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Soybean isoflavones inhibit tumor necrosis factor-α-induced apoptosis and the production of interleukin-6 and prostaglandin E_2 in osteoblastic cells

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Abstract

The effects of individual soybean isoflavones, genistein (4′,5,7-trihydroxyisoflavone) and daidzein (4′,7-dihydroxyisoflavone), on tumor necrosis factor-α (TNF-α)-induced apoptosis and the production of local factors in osteoblastic cells has been investigated. Soybean isoflavones increased DNA synthesis and the number of viable cells. When cells were treated with TNF-α, the number of viable cells dose-dependently decreased. The decrease in cell number caused by TNF-α treatment was due to apoptosis, which was confirmed by TUNEL and cell death ELISA analyses. Soybean isoflavones inhibited apoptosis of osteoblastic cells subjected to TNF-α treatment. MC3T3-E1 osteoblastic cells secrete interleukin-6 (IL-6), interleukin-1β (IL-1β), nitric oxide (NO) and prostaglandin E_2 (PGE_2) constitutively, but at low levels. Soybean isoflavones had no effect on the constitutive production of these local factors. When cells were treated with TNF-α (10^{-10}M), the production of IL-6 and PGE_2, but not that of IL-1β and NO, significantly increased. Treatment with soybean isoflavones (10^{-5}M), in the presence of TNF-α (10^{-10}M), for 48 h inhibited production of IL-6 and PGE_2, suggesting the antiresorptive action of soy phytoestrogen may be mediated by decreases in these local factors. The findings of this study thus suggest that soybean isoflavones may promote the function of osteoblastic cells and play an important role in bone remodeling.

Keywords: Soybean isoflavones; Genistein; Daidzein; Estrogen; Osteoblast; Tumor necrosis factor-alpha; Apoptosis; Cytokines

1. Introduction

Bone cells are regulated by a number of systemic and local factors that affect bone metabolism. Among the systemic factors are various hormones involved in calcium homeostasis. In addition, certain local factors such as cytokines and prostaglandins can mediate the effect of systemic factors, acting in a paracrine or autocrine manner (Manolagas, 1995).

Tumor necrosis factor-α (TNF-α) has been shown to play an important role in local control of bone remodeling. TNF-α exerts pleiotropic effects on bone under pathological conditions such as estrogen deficiency, leading to osteoporosis (Kassem et al., 1996). TNF-α-mediated osteoporosis can be explained by two lines of evidence. Firstly, TNF-α stimulates osteoblasts to secrete other cytokines (interleukin-1β and interleukin-6) and prostaglandin E_2 (PGE_2) as well as TNF-α itself that act directly on osteoclasts to cause bone resorption (Franchimont et al., 1997; Jilka, 1998). Secondly, TNF-α also induces the apoptosis of osteoblasts that is mediated by the transcription factor nuclear factor kappa B (NF-κB) (Bodine et al., 1999), the cell surface molecule Fas (Tsuboi et al., 1999), and nitric oxide (Mogi et al., 1999). Thus, an increase of cytokine production and a decrease in osteoblast cell numbers via apoptosis could be responsible for the bone loss.

Estrogen (E_2) is the most important factor involved in the balanced coupling of osteoblast bone formation and osteoclastic bone resorption. While the major effect of

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E_2 is thought to be a suppression of osteoclastic bone resorption (Turner et al., 1994), E_2 also has an indirect inhibitory effect on the bone resorption via the inhibition of cytokines in osteoblasts (Kassem et al., 1996). Treatment with E_2 partially suppresses the activation of NF-κB by TNF-α in the human osteoblast cell line, HOB-O3-CE6 (Bodine et al., 1999). In addition, E_2 prevents glucocorticoid-induced apoptosis in primary rat and mouse osteoblasts (Gohel et al., 1999), and TNF-α-induced apoptosis in U937 monoblastoid cells (Vegeto et al., 1999), MCF-7 breast carcinoma cells (Burow et al., 1999), and endothelial cells (Spyridopoulos et al., 1997).

Traditional steroid estrogen replacement therapy (ERT) reduces the bone loss and the incidence of fracture in postmenopausal women (Gennari et al., 1998; Michaelsson et al., 1998) and remains the "gold standard" for the prevention and treatment of osteoporosis in this population. However, ERT may be associated with an increased risk of developing breast cancer and side effects (Barrett-Connor and Grady, 1998).

Soybean isoflavones are naturally occurring plant chemicals belonging to the phytoestrogen class. They are strikingly similar in chemical structure to mammalian estrogen (Leclerq and Heuson, 1979) and are currently heralded as offering potential alternative therapies for osteoporosis. Data from human studies provide convincing evidence for the potential role of soybean isoflavones in preventing osteoporosis (Alekel et al., 2000). Similar benefits have been reported in animal studies (Harrison et al., 1998; Fanti et al., 1998). Moreover, the role of isoflavones in bone health is further supported by in vitro studies investigating the effect of soybean isoflavones on osteoblastic cells, suggesting that this effect is mediated partly through estrogen action (Yamaguchi and Sugimoto, 2000). Our previous studies also showed that soybean ethanol extract increases the function of osteoblastic MC3T3-E1 cells (Choi et al., 2001). However, the effects of soybean isoflavones on the secretion of local factors and apoptosis in osteoblastic cells have not been reported.

The objectives of this study were to evaluate whether individual soybean isoflavones, genistein (4′,5,7-trihydroxyisoflavone) and daidzein (4′,7-dihydroxyisoflavone), have a potential effect on the production of local factors and apoptosis induced by TNF-α in MC3T3-E1 osteoblastic cells.

2. Results

2.1. Effect of soy isoflavones on the proliferation of MC3T3-E1 cells

When cells were treated with genistein or daidzein (10^{-6}–10^{-5} M), DNA synthesis was dose-dependently increased. However, at high concentration (10^{-4} M), an inhibitory effect was also observed. When cells were treated with E_2 (10^{-10}–10^{-7} M), DNA synthesis dose-dependently increased, but never exceeded those treated with genistein or daidzein (Fig. 1). For a more precise estimation of the amount of cell proliferation, the number of viable cells was directly determined by the ability of cells with uncompromised membrane integrity to exclude Trypan blue dye. When cells were treated with TNF-α (10^{-11}–10^{-9} M), the number of cells was dose-dependently decreased (Fig. 2a). TNF-α-induced decrease of cell numbers was recovered by treatment with soybean isoflavones (10^{-5} M) or E_2 (10^{-8} M) (Fig. 2b).

2.2. Effect of soy isoflavones on the apoptosis of MC3T3-E1 cells

As indicated by the TUNEL assay, the number of apoptotic cells dramatically increased following treatment

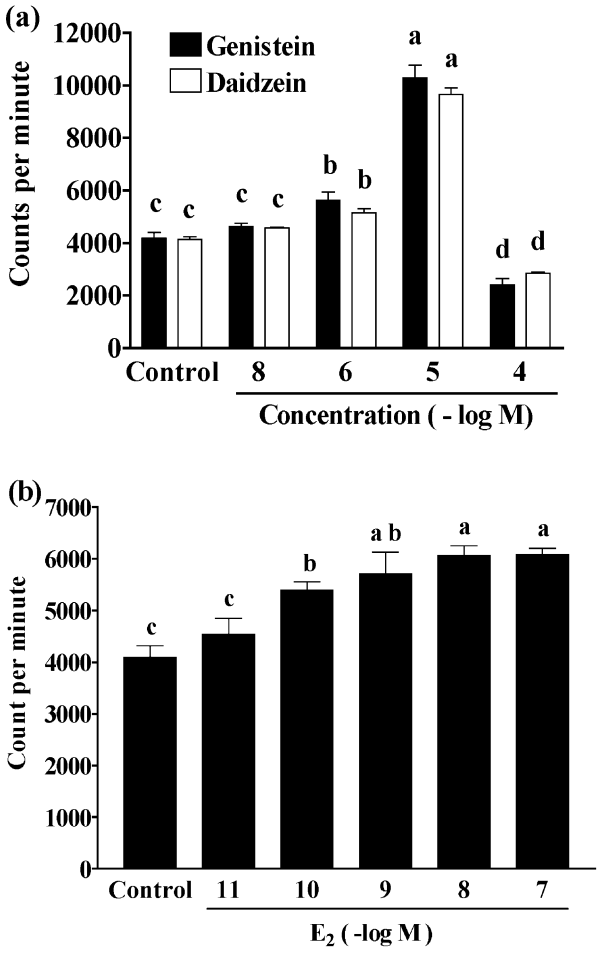

Fig. 1. Effect of soybean isoflavones or E_2 on [^{3}H]-thymidine incorporation into osteoblastic cells. Cells were cultured in the absence or presence of increasing concentrations of soybean isoflavones (a) or E_2 (b). Vehicle (α-MEM containing 0.3% BSA) was used as a control. Data are expressed as mean±S.D. of six cultures. Groups with different letters at the top of the bars are significantly different from each other by analysis of variance ($P<0.05$).

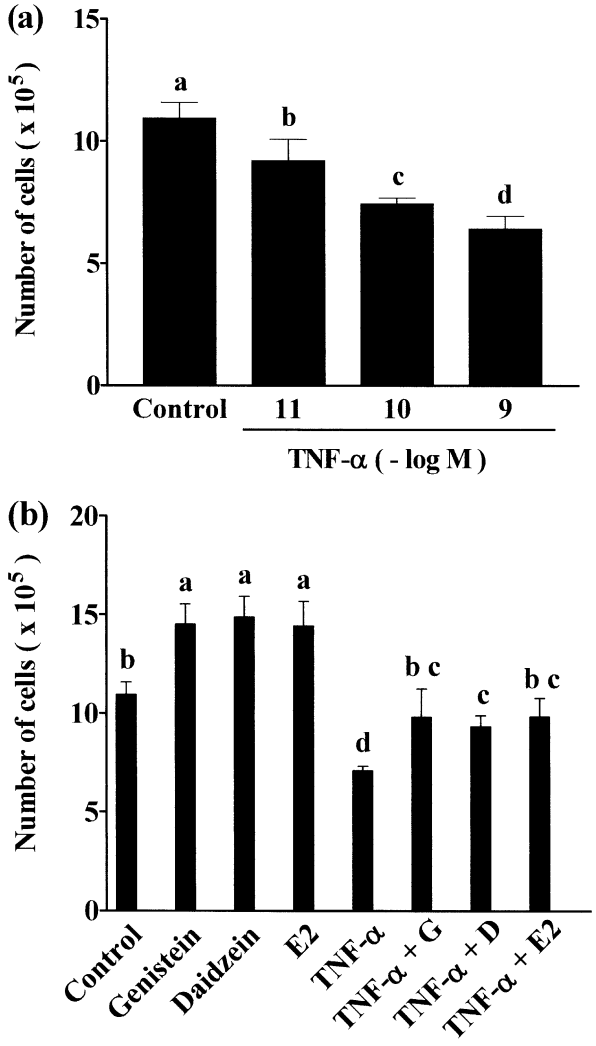

Fig. 2. Effect of genistein (G), daidzein (D) or 17β-estradiol (E_2) on the number of viable osteoblastic cells. Cells were cultured in the absence or presence of increasing concentrations of TNF-α (a), and cultured in the presence of TNF-α (10^{-10} M) alone or in combination with genistein (10^{-5} M), daidzein (10^{-5} M) or E_2 (10^{-8} M) (b). To measure cell numbers, cells were released from the culture surface with 0.05% Trypsin–0.05% EDTA and counted in a fixed volume hemocytometer. Vehicle (α-MEM containing 0.3% BSA) was used as a control. Data are expressed as mean±S.D. of six cultures. Groups with different letters at the top of the bars are significantly different from each other by analysis of variance ($P<0.05$).

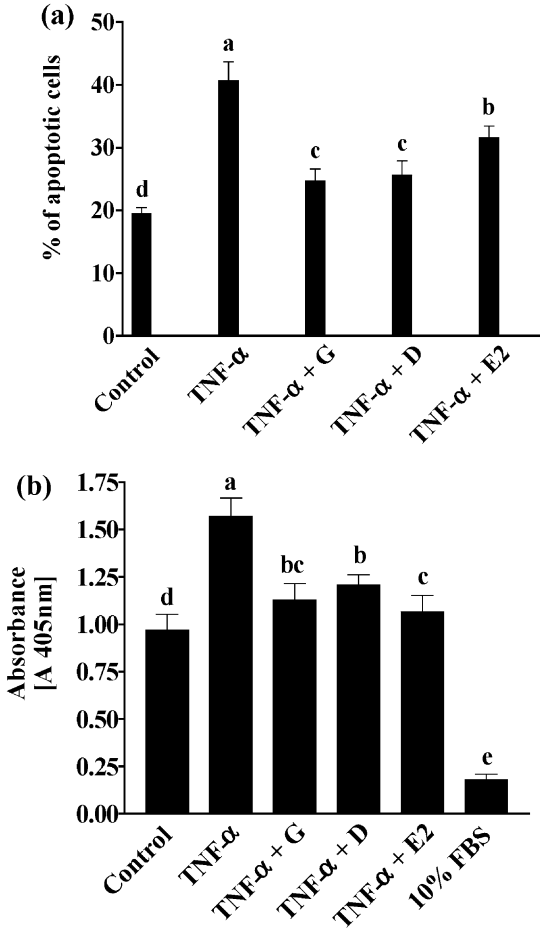

Fig. 3. Effect of genistein (G), daidzein (D) or 17β-estradiol (E_2) on TNF-α-induced apoptosis. Cells were cultured in the presence of TNF-α (10^{-10} M) alone or in combination with genistein (10^{-5} M), daidzein (10^{-5} M) or E_2 (10^{-8} M). Apoptosis was assayed by TUNEL (a) and cell death ELISA (b). Vehicle (α-MEM containing 0.3% BSA) was used as a control. Data are expressed as mean±S.D. of six cultures. Groups with different letters at the top of the bars are significantly different from each other by analysis of variance ($P<0.05$).

of TNF-α (10^{-10} M). TNF-α-induced apoptosis was significantly decreased by treatment of soybean isoflavones or E_2, respectively (Fig. 3a).

We also quantitated the apoptosis induced by TNF-α (10^{-10} M) using a one-step sandwich immunoassay. When MC3T3-E1 cells were treated with TNF-α, apoptosis was significantly increased. Soybean isoflavones or E_2 inhibited TNF-α-induced apoptosis. A small portion of MC3T3-E1 cells underwent apoptosis when the cells were maintained in the presence of 10% fetal bovine serum (FBS) (Fig. 3b).

2.3. Effect of soy isoflavones on IL-6, IL-1β, PGE_2 and NO production

MC3T3-E1 cells synthesized IL-6 and PGE_2 constitutively, but at very low levels.

However, when TNF-α (10^{-10} M) was added to cells, production of IL-6 and PGE_2 increased significantly. When cells were treated with soybean isoflavones (10^{-5} M) or E_2 (10^{-8} M) for 48 h, TNF-α-induced IL-6 and PGE_2 production was significantly inhibited. However, isoflavones or E2 had no effect on the basal level of IL-6 and PGE_2 production (Fig. 4a and b).

When TNF-α (10^{-10} M) was added to cells, production of IL-1β and NO slightly increased, but this effect was not statistically significant. In addition, treatment with soybean isoflavones (10^{-5} M) or E_2 (10^{-8} M) had no significant effect on the constitutive and TNF-α-induced IL-1β and NO production, respectively (Fig. 4c and d).

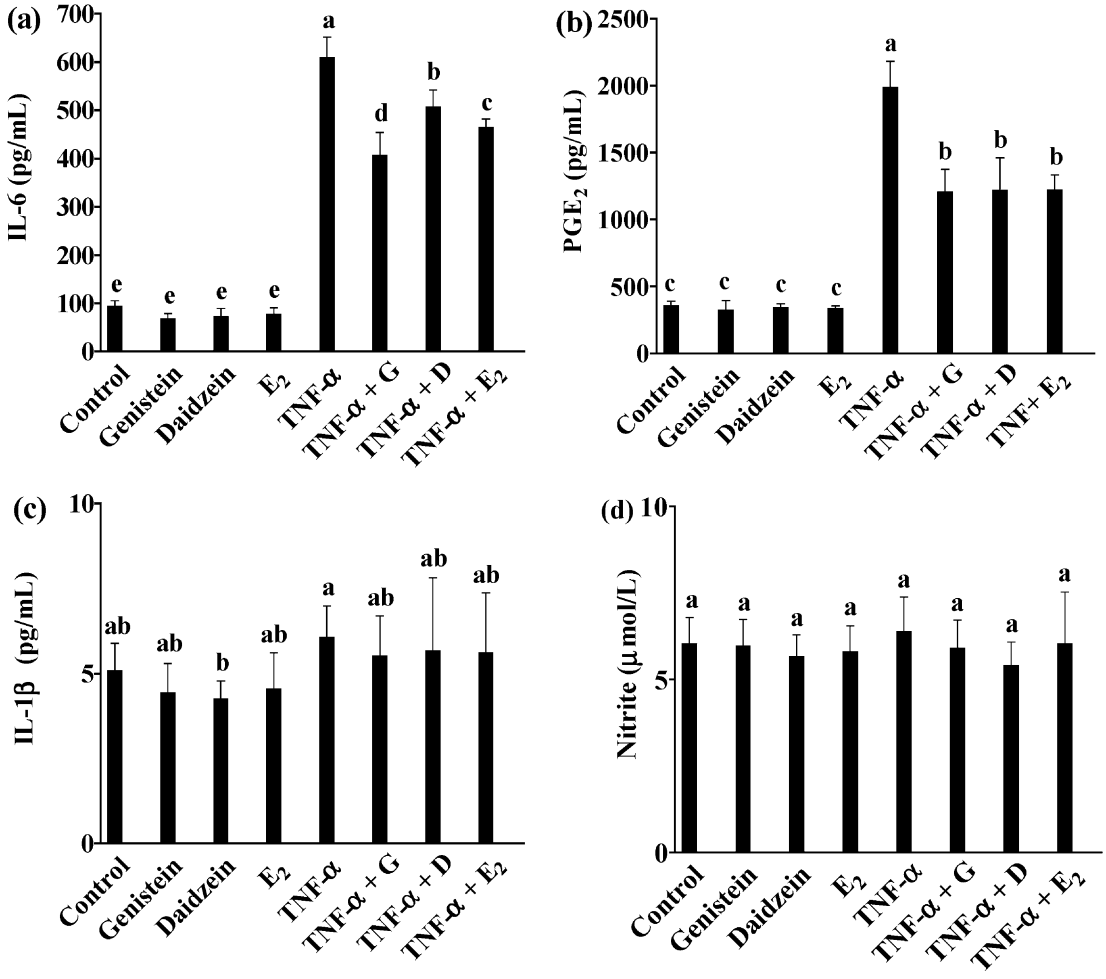

Fig. 4. Effect of genistein (G), daidzein (D) or 17β-estradiol (E_2) on TNF-α-induced IL-6, IL-1β, PGE_2, and NO production in osteoblastic MC3T3-E1 cells. Cells were cultured in a medium containing either vehicle or genistein (10^{-5} M), daidzein (10^{-5} M) or E_2 in the absence or presence of TNF-α (10^{-10} M). Vehicle (α-MEM containing 0.3% BSA) was used as a control. The conditioned media were collected and assayed by ELISA. Data are the mean±S.D. of six cultures. Groups with different letters at the top of the bars are significantly different from each other by analysis of variance ($P<0.05$).

3. Discussion

The present results demonstrate that soybean isoflavones not only stimulate cell proliferation but also inhibit TNF-α-induced apoptosis in MC3T3-E1 osteoblast cells. In addition, these isoflavones inhibit TNF-α-induced IL-6 and PGE_2 production. It has been reported that soy isoflavones can stimulate bone formation and mineralization in bone tissue culture in vitro (Gao and Yamaguchi, 1999). Furthermore, Sugimoto and Yamaguchi (2000) demonstrated that genistein and daidzein could increase protein content, alkaline phosphatase activity, and DNA content in osteoblastic MC3T3-E1 cells in vitro, indicating an anabolic effect. This finding may support the view that soy isoflavones can stimulate osteoblastic bone formation. However, the effects of soybean isoflavones on the secretion of local factors and apoptosis in osteoblastic cells had not been reported.

TNF-α can inhibit replication of abnormal cells or stimulate normal cell division in a variety of tissues (Sugarman et al., 1985). Consistent with these findings, TNF-α significantly inhibits proliferation in a dose dependent manner. The decrease in the cell number caused by TNF-α treatment was due to apoptosis. It has been shown that TNF-α directly mediated apoptosis in MC3T3-E1 osteoblast cells (Kitajima et al., 1996; Hill et al., 1997). The fact that soybean isoflavones or E_2 administration inhibited the apoptosis of osteoblast cells subjected to TNF-α treatment suggests that these agents may prolong the lifespan, and thereby instigate an increase in the number of osteoblasts. On the other hand, soybean isoflavone has been known to have an apoptosis-inducing anticancer effect. Especially, genistein has attracted considerable attention as it is one of the major soybean isoflavones and has been identified as an apoptosis inducer in cancer cells (Peterson, 1995; Gescher et al., 2001). However, the mechanisms through

which soybean isoflavones may exert the multidirection action remain to be clarified. Soybean isoflavones or E_2 also has a stimulatory effect on cell proliferation in this study. Thus, it can be suggested that soybean isoflavones are involved in maintaining the viability and proliferation of osteoblasts, which may be clearly associated with the regulation of bone remodeling. Nonetheless, the action of soybean isoflavones on bone and the most efficacious dose need further investigation.

This study demonstrated that genistein and daidzein dramatically stimulated the rate of DNA synthesis at a concentration of 10^{-5} M, whereas at a high concentration ($\sim 10^{-4}$ M), an inhibitory effect was noted, which may be due to cytotoxicity. This finding is supported by an in vivo study that low-dose genistein treatment in ovariectomized rats is effective in maintaining trabecular bone tissue, but at high doses, the bone-sparing effect of genistein was reversed (Anderson et al., 1998). This biphasic effect of soybean isoflavones on DNA synthesis remains to be clarified.

TNF-α stimulates osteoblasts to secrete other cytokines (IL-1β and IL-6) and prostaglandin E_2 (PGE_2) as well as TNF-α itself that act directly on osteoclasts to cause bone resorption (Franchimont et al., 1997; Jilka, 1998). Estrogen inhibits the IL-1-and TNF-α-stimulated biosynthesis of IL-6 in stromal and osteoblastic cells (Galien et al., 1996; Girasole et al., 1992). It is hypothesized that a blockage of any one of these cytokines may suppress the stimulatory effect of estrogen deficiency on bone metabolism. In this study, soybean isoflavones (10^{-5} M) or E_2 (10^{-8} M) inhibited TNF-α-induced secretion of IL-6. Consistent with this finding, Girasole et al. (1992) and Kassem et al. (1996) reported that E_2 had no effect on the constitutive production of IL-6. However, IL-1 β plus TNF-α-stimulated production of IL-6 was reduced by E_2, supporting the hypothesis that the antiresorptive action of E_2 may be mediated by decreased production of IL-6 by osteoblastic cells.

The antioxidative and free radical scavenging properties of polyphenolic compounds in several plant extracts have recently been reported (Kim et al., 1998). However, in the present study, soybean isoflavones or E_2 had no effect on the constitutive and TNF-α induced nitrite production in MC3T3-E1 osteoblastic cells. Unstimulated cells produce little NO, and this is not altered by using one of bone-resorbing inflammatory cytokines alone (Lowik et al., 1994). This result suggests that soy isoflavones may not regulate TNF-α induced NO production.

Osteoblasts produce prostaglandins in response to a wide variety of stimuli. It has been reported that TNF-α stimulates cyclo-oxygenase-2 (COX2) activity in osteoblastic cells, resulting in an increase in the production of prostaglandin E_2 (Raisz, 1995; Wadleigh and Herschman, 1999). PGE_2 has been reported to play an important role in postmenopausal bone loss associated with estrogen deficiency (Kanematsu et al., 2000; Kawaguchi et al., 1995). Treatment with PGE_2 increased the number of osteoclast-like cells in bone marrow cells derived from ovariectomized mice (Kanematsu et al., 2000). E_2 has been reported to inhibit the production of PGE_2 (Raisz et al., 1993). In this study, soybean isoflavones inhibited the TNF-α-induced PGE_2 production.

In summary, soybean isoflavones, genistein and daidzein, inhibited TNF-α-stimulated apoptosis, and production of IL-6 and PGE_2 by osteoblastic MC3T3-E1 cells in vitro. These studies suggest that soybean isoflavones may promote the function of osteoblastic cells and play an important role in bone remodeling.

4. Experimental

4.1. Cell culture

The clonal murine osteoblastic MC3T3-E1 cells (provided by Dr. Suda, Tokyo Women's Medical University, Tokyo, Japan) were cultured in alpha-minimum essential medium (α-MEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA), 100 μU/ml penicillin and 100 μg/ml streptomycin (DPBS, Gibco BRL Co., USA). The cultures were maintained at 37 °C with a gas mixture of 5% CO_2–95% air. Subcultures were performed with 0.05% trypsin-0.02% EDTA in Ca^{2+}, Mg^{2+} free phosphate-buffered saline (DPBS, Gibco BRL Co., USA). The cells were cultured in α-MEM supplemented with 10% FBS for 48 h. The medium was replaced with fresh medium containing 0.3% BSA with soybean isoflavones (genistein, daidzein) or 17β-estradiol (Sigma, St. Louis, MO, USA) dissolved in DMSO and incubated for 48 h [final DMSO concentration was 0.05% (vol/vol)]. For experiments evaluating the effects of TNF-α (Roche molecular biochemicals, Germany)-stimulated apoptosis and production of local factors, the medium was replaced with fresh medium containing test substances with or without TNF-α (10^{-10} M), and the incubation was continued for a further 48 h.

4.2. [^{3}H] thymidine incorporation and cell proliferation assay

MC3T3-E1 cells were seeded in 24 well culture plates (Falcon, Becton Dickinson, NJ, USA) at a density of 2×10^4 cells. The cells were labeled with 1 μCi of [^{3}H]-methyl thymidine (Amersham, Arlington Heights, IL, USA) for the last 6 h of treatment, and then were rinsed twice with DPBS and 10% trichloroacetic acid (Sigma, St. Louis, MO, USA). Finally, the cells were dissolved in 2 M perchloric acid (500 μl) (Sigma, St. Louis, MO, USA). Radioactivity was determined by β-scintillation counting (LS 3000, Beckman, USA). For proliferation

studies, cells from each treatment were harvested and counted in a hemocytometer (Bright-Line, PA, USA) using 0.4% Trypan blue vital staining.

4.3. Analysis of apoptosis in cultured cells

4.3.1. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

Apoptotic cell death was assessed using the in situ Apoptosis Detection Kit (Takara Shuzo Co., Japan). Cells were cultured at a density of 1×10^4 /well on Lab-Tek chamber slides (Nunc, Copenhagen, Denmark) in serum free medium (SFM) containing test substances and thymidine (10^{-3} M) in the presence or absence of TNF-α (10^{-10} M). After 2 days in culture, apoptotic cells were fixed in 4% paraformaldehyde for 10 min, washed in 10 mM Tris–HCl, pH 8.0, and then permeabilized in 0.1% Triton X-100, pH 8.0. Apoptotic cells were detected by labeling free 3′-OH DNA ends with fluorescein-labeled dUTP, using the enzyme terminal deoxynucleotidyl transferase (TdT). Cells were subsequently exposed to peroxidase-labeled anti-fluorescein antibody. Cells were incubated with diaminobenzidine at room temperature for 15 min, and counterstained with 3% methyl green and examined under light microscopy (Olympus, Japan). The total number of apoptotic cells in each slide was summed and expressed as a percentage of total cell number.

4.3.2. Cell death ELISA

A cell death ELISA kit (Roche molecular biochemicals, Mannheim, Germany), which quantitatively detects cytosolic histone-associated DNA fragments, was employed to assess apoptosis in osteoblastic cells. DNA fragments were measured according to the procedures described in the kit. Briefly, cells were seeded at a density of 2×10^4 cells in 24-well culture plates. The culture conditions were the same as those described for the cell proliferation assay. After the incubation, the cells were lysed and intact nuclei were pelleted by centrifugation. An aliquot of the supernatant was used as antigen source in a sandwich ELISA with a primary anti-histone monoclonal antibody coated to the streptoavidin-coated well of a microtiter plate. Subsequently, a second anti-DNA monoclonal antibody coupled to peroxidase was added. The amount of nucleosomes was quantified by the peroxidase retained in the immunocomplex. Peroxidase was determined photometrically at 405 nm with ABTS (2,2′-azino-di[3-ethylbenzthiazolin-sulfonate]) as substrate.

4.4. Immunoassay of IL-6, IL-1β, PGE_2 and NO production

Cells were seeded at a density of 2×10^4 cells in a 24-well plate, and cultured for 2 days. Cells were then cultured with soy isoflavones (10^{-5} M) or E_2 (10^{-8} M) in the presence or absence of TNF-α (10^{-10} M) for 48 h. IL-6, IL-1β, PGE_2 and NO were measured in the conditioned medium by commercially available immunoassay (R&D system, Minneapolis, MN, USA) according to the manufacturer's instructions. The data were obtained from the standard curve using computer software capable of generating a log–log curve-fit or linear curve-fit (Prism 3, GraphPad software, CA, USA).

4.5. Statistics

Experiments were carried out in six cultures and expressed as mean ± S.D. Differences between the means were calculated using one-way analysis of variance (ANOVA) with a subsequent Duncan's multiple range test ($P<0.05$). The analysis was performed using SAS statistical software.

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