Promoting Effect of Kaempferol on the Differentiation and Mineralization of Murine Pre-osteoblastic Cell Line MC3T3-E1

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A number of agents have been reported to influence osteoblastic differentiation and to prevent and treat bone loss. We found that kaempferol, a flavonoid identified in extracts of the medicinal plant, Polygonum tinctorium. Lour, had stimulatory effects on the differentiation and mineralization of the murine pre-osteoblastic cell line, MC3T3-E1. After enhancing the alkaline phosphatase activity, significant augmentation of calcification by kaempferol was observed between concentrations of 10 and 20 μM, without any marked effect on cell proliferation. When kaempferol was combined with ipriflavone, which is clinically applied to treat bone loss, calcification was synergistically augmented, suggesting that these two flavonoids may have different mechanisms of action.

These results suggest that kaempferol may be a promising agent for the prevention or treatment of bone loss, especially when combined with ipriflavone.

Key words: kaempferol; osteoblasts; alkaline phosphatase; mineralization; differentiation

Many plant-derived substances have been used as drugs for the treatment of various diseases since ancient times, and traditional Asian therapies are rich in phytotherapeutic regimens. We have long been interested in Indigo plants as a source of biologically active ingredients, since these plants have been used as a source of dye and a form of traditional medicine. During our investigations, we and others have found Polygonum tinctorium Lour. (P. tinctorium), a member of the Indigo plant family, to be a source of a variety of biologically active substances. Various biological activities attributed to crude extracts and compounds isolated from P. tinctorium have been described. Anti-oxidative and anti-tumor activities in an ethyl acetate extract of this plant have been reported by Kimoto et al., which were found to be caused by gallic acid and caffeic acid, and tryptanthrin, respectively. We have recently reported that tryptanthrin exerted a potent anti-inflammatory effect on murine macrophages in an in vitro system by inhibiting the synthesis of NO and PGE2, and this molecule was found to have a therapeutic effect in an in vivo model of inflammatory disease. Furthermore, it has been reported that tryptanthrin, kaempferol and other compounds in P. tinctorium extracts possessed anti-microbial and anti-viral activities against a variety of microorganisms.

Many investigators have shown that a class of plant-derived compounds such as kaempferol had estrogenic activities. These compounds have shown the capacity to bind to estrogen receptors, increase uterine weight and protect against beta amyloid-induced toxicity in a neuroblastoma cell line. Members of this class are the so-called phytoestrogens. It has been extensively reported that estrogen can protect against bone loss or decreasing mineral density based on many clinical studies on postmenopausal women. Considering these reports, we considered the possibility that kaempferol could show a protective effect against bone loss. We therefore examined the effect on differentiation and mineralization induced by kaempferol by using the pre-osteoblastic target cell line, MC3T3-E1, which has been a well-characterized as an in vitro model for osteoblast differentiation.

Materials and Methods

Cell culture. Murine pre-osteoblastic MC3T3-E1 cells were cultured in the growth medium, Eagle's alpha minimum essential medium (MEM, Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Whittaker, Walkersville, MD, USA), 100 U/ml of penicillin G and 100 μg/ml of streptomycin at 37°C in a humidified 5% CO2/air mixture. The MC3T3-E1 cells were plated at a density of 5 × 10^4 cells/ml in 24-well plastic dishes for 3 days to reach semi-confluency. In order to sustain the in vitro differentiation and mineralization, the culture medium was replaced with a
differentiation-inducing medium of MEM supplemented with 10% FBS containing 10 mM beta glycerophosphate (Sigma, St. Louis, MI, USA), 10 mM HEPES at pH 6.8, and various concentrations of kaempferol (3,4',5,7-tetrahydroxyflavone, Funakoshi Co., Tokyo, Japan) and/or iripriflavone (7-isopropyloxy-3-phenyl-4H-1-benzopyran-4-one; Daito Corporation, Toyama, Japan). The spent medium was replaced with one containing the fresh test agents every 3 days. The chemicals were prepared as stock solutions in ethanol and diluted with the medium to the desired concentrations. Control wells received equivalent amounts of the solvent only.

Influence of cell proliferation. The influence of cell proliferation was evaluated by a semi-automated microfluorometric method with Alamar Blue, a specific stain for enzyme activity in the mitochondria of viable cells. MC3T3-E1 cells were plated at a density of 1 × 10^4 cells per well in 96 flat-bottomed well plates and cultured for 3 days in the growth medium to reach semi-confluency. On the 3rd day, the medium was replaced with the differentiation-inducing medium containing various chemical stimuli, and the cells incubated at 37°C for the next successive 3, 6, or 9 days. The medium was again replaced with one containing fresh test agents every 3 days. After incubating, the cells were washed twice with 10 mM phosphate-buffered saline (PBS, pH 7.2), and then incubated at 37°C for 1 h with the growth medium containing 10% (v/v) Alamar Blue. The optical density was determined automatically by a colorimetric microtiter plate reader at a wavelength of 544 nm and a reference wavelength of 590 nm. Each result is expressed as the mean of triplicate wells ± the standard deviation (SD).

Alkaline phosphatase activity. The alkaline phosphatase enzyme activities within the cells and matrices were measured by an assay based on the method of Kajii et al. Briefly, the cells and matrices attached to the wells were washed three times with 0.25 M sucrose. After adding 0.9 ml of a reaction mixture containing a 50 mM carbonate buffer (pH 9.8), 25 mM sucrose and 1 mM MgCl₂ at 25°C, the enzyme reaction was initiated by adding 0.1 ml of a 2.5 mM p-nitrophenyl phosphate disodium (pNPP) substrate. After 10 min, 50 μl of the reaction mixture was extracted and mixed with 150 μl of a 0.6 N NaOH solution to develop the color. The hydrolytic activity of pNPP was measured colorimetrically at 420 nm by the microtiter plate reader. One unit of enzyme activity is defined as the amount of the enzyme that caused the release of 1 μmol of the product per min at 25°C.

Quantitation of Ca²⁺. We measured the amount of calcium on the cells as a component of hydroxyapatite by using an assay based on the method of Hagiwara et al. The cell culture in 24-well plates (2.0 cm²/well) were washed with PBS and then incubated with 0.5 ml of 2 N HCl while gently shaking. The Ca²⁺ in the samples was quantitated by the o-cresolphthalein complexone method with a Calcium C kit (Wako Pure Chemical Industries, Osaka, Japan) as described by the manufacturer. This kit is specific for Ca²⁺ and has a limit of detection of 1 μg/ml. Dilutions of the standard solution of Ca²⁺ (10 mg/ml) provided with the kit were used for titration purposes.

Alizarin red-S staining. Calcified nodules on the cells were demonstrated by using an assay based on the method of Prabhakar et al. Briefly, cell cultures were washed twice with PBS, fixed for 10 minutes in 50% ethanol in distilled water, and rehydrated with 1 ml of distilled water for 5 minutes. Cultures were then stained for 1 minute with 200 μl of 1% Alizarin red-S. The cell monolayers were next washed twice with 1 ml of distilled water per wash. Calcified nodules that appeared bright red in color were identified by light microscopy and recorded by photography.

Statistical analysis. Data were analyzed by a one-way analysis of variance (ANOVA). When ANOVA indicated differences among the groups, a pairwise comparison of each experimental group versus the control group was performed by Scheffe’s test.

Results

Effects on MC3T3-E1 cell proliferation
Figure 1 shows the effect of kaempferol and/or iripriflavone on the MC3T3-E1 cell proliferation. The semi-confluent MT3T3-E1 cells were exposed to various concentrations of kaempferol and/or iripriflavone, and incubated for a further 3, 6, or 9 days. As shown in Fig. 1, there was slightly increased cell proliferation on successive days in all groups. Kaempferol and/or iripriflavone did not show any remarkable cytotoxicity at the concentrations used, although the proliferation of the cells was slightly inhibited by the combination of kaempferol and iripriflavone at 20 μM.

Stimulation of the alkaline phosphatase activity in MC3T3-E1 cells
To evaluate the effect of kaempferol on the differentiation of MC3T3-E1 cells along the osteoblastoid lineage, we examined the activity of ALP, a well-known marker of osteoblastic differentiation. The seeded semi-confluent cells were incubated in the presence of different concentrations of kaempferol and/or iripriflavone for 3, 6, or 9 days. At an early stage on day 3, increasing ALP activity in the cells stimulated with iripriflavone was observed in a dose-dependent manner (Fig. 2). Iripriflavone at 20 μM
Fig. 1. Effects of Kaempferol, Ipriflavone and Their Combination on MC3T3-E1 Cell Proliferation in vitro.
Cells were cultured in the presence of the agents for various times as described in the text, and cell proliferation was determined by the alamar blue assay with a commercially available kit. The spent medium was replaced with fresh medium every 3 days. Each result represents the mean from triplicate wells and the standard deviation (S.D.). Statistically significant difference between the means of the experimental and control groups: *P<0.05, **P<0.01.

Fig. 2. Effects of Kaempferol, Ipriflavone and Their Combinations on the Kinetics of Induced MC3T3-E1 Cell Alkaline Phosphatase Activity.
Cells were cultured for a maximum of 9 days in the presence of the flavonoids, and the enzyme activity was determined as described in the text after 3, 6 and 9 days of culture. Each result represents the mean from triplicate wells and the S.D. Statistically significant difference between the means of the experimental and control groups: *P<0.05, **P<0.01.
enhanced the activity of ALP to approximately 221% of that of the control culture. As shown in Fig. 2, the differentiation-inducing effect of ipriflavone was evident earlier than that of kaempferol, and the combination of kaempferol and ipriflavone showed the most potent induced level of ALP on day 3. At an intermediate stage on day 6, in all the cell cultures stimulated with kaempferol and/or ipriflavone, an increased level of ALP activity was also observed in a dose-dependent manner. Interestingly, kaempferol at 20 μM enhanced the ALP activity to approximately 202% that of the control, and showed more potent induction of ALP activity than ipriflavone. Nevertheless, the combination of kaempferol and ipriflavone, each at 10 μM, significantly enhanced the activity of ALP to approximately 237% that of the control. The combination also showed the most potent ALP-inducing capacity on day 6. In contrast, in the later stage on day 9, the enhancing effect on ALP activity of the combination was reduced, while that of the single agents remained at the same level as that on day 6.

Augmented mineralization of MC3T3-E1 cells

Figure 3 shows the measured results of the amount of calcium in the mineralized cell layers. The semi-confluent cells were cultured in the presence of kaempferol and/or ipriflavone for 3, 6, or 9 days. On day 3, at the early stage, there was no increase in the deposition of calcium induced by any of the agents. However, on day 6, at the intermediate stage, kaempferol at 20 μM showed an approximately 323% increase in calcium deposition when compared to the control, and the inducing activity was more potent than that of ipriflavone (Fig. 3). The combined use of the agents, each at 20 μM, produced mineralization that was approximately 942% that of the control. This augmented mineralization induced by the combination was markedly greater than that induced by either of the single agents.

Figure 4 shows the results of Alizarine red-S staining of mineralized nodules that were formed by MC3T3-E1 cells on day 9 in the presence of 10 μM kaempferol and/or ipriflavone, showing that combined use of these agents augmented the formation of mineralized nodules on MC3T3-E1 cells.

Discussion

We have shown in this study that the estrogenic plant product, kaempferol, potently enhanced calcium deposition on murine pre-osteoblastic cell line MC3T3-E1, after inducing the activity of ALP, and that both activities were synergistically augmented by combining kaempferol with ipriflavone.

In general, pre-osteoblastic cells are known to produce protein of the extra-cellular matrix, including type I collagen at first, and then to successively produce ALP, osteopontin and osteocalcin during differentiation to osteoblasts. Finally, the osteoblasts deposit calcium.18)

Kaempferol and ipriflavone belong to the flavonoid family, being respectively chemically classified as flavonol and isoflavone derivatives. In addition,
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Kaempferol is known to up-regulate the expression of the estrogen receptor, not but to bind directly to it. Ipriflavone, which has been used as an anti-osteoporotic drug, reportedly enhanced the ALP activity in rat osteoblastic cell line UMR-106 cells, ROB cells, and in rat bone marrow stromal cells. Ipriflavone has also stimulated the differentiation and mineralization of stromal osteoprogenitor cells in the human bone marrow and trabecular bone osteoblasts, ROB cells, and in rat bone marrow stromal cells. Ipriflavone regulates the mechanism of bone formation via endothelin receptors and via estrogen receptors.

As shown in Fig. 2, ipriflavone induced the ALP activity of MC3T3-E1 cells from an early stage after exposure, on day 3, and kaempferol induced the same activity from the intermediate stage, on day 6. Thus, the kinetics of ALP activation induced by kaempferol and ipriflavone were different. In addition, when used together, the two agents showed a synergistic effect on the induction of ALP activity and calcium deposition (Figs. 2 and 3). These results suggest that these agents probably induced differentiation of the pre-osteoblastic cells along different pathways. However, the precise mechanism for osteoblast differentiation and calcification induced by kaempferol remains to be resolved.

Clinical results have shown that the plasma concentration of ipriflavone and its derivatives totaled approximately 150 ng/ml 24 h after ipriflavone at 200 mg had been orally administered. The plasma concentration of kaempferol was reportedly 15 ng/ml 24 hr after an oral administration of kaempferol at 27 mg. Thus, it seems that the absorption and metabolism of the two agents were similar. In addition, the effective in vitro concentrations of kaempferol and ipriflavone used in this and other reports were approximately the same, i.e. ranging from 10 to 20 μM. These results suggest that kaempferol and ipriflavone alone or in combination may be clinically effective.

Further in vitro studies are necessary to clarify the mechanisms for the diverse biological effects of flavonoids, including kaempferol and ipriflavone, on the proliferation and differentiation of osteoblasts that lead to the mineralization of pre-osteoblastic cells. In vivo studies will also shed light on what effects one may expect when applying such biologically active
compounds in complex cellular networks in vivo, especially the effects arising after long-term treatment. In conclusion, the results from this study suggest that kaempferol promoted osteoblastic differentiation and may be useful as a pharmacological agent alone or in combination with ipriflavone for the treatment of osteoporosis.

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References

