...
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cytotoxicity of some phytoestrogens, especially (±)-equol, against human monoblastic leukemia U937 cells that have been used as an in vitro model for macrophage. As is well known, macrophages exist in intestine and play significant roles to maintain intestinal homeostasis (Bain and Schridde, 2018). These findings suggested that conversion from daidzein to equol may affect the intestinal homeostasis. Moreover, these results in this paper propose that combination of RA and (±)-equol has the potential to serve as an effective tool in therapy for leukemia. However, further studies are needed before practical application as therapy for leukemia. Studies on cytotoxicity of phytoestrogens may be more important for both maintenance of intestinal homeostasis and development of tumor treatment in the future.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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