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Apigenin increases osteoblastic differentiation and inhibits tumor necrosis factor-α-induced production of interleukin-6 and nitric oxide in osteoblastic MC3T3-E1 cells

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Many plant-derived substances have estrogenic activities. Due to their ability to bind the estrogen receptor, these compounds have the potential to counteract the deleterious effects of estrogen deficiency on bone. In this study, the effects of apigenin on the function of osteoblastic MC3T3-E1 cells and the production of local factors in osteoblasts were investigated. Apigenin (0.01 μM) significantly increased the growth of MC3T3-E1 cells and caused a significant elevation of alkaline phosphatase (ALP) activity and collagen content in the cells ($P < 0.05$). The effect of apigenin in increasing ALP activity and collagen content was completely prevented by the presence of 10^{-6} M cycloheximide and 10^{-6} M tamoxifen, suggesting that apigenin's effect results from a newly synthesized protein component and might be partly involved in estrogen action. Locally derived mediators in bone play a crucial role in the regulation of bone remodeling, i.e., bone formation and bone resorption processes. The effect of apigenin on the TNF-α-induced production of IL-6 and nitric oxide (NO) in osteoblasts was examined. Treatment with apigenin (10 μM) decreased the TNF-α-induced production of IL-6 and NO in osteoblasts. Taken together, these results suggest that apigenin may represent new pharmacological tools for the treatment of osteoporosis.

1. Introduction

The process of bone remodeling is controlled by a balance of bone formation and bone resorption (Roodman 1996). Excessive bone resorption that overcomes bone formation results in abnormalities such as osteoporosis, which is characterized by a reduction in bone mass and a higher incidence of bone fractures (Weinreb et al. 1989). Osteoporosis is a major health concern for aging communities. The progressive decrease in bone mass leads to an increased susceptibility to fractures, which results in substantial morbidity and mortality (Riggs and Melton 1992). The modern era of therapy for osteoporosis has become firmly established with the advent of antiresorptive agents.

Phytoestrogens are non-steroidal plant-derived compounds possessing estrogenic activity that are implicated in protecting against cancer progression (Strom et al. 1999). The phytoestrogens may display estrogenic or anti-estrogenic activity by binding to the various forms of the estrogen receptor (ER) or by competing for the binding site of estrogen biosynthesizing or metabolizing enzymes (Santti et al. 1998). There is a higher affinity of phytoestrogens typically for ERβ over ERα. Apigenin (4′,5,7-trihydroxyflavone), a common dietary flavonoid, is an ERβ selective phytoestrogen (Jarry et al. 2003). Apigenin is a non-toxic and non-mutagenic flavone. Apigenin is widely distributed in many fruits and vegetables such as parsley, onions, orange, tea, chamomile, and wheat sprouts and in some seasonings (Fernandez-de-Simon et al. 1992). More recently it has been suggested that apigenin inhibits NF-kB and acts through a mitochondrial mediated cell death pathway in cancer cells (Shukla et al. 2004). Apigenin has previously been described to inhibit the inflammatory mediators nitric oxide and prostaglandin E_2 in an *in vitro* model using two different murine macrophage cell-lines (RAW264.7 and J774A.1) that are activated with lipopolysaccharide (Raso et al. 2001).

In the initiation and development of rheumatoid arthritis (RA) and osteoarthritis (OA) disease processes, pro-inflammatory cytokines are throught to play a pivotal role (Pelletier et al. 1995). Interleukin-6 (IL-6) is pro-inflammatory cytokine and is important in the inflammatory stages of several chronic inflammatory diseases. RA and OA, both chronic joint diseases, share the characteristics of an inflamed synovium during certain stages of the disease. During inflammation, the synovium is infiltrated with mononuclear cells (Haraoui et al. 1991), producing a range of inflammatory mediators. Nitric oxide (NO) can be generated endogenously in several types of cells and plays diverse biological roles. When produced in large excess or concurrently produced with tumor necrosis factor-α (TNF-), NO also displays cellular toxicity and can induce apoptotic cell death in different types of cells. NO has been shown to modulate osteoclast recruitment and

activity (Mayer and Hemments 1997). *In vitro* studies suggest that high levels of NO suppress osteoblast proliferation and differentiation (Ralston et al. 1994). When certain flavonoids appear to be capable of inhibiting the production of the above-mentioned pro-inflammatory mediators, they could be of potential use as anti-inflammatory agents in chronic inflammatory diseases such as RA or OA.

MC3T3-E1 cells, an osteoblast-like cell line, have been reported to retain the capacity to differentiate into osteoblasts (Kodama et al. 1981) and to have both ERα and ERβ (Chen et al. 2003; Ikegami et al. 1993). Those cells may provide very useful information about the effects of phytoestrogens on the differentiation of osteoblasts. In the present study, the effects of apigenin on the differentiation of osteoblastic MC3T3-E1 cells were evaluated. Also, the effects of apigenin on TNF-α-induced production of IL-6 and NO in osteoblasts were investigated.

2. Investigations and results

2.1. Effect of apigenin on the growth of MC3T3-E1 cells

MC3T3-E1 cells were incubated with apigenin and cell growth was measured. MC3T3-E1 cell growth was promoted by stimulation with apigenin at $0.01 \sim 0.1$ μM (Fig. 1). Based on this preliminary observation, we evaluated the differentiation-inducing activities of apigenin on MC3T3-E1 cells by assessing for intracellular ALP activity and collagen synthesis.

2.2. Effect of apigenin on ALP activity in MC3T3-E1 cells

ALP activity was measured to study the effect of apigenin on the osteoblastic differentiation in MC3T3-E1 cells (Fig. 2). Culture in the presence of apigenin (0.01 μM) caused a significant increase in the ALP activity of osteoblastic cells (Fig. 2A). The effect of apigenin (0.01 μM) in increasing ALP activity was not seen in the presence of cycloheximide (10^{-6} M) or tamoxifen (10^{-6} M) (Fig. 2B).

2.3. Effect of apigenin on collagen synthesis in MC3T3-E1 cells

The effect of apigenin on collagen synthesis in osteoblastic MC3T3-E1 cells is shown in Fig. 3. The collagen synthesis

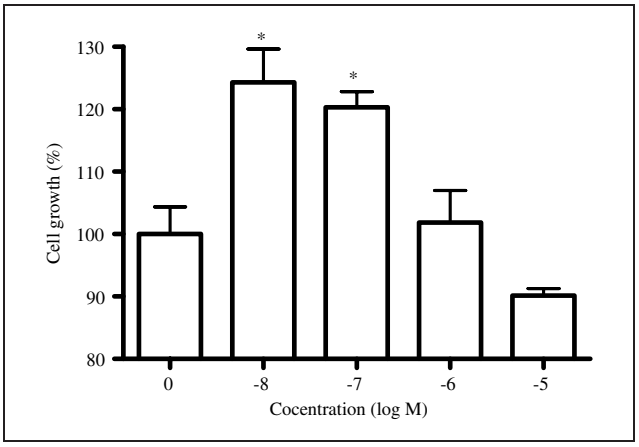

Fig. 1: Effect of apigenin on the growth of MC3T3-E1 cells. Data shown are mean ± SEM, expressed as a percentage of control. The control value for MTT assay was 0.247 ± 0.004 OD. * $P < 0.05$ vs. control

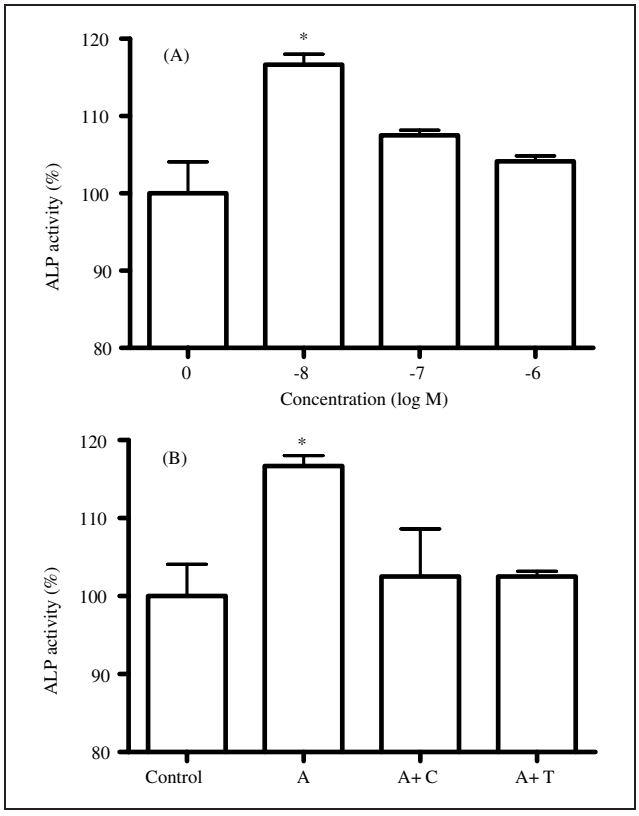

Fig. 2: Effect of apigenin on the alkaline phosphatase activity of MC3T3-E1 cells. After the cells reached confluence, the medium was replaced with α-MEM containing 5% CD-FBS in the presence or absence of apigenin (Fig. 2A), and in combination with 0.01 μM apigenin (A) and 10^{-6} M cycloheximide (C) or 10^{-6} M tamoxifen (T) (Fig. 2B). Data shown are mean ± SEM, expressed as a percentage of control. The control value for ALP activity was 1.88 ± 0.09 Unit/mg protein. * $P < 0.05$ vs. control

of MC3T3-E1 cells was increased by the addition of $0.01 \sim 1$ μM apigenin (Fig. 3A). The apigenin (0.01 μM)-induced increase in collagen synthesis was clearly eliminated by the presence of cycloheximide (10^{-6} M) or tamoxifen (10^{-6} M) (Fig. 3B).

2.4. Effect of apigenin on IL-6 and NO production in MC3T3-E1 cells

We investigated whether apigenin modulates osteoblast production of IL-6 and NO (Fig. 4). When 10^{-10} M TNF-α was added to cells, production of IL-6 and NO increased significantly. However, TNF-α-induced IL-6 and NO productions were inhibited by treatment of apigenin (10 μM).

3. Discussion

It has often been suggested that the consumption of dietary phytoestrogens may have beneficial effects on bone health at all stages of life. But the experimental evidence supporting these assertions is often equivocal or less than definitive. Osteoblasts are the bone-forming cells of the skeleton; they synthesise and regulate the deposition and mineralization of the extra-cellular matrix of bone. The three principal periods of the development are proliferation, matrix development and maturation, and mineralization. It is well known that osteoblasts produce type I collagen and ALP, which are associated with matrix maturation and mineralization (Stein et al. 1990). Thus we examined cell growth,

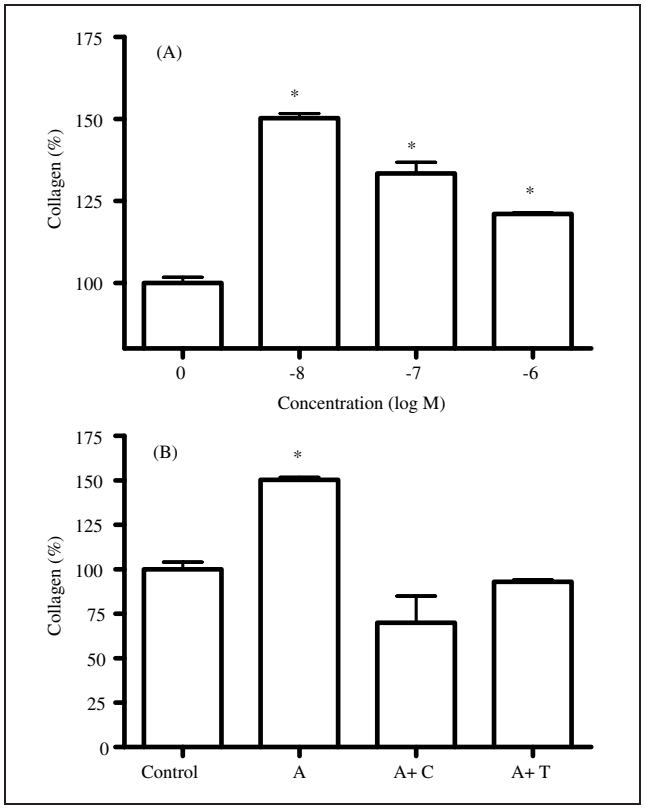

Fig. 3: Effect of apigenin on the collagen synthesis of MC3T3-E1 cells. After the cells reached confluence, the medium was replaced with α-MEM containing 5% CD-FBS in the presence or absence of apigenin (Fig. 3A), and in combination with 0.01 μM apigenin (A) and 10^{-6} M cycloheximide (C) or 10^{-6} M tamoxifen (T) (Fig. 3B). Data shown are mean ± SEM, expressed as a percentage of control. The control value for collagen content was 2.83 ± 0.43 μg per 10^7 cells. * $P < 0.05$ vs. control

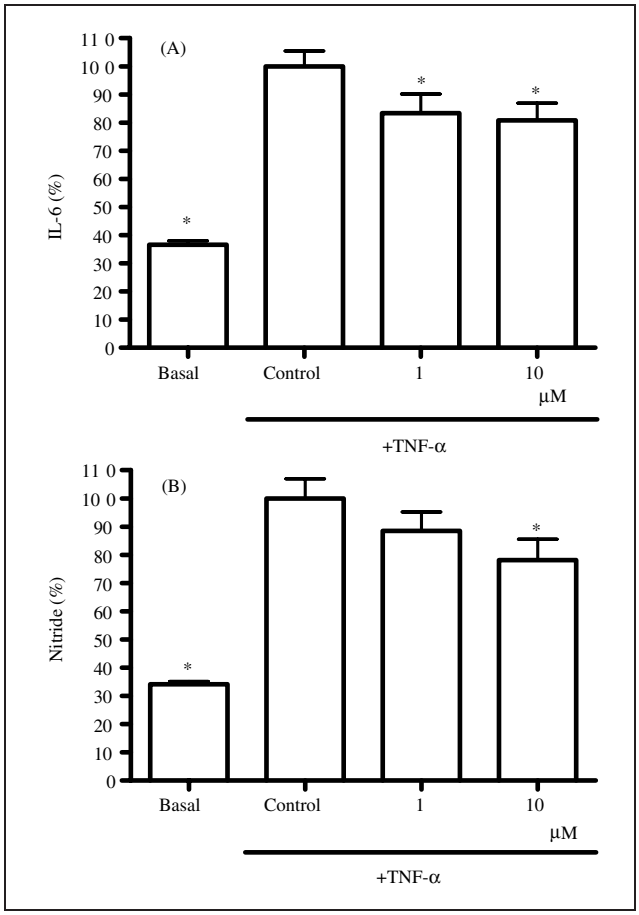

Fig. 4: Effect of apigenin on TNF-α-induced IL-6 and NO production of MC3T3-E1 cells. MC3T3-E1 cells were cultured with vehicle or apigenin in the presence of 10^{-10} M TNF-α for 48 h. Data shown are mean ± SEM, expressed as a percentage of control. Control values of IL-6 and NO production were 12.77 ± 0.69 pg/ml and 47.06 ± 3.27 mM, respectively, per 10^5 cells. * $P < 0.05$ vs. control

ALP activity, and collagen contents to investigate the effects of apigenin on the growth and differentiation of osteoblast-like cells. The present study demonstrates that apigenin (10^{-8} M) can significantly increase cell growth, ALP activity, and collagen synthesis in osteoblastic MC3T3-E1 cells *in vitro* ($P < 0.05$), indicating its anabolic effect at low concentration. The enhancing effect of apigenin on ALP activity and collagen synthesis in osteoblastic MC3T3-E1 cells was blocked completely by the presence of cycloheximide, an inhibitor of protein synthesis. The anabolic effect of apigenin may be based partly on a newly synthesized protein component. Moreover, the effect of apigenin in elevating ALP activity and collagen synthesis in osteoblastic cells was blocked completely by the anti-estrogen tamoxifen. This result may support the view that the effect of apigenin is partly involved in the mechanism of estrogen action in osteoblastic cells. Although the mechanism of action of apigenin on osteoblast differentiation is unknown at the moment, the increased ALP activity and collagen synthesis suggests that this compound may act directly on osteoblastic cells in this model. The molecular mechanism of apigenin's action remains to be elucidated.

TNF-α stimulates osteoblasts to secrete other bone resorbing cytokines (IL-1β and IL-6) and NO as well as TNF-α itself that act directly on osteoclasts to cause bone resorption (Franchimont et al. 1997). Considering that production of TNF-α in the bone microenvironment occurs in disease state, we aimed to investigate the effect of apigenin addition on the TNF-α-induced production of IL-6 and NO in osteoblastic MC3T3-E1 cells. We found that TNF-α increases the production of these inflammatory mediators, and apigenin (10 μM) inhibits TNF-α-induced IL-6 and NO production in MC3T3-E1 osteoblastic cells. Girasole et al. (1992) and Kassem et al. (1996) reported that IL-1β plus TNF-α-stimulated production of IL-6 was reduced by E_2, supporting the hypothesis that the antiresorptive action of the phytoestrogen may be mediated by a decreased production of IL-6 by osteoblastic cells. Moreover, the result of NO production in osteoblasts suggests that apigenin may regulate TNF-α-induced NO production. Hukkanen et al. (1995) suggested that NO formation is an important postreceptor effect mechanism of these processes. NO mediates many of cytokine actions. It is possible that cytokines in the circulation or produced by peripheral monocytes as a result of systemic inflammation may induce inducible NO synthase expression in osteoblasts, and that the large amount of NO produced are responsible for the suppression of bone formation observed in systemic inflammation.

Previous studies have shown that TNF production by peripheral blood monocytes increased in women after natural or surgical menopause, and this increase was blocked by estrogen replacement (Pacifini et al. 1991). In addition, treatment of rodents with TNF inhibitor blocked bone loss and osteoclastogenesis in ovariectomized animals (Kimble et al. 1995), which implies that TNF production in premenopausal bone may not be significant and that only in the postestrogen withdrawal state do this cytokine becomes

involved in bone metabolism. Our data imply that apigenin may prevent TNF-α-induced bone resorption through inhibiting osteoblasts to release IL-6 and NO. These mediators act in concert to promote matrix metalloproteinases expression in the adjacent osteoblasts. The results further suggest that apigenin may have an important role in the regulation of localized bone destruction associated with inflammatory bone diseases such as rheumatoid arthritis by inhibition of NO production in osteoblasts.

In summary, apigenin increased osteoblastic differentiation at low concentration and inhibited TNF-α-stimulated production of IL-6 and NO by osteoblastic MC3T3-E1 cells. These findings suggest that apigenin might be useful for diseases associated with the excessive production of IL-6 and NO, and skeletal tissues may benefit from the consumption of apigenin-containing plants.

4. Experimental

4.1. Reagents

Apigenin was purchased from Wako Pure Chemicals, Industries, Ltd. (Japan). Apigenin was dissolved in dimethylsulfoxide (DMSO) and then diluted with the medium (final DMSO concentration $\leq 0.05\%$ (v/v)). All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

4.2. Cell cultures

MC3T3-E1 cells (RCB1126, an osteoblast-like cell line from C57BL/6 mouse calvaria) were obtained from the RIKEN Cell Bank (Tsukuba, Japan). MC3T3-E1 cells were cultured at 37 °C in 5% CO_2 atmosphere in α-modified minimal essential medium (α-MEM; GIBCO). Unless otherwise specified, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin.

4.3. Cell viability; MTT assay

The cells were suspended in medium and plated at a density of 7.0×10^3 cells/well into a 96-well culture dish (Costar, Cambridge, MA). After 24 h, the medium was replaced with phenol red-free media containing 5% charcoal-dextran-treated FBS (CD-FBS) supplemented with sample. After 2 days of culture, cell proliferation was measured by MTT assay. This assay is based on the ability of viable cells to convert soluble 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble dark blue formazan reaction product. In the bulk cell photometric MTT assay, the bulk conversion of MTT in the well plate was measured photometrically. MTT was dissolved in DPBS at a concentration of 5 g/l and sterilized by passage through a 0.22 mm filter. This stock solution was added (one part to 10 parts medium) to each well of culture plate, and the plate was incubated at 37 °C for 2 h. DMSO was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature, to ensure that all the crystals were dissolved, the plates were read on a microplate reader at a wavelength of 570 nm.

4.4. Alkaline phosphatase (ALP) activity

After the cells were cultured at a density of 10^6 cells into culture dish for 7 days, the medium was replaced with phenol red-free α-MEM containing 5% CD-FBS. Then, the cells were cultured with sample in the presence of 10 mM β-glycerophosphate (β-GP); β-GP was added to initiate in vitro mineralization (Kanno et al. 2001). After 3 days, the medium was removed and the cell monolayer was gently washed twice with PBS. The cells were lyzed with 0.2% Triton X-100 and the lysate was centrifuged at $14{,}000 \times g$ for 5 min. The clear supernatant was used for the measurement of ALP activity and protein concentration. ALP activity and protein concentration were determined using an ALP activity assay kit (Wako) and a BCA-protein assay kit (Pierce, Rockford, IL), respectively.

4.5. Collagen contents

After the cells were cultured at a density of 10^6 cells into culture dish for 7 days, the medium was replaced with α-MEM containing 5% CD-FBS. Then, the cells were cultured with sample in the presence of 10 mM β-GP for 3 days and cellular collagen content was measured using Sircol Collagen Assay kit (Biocolor Ltd. Northern Ireland). This assay is a quantitative dye-binding method designed for the analysis of collagens extracted from mammalian tissues and cells during *in vitro* culture. The dye reagent binds specifically to the $[Gly\text{-}X\text{-}Y]_n$ helical structure found in mammalian collagens (types I to V).

4.6. IL-6 immunoassay

After cells were treated with sample and 10^{-10} M TNF-α for 48 h, IL-6 content in the medium was measured with an enzyme immunoassay system (R&D system Inc., Minneapolis, MN, USA) according to the manufacturer's recommendation. In brief, cytokine present is bound by immobilized antibody pre-coated onto a microplate. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for cytokin is added to the well. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when stop solution is added. The intensity of the color measured is in proportion to the amount of cytokine bound.

4.7. Determination of nitrite production

After cells were treated with sample and 10^{-10} M TNF-α for 48 h, nitrite production, an indicator of NO synthesis, was measured in the culture supernatant of osteoblasts, as described previously by Kleinerman et al. (1987). Briefly, after mixing 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) with 100 μl culture supernatant, optical density at 540 nm was measured using a microplate reader. Nitrite concentrations were calculated from the standard curve of sodium nitrite prepared in culture medium.

4.8. Statistics

The results are expressed as mean ± SEM ($n = 6$). Statistical analysis was performed using one-way ANOVA ($P < 0.05$). The analysis was performed using SAS statistical software.

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