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Han, Dal-Ho
Laboratory of Food Chemistry, Department of Bioscience and Biotechnology, Division of Bioresource and Bioenvironmental Science, Graduate School, Kyushu University

Miyazaki, Yoshiyuki
Laboratory of Food Chemistry, Department of Bioscience and Biotechnology, Division of Bioresource and Bioenvironmental Science, Graduate School, Kyushu University

Tachibana, Hirofumi
Laboratory of Food Chemistry, Department of Bioscience and Biotechnology, Division of Bioresource and Bioenvironmental Science, Graduate School, Kyushu University

Yamada, Koji
CREST, Japan Science and Technology | Laboratory of Food Chemistry, Department of Bioscience and Biotechnology, Division of Bioresource and Bioenvironmental Science, Graduate School, Kyushu University

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Optimization of Estrogenic Activity Detection Method Using Human Breast Cancer MCF–7 Cells

Dal–Ho Han[1], Yoshiyuki Miyazaki[1], Hirofumi Tachibana[1] and Koji Yamada[*1, 2]

[1] Laboratory of Food Chemistry, Department of Bioscience and Biotechnology, Division of Bioresource and Bioenvironmental Science, Graduate School, Kyushu University Fukuoka 812–8581, Japan
[2] CREST, Japan Science and Technology
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We tried to optimize the assay system using human breast cancer MCF–7 cells for detection of estrogenic activity of environmental estrogens. Since fetal bovine serum (FBS) contains various growth factors including estrogen, 100 ml of FBS was treated with 5 g of activated charcoal to remove estrogen. MCF–7 cells were cultured with RPMI 1640 medium supplemented with FBS or charcoal treated FBS (cFBS), in the presence of 17β-estradiol or flavonoids. Then, the cells were trypsinized and cell number was counted using a Coulter Counter. When the cells were cultured in the medium containing 0 to 5% FBS, proliferation-stimulating activity of daidzein and genistein was detected most strongly in the medium containing 1% FBS. In the case cFBS–supplemented medium, the effect of flavonoids was detectable, in all cFBS concentrations. The proliferation–stimulating effect of daidzein and genistein was detectable after a 72–hr lag period. In the presence of 1% FBS or 1% cFBS, 17β–estradiol enhanced proliferation of the cells at the concentrations between 10^{-11} and 10^{-8}M, while daidzein and genistein enhanced it only at 10^{-8}M. On the other hand, quercetin and luteolin exerted a proliferation–inhibiting activity against MCF–7 cells at the concentrations over 10^{-10}M. These results indicate that estrogenic activity of flavonoids against MCF–7 cells was detectable in the medium supplemented with 1% FBS or 1 to 5% cFBS. Proliferation–inhibitory activity of quercetin and luteolin suggests that these compounds exert anti–estrogenic and anti–cancer activities.

INTRODUCTION

Reports on environmental estrogens have been widespread in the last few years. Many environmental estrogens and natural compounds have been shown to modify the endocrine system in wildlife animals and even human beings (Guillette, 1995). As endocrine disruptor chemicals, many kinds of chemicals have been targeted for study in hormonally response assays. It has been shown that many natural and synthetic chemicals exhibit estrogenic activity (Sharpe et al., 1993; Safe et al., 1995; Colborn et al., 1993). When we deal with the problems associated with environmental estrogens, we have to pay an attention to flavonoids (Bradbury et al., 1954). Besides environmental estrogens, there are at least 20 types of flavonoids, the substances derived from plant with estrogenic activity (Farnsworth et al., 1975; Cheng et al., 1954). When such a substance is taken by animals, it will influence estrogen synthesis and metabolism, and

* To whom correspondence should be addressed.

may exert estrogenic or anti-estrogenic effects (Stob, 1983). The amount of flavonoids taken from the diet is much larger than the amount of environmental estrogens. In addition to their weak estrogenic and anti-estrogenic activities, they possess a variety of characteristics, such as antioxidative, anti-proliferative, anti-virus, anti-bacteria, and differentiation-inducing activities (Setchell et al., 1985; Price et al., 1985; Kenneth et al., 1998; Leclerg et al., 1979). Thus, they can affect many biological and physiological processes, especially as a candidate for cancer prevention (Adlercrentz et al., 1990). Recently, studies have been carried out on absorption and metabolism of flavonoids in human body as well as on their biological effects (Messina et al., 1991; Steinmetz et al., 1991; Messina et al., 1990; Setchell et al., 1984; Barnes et al., 1990; Hawryewicz et al., 1995).

It is important to establish screening and testing methods to assay for endocrine disrupting activity. However, the assay system for environmental estrogen is few and not so sophisticated. Thus, we tried to optimize the assay system for the detection of estrogenic activity using human breast cancer MCF-7 cells. In addition, we evaluated estrogenic or anti-estrogenic activity of natural estrogens including flavonoids using the optimized assay system.

METERIALS AND METHODS

Chemicals

The steroids, 17β-estradiol and activated charcoal were obtained from Sigma Chemicals (St. Louis, MO). Daidzein, genistein, quercetin and luteolin were obtained from Fujicco Co. (Kyoto, Japan). These chemicals were dissolved in 70% ethanol solution bufferized with a phosphate-buffered saline, pH 7.4 (70% EtOH/PBS). Fatal bovine serum (FBS) was obtained from Bio Whittaker (Maryland, U. S. A) and all other chemicals used were reagent grade.

Charcoal treatment of FBS

To remove endogeneous estrogens, 100 mL of FBS was treated with twice 5 g each of activated charcoal at 55 °C for 30 min. Then, activated charcoal was removed by a centrifugation at 450×g and 4 °C for 20 min and a filtration using cellulose acetate filter (0.45 μm). Then, the charcoal-treated FBS (cFBS) was stored at -20 °C until use.

Cells and cell culture

Human breast cancer MCF-7 cells were grown in RPMI 1640 medium supplemented with N-2-hydroxyethylpiperazine-N' 2 ethanesulfonic acid (HEPES, 2.3 mg/mL), penicillin G (100 unit/mL), streptomycin (0.1 mg/mL), sodium bicarbonate (2.08 mg/mL) and 5% FBS. The cells were cultured using 100 mm plastic tissue culture dishes (Nunc Roskilde, Denmark) at 37 °C with 5% CO_2 in a humidified incubator.

MCF-7 cells from stock cultures were washed twice with PBS. Then, the cells were treated with 0.2% trypsin/PBS and washed once with RPMI 1640 medium by centrifuging at 150×g at 4 °C for 3 min. The cell pellet was resuspended in RPMI 1640 medium and washed twice with the medium. The finally obtained cell pellet was resuspended in RPMI 1640 medium and cell number was counted using a Coulter Counter (model Z1; Coulter

Electronics, Hialeah, FL). The cells ($0.8–1.0\times10^5$ cells/mL) were cultured in 24–well plastic dishes (Nunc) in the medium containing FBS or cFBS, and flavonoids or 17β–estradiol were added at 24–hr after the inoculation.

Statistics

Results are expressed as the ratio of compound tested over the vehicle control and as the mean ± standard deviation (SD) of 3 or 4 independent assays (two–tailed) with Sigma–plot software (SPSS Inc, IL, U.S.A.)

RESULTS

Effect of flavonoids on proliferation of MCF–7 cells

As a preliminary experiment, proliferation inhibiting activity of flavonoids was examined. Figure. 1 shows the time–dependent effect of 10^{-5} M flavonoids, such as daidzein, genistein, quercetin and luteolin on proliferation of MCF–7 cells cultured in 5% FBS/RPMI 1640 medium for 144 hr. In this condition, doubling time of the cells was around 72 hr and all flavonoids exerted a proliferation–inhibiting activity. Among them, luteolin exerted the strongest inhibitory activity and the effect of daidzein was weakest.

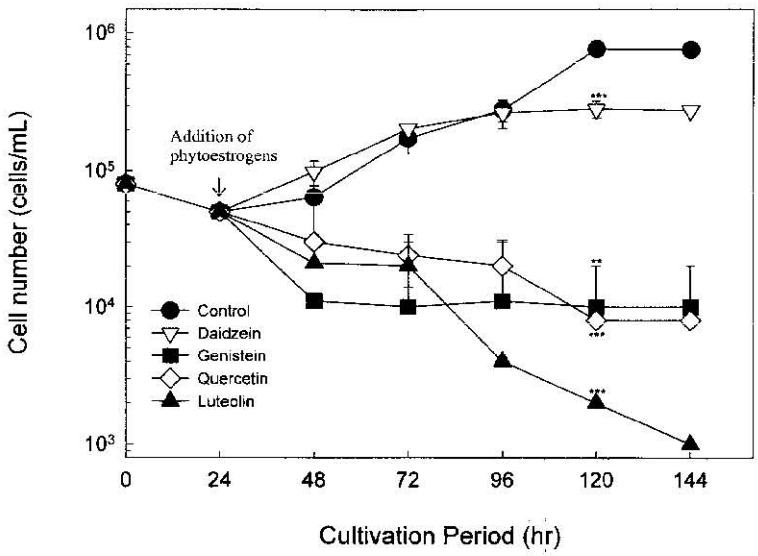

Fig. 1. Time–dependent effect of flavonoids on proliferation of MCF–7 cells. Cells were cultured with 5% FBS/RPMI 1640 medium at 37°C for 24 hr and cultured further in the presence of 10^{-5}M flavonoids. Cell number was counted using a Coulter Counter and data are means ± SD (n=4, $^{**}0.001<p<0.01$; $^{***}p<0.001$)

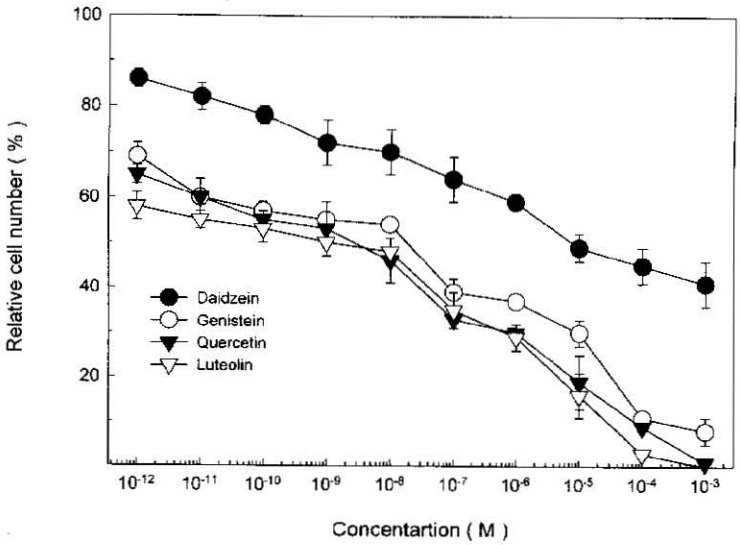

Fig. 2. Dose-dependent effect of flavonoids on proliferation of MCF-7 cells. Cells were cultured with 5% FBS/RPMI 1640 medium containing flavonoids between 10^{-12}M and 10^{-3}M for 96 hr. Results are expressed as percentages of control, which were treated with 70% EtOH/PBS. Data are means ± SD (n=4).

Dose-dependent effect of flavonoids on proliferation of MCF-7 cells

Because the concentration of flavonoids used above was very high, we examined the dose-dependent effect of flavonoids on proliferation of MCF-7 cells. The cells were treated with 10^{-12} to 10^{-3}M flavonoids for 96 hr. As shown in Fig. 2, these flavonoids did not enhance proliferation of MCF-7 cells, but inhibited it at all concentrations. Proliferation-suppressing activity of daidzein was significantly weaker than those of other flavonoids.

Effect of FBS concentration on proliferation-regulating activity of flavonoids

Endogeneous estrogen in FBS may inhibit the expression of estrogenic activity of flavonoids. Thus, the effect of FBS concentration on proliferation of MCF-7 cells in the presence of various concentrations of flavonoids was examined (Fig. 3). In the presence of 3 or 5% FBS, flavonoids inhibited proliferation of the cells at all concentrations used here (10^{-11} M to 10^{-4}M). On the other hand, daidzein significantly enhanced proliferation of the cells in 1% FBS/RPMI 1640 medium at 10^{-8}M.

Since 10^{-8}M daidzein significantly stimulated proliferation of MCF-7 cells in 1% FBS/RPMI 1640 medium, time-dependent effect of flavonoids was examined at the condition. As shown in Fig. 4, daidzein and genistein exerted a stimulatory effect on proliferation of MCF-7 cells after a treatment for 72 hr. On the contrary, 10^{-8}M quercetin

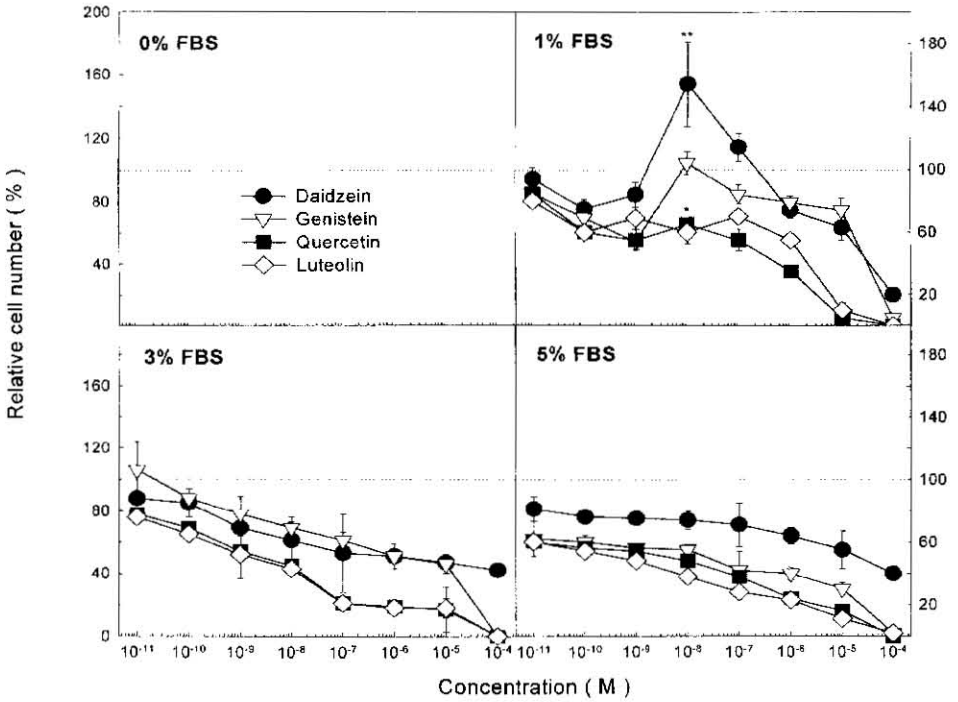

Fig. 3. Effect of FBS concentration on proliferation regulating activity of flavonoids.
Cells were cultured with flavonoids between 10^{-11}M and 10^{-4}M in FBS/RPMI 1640 medium containing 1 to 5% FBS for 96 hr. Results are expressed as percentage of control, which were treated with 70% EtOH/PBS. Data are means ± SD (n=3, *$0.01<p<0.05$; **$0.001<p<0.01$).

and luteolin exerted a strong proliferation–inhibiting activity.

Effect of FBS and cFBS concentrations on proliferation of MCF-7 cells

Since FBS contains various growth factors including estrogens, FBS was treated with activated charcoal to remove them. MCF-7 cells were cultured with RPMI 1640 medium containing FBS or cFBS for 96 hr, as shown in Fig. 5. Number of the cells in cFBS supplemented medium was significantly smaller than that in FBS supplemented medium, at all serum concentrations.

Time-dependent effect of flavonoids on proliferation of MCF-7 cells in 1% cFBS/RPMI 1640 medium

Then, time-dependent effect of flavonoids on proliferation of MCF-7 cells in 1% cFBS/RPMI 1640 medium was tested (Fig. 6). After an 120-hr cultivation, proliferation-stimulating activity was observed in daidzein and genistein. Genistein exerted a stronger cell proliferation-stimulating activity than daidzein in 1% FBS/RPMI 1640 medium.

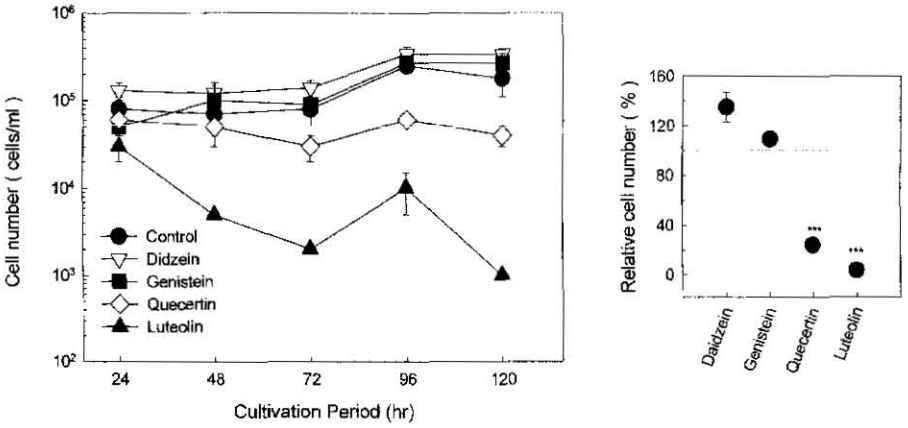

Fig. 4. Time-dependent effect of flavonoids on proliferation of MCF-7 cells in 1% FBS/RPMI 1640 medium.
Cells were treated with 10^{-5}M flavonoids in the presence of 1% FBS/RPMI 1640 medium for 120 hr. Right figure is expressed as percentage of the control treated with 70% EtOH/PBS at 96 hr. Data are means ± SD (n=4, ***$p<0.001$).

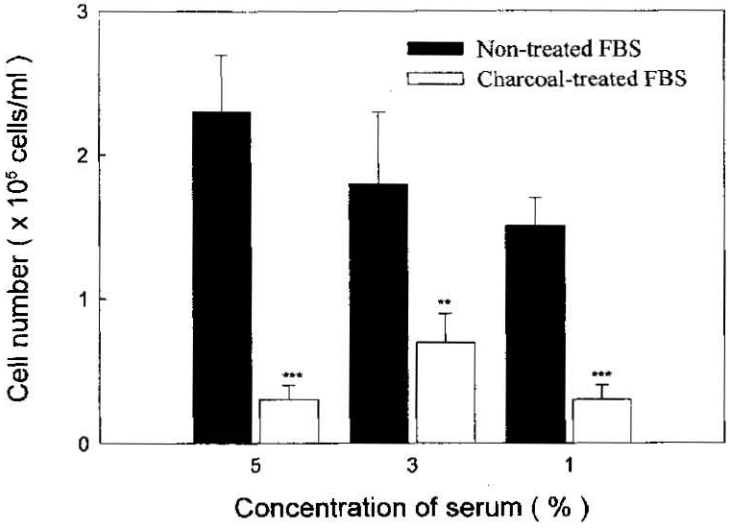

Fig. 5. Effect of serum concentration on proliferation of MCF-7 cells.
Cells were cultured in the presence of various concentrations of cFBS or FBS for 96 hr. Data are means ± SD (n=4, **$p<0.01$; ***$p<0.001$).

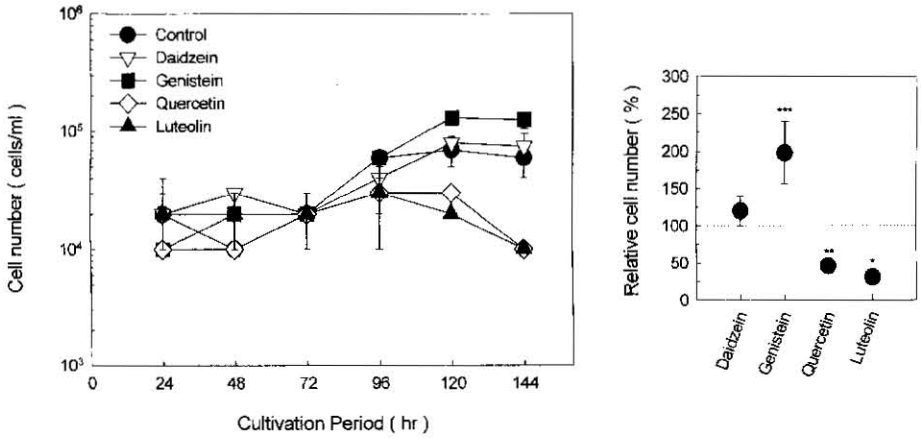

Fig. 6. Time-dependent effect of flavonoids on proliferation of MCF-7 cells in 1% cFBS/RPMI 1640 medium.
Cells were treated with 10^{-8}M flavonoids in the presence of 1% cFBS/RPMI 1640 medium for 144 hr. Right figure is expressed as percentage of the control treated 70% EtOH/PBS at 120 hr. Data are means ± SD (n=4, *$0.01<p<0.05$; **$0.001<p<0.01$; ***$p<0.001$).

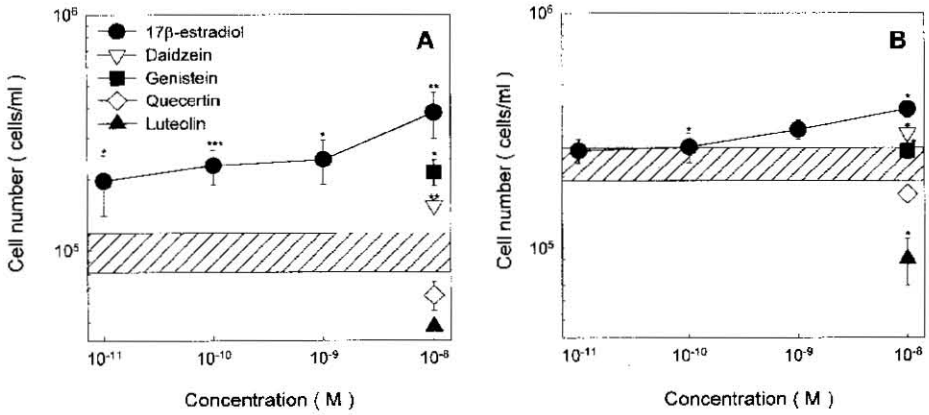

Fig. 7. Effect of 17β-estradiol and flavonoids on proliferation of MCF-7 cells in the medium containing 1% cFBS or FBS.
Cells were inoculated at 1×10^5cells/mL in 24-well plate and cultured with 1% cFBS/RPMI 1640 medium for 120 hr (A) or 1% FBS/RPMI 1640 medium for 96 hr (B). At 24 hr after inoculation, 17β-estradiol between 10^{-11}M and 10^{-8}M and 10^{-8}M flavonoids were treated. The horizontal hatched areas indicate the range of the control values. Data are means ± SD (n=4, *$0.01<p<0.05$; **$0.001<p<0.01$; ***$p<0.001$).

Effect of 17β–estradiol and flavonoids on proliferation of MCF–7 cells in the medium containing 1% cFBS or FBS

Since proliferation–stimulating activity of daidzein was detectable in the medium supplemented 1% FBS or cFBS, we examined the effect of 17β–estradiol at various concentrations from 10^{-11} to 10^{-8}M or 10^{-8}M flavonoids (Fig. 7). Cells were treated with these estrogenic compounds for 120 hr in the medium containing 1% cFBS (Fig. 7A) or 96 hr in the medium containing 1% FBS (Fig. 7B). Proliferation–stimulating activity of 17 β–estradiol is more marked in 1% cFBS containing medium than that in 1% FBS medium. In the presence of 1% cFBS, proliferation–stimulating activity of 17β–estradiol was significantly higher than daidzein and genistein.

DISCUSSION

Reproductive dysfunction involving exposure to hormonally action compounds has been extensively documented in wildlife animals and implicated in human breast cancer. As endocrine disruptors, many kinds of environmental estrogens have been targeted for study in hormonal response bioassays (Wang et al., 1997). Recently, flavonoids have received an increasing attention, because of their possible role in human cancer prevention and their high bioavailability. There are numerous reports on the effectiveness as an anti–cancer activities in a variety of tumor cells (Yolanddle et al., 1983; Darbre et al., 1983; Andreas et al., 1998; Theodore et al., 1997).

Although proliferation–inhibiting activity of flavonoids have been reported in MCF–7 cells, most of these studies were performed at high dose of daidzein and genistein (Barkly et al., 1981; Vignon et al., 1979; Katsuzo et al., 1975; Richard et al., 1981). In the present study, we found that flavonoids, such as daidzein, genistein, quercetin, and luteolin inhibited proliferation of MCF–7 cells in the medium supplemented with 1 to 5% FBS at range from 10^{-7}M to 10^{-3}M. In this assay system, genistein, quercetin, and luteolin exerted a stornger proliferation–inhibiting activity on MCF–7 cells than daidzein. On the other hand, proliferation–stimulating activity of daidzein and genistein was also detectable in the medium containing 1% FBS. These results suggest that one factor affecting the inhibition of cell proliferation by flavonoids is serum concentration in culture medium as reported by other researchers (Osborne et al., 1976; White et al., 1994; US EPA Report, 1997).

As part of a study focusing on our *in vitro* assay system, we have treated MCF–7 cells with 17β–estradiol or flavonoids in the presence of cFBS. In the studies evaluating hormonal action, researchers have eliminated estrogens from sera, a prerequisite for cell culture experiments, and considerable efforts have been applied towards the development of serum–free media. MCF–7 cells have been reported to possess the receptors for estrogen, androgen, progesterone, glucocorticoid, and insulin. This means that the cells may demand serum for their growth as these hormone supplementation. The complex and undefined nature of serum adds a complicating aspect to the designed interpretation of any experiments aimed at understanding the interactions of hormones or drugs with mammary epithelium (Vignon et al., 1979; Katsuzo et al., 1975; Richard et al., 1981; Osborne et al 1976; US EPA Report, 1997; UK Environmental Agency, 1998; Vera et al., 1999). Although estrogen in serum was not totally removed from cytosol by the

charcoal treatment, we found that cFBS was superior than FBS for detection of estrogenic activity of isoflavones such as daidzein and genistein. And the proliferation–stimulating effect of daidzein and genistein was detectable after a 72–hr lag period. Moreover, 17 β–estradiol in the presence of 1% FBS or 1% cFBS sufficiently enhanced proliferation of the MCF–7 cells at the concentrations between 10^{-11}M and 10^{-8}M, while daidzein and genistein enhanced it only at 10^{-8}M. On the contrary, quercetin and luteolin exerted proliferation inhibiting activity against MCF–7 cells at the concentrations over 10^{-11}M. These results indicate that estrogenic activity of flavonoids such as daidzein and genistein against MCF–7 cells was detectable in the RPMI 1640 medium containing 1% FBS or 1–5% cFBS after a 96–hr treatment. On the other hand, proliferation–inhibiting activity of quercetin and luteolin suggests that these compounds exert anti–estrogenic and anti–cancer activities against human mammary cancer MCF–7 cells.

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