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Stimulatory Effect of Daidzein in Osteoblastic MC3T3-E1 Cells

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ABSTRACT. Daidzein is a natural isoflavone found in *Leguminosae*. The effect of daidzein on osteoblastic MC3T3-E1 cells was investigated. Cells were cultured in a serum-free medium for 48 hr in the presence of daidzein (10^{-7}–10^{-5} M). Daidzein (10^{-6} and 10^{-5} M) caused a significant elevation of protein content, alkaline phosphatase activity, and DNA content in cells; those increases were about 1.4-, 1.5-, and 2.0-fold, respectively. The ability of daidzein (10^{-5} M) to increase protein content, alkaline phosphatase activity, and DNA content in cells was prevented completely by the presence of cycloheximide (10^{-6} M), an inhibitor of protein synthesis, indicating that the isoflavone has a stimulatory effect on cellular protein synthesis. The anabolic effect of daidzein (10^{-6} or 10^{-5} M) in cells was not distinguishable from that of genistein (10^{-6} or 10^{-5} M). Meanwhile, cell protein showed an additive effect of 17β-estradiol and daidzein, but their effects on alkaline phosphatase were not additive. Moreover, the effect of daidzein (10^{-5} M) in elevating cellular protein content and alkaline phosphatase activity was inhibited completely by the presence of tamoxifen (10^{-6} M), suggesting that the effect of the isoflavone is mediated partly through estrogen action. This study demonstrates that daidzein has an anabolic effect in osteoblastic MC3T3-E1 cells. BIOCHEM PHARMACOL **59**;5:471–475, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. daidzein; genistein; isoflavone; 17β-estradiol; osteoblast; bone formation

Genistein and daidzein are natural isoflavones found in *Leguminosae*. Genistein has been shown to have an inhibitory effect on protein tyrosine kinases [1, 2], whereas the biological effect of daidzein has not been clarified fully. It has been shown recently that genistein has an anabolic effect on bone metabolism [3, 4]; the isoflavone can increase alkaline phosphatase activity, DNA, and calcium contents in bone tissues. Blair *et al.* [3] have reported that genistein inhibits bone loss in ovariectomized rats. Dietary soybean protein also has been shown to prevent bone loss in ovariectomized rats [4]. Moreover, it has been demonstrated that genistein has a direct inhibitory effect on bone resorption [5] and a stimulatory effect on bone formation [6] in tissue culture system *in vitro*. Thus, genistein may prevent ovariectomy-induced bone loss due to inhibiting bone resorption and stimulating bone formation.

On the other hand, the effect of daidzein on bone metabolism is unknown. More recently, it has been found that daidzein can stimulate bone formation and mineralization in tissue culture system *in vitro* [7]. The anabolic effect of daidzein on bone metabolism seems to be equal to that of genistein [7]. When daidzein is hydroxylated, its chemical structure is similar to that of genistein. Genistein and daidzein may have an anabolic effect on bone metabolism, and the isoflavones may have pharmacological and nutritional roles in the prevention of osteoporosis. The cellular mechanism by which an isoflavone exerts an anabolic effect on bone metabolism, however, remains to be elucidated.

The present study was undertaken to clarify the effect of daidzein in osteoblastic cells *in vitro*. It was found that daidzein has an anabolic effect in osteoblastic MC3T3-E1 cells, supporting the view that daidzein has a stimulatory effect on osteoblastic bone formation.

MATERIALS AND METHODS

Chemicals

α-Modification of Eagle's Minimal Essential Medium was obtained from Flow Laboratories, Inc. FBS† was obtained from Bioproducts Inc. BSA (fraction V), daidzein, genistein, tamoxifen, cycloheximide, and 17β-estradiol were purchased from the Sigma Chemical Co. All other chemicals were reagent grade and obtained from Wako Pure Chemical Industries, Ltd. Tissue culture plastic dishes were purchased from Falcon Plastics. Other materials used were commercial products of the highest grade available.

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† *Abbreviations:* FBS, fetal bovine serum; and α-MEM, α-minimal essential medium.

Cell Culture

Osteoblastic MC3T3-E1 cells were provided by Drs. Y. Amagai and S. Kasai. The cells were cultured at 37° in a CO_2 incubator in plastic dishes containing α-MEM supplemented with 10% FBS. They were subcultured every 3 days using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free PBS. For experiments, about 1×10^4 cells per dish were cultured for 3 days to obtain confluent monolayers in 35-mm plastic dishes containing 2 mL α-MEM with 10% FBS. After the cells were rinsed with PBS, the medium was exchanged for medium containing 0.1% BSA plus various concentrations of daidzein (10^{-7}–10^{-5} M), genistein (10^{-6} and 10^{-5} M), cycloheximide (10^{-6} M), or tamoxifen (10^{-7} and 10^{-6} M), and the cells were cultured further for appropriate periods of time. Cell viability was estimated by staining with trypan blue.

Determination of Cell Numbers

After trypsinization using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free PBS, cell numbers were determined by an electronic particle counter.

Analytical Procedures

To determine the protein concentration in osteoblastic cells, the cells were washed three times with PBS, scraped into 0.5 mL of ice-cold 6.5 mM barbital buffer (pH 7.4) containing 0.2% polyoxyethylene (10) octylphenyl ether (Triton X-100) solution, and disrupted for 60 sec with an ultrasonic device. Protein concentration in the cell homogenate was determined by the method of Lowry *et al.* [8] and expressed as the amount of protein (μg) per dish.

To assay alkaline phosphatase activity in the cells after appropriate treatment periods, the cells were washed three times with PBS, scraped into 0.5 mL of ice-cold 6.5 mM barbital buffer (pH 7.4), and disrupted for 60 sec with an ultrasonic device. The supernatant, centrifuged at 600 *g* for 5 min, was used to measure enzyme activity. The enzyme assay described below was carried out under optimal conditions. Alkaline phosphatase activity was determined by the method of Walter and Schutt [9]. The enzyme activity was expressed as nanomoles of *p*-nitrophenol liberated per minute per milligram of protein.

To measure DNA content in the cells, the cells were detached by using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free PBS and washed with PBS. The cells were shaken with 2.0 mL of ice-cold 0.1 N NaOH solution for 24 hr after disruption [10]. After alkali extraction, the samples were centrifuged at 10,000 *g* for 5 min, and the supernatant was collected. DNA content in the supernatant was determined by the method of Ceriotti [11] and expressed as the amount of DNA (μg) per dish.

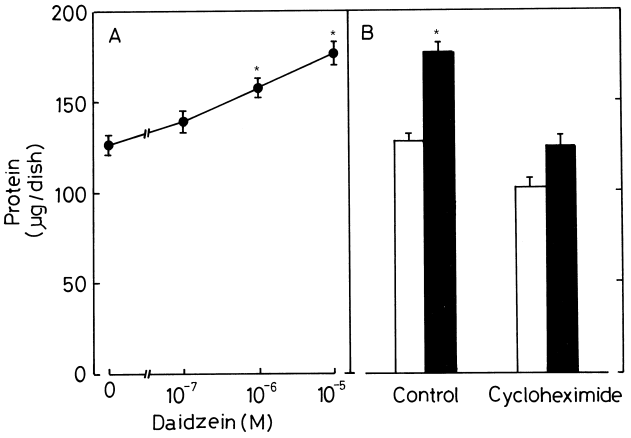

FIG. 1. Effect of daidzein on protein content in osteoblastic MC3T3-E1 cells. (A) Cells were cultured for 48 hr in a medium containing either vehicle (1% ethanol) or daidzein (10^{-7}–10^{-5} M). (B) Cells were cultured for 48 hr in a medium containing either vehicle or daidzein (10^{-5} M) in the presence or absence of cycloheximide (10^{-6} M). Each value is the mean ± SEM of 6 dishes. Key (*) $P < 0.01$, compared with the control value without daidzein. Open bars, control; and solid bars, daidzein.

Statistical Methods

Data are expressed as means ± SEM. Statistical differences were analyzed using Student's *t*-test; *P* values less than 0.05 were considered to indicate statistical differences.

RESULTS

Effect of Daidzein in Osteoblastic Cells

The effect of daidzein on protein content in osteoblastic MC3T3-E1 cells is shown in Fig. 1. Cells were cultured for 48 hr in a medium containing either vehicle (1% ethanol) or daidzein (10^{-7}–10^{-5} M). The presence of 10^{-6} and 10^{-5} M daidzein caused a significant increase in protein content, whereas 10^{-7} M daidzein had no effect (Fig. 1A). When cells were cultured for 48 hr in a medium containing either vehicle, daidzein (10^{-5} M), cycloheximide (10^{-6} M), or cycloheximide (10^{-6} M) plus daidzein (10^{-5} M), the daidzein (10^{-5} M)-induced increase in the cellular protein content was prevented completely by the presence of cycloheximide (10^{-6} M) (Fig. 1B).

The effect of daidzein on alkaline phosphatase activity in osteoblastic MC3T3-E1 cells is shown in Fig. 2. Culture in the presence of daidzein (10^{-7}–10^{-5} M) for 48 hr caused a significant increase in alkaline phosphatase activity in osteoblastic cells (Fig. 2A). The effect of daidzein (10^{-5} M) in increasing alkaline phosphatase activity in cells was not seen in the presence of cycloheximide (10^{-6} M) (Fig. 2B).

DNA content in osteoblastic MC3T3-E1 cells was elevated significantly by the presence of daidzein (10^{-7}–10^{-5} M), when the cells were cultured for 48 hr (Fig. 3A). Such an increase was not seen in medium containing cycloheximide (10^{-6} M) in the presence of daidzein (10^{-5} M) (Fig. 3B).

The effect of the combination of daidzein and genistein

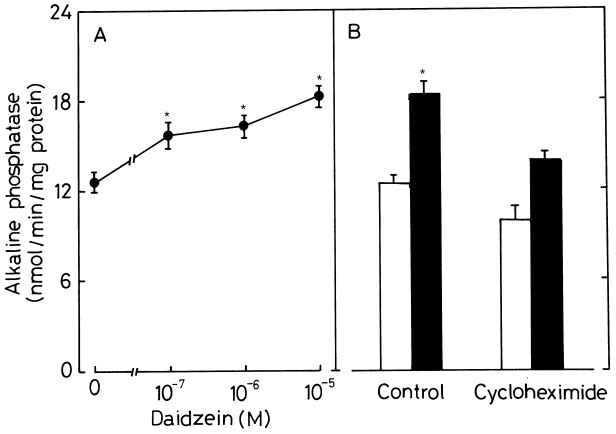

FIG. 2. Effect of daidzein on alkaline phosphatase activity in osteoblastic MC3T3-E1 cells. (A) Cells were cultured for 48 hr in a medium containing either vehicle (1% ethanol) or daidzein (10^{-7}–10^{-5} M). (B) Cells were cultured for 48 hr in a medium containing either vehicle or daidzein (10^{-5} M) in the presence or absence of cycloheximide (10^{-6} M). Each value is the mean ± SEM of 6 dishes. Key: (*) $P < 0.01$, compared with the control value without daidzein. Open bars, control; and solid bars, daidzein.

on protein content in osteoblastic MC3T3-E1 cells was examined, and the result is shown in Fig. 4. The presence of genistein (10^{-6} M) caused a significant increase in protein content in cells; this increase was not enhanced further by the addition of daidzein (10^{-6} M) (Fig. 4A). In addition, the effect of genistein at 10^{-5} M in increasing the cellular protein content was not appreciably enhanced by the presence of daidzein at 10^{-5} M (Fig. 4B).

The effect of genistein on daidzein-increased alkaline phosphatase activity in osteoblastic MC3T3-E1 cells is shown in Fig. 5. Alkaline phosphatase activity in cells was increased significantly by the presence of genistein (10^{-6} or

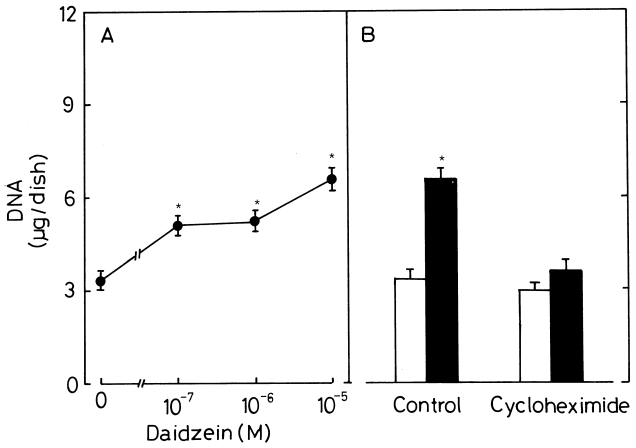

FIG. 3. Effect of daidzein on DNA content in osteoblastic MC3T3-E1 cells. (A) Cells were cultured for 48 hr in a medium containing either vehicle (1% ethanol) or daidzein (10^{-7}–10^{-5} M). (B) Cells were cultured for 48 hr in a medium containing either vehicle or daidzein (10^{-5} M) in the presence or absence of cycloheximide (10^{-6} M). Each value is the mean ± SEM of 6 dishes. Key: (*) $P < 0.01$, compared with the control value without daidzein. Open bars, control; and solid bars, daidzein.

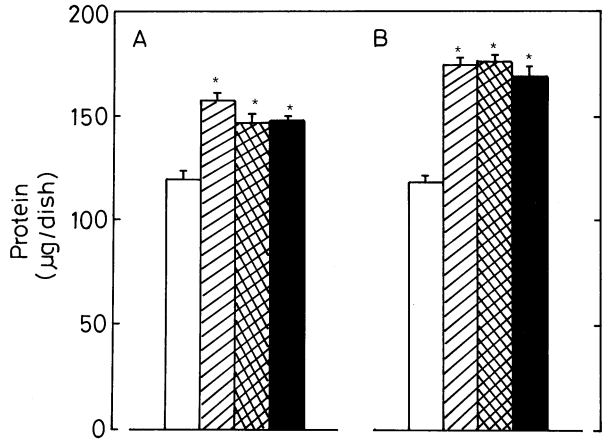

FIG. 4. Effect of the combination of daidzein and genistein on protein content in osteoblastic MC3T3-E1 cells. (A) Cells were cultured for 48 hr in a medium containing either vehicle (1% ethanol), daidzein (10^{-6} M), genistein (10^{-6} M), or daidzein (10^{-6} M) plus genistein (10^{-6} M). (B) Cells were cultured for 48 hr in a medium containing either vehicle, daidzein (10^{-5} M), genistein (10^{-5} M), or daidzein (10^{-5} M) plus genistein (10^{-5} M). Each value is the mean ± SEM of 6 dishes. Key: (*) $P < 0.01$, compared with the control value without isoflavone. Open bars, control; hatched bars, daidzein; cross-hatched bars, genistein; and solid bars, daidzein and genistein.

10^{-5} M). This elevation was not enhanced further by the addition of daidzein [10^{-6} M (panel A) or 10^{-5} M (panel B)].

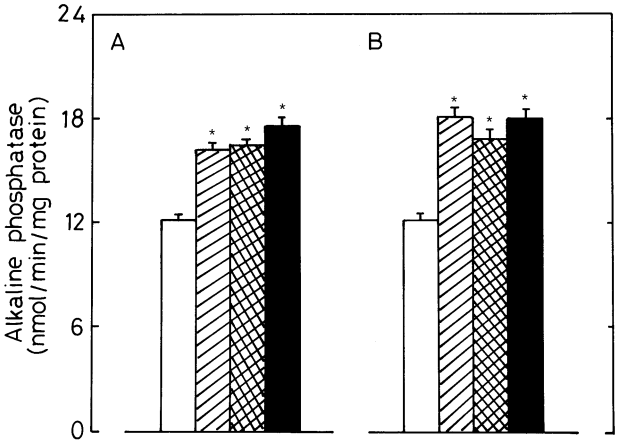

FIG. 5. Effect of the combination of daidzein and genistein on alkaline phosphatase activity in osteoblastic MC3T3-E1 cells. (A) Cells were cultured for 48 hr in a medium containing either vehicle (1% ethanol), daidzein (10^{-6} M), genistein (10^{-6} M), or daidzein (10^{-6} M) plus genistein (10^{-6} M). (B) Cells were cultured for 48 hr in a medium containing either vehicle, daidzein (10^{-5} M), genistein (10^{-5} M), or daidzein (10^{-5} M) plus genistein (10^{-5} M). Each value is the mean ± SEM of 6 dishes: Key: (*) $P < 0.01$, compared with the control value without isoflavone. Open bars, control; hatched bars, daidzein; cross-hatched bars, genistein; and solid bars, daidzein and genistein.

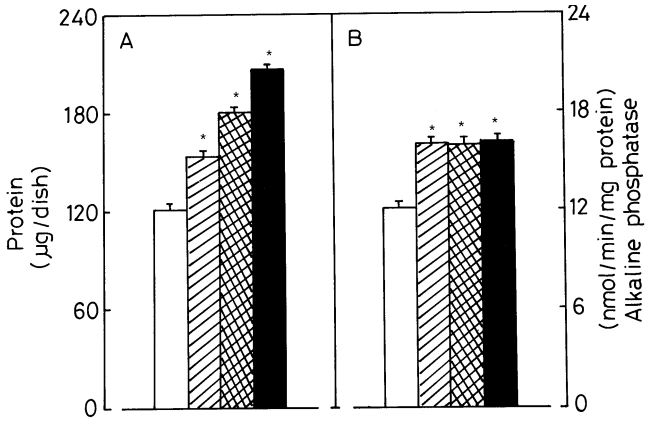

FIG. 6. Effect of the combination of daidzein and 17β-estradiol on protein content and alkaline phosphatase activity in MC3T3-E1 cells. Cells were cultured for 48 hr in a medium containing either vehicle (1% ethanol), daidzein (10^{-6} M), 17β-estradiol (10^{-9} M), or daidzein (10^{-6} M) plus 17β-estradiol (10^{-9} M). Each value is the mean ± SEM of 6 dishes. Key: (*) $P < 0.01$, compared with the control value without daidzein and tamoxifen. Open bars, control; hatched bars, daidzein; cross-hatched bar, 17β-estradiol; and solid bars, daidzein plus 17β-estradiol.

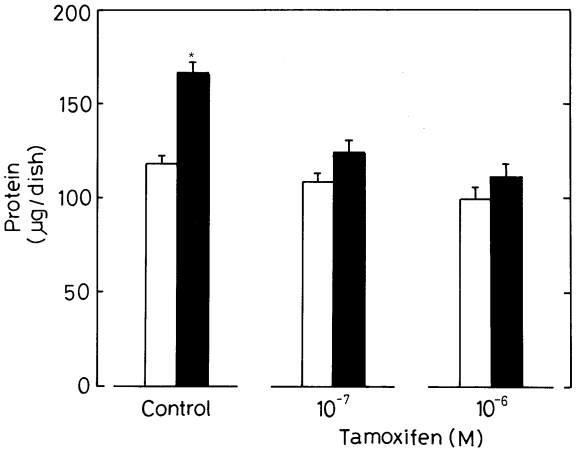

FIG. 7. Effect of the anti-estrogen tamoxifen on daidzein-increased protein content in osteoblastic MC3T3-E1 cells. Cells were cultured for 48 hr in a medium containing either vehicle (1% ethanol) or daidzein (10^{-5} M) in the presence or absence of tamoxifen (10^{-7} or 10^{-6} M). Each value is the mean ± SEM of 6 dishes. Key: (*) $P < 0.01$, compared with the control value without daidzein and tamoxifen. Open bars, control; and solid bars, daidzein.

Effects of Estradiol and the Anti-estrogen Tamoxifen on the Action of Daidzein in Osteoblastic Cells

The presence of 17β-estradiol (10^{-9} M) at a physiological concentration caused a significant increase in protein content in cells (Fig. 6A). This elevation was enhanced significantly by the addition of daidzein (10^{-6} M) (Fig. 6A). Meanwhile, the effect of 17β-estradiol (10^{-9} M) in elevating alkaline phosphatase activity in cells was not enhanced significantly by the presence of daidzein (10^{-6} M) (Fig. 6B).

Osteoblastic MC3T3-E1 cells were cultured for 48 hr in a medium containing either vehicle (1% ethanol) or daidzein (10^{-5} M) in the presence or absence of the anti-estrogen tamoxifen [12, 13] (10^{-7} and 10^{-6} M), and the result is shown in Fig. 7. The daidzein (10^{-5} M)-induced increase in protein content in cells was prevented completely by the presence of the anti-estrogen tamoxifen (10^{-7} or 10^{-6} M), although tamoxifen alone did not have an appreciable effect.

The effect of tamoxifen on the daidzein-induced increase in alkaline phosphatase activity in osteoblastic cells is shown in Fig. 8. The daidzein (10^{-5} M)-increased alkaline phosphatase activity in cells was abolished completely by the presence of tamoxifen (10^{-7} or 10^{-6} M).

DISCUSSION

Nutritional and pharmacological factors are needed to prevent bone loss with aging [14]. Genistein and daidzein are natural isoflavones found in *Leguminosae*. Recently, it has been reported that genistein can inhibit ovariectomy-induced bone loss, suggesting a role for it in the prevention of osteoporosis [3, 4, 6]. The effect of daidzein on bone metabolism, however, is poorly understood. More recently, it has been found that daidzein can stimulate bone formation and mineralization in bone tissue culture *in vitro* [7]. Furthermore, the present study demonstrates that daidzein can increase protein content, alkaline phosphatase activity, and DNA content in osteoblastic MC3T3-E1 cells *in vitro*, indicating its anabolic effect. This finding may support the view that daidzein can stimulate osteoblastic bone formation.

The effect of daidzein in increasing protein content, alkaline phosphatase activity, and DNA content in osteo-

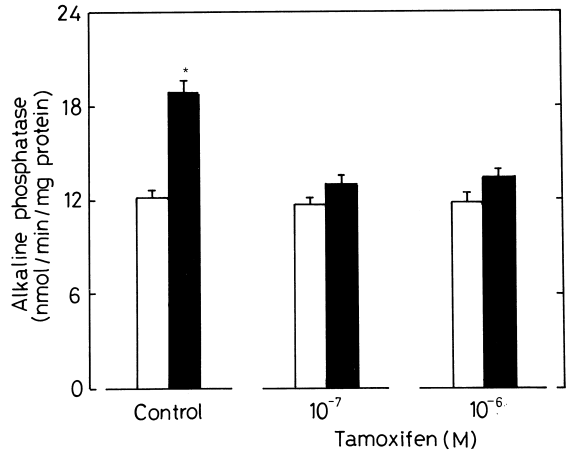

FIG. 8. Effect of the anti-estrogen tamoxifen on daidzein-increased alkaline phosphatase activity in osteoblastic MC3T3-E1 cells. Cells were cultured for 48 hr in a medium containing either vehicle (1% ethanol) or daidzein (10^{-5} M) in the presence or absence of tamoxifen (10^{-7} or 10^{-6} M). Each value is the mean ± SEM of 6 dishes. Key: (*) $P < 0.01$, compared with the control value without daidzein and tamoxifen. Open bars, control; and solid bars, daidzein.

blastic MC3T3-E1 cells was blocked completely by the presence of cycloheximide, an inhibitor of protein synthesis. The anabolic effect of daidzein may be based partly on a newly synthesized protein component. The effect of daidzein in osteoblastic cells seems to be equal to that of genistein. When daidzein is hydroxylated, its chemical structure is similar to that of genistein. The combination of daidzein and genistein did not enhance the effect of each other in osteoblastic cells. Presumably, the mechanism by which daidzein produces an anabolic effect does not differ from that of genistein in osteoblastic cells.

The presence of 17β-estradiol caused a significant increase in protein content and alkaline phosphatase activity in osteoblastic cells. Daidzein enhanced the effect of 17β-estradiol in increasing protein content in cells, although the isoflavone did not have an additive effect on alkaline phosphatase activity. This suggests that the mechanisms responsible for cell protein increase due to daidzein are different from those involved in promoting alkaline phosphatase expression. Presumably the effect of daidzein is mediated partly through the mode of action of 17β-estradiol in osteoblastic cells. Moreover, the effect of daidzein in elevating protein content and alkaline phosphatase activity in osteoblastic cells was blocked completely by the anti-estrogen tamoxifen [12, 13]. This result may support the view that the effect of daidzein is involved partly in the mechanism of estrogen action in osteoblastic cells. The receptors of estrogen are found in osteoblastic cells [15, 16]. Genistein has been shown to bind to estrogen receptor β in osteoblastic cells [17], although it has not been reported whether daidzein can bind to estrogen receptors. It is speculated, however, that daidzein may bind to estrogen receptor β in osteoblastic cells. The molecular mechanism of daidzein's action remains to be elucidated.

DNA content in osteoblastic cells was increased significantly by the presence of daidzein, suggesting that the isoflavone stimulates cell proliferation. This effect was abolished completely by cycloheximide. The effect of daidzein in elevating cellular DNA content may be based partly on newly synthesized protein. Also, the isoflavone can increase alkaline phosphatase, which is a marker enzyme in the differentiation of osteoblastic cells. Thus, daidzein may have a stimulatory effect on the proliferation and differentiation of osteoblastic MC3T3-E1 cells.

In conclusion, it has been demonstrated that daidzein has an anabolic effect in osteoblastic MC3T3-E1 cells. Daidzein may be able to stimulate osteoblastic bone formation.

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