Anabolic Effects of Bee Pollen *Cistus ladaniferus* Extract in Osteoblastic MC3T3-E1 Cells *In Vitro*

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The effect of bee pollen extract on osteoblastic MC3T3-E1 cells was investigated. The water-solubilized extracts, which were obtained from the bee pollen of *Cistus ladaniferus*, was purified using the membrane fractionation method with molecular weight (MW) less than 1000. Osteoblastic cells were cultured for 72 hr in a medium containing either vehicle or cistus extract of less than MW 1000 (10, 25, or 50 μg/ml of medium) in the presence of 10% fetal bovine serum (FBS). The proliferation of osteoblastic cells was significantly enhanced in the presence of cistus extract (25 or 50 μg/ml). The stimulatory effect of cistus extract on cell proliferation was also observed when the cells with subconfluency were cultured for 24 or 72 hr without FBS. Cells with subconfluency were cultured for 24 or 72 hr in a medium containing either vehicle or cistus extract without FBS. DNA content or alkaline phosphatase activity in osteoblastic cells was significantly increased after culture with cistus extract (50 μg/ml) for 24 hr. The effect of 25 μg/ml of cistus extract on these components was seen after culture for 72 hr. This study demonstrates that the cistus extract fraction of less than MW 1000 has anabolic effects in osteoblastic MC3T3-E1 cells which involve in bone formation.

Key words —— *Cistus ladaniferus*, bee pollen, osteoblast, bone formation

INTRODUCTION

Bone loss with aging induces osteoporosis, which is widely recognized as a major public health problem. Bone loss may be due to decreased bone formation and increased bone resorption. Pharmacologic and nutritional factors are needed to prevent bone loss with aging.[1–3] Chemical compounds in food that act on bone metabolism, however, are poorly understood. Isoflavones,[2,4,5] which are contained in soybeans, menaquinone-7,[6,7] an analogue of vitamin K_2 abundant in fermented soybeans, and carotenoid β-cryptoxanthin,[8,9] which is present in Satsuma mandarins (*Cistrus unshiu*, MARC.), have been demonstrated to have stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption, thereby increasing bone mass. Chemical factors in food and plants play a role in bone health and may be important in the prevention of bone loss with aging.

Our recent studies demonstrated that water-solubilized extract of bee pollen *Cistus ladaniferus* has anabolic effects on bone components in rats *in vitro* and *in vivo*.[10] The extract of bee pollen cistus has stimulatory effects on bone formation and inhibitory effects on bone resorption *in vitro*.[10,11] The extract stimulates bone calcification as potently as propolis.[10] Royal jelly does not have an inhibitory effect on osteoclastogenesis in mouse marrow culture *in vitro*,[12] so that the effect of bee pollen on bone metabolism may thus be unique among bee-related products. The intake of bee pollen cistus extract has been demonstrated to have preventive effects on bone loss induced in the diabetic state[13] and ovariectomized rats,[14] suggesting a useful role in the prevention of osteoporosis with aging.

The active component of bee pollen cistus extract, which stimulates bone formation and inhibits osteoclastic bone resorption, has been shown to be a fraction with molecular weight (MW) of less than 1000.[15]

This study was undertaken to determine whether the cistus extract with the fraction of less than MW 1000 has anabolic effects on osteoblastic MC3T3-E1 cells, which involve in bone formation.

MATERIALS AND METHODS

Chemicals —— α-Minimal essential medium (α-MEM) and penicillin-streptomycin (5000 U/ml penicillin; 5000 μg/ml streptomycin) were obtained

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from Gibco Laboratories. Fetal bovine serum (FBS) was obtained from Bioproducts, Inc. Other chemicals were of reagent grade and were obtained from Wako Pure Chemical Industries (Osaka, Japan). All water used were glass distilled.

Bee Pollen Extracts —— Bee pollen was obtained from *Cistus ladaniferus*. The powder of bee pollen (5 g) was suspended in distilled water (20 ml) and mixed vigorously, and the suspension was centrifuged at 10000 *g* in a refrigerated centrifuge for 20 min. The 10000 *g* supernatant fraction was collected and filtered, and the filtrate was freeze-dried.[10] The powder of the water-solubilized extract was dissolved in ice-cold distilled water for use in experiments. The water-solubilized extract from bee pollen was purified using the membrane fractionation method with less than MW 1000.[15]

Cell Culture —— Osteoblastic MC3T3-E1 cells were cultured at 37°C 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 phosphate-buffered saline (PBS). For experiments, about 1.0×10^5 cells per dish were cultured for 72 hr to obtain subconfluent monolayers in 35-mm plastic containing 2 ml α-MEM with 10% FBS. After the cells were rinsed with PBS, the medium was exchanged for medium without FBS containing either vehicle or cistus extract fraction with less than MW 1000 (10, 25, or 50 μg/ml of medium) and the cells were cultured further for 24 or 72 hr. Cell viability was estimated by staining with trypan blue.

Cell Counting —— After trypsinization of the cells in each culture dish using a Ca^{2+}/Mg^{2+}-free PBS containing 0.2% trypsin and 0.02% EDTA for 2 min at 37°C, cells were collected and wash-centrifuged in a PBS solution at $100 \times g$ for 5 min. The cells were resuspended in a 0.5 ml PBS solution, and an aliquot was stained with eosin. The cells were counted under a microscope using a Hemacytometer plate. For each dish, we took the average of two counts.

Analytical Procedures —— 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 6 hr after disruption.[16] After alkali extraction, the samples were centrifuged at $10000 \times g$ for 5 min, and the supernatant was collected. DNA content in the supernatant was determined by the method of Cerriotti[17] and expressed as the amount of DNA (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 0.25 M sucrose solution, and disrupted for 30 s with an ultrasonic device. The supernatant, centrifuged at $600 \times g$ 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 using the method of Walter and Schutt.[18] Protein concentration in the cell homogenate was determined using the method of Lowry *et al.*[19] and expressed as the amount of protein (μg) per dish. The enzyme activity was expressed as nanomoles of *p*-nitrophenol liberated per minute per milligram of protein.

Statistical Analysis —— Data are expressed as the mean ± standard error of the mean (SEM). Statistical differences were analyzed using Student's *t*-test. p-value < 0.05 was considered to indicate a statistically significant difference. The analysis of variance (ANOVA) multiple comparison test was used to compare the treatment groups.

RESULTS

Effects of Cistus Extract on Proliferation of Osteoblastic Cells

The effect of cistus extract on proliferation of osteoblastic MC3T3-E1 cells reaching subconfluent monolayers was examined. Osteoblastic cells were cultured for 72 hr in a medium containing either vehicle or cistus extract with less than MW 1000 (10, 25, or 50 μg/ml of medium) in the presence of 10% FBS. The proliferation of osteoblastic cells was progressively increased after culture for 72 hr to reach confluent monolayers. This increase was significantly enhanced in the presence of cistus extract (25 or 50 μg/ml; Fig. 1). The stimulatory effect of cistus extract (25 or 50 μg/ml) on cell proliferation was also observed when the cell with subconfluency were cultured for 24 or 72 hr in the absence of 10% FBS (Fig. 2).

Effect of Cistus Extract on Biochemical Component in Osteoblastic Cells

Osteoblastic cells were cultured for 72 hr in a medium containing 10% FBS. Cells with subconfluency were cultured for 24 or 72 hr in a medium con-

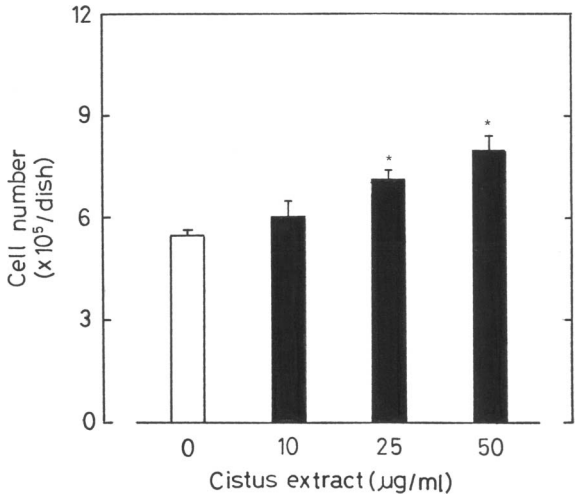

Fig. 1. Effect of Cistus Extract on Growth of Osteoblastic MC3T3-E1 Cells *In Vitro*

Osteoblastic cells (1×10^5 cells) were cultured for 72 hr in a medium containing either vehicle or cistus extract (10, 25, or 50 μg/ml of medium) with less than MW 1000 in the presence of 10% FBS. After trypsinization of the cells in each culture dish, cells were collected and counted. Each value is the mean ± SEM of six cultures. $^*p < 0.01$ compared with the control (none) value.

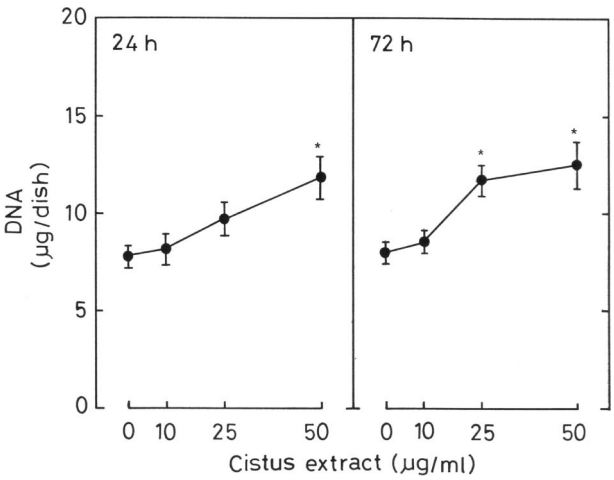

Fig. 3. Effect of Cistus Extract on DNA Content in Osteoblastic MC3T3-E1 Cells

Osteoblastic cells (1×10^5 cells) were cultured for 72 hr in a medium containing 10% FBS. After culture, the medium was changed, then cistus extract (10, 25, or 50 μg/ml of medium) with less than MW 1000 was added in the culture medium without FBS, and the cells were cultured for an additional 24 or 72 hr. Cells were washed with PBS and scraped to determine DNA content. Each value is the mean ± SEM of six cultures. $^*p < 0.01$ compared with the control (none) value.

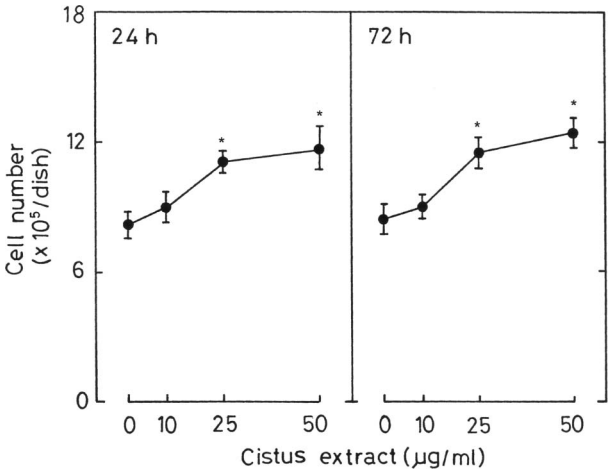

Fig. 2. Effect of Cistus Extract on Cell Number of Osteoblastic MC3T3-E1 Cells with Subconfluency *In Vitro*

Osteoblastic cells (1×10^5 cells) were cultured for 72 hr in a medium containing 10% FBS. After culture, the medium was changed, then cistus extract (10, 25, or 50 μg/ml of medium) with less than MW 1000 was added in the culture medium without FBS, and the cells were cultured for an additional 24 or 72 hr. After trypsinization of the cells in each culture dish, cells were collected and counted. Each value is the mean ± SEM of six cultures. $^*p < 0.01$ compared with the control (none) value.

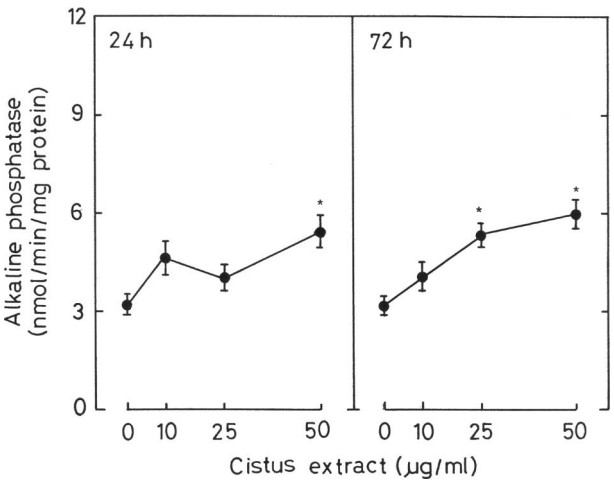

Fig. 4. Effect of Cistus Extract on Alkaline Phosphatase Activity in Osteoblastic MC3T3-E1 Cells

Osteoblastic cells (1×10^5 cells) were cultured for 72 hr in a medium containing 10% FBS. After culture, the medium was changed, then cistus extract (10, 25, or 50 μg/ml of medium) with less than MW 1000 was added in the culture medium without FBS, and the cells were cultured for an additional 24 or 72 hr. Cells were washed with PBS and scraped to assay the enzyme activity. Each value is the mean ± SEM of six cultures. $^*p < 0.01$ compared with the control (none) value.

taining either vehicle or cistus extract with less than MW 1000 (10, 25, or 50 μg/ml of medium) without FBS. The change in DNA content or alkaline phosphatase activity in osteoblastic cells was examined. DNA content in osteoblastic cells was significantly increased after culture with cistus extract (50 μg/ml) for 24 hr (Fig. 3). The effect of 25 μg/ml of cistus extract was seen after culture for 72 hr.

Alkaline phosphatase activity in osteoblastic cells was significantly increased after culture with

cistus extract (50 μg/ml) for 24 hr (Fig. 4). Culture with 25 μg/ml of cistus extract for 72 hr caused a significant increase in alkaline phosphatase activity in osteoblastic cells.

DISCUSSION

Bee pollen *Cistus ladaniferus* extract has been shown to have stimulatory effects on bone formation and inhibitory effects on bone resorption in rat femoral tissues *in vitro*,[10, 11] thereby increasing bone mass. The active component, which stimulates bone formation and inhibits bone resorption *in vitro*, is found to be in the extract fraction with less than MW 1000.[15] The extract has been demonstrated to inhibit osteoclastogenesis in mouse marrow culture *in vitro*. This study, moreover, demonstrates that the extract fraction with less than MW 1000 has anabolic effects on osteoblastic MC3T3-E1 cells *in vitro*.

Cistus extract with molecules less than MW 1000 was found to have stimulatory effects on proliferation of osteoblastic cells. Culture with extract stimulated cell proliferation in reaching confluent monolayers in the presence of FBS. Moreover, the extract increased the number of osteoblastic cells with subconfluency in the absence of FBS. These observations suggest that the cistus extract with molecules less than MW 1000 has a role as growth factor in osteoblastic cells.[20]

DNA content in osteoblastic cells was increased after culture with cistus extract fraction of less than MW 1000, suggesting that the extract has mitogenic effect in osteoblastic cells.

Alkaline phosphatase is involved in mineralization in osteoblastic cells.[21] Culture with cistus extract caused a significant increase in alkaline phosphatase activity in osteoblastic cells. It is speculated that cistus extraction has stimulatory effects on differentiation and mineralization in osteoblastic cells.

Bee pollen cistus extract with the fraction of less than MW 1000 has been shown to have stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption, thereby increasing bone mass. The intake of cistus extract has preventive effects on bone loss in diabetic state and ovariectomized rats,[13, 14] suggesting a role in the prevention of osteoporosis.

In conclusion, it has been demonstrated that the extract fraction with less than MW 1000, which was obtained from bee pollen *Cistus ladaniferus*, has anabolic effects in osteoblastic MC3T3-E1 cells *in vitro*, supporting the view that cistus extract can stimulate osteoblastic bone formation.

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