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Effects of Eucommiae Cortex on Osteoblast-like Cell Proliferation and Osteoclast Inhibition

Hyekyung Ha, Jinnyung Ho[1], Sunmi Shin, Hyejin Kim, Sungja Koo[1], In-Ho Kim[2], and Chungsook Kim

Drug Research and Development Team, Korea Institute of Oriental Medicine, 129-11 Chungdam-dong, Kangnam-ku, Seoul 135-100, Korea, [1]Graduate School of East-West Medical Sciences, Kyung Hee University, Seoul, Korea, and [2]Material Processing Technology Division, Korea Food Research Institute, Songnam, Korea

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Methanol extract (MeOH), *n*-hexane (Hx), chloroform ($CHCl_3$), ethyl acetate (EA), butanol (BuOH) and aqueous (H_2O) fractions of Eucommiae Cortex including geniposidic acid (GA), geniposide (GP) and aucubin (AU) were tested for their therapeutic efficacy on osteoporosis. The contents of GA, GP and AU in the cortex and leaf of *Eucommia ulmoides* Oliver were quantified by HPLC. The effect of Eucommiae Cortex on the induction of growth hormone (GH) release was studied by using rat pituitary cells. The proliferation of osteoblast-like cells increased by herbal extracts was assayed using a tetrazolium (MTT), alkaline phosphatase (ALP) activity, and [^{3}H]-proline incorporation assays. The inhibition of osteoclast was studied by using the coculture of mouse bone marrow cells and ST-2 cells. As a result, the GA, GP and AU were present in the cortex more than in the leaf of *E. ulmoides* Oliver. The MeOH (1 mg/mL), Hx, $CHCl_3$ and EA fractions (each 20 μg/mL) had potent induction of GH release. The $CHCl_3$ exhibited the potent proliferation of osteoblasts. The AU, GP and GA were increased proliferation of osteoblasts. In addition, GA (IC_{50}: 4.43×10^{-7} M), AU and GP were significantly inhibited proliferation of osteoclast. In summary, it is thought that the components in a part of the fractions of Eucommiae Cortex participate in each step of mechanism for activating osteoblast to facilitate osteogenesis, and suppress osteoclast activity to inhibit osteolysis.

Key words: Eucommiae cortex, Osteoblast, Osteoclast, Growth hormone, Proline incorporation, Osteoporosis

INTRODUCTION

Eucommiae Cortex is at least 15-year-old dried cortex of *Eucommia ulmoides* Oliver and used as a medicinal herb. The main components of Eucommiae Cortex are 6-10% gutta percha (plant gum), glycoside, alkaloid, pectin, chlorogenic acid, iridoid, cyclopentanoid and dilignan-glycoside (Hong *et al.*, 1988). Eucommiae Cortex displays pharmacological actions of hypotension, myocardial inhibition, vasodilation, uresis, cholagogue, glycosuria inhibition, ataralgesia and anti-inflammation (Chien, 1957; Kin, 1956; Au *et al.*, 1986; Xu *et al.*, 1986). Especially, the extracts of the cortex and leaf of *E. ulmoides* Oliver were reported to have effects on the prevention of osteolysis and bone mineral loss, and osteogenesis, thus it has been raised to study the action-mechanisms of Eucommiae Cortex.

Bone is a tissue maintaining itself through continuous osteogenesis and osteolysis by osteoblast and osteoclast (Dempster and Lindsay, 1993) respectively. The unbalance between osteoblast and osteoclast activities is caused by the reduction of estrogen in a woman at the menopause, aging, administration of corticoid preparations, smoking, drinking and the like. It increases osteolysis rather than osteogenesis and consequently induces osteoporosis (Dempster and Lindsay, 1993; Ryan *et al.*, 1992; Spencer *et al.*, 1986). The osteogenesis is performed through the steps of: synthesis and secretion of type-I collagen; formation of fiber and maturation of collagen matrix; and formation of hydroxyapatite (Thomas *et al.*, 1989). Type-I collagen synthesized in osteoblast accounts for about 85-90% of total bone proteins. Osteoblast is regulated by various kinds of hormones (parathyroid hormone, thyroid

Correspondence to: Chungsook Kim, Ph.D., Drug Research and Development Team, Korea Institute of Oriental Medicine, 129-11 Chungdam-dong, Kangnam-ku, Seoul, 135-100, Korea
Tel: 82-2-3442-1994-223, 82-2-3442-2120, Fax: 82-2-3442-1030
E-mail: cskim@kiom.re.kr

hormone, $1,25(OH)_2D_3$, sex hormone, insulin and the like) (Canalis, 1983; Canalis, 1985; Noriyoshi *et al.*, 1986) and minor factors (prostaglandin and various kinds of cytokines) (Canalis *et al.*, 1989). The synthesis of collagen is facilitated through the increase of IGF-1 induced by GH released from pituitary gland (Canalis *et al.*, 1989). Osteoblast and osteoclast affect each otherís differentiation and activation (Jimi *et al.*, 1996). Therefore, in this study, the contents of the extract of Eucommiae cortex and main components such as iridoid glucoside including GA, GP and AU were quantitatively analyzed by HPLC. And their effects on the induction of GH release, and the activities of osteoblast and osteoclast were also analyzed, in order to find out the action-mechanism of Eucommiae Cortex which was reported to have an effect on the increase of bone in ovariectomized rats.

MATERIALS AND METHODS

Reagents and materials

The Cortex and leaf of *E. ulmoides* Oliver (Deockjuckdo, Incheon, Korea) were purchased at Kyungdong-Market (Seoul, Korea). GP, GA and AU (WAKO Pure Chem., Japan), methanol for HPLC (Merck Co., Darmstadt, Germany) and others were highly purified reagents. A cell growth medium such as DMEM (Dulbeccoís modified eagle medium), RPMI 1640 medium, α-MEM (α-minimal essential medium), FBS (fetal bovine serum), and collagenase, was purchased from Gibco BRL. (U.S.A). Rat growth hormone (rGH) radioimmunoassay (RIA) kit and [^{3}H]-proline were purchased from Amersham Pharmacia Biotech. (England). The dimethylsulfoxide (DMSO), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), TRAP (tartrate resistance acid phosphatase) staining kit (Sigma Chemical Co., MO, U.S.A.), Human osteoblast-like cell lines, MG-63 and Saos-2 cells (Korean Cell Line Bank, Seoul, Korea), mouse stromal cell line, ST-2 cell (RIKEN Cell Bank, Tsukuba, Japan), Sprague Dawley (SD) rat and ICR mouse (Daehan Biolink, Umsung-gun, chungbuk, Korea) were purchased.

Preparation of extracts and HPLC analysis

Eucommiae Cortex was deposited in our Institute (KIOM-00-3-0021) after being examined by professor, Jae-Hyun Lee (College of Pharmacy, KyungHee University, Seoul, Korea). Dried Eucommiae Cortex powder was extracted with 70% methanol, of which quantity is 5 times larger than that of the dried Eucommiae Cortex powder, for 48 h at room temperature, and the extraction was repeated four times. Eucommiae Cortex MeOH extract was concentrated and dried, then fractionated with *n*-hexane, chloroform, ethylacetate, butanol and water, sequentially. Each solvent fraction was concentrated and dried.

The quantitative analysis of iridoid glucoside including GA, GP and AU was carried out using HPLC Spectra System P1000 (Thermo Separation Products, Fremont, CA, U.S.A.). The column used in HPLC was LUNA 5 μ, C18 (250×4.6 mm) (Phenomenex Co., Torroance, CA, U.S.A.). The mobile phase used in HPLC was the mixture of 5 mM NaH_2PO_4 solution (pH 2.5) and methanol in the ratio of 85:15 and its flow rate was 1 mL/min. The absorbencies of GA and GP were measured at 238 nm and the absorbence of AU was measured at 210 nm. The heights of the chromatographic peaks were measured by valley to valley method without internal standard substances then the mean height was calculated. A standard calibration curve was plotted by using various concentration ranges of GA (1-1000 μg/mL), GP (10-1000 μg/mL) and AU (5-1000 μg/mL). The contents of GA, GP and AU in each solvent fraction were determined by using the standard calibration curve.

GH inducing test

According to Cheng *et al.* (Cheng *et al.*, 1989; Cheng *et al.*, 1991), the pituitary gland of SD rat was separated and 0.02% collagenase/0.02% hylauronidase were added thereinto to separate cells. After the separated cells were primary cultured in 24-well plate for 3 days, various concentration ranges of GA, GP and AU were added into each well and they incubated at 37°C for 15 minutes. After that, the concentration of rGH in the cultured medium was measured by RIA method.

Proliferation of osteoblast-like cells : MG-63 and Saos-2

MTT test. MG-63 and Saos-2 were incubated in a DMEM containing 10% FBS and a RPMI 1640 medium containing 10% FBS respectively under partial pressure of 5% CO_2 at 37°C. MTT assay was carried out by: placing each cell into each well of 96-well plate; adding 0.05% DMSO containing samples (1×10^{-1} to 1×10^{-8} mg/mL) into each well and incubating them for 72 h; adding 0.5 mg/mL MTT into each well and further incubating them for 4 h; dissolving produced formazan crystals in DMSO; and measuring their absorbencies at 550 nm using ELISA reader (Ceres UV 900C, Bio-tech instrument, U.S.A.) (Carmichael *et al.*, 1987).

ALP activity assay. Saos-2 cell line was incubated for 72 h in the same way as MTT test above. 1% Triton X-100 was added into a fermented medium and the medium was centrifuged at 4°C, 14,000×g for 20 minutes to recover the supernatant (Kassem *et al.*, 1993). The change in absorbency was measured at 405 nm by the method of Bowers and McComb (Raab, 1972).

Proline Incorporation test. According to Yoon *et al.* (Yoon *et al.*, 1993), each of the samples with various

concentrations was added to MG-63 then incubated for 48 h. 1 mL of DMEM containing 3 μCi [^{3}H]-proline, 50 mg sodium ascorbate, and β-aminopropionitrile was added into the MG-63 then incubated for 16 h. 10% TCA (trichloroacetic acid) and 1% tannic acid were added into the fermented MG-63 and centrifuged for 10 minutes to precipitate proteins. The precipitates were washed with cool acetone twice and dissolved in 1 N NaOH. 0.1 M HEPES buffer (pH 7.6) containing 10 mM $CaCl_2$, 20 mM *N*-ethylmalrimide, 100 μL/mL bacterial collagenase was added into the dissolved precipitates and they reacted at 37°C for 1 h. 10% TCA and 1% tannic acid were added into the reaction solution to separate it into an upper layer containing collagenase digestible protein (CDP) and a lower layer containing non-collagen protein (NCP). The radioactivity of each layer was measured by using liquid scintillation counter. The percentage of collagen synthesized (PCS) was calculated by the following equation (Shioi *et al.*, 1994):

$$PCS = \frac{\text{cpm of CDP}}{(\text{cpm of NCP} \times 5.4)} \times 100$$

Inhibition of osteoclast

According to Shioi *et al.* (Shioi *et al.*, 1994), the expression of osteoclast was carried out by using ICR mouse bone marrow separated and collected from tibia and femur of the mouse. The bone marrow cell was cultured in α-MEM containing 10% FBS at 37°C for 24 h. Mouse stromal cell, ST-2, was added into each well including 10% FBS-containing α-MEM in an amount of 1×10^5 cells/well and cultured for 24 h. The bone marrow cells separated above were added thereinto in an amount of 1×10^6 cells/well and cocultured for 6 days. When osteoclast was expressed, GA, GP and AU samples were added into the wells in an amount of 1×10^{-1} – 1×10^{-5} mg/mL and cultured for 2 days. The number of osteoclasts was determined by the number of multiple-nuclei cells having at least 3 nuclei, using TRAP staining method (Shioi *et al.*, 1994).

Statistics

The results of the control groups without addition of samples and sample-adding groups compared with each other by using Systat® program. According to ANOVA method or Bonferroni multiple comparison analysis, it was defined that they were different significantly from each other at P<0.05 (Rosner, 1990).

RESULTS

Preparation of extracts and HPLC analysis

The separation yields (% of dried herb) of 70% MeOH extract of Eucommiae Cortex and each solvent fraction was as follows: Hx (0.32%) < EA (0.36%) < $CHCl_3$ (1.60 %) < BuOH (1.78%) < H_2O (7.98%) < MeOH extract (12.04 %). As shown in Fig. 2, each retention time of GA, GP and AU was 10.587 minutes, 50.989 minutes and 5.001 minutes respectively and their standard calibration curves were Y = 71380.03X + 928.72 (γ^2 = 1.00), Y = 19577.00X + 1038.30 (γ^2 = 0.99) and Y = 21117.98X + 3164.70 (γ^2 = 0.99) each. As shown in Table I, the content of GA in the cortex of *E. ulmoides* Oliver was about 70 fold higher than that in the leaf. Although GP and AU in the leaf of *E. ulmoides* Oliver were not detected, the contents of GP and AU in the cortex were 613.27±130.26 μg/g and 721.55±176.37 μg/g respectively. As the result of the quantitative analysis of GA, GP and AU in solvent fractions including Hx, $CHCl_3$, EA, BuOH and H_2O, the contents of GA in BuOH and H_2O fractions were 1.48±0.07 ($\times10^3$) mg/g and 1.30±0.08 ($\times10^3$) mg/g respectively, which were as much as higher than other fractions (Table I).

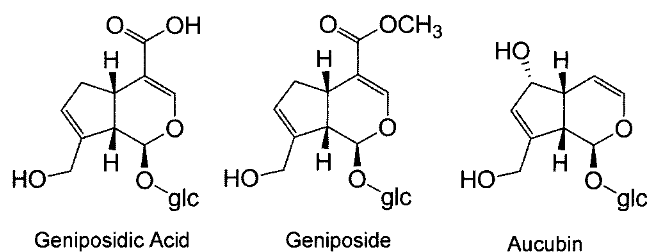

Fig. 1. The structures of iridoid glucosides

GH inducing test

When various concentrations (0.1~1.0 mM) of GH releasing factors (GRF) were added into rat pituitary gland cells, the amount of GH produced was increased depend

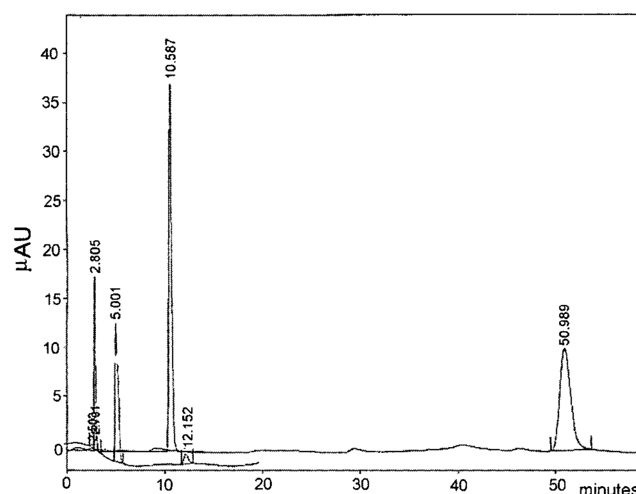

Fig. 2. A representative HPLC Chromatogram of geniposidic acid (10.59 min), geniposide (50.99 min) and aucubin (5.00 min).

Table I. The amount of Geniposidic acid (GA), Geniposide (GP) and Aucubin (AU) in various fractions and methanol extracts of Eucommiae Cortex (Mean±SE : μg/g[1])

	Cortex	Leaves	He	$CHCl_3$	EA	BuOH	H_2O
GA	2.25±0.17(x10³)	30.54±0.98	0.16±0.01	7.43±0.83	32.54±2.31	1.30±0.08(x10³)	1.48±0.07(x10³)
GP	613.27±130.26	ND [2]	ND	11.23±1.83	283.82±9.29	468.97±32.00	ND
AU	721.55±176.37	ND	ND	ND	ND	330.85±31.66	121.38±4.35

[1] weight of dried Eucommiae Cortex (g)
[2] ND: Not Detected

on the concentration of GRF, thereby using it as a standard curve, Y = 76.22X + 23.39 (γ^2 = 0.99) (Cheng *et al.*, 1989). When MeOH extract (1 mg/mL) was treated with the same process described above, the concentration of GH induced was nearly the same as that of GRF 1.0 mM. Hx, $CHCl_3$ and EA fractions (each 20 μg/mL) induced the release of GH in the same extent as GRF 0.5 mM (P< 0.05), however, the induction of GH release by BuOH and H_2O was not different from that of a control (Fig. 3). AU, GP and GA (10 μg/mL) did not induce the release of GH (Fig. 3).

Proliferation of osteoblast-like cells: MG-63 and Saos-2

MTT test. As the result of MTT test of MG-63, the MeOH extract (1×10^{-8} mg/mL) was 132±10% of control, BuOH fraction (1×10^{-1} mg/mL) was 138±8% of control, and H_2O fraction (1×10^{-6} mg/mL) was 126±6% of control. However, there were no difference in cell proliferation activity between Hx, $CHCl_3$ and EA fractions and the control (Fig. 4). In addition, the cell proliferation activities of $1,25(OH)_2D_3$ (1×10^{-8} – 1×10^{-4} mg/mL) and E_2 (1×10^{-8} – 1×10^{-4} mg/mL) were 110±7% of control and 123±7% of control respectively. Consequently, it was known that the cell proliferation activities of BuOH and H_2O fractions were higher than that of $1,25(OH)_2D_3$ and similar to that of E_2. The cell proliferation activities of GA, GP and AU were as follows: GP > AU > GA. The cell proliferation activities of GP (1×10^{-4} mg/mL) and AU (1×10^{-4} mg/mL) were 135± 5% and 134±8% of control respectively, which were higher than that of $1,25(OH)_2D_3$ and E_2. However, the cell proliferation activity of GA was not different from that of the control (Fig. 4, Fig. 5).

ALP activity assay. The ALP activity of the MeOH extract (1×10^{-1} mg/mL) was 110±3% of control and $CHCl_3$ fraction (1×10^{-6} mg/mL) displayed the highest ALP activity, 138±3% of control (Fig. 6) among the fractions. $1,25(OH)_2D_3$ (1×10^{-5} mg/mL) showed high ALP activity, 205±6% of control. The ALP activities of BuOH (1×10^{-5} mg/mL) and H_2O fractions (1×10^{-4} mg/mL) were 117±1% and 107±1% of control respectively, which were not different from those of the control. Regarding to GA, GP and AU, the ALP activity of AU (1×10^{-11} mg/mL) was 129±1% of control, while GA and GP had no effect on the increase of ALP activity (Fig. 6).

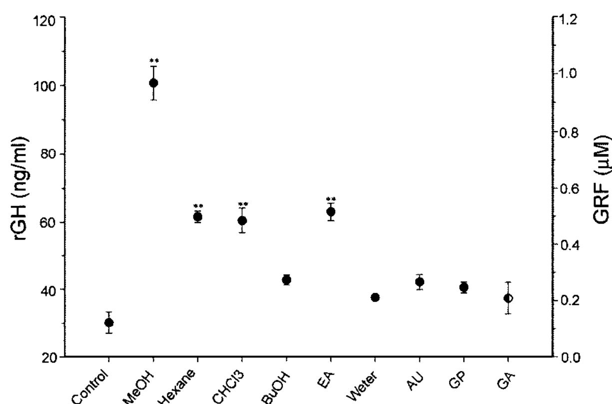

Fig. 3. rGH concentrations induced by Eucommiae Cortex. MeOH; 70% methanol extract of Eucommiae Cortex, Hx, $CHCl_3$, BuOH, EA and H_2O; hexane, chloroform, butanol, ethylacetate, and water fractions from 70% methanol extract of Eucommiae Cortex, AU; aucubin, GP; geniposide, and GA; geniposidic acid. Comparison to control by Bonferroni multiple adjustment method, *P<0.05, **P<0.01.

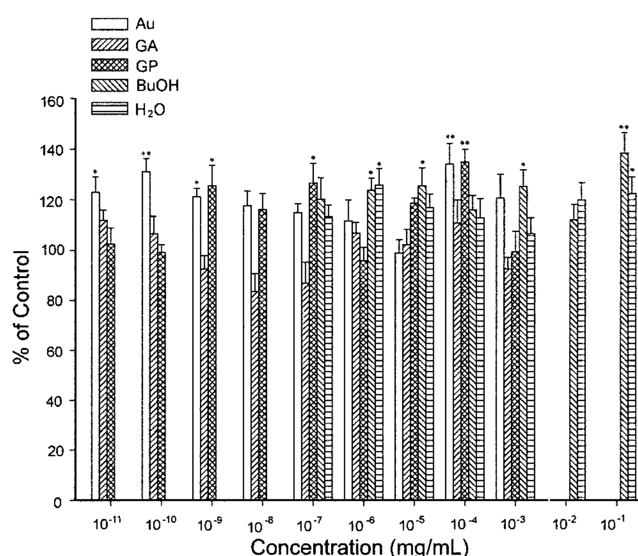

Fig. 4. Cell proliferation effects of Geniposidic acid, Aucubin, Geniposide and the fractions of Eucommiae Cortex on MG-63, human osteoblast-like cell line. Comparison to control by Bonferroni multiple adjustment method, *P<0.05, **P<0.01.

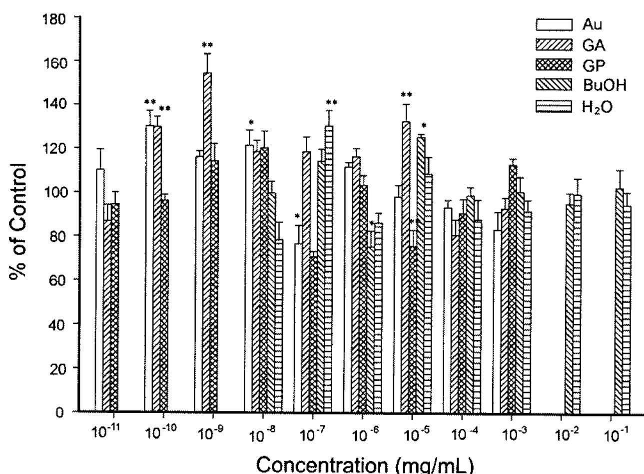

Fig. 5. Cell proliferation effects of Geniposidic acid, Aucubin, Geniposide and the fractions of Eucommiae Cortex on Saos-2, human osteoblast-like cell line. Comparison to control by Bonferroni multiple adjustment method, *P<0.05, **P<0.01.

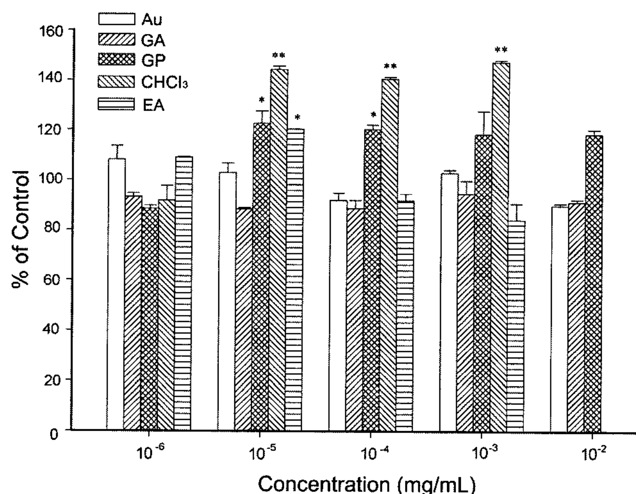

Fig. 7. Proline incorporation effects of Geniposidic acid, Aucubin, Geniposide, and the fractions of Eucommiae Cortex on MG-63, human osteoblast-like cell line. Comparison to control by Bonferroni multiple adjustment method, *P<0.05, **P<0.01.

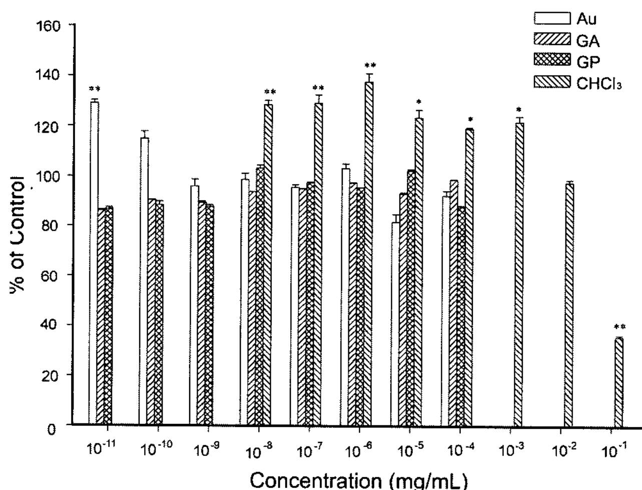

Fig. 6. Changes of alkaline phosphatase activities of Geniposidic acid Aucubin, Geniposide and the fractions of Eucommiae Cortex on Saos 2, human osteoblast-like cell line. Comparison to control by Bonferror multiple adjustment method, *P<0.05, **P<0.01.

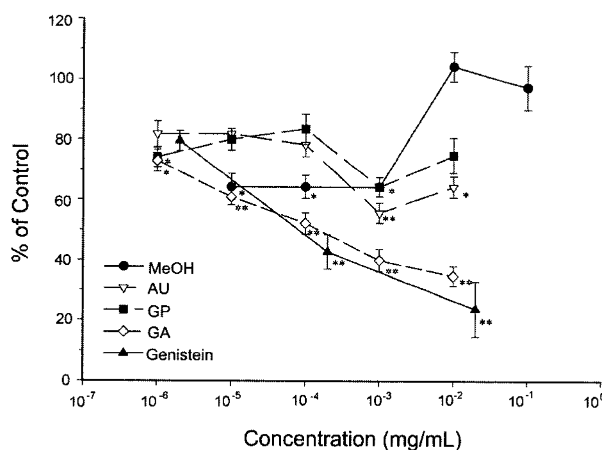

Fig. 8. Cell inhibition effects of Eucommiae cortex extracts on osteoclasts. Comparison to control by Bonferroni multiple adjustment method, *P<0.05, **P<0.01.

Proline incorporation test. Through [^{3}H]-proline incorporation as indirect measurement of collagen synthesis, the activity of 1,25$(OH)_2D_3$ (1×10^{-5} mg/mL) and E_2 (1×10^{-3} mg/mL) were 107±2% and 107±3% of control respectively. The proline incorporation activities of $CHCl_3$ (1×10^{-3} – 1×10^{-5} mg/ml) and EA fractions (1×10^{-5} mg/mL) were over 140% and over 120% of control respectively (Fig. 7). However, the proline incorporation activities of BuOH (1×10^{-3} mg/mL) and H_2O fractions (1×10^{-5} mg/mL) were 110±1% and 92±2% of control respectively, which were not significantly different from those of the control. In addition, the proline incorporation activity of GP (1×10^{-5} – 1×10^{-3} mg/mL) was 120% of control, while the proline incorporation activities of AU (1×10^{-6} mg/mL) and GA (1×10^{-3} mg/mL) were 108±5% and 94±5% of control respectively, which were not different from those of the control (p=1.00) (Fig. 7).

Inhibition of osteoclast

The number of osteoclasts expressed in the MeOH extract (1×10^{-5} – 1×10^{-3} mg/mL) was 65% of control, 1,509 ±74 cells/well, that is, the MeOH extract inhibited the cell proliferation of osteoclast 35%. In addition, AU (1×10^{-3} mg/ml) and GA (1×10^{-4} mg/mL) inhibited osteoclast approximately 40% and over 50%, respectively and their inhibition activities on osteoclast were increased depending on the increase of their concentration (IC_{50} : 4.43×10^{-7} M) (Fig. 8).

DISCUSSION

The cortex of *E. ulmoides* Oliver (Eucommiae Cortex) has been used as a medicinal herb rather than its leaf. According to this study, the Eucommiae Cortex had a larger amount of iridoid glucoside including GA, GP and AU than the leaf of *E. ulmoides* Oliver (Table I). Particularly, it was revealed that the content of GA was much higher than that of the others, GP and AU, through HPLC. In addition, GA, GP and AU were mainly present in the BuOH and H_2O fractions of Eucommiae Cortex.

The GH increases IGF-I concentration, thereby inducing the synthesis of collagen that accounts for about 95% of organic matrix in the components of bone (Finkenstedt *et al.*, 1997; Johansson *et al.*, 1996; Higashi *et al.*, 1996). It was reported that the deficiency of GH in childhood and adulthood resulted in the decrease of bone mass and the GH replacement therapy facilitated both osteogenesis and osteolysis, thereby improving bone turnover (Finkenstedt *et al.*, 1997; Higashi *et al.*, 1996). In this study, the effects of Eucommiae cortex and its components on the release of GH were studied. Consequently, the MeOH extract of Eucommiae Cortex (1 mg of dried herb/ml) had the highest GH releasing activity among iridoid glucosides, corresponding to GRF 1.0 μM. Hx, $CHCl_3$ and EA fractions of Eucommiae Cortex (each 20 μg of each fraction/mL) had GH releasing activities less than the MeOH.

Osteoporosis is usually caused by the unbalance between osteoblast and osteoclast activities induced by multiple reasons. Thus, medicines, which suppress the osteoclast activity to delay osteolysis, or facilitate both osteoblast and osteoclast activities to activate osteogenesis, are commonly used for the treatment of osteoporosis. It is accordingly thought that the medicines, which improves the activity of osteoblast to facilitate osteogenesis and inhibits the activity of osteoclast to delay osteolysis, are ideal therapeutic agents in osteoporosis. The osteoblast relating to osteogenesis, secretes bone matrix consisting of collagen and cohesive polysaccharides to form new bone, and has receptors for parathyroid hormone, active vitamin D, estrogen, and prostaglandin E_2, thereby continuously proceeding osteogenesis (MMWR Morb Mortal Wkly Rep., 1996). In order to analyze the effects of osteoblast, MTT assay, ALP activity and [^{3}H]-proline incorporation assay are used according to the activation mechanism of osteoblast (Stein and Lian, 1995). MTT assay shows the differentiation and proliferation of osteoblast using osteoblast-like cells (MG-63 and Saos-2). ALP activity detection assays the activity of ALP, a specific enzyme relating to the maturation step of osteoblast, and [^{3}H]-proline incorporation assay shows the effects on the synthesis of collagen participating in the matrix formation of osteoblast. In this study, following mineralization step of osteoblast was, however, skipped because it might be easily carried out *in vivo*.

In ALP detection showing the cell activity of osteoblast, NaF and $1,25(OH)_2D_3$, which were used as a therapeutic agent in osteoporosis, had much higher ALP activation activity, while E_2 had a little ALP activity. These results were nearly identical with the prior report that NaF increased the cell proliferation approximately 220% and $1,25(OH)_2D_3$ increased ALP activity 170% in human bone marrow stromal osteoblast-like cell (Kassem *et al.*,1993). The $CHCl_3$ fraction (123%~138%) and BuOH fraction also had higher ALP activity. The components in the $CHCl_3$ fraction were thought to participate in and activate the maturation of osteoblasts on the basis of a result that the $CHCl_3$ fraction had no effect on the cell proliferation while it had the higher ALP activity. In addition, it is required to study the BuOH and H_2O fractions since GA in the BuOH and H_2O fractions had both cell proliferation and ALP activities. In the [^{3}H]-proline incorporation assay as an index of the matrix formation of osteoblast, the $CHCl_3$ fraction also had the highest [^{3}H]-proline incorporation activity. The $CHCl_3$ fraction was assumed to participate in and facilitate the maturation and matrix formation of osteoblast, a though it did not induce the cell proliferation in the activation mechanism of osteoblast.

The inhibition of osteoclast causing osteolysis showed an increase of about 30~40% and 60% by AU and GP, and by GA, respectively. In particular, IC_{50} of GA on osteoclast was 4.43×10^{-7} M and the inhibition was dose-independent.

Synthetically, the ALP and proline incorporation activities as an index of the maturation of osteoblast, were the highest in the $CHCl_3$ fraction. It was nearly identical with that the $CHCl_3$ fraction had the highest effect on the release of GH. Meanwhile, in MTT assay on MG-63, the BuOH fraction had the highest activity. The BuOH fraction participated in the cell proliferation step of osteoblast, but it had no effect on the maturation and matrix formation steps of osteoblast. The main components, AU, GP and GA were mainly present in the BuOH and H_2O fractions. They had effects on the cell proliferation of MG-63 and Saos-2, especially GA had much higher cell proliferation effect on Saos-2, while they had no effect on the release of GH. The main components of Eucommiae Cortex, GA, GP and AU, were thought to have an effect on the treatment of osteoporosis because they participated in the cell proliferation of osteoblast rather than the maturation of osteoblast, and inhibited the cell proliferation of osteoclasts.

It is known that the GA and GP in Eucommiae Cortex have the anticarcinomic activity (Hsu *et al.*, 1997), and GA improves the turnover rate of the corneal layer of the skin (Li *et al.*, 1999). In addition, AU is known to have effects on the protection of liver (Chang, 1998) and inhibition of

muscle convulsion (Oritz *et al.*, 1994) because it has solubility in water and in methanol but not in chloroform and in ether. Although there was no result relating to the synthesis of collagen *in vitro*, Li *et al.* (Li *et al.*, 1998) reported that GA and AU in the leaf of *E. ulmoides* Oliver increased the synthesis of collagen *in vivo* (Li *et al.*, 2000) and Eucommiae Cortex increased the synthesis of collagen, too. Similarly, in an *in vitro* experiment using osteoblast-like cell, MG-63 and Saos-2, GA had markedly higher effect on the collagen synthesis compared to $1,25(OH)_2D_3$ and E_2 as a control.

In conclusion, it is thought that the activity of osteoblast is increased by that the BuOH and H_2O fractions have the higher cell proliferation effect on osteoblast, MG-63 and Saos-2, then by that the $CHCl_3$ fraction activates the ALP activity relating to the maturation of osteoblast and increases the synthesis of collagen to activate the matrix formation of osteoblast. They have shown nearly identical effect on the cell-proliferation of osteoblast with $1,25(OH)_2D_3$ used as a therapeutic agent in osteoporosis. In the collagen synthesis using MG-63, the $CHCl_3$ and EA fractions and GP had higher effects on the synthesis of collagen and GA, AU and GP had excellent effects on the inhibition of osteoclast. Therefore, it is thought that Eucommiae Cortex extract may be used in the prevention and treatment of osteoporosis because its components in part of fractions participate in each step of a mechanism for activating osteoblast to facilitate osteogenesis, and suppress the activity of osteoclast to inhibit osteolysis. In addition, Eucommiae cortex extract induces the release of GH regulating bone maturation and bone remodeling. Therefore, Eucommiae cortex extract can be developed as a therapeutic agent in osteoporosis through further studies thereon.

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