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Original Article

Inhibitory Action of Ulmus Davidiana Planch Extract Solution to Osteoclast Cell Proliferation and Prostaglandin E2 Synthesis in Mice

Park Sang-dong, Kim Kap-sung, Cho Hyun-seok, Lee Seung-deok and Kim Kyung-ho

Department of Acupuncture & Moxibustion, College of Oriental Medicine, Dongguk University

Abstract

Objectives : Ulmus davidiana Planch (UD) has long been known to have anti-inflammatory and protective effects on damaged tissue, inflammation and bone among other functions.

Methods : This study was undertaken to address whether the water extract of the bark of UD could modulate proliferation of mouse osteoclasts in vitro and to investigate its effect on cyclooxygenase-2 (COX-2), which converts arachidonic acid to prostaglandin E2 (PGE2) and is highly expressed in osteoclasts. Mouse osteoclasts were tested in vitro for growth inhibition, proliferation cell nuclear antigen expression, and COX-2 activity and expression after treatment with UD extract.

Results : Its effects were compared with those of indomethacin (a nonselective COX inhibitor) and celecoxib (a selective COX-2 inhibitor) by Cell viability assay, Cell cycle analysis, Immunohistochemical analysis of PCNA expression, Western blot analysis and PGE2 Enzyme immunoassay (EIA).

UD demonstrated a strong growth inhibitory action in both tested osteoclasts cells. The IC50s were 10 μg/ml for UD, 6 μM for celecoxib and 42 μM for indomethacin. UD, as well as celecoxib and indomethacin, suppressed proliferation cell nuclear antigen expression and PGE2 synthesis in osteoclasts. UD inhibited COX-2 expression, whereas celecoxib inhibited COX-2 activity directly.

Conclusion : UD selectively and effectively inhibits osteoclasts cell growth in vitro. Inhibitory action of PGE2 synthesis via suppression of COX-2 expression may be responsible for its anti-inflammatory activity.

Key words : Ulmus davidiana Planch (Ulmaceae), prostaglandin, cyclooxygenase

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· **Corresponding author** : Kim Kyung-ho, Department of Acupuncture & Moxibustion, Dongguk University Oriental Hospital, #87-2, Sunae-dong, Bundang-gu, Sungnam-si, Kyunggi-do, Republic of Korea
Tel. 82-31-710-3746 E-mail : omdkkh@hanmail.net

Ⅰ. Introduction

UD is a deciduous tree which is widely distributed in Korea. The barks of the stem and the root of this plant have been used in traditional Korean medicine for the treatment of oedema, mastitis, gastric cancer, and inflammation[1-2]. UD has been used for protection against degeneration of cartilage and regeneration of damaged tissue[3]. UD is known for their functions in maintaining or assisting blood circulation. UD water extract has been developed on the basis of the known function of the herb, as described in the literature of traditional Chinese and Korean medicine[4-5].

Early experiments revealed that Prostaglandin (PG)s are often highly produced in head and neck cancer patients[6]. PGE2 is the predominant type of PGs derived from the cell membrane arachidonic acid, the release of which, in turn, is controlled by phospholipases[7]. Increased PGE2 levels in inflammatory tissue are mainly because of the increase of the expression of COX, a key enzyme involved in the conversion of arachidonic acid to PGs[8]. There are two isoforms of COX: COX-1 is constitutively expressed in all eukaryotic cells, whereas COX-2 is inducible and is increased under the certain conditions such as inflammation.

Although UD has been known to have anti-inflammatory and protective effects on damaged tissue, inflammation and bone among other functions, little is still known about the action mechanisms of UD. As a part of our search for new biologically active substances from traditional medicines, we evaluated whether extracts of UD could modulate the induction of rheumatoid arthritis (RA) in mice. UD has a strong anti-inflammatory effect, we hypothesized that its anti-inflammatory activity may derive from inhibition of COX-2 pathway.

The purpose of this study is to examine the anti-inflammatory activity of UD on mouse osteoclasts in vitro and to understand its molecular mechanisms with special emphasis on its effect on COX-2 pathway.

Ⅱ. Experiments

1. Material

1) Plant

The stem barks of UD were collected from Mt. Phal-gong, Kyungbuk Province, South Korea in May 2002, and identified by Professor Kim Kap-sung, College of Oriental Medicine, Dongguk University, South Korea. Fresh stems were dried in a dark, well ventilated place. The voucher specimen (No. UD-W-57) is deposited in the Herbarium of this college.

2) Chemicals and Drugs

50 mM stock solutions of indomethacin (Sigma CO., USA) and 10mM stock solution of celecoxib [SC-58635;4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1]-enzene-ulfonamide; Searle Research and Development, St. Louis, MO. USA] were prepared with DMSO (Sigma CO., USA) and diluted with culture medium to final concentrations of 5-400 μM. Control cells received DMSO (0.25 %) or culture medium only.

3) Animals

Inbred Swiss albino mice weight between 18 and 25 g were used. The animals were housed under standard conditions of temperature (23±1 ℃), relative humidity (55±10%), 12/12 h light/dark cycles and fed with standard pellet diet (Daehan Experimental Animals Ltd, Seoul, Korea) and tap water ad libitum.

2. Methods

1) Preparation of Herb Extract and Fractions

UD was purchased from a market specializing in herbs (Kyungju herb market, Kyungju, Korea). The herb had a moisture content of <10 % by weight, and was air-dried. Air-dried barks (totalling 70 g dry weight) were mixed, minced with a grinder, and extracted by storing in 1L of boiling water for 3 hours. The supernatant was filtered with 10 μm cartridge paper and water was removed by rotary evaporation (Eyela, Tokyo, Japan) and concentrated extracts were freeze-dried. This process generally produced 15 g of brown powder. The powder form of the extract was dissolved in medium to 20 mg/ml, vortexed at room temperature for 1 min, and incubated at 37 °C for 1 h while rotating before use. This solution was centrifuged at 5000 rpm for 10min to remove any insoluble ingredients. The supernatant was passed through a 0.22 μm filter for sterilization and diluted with culture medium to final concentrations of 1-40 μg/ml UD extract. A voucher specimen has been deposited at the Kyungju Oriental Medical Hospital, Dongguk University, Kyungju city, Kyungbuk, Korea under acquisition number UD-13.

2) Cell Lines and Osteoblast Cultures

Cultures of osteoblast cells were established from fetal calvarial bone obtained, as described by Hong et a[9]. Previously, minced pieces of tissue were explanted to 25 cm^2 Falcon tissue culture flasks containing 5 ml of Eagle's basal medium (BME). The osteoblasts were obtained, as previously described by Y. Kuroki. et al.[10]. Calvarial osteoblasts were seeded and grown in BME supplemented with 5 % fetal calf serum, L-glutamine, penicillin-streptomycin and HEPES for 24 h. Cells were also cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). For the experiments, cells were seeded in 6 well plates(1.8 x 10^5 cells/well). The cells were examined under a microscope every day and photographs were obtained. After 3 days, the cells were trypsinized, stained using Trypan blue, and counted with a hemocytometer.

Mouse osteoblast cells were grown in a 50:50 mixture of DMEM (Life Technologies, Inc., Rockville, MD, USA) and F12 (Life Technologies, Inc. Rockville, MD, USA) containing 10 % FBS (Life Technologies, Inc., Rockville, MD, USA) and 1 % Antibiotic-Antimycotic (Life Technologies, Inc., Rockville, MD, USA). Cell lines were maintained at 37 °C in a humidified atmosphere of 5 % CO_2.

3) Cell Viability Assay

The percentage of growth inhibition was determined by using a MTT (Sigma CO., USA) assay to measure viable cells. A total of 2.5 x 10^3 cells/well was seeded onto a 96 well plate for 24 h, treated with various concentrations of UD, indomethacin, and celecoxib, and incubated for an additional 3 days at 37 °C. Subsequently, 10 μl of MTT at a concentration of 5 mg/ml was added to each well, and cells were incubated for an additional 4-6 h. The supernatant was aspirated, and 100 μl of DMSO were added to the wells to dissolve any precipitate present. The absorbance was then measured at a wavelength of 570 nm using an ELX800 reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

For cell viability assessment, cells were seeded in 6 well plates at 6 x 10^4 cells/well and grown to confluency for 3 days. Cells in duplicate wells were deprived of serum for 1 day and subsequently treated with increasing concentrations of agent or corresponding concentrations of DMSO for 3 days. The cells were examined under the microscope each day and photographs were obtained. After 3 days, cells were trypsinized, stained with Trypan blue, and counted with a hemocytometer.

4) Cell Cycle Analysis

A total of 1.5 x 10^5 cells/well was plated onto

6 well plates and incubated for 24 h at 37 °C. Various concentrations of UD, indomethacin, and celecoxib were added to the wells and incubated for an additional 3 days. Cells were then washed, pelleted, fixed with cold 70 % ethanol for at least 30 min, and incubated with 100 μg/ml RNase A and 50 μg/ml propidium iodide in PBS at room temperature for 30 min. Samples were immediately analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA). Cell cycle phase distribution was determined using Modfit software (Verity Software House, Topsham, ME, USA).

5) Immunohistochemical Analysis of PCNA Expression

Harvested cells were applied to polylysine-coated slides by centrifugation at 800 rpm for 5 min in a Cytospin instrument (Sakura Finetek, Torrance, CA, USA). Cells were air dried and fixed in 100 % acetone for 10 min at 4 °C. Slides were then incubated with 3 % hydrogen peroxide to quench endogenous peroxidase activity for 5 min. After three washings with PBS, slides were incubated with serum blocking solution for 20 min at room temperature. The solution was blotted, and 2 μg/ml primary mouse monoclonal anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added. Cells were then incubated at 4 °C overnight. After three washings with PBS, the slides were incubated for 10 min with biotinylated goat antimouse antibodies at room temperature (Zymed Laboratory, San Francisco, CA, USA). Slides were washed three more times with PBS and then incubated for 10 min with streptavidin-peroxidase conjugate at room temperature (Zymed Laboratory, San Francisco, CA, USA). Color was developed by the addition of diaminobenzoate chromogen (Zymed Laboratory, San Francisco, CA, USA), and slides were counterstained with hematoxylin. Cells with brown nuclear precipitations were regarded as positive. A minimum of 500 cells/slide was analyzed in a high-powered field using a light microscope, and the percentage of positive cells was determined. Inhibitory action of UD, indomethacin and celecoxib on osteoclast cells was confirmed using the immunohistochemical marker, PCNA. PCNA/cyclin regulates the initiation of cell proliferation by mediating DNA polymerase and is elevated in the G1 and S phases of cell cycle[11].

6) Western Blot Analysis

Cells were treated with UD, and the proteins were extracted from the cells using a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 % SDS, 1 % NP 40, and 1x protease inhibitors (Roche Applied Science, Indianapolis, IN, USA). 15 μg of protein were fractionated by electrophoresis through a 10 % SDS polyacrylamide gel, and the proteins then transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h in a blocking buffer containing 5 % dry milk, 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1 % Tween 20 and then incubated with a polyclonal anti-COX-2 antibody (1:400 dilution; Cayman Chemical, Ann Arbor, MI, USA) in the blocking buffer at 4 °C overnight. The membrane was then incubated with an antirabbit antibody conjugated with horseradish peroxidase (Amersham, Arlington Height, IL, USA), and the protein was detected using chemiluminescence method followed by autoradiography. The same membrane was then used to detect ß-actin protein using a monoclonal anti-ß-actin antibody (1:10,000 dilution; Sigma CO., USA) as described above.

7) PGE2 EIA

A competitive PGE2 EIA was used to quantify the level of PGE2 released into culture media and was performed according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The supernatants of control and treated cells were collected, centrifuged at 2500 rpm for 2 min, and stored at -70 °C until tested. 50 μl aliquots of each sample were assayed in triplicate.

Absorbance was measured at 450 nm on an ELX800 reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The minimal detectable concentration of PGE2 by the assay is 50 pg/ml. The PGE2 produced is expressed as pg/10^6 cells.

The effect of celecoxib and UD on COX-2 activity was examined using a cellular arachidonic acid conversion assay. By providing exogenous arachidonic acid, which is the substrate of COX-2, the effect of arachidonic acid release from membrane (i.e., protein kinase and phospholipase A2 activities) can be eliminated[12]. The cells were incubated for 12 h, and the medium was aspirated and replaced with fresh medium containing UD or celecoxib at IC50 concentrations (150 μg/ml for UD or 25 μM for celecoxib). The cells were additionally incubated for various periods of time, and the medium was aspirated. Fresh medium containing 100 μM arachidonic acid(Cayman) was added, and the cells were incubated for additional 30 min. PGE2 in the medium was measured as described above.

8) Statistical Analysis

The results are presented as Mean±SD. Statistical significance between the groups was analyzed by Student's t-test. $P < 0.05$ was considered to be significant.

Ⅲ. Results

1. Inhibitory Action of Osteoclast Cell Growth

The percentage of growth inhibition of different agents on osteoclast cells was determined as the percentage of viable-treated cells in comparison with viable cells of untreated controls. UD displayed a dose-dependent inhibitory action on the growth of both cell lines(Fig. 1-A). Osteoclast cells exhibited equal sensitivity to UD and IC50s of both cell lines were determined to be 10 μg/ml. The maximal inhibition of cell growth (>90 %) was achieved at 40 μg/ml. The inhibitory action of indomethacin

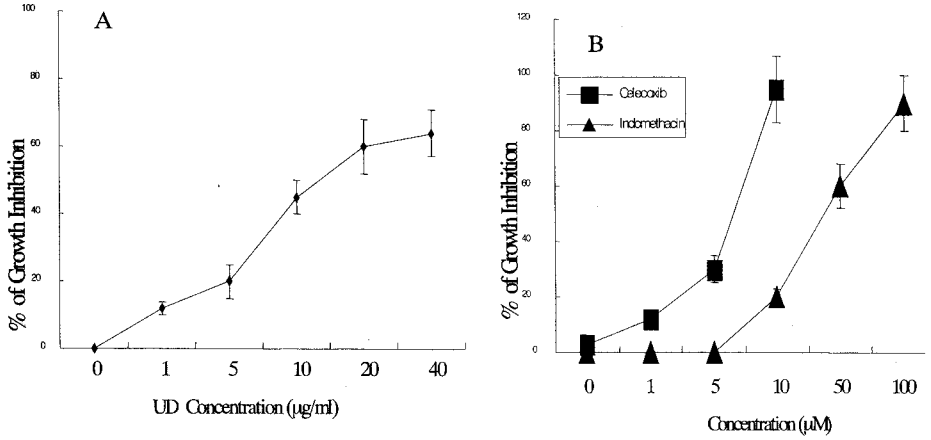

Fig. 1. Inhibitory Action of Osteoclast Cell Growth

Cells were seeded onto 96 well plate at 2.5 x 10^3 cells/well and were treated with various agents at different concentrations, and percentage of growth inhibition was determined by MTT assay after 72 h treatment.

(A) Dose-dependent growth inhibition of osteoclast cells was observed after treatment with UD at concentrations ranging from 1 to 40 μg/ml.

(B) Inhibition of osteoclast cell growth was observed after treatment with indomethacin, and celecoxib at concentrations ranging from 1 to 100 μM. Results are mean values±SD of independent experiments performed in triplicate (bars not evident because of minimal SD).

Table 1. Cell Cycle Distribution of Osteoclast Cells

Group	G0-G1 (%)	S (%)	G2-M (%)
Control	60.2	24.4	15.4
UD 5 μg/ml	66.6	16.1	17.3
10 μg/ml	67.6	17.2	15.2
20 μg/ml	71.5	14.4	14.1
Celecoxib (5 μM)	73.0	14.9	12.1
Indomethacin (40 μM)	61.9	23.2	14.9

G0, Gap0; G1, Gap1; S, DNA synthysis; G2, Gap2 ; M, mitosis.
Control; Group was untreated
UD ; Group was treated with UD(5, 10, 20 μg/ml)
Indomethacin; Group was treated with Indomethacin(5 μM)
Celecoxib; Group was treated with Celecoxib(40 μM)

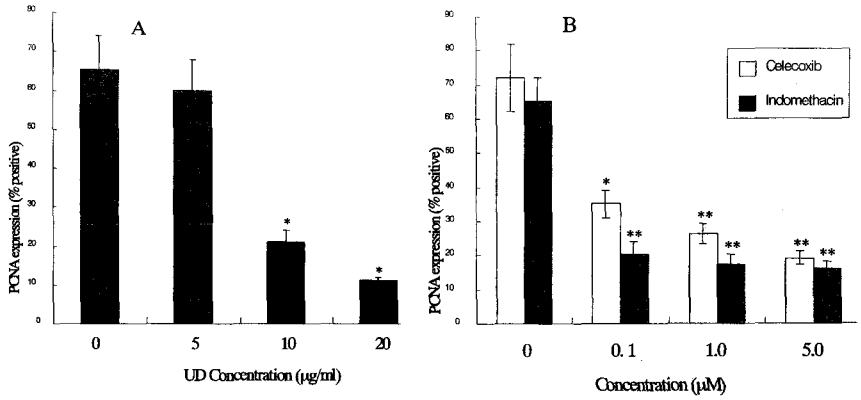

Fig. 2. Inhibitory Action of PCNA Expression

Osteoclast cells were treated with various agents for 72 h, and percentage of cells staining positive for PCNA were determined (n = 500). A significant inhibitory action of PCNA expression in both cells was observed at 10 μg/ml for UD (A) and 0.1 μM for celecoxib and indomethacin (B). Results are mean values ±SD (bars) of independent experiments performed in triplicate. Student's t-test was used to analyze the data.
*, $P < 0.05$; **, $P < 0.001$.

(nonselective COX inhibitor) and celecoxib (COX-2-specific inhibitor) was tested and compared. The results show that osteoclast cells are sensitive to these two agents (Fig. 1-B). However, celecoxib exhibited stronger growth inhibition (IC50 = 6 μM) than indomethacin (IC50 = 42 μM).

2. Cell Cycle Arrest

The effect of these agents on cell cycles was analyzed. These results show that UD caused a significant G0-G1 phase arrest with concurrent decrease of S phase in osteoclast cells. Celecoxib caused a significant arrest at G0-G1 phase, whereas indomethacin exerted mild arrest at G0-G1 phase(Table1).

3. Inhibitory Action of PCNA Expression

These results show that there was a significant decrease of PCNA-positive cells after 72 h treatment with 150 μg/ml UD, indicating a suppression of cell proliferation (Fig. 2-A). Osteoclast cells displayed similar dose-dependent

decreases in PCNA expression and had equal sensitivity to UD. Osteoclast cells exhibited a dose-dependent reduction of PCNA expression after treatment with indomethacin, and celecoxib (Fig. 2-B). However, COX-specific inhibitors, indomethacin and celecoxib, caused a dramatic decrease of PCNA expression in osteoclast cell. These observations additionally confirmed the supportive role of PGE2 in osteoclast cell proliferation and indicated that different mechanisms of cell growth inhibition are involved. COX-specific inhibitors, indomethacin and celecoxib, reduce PCNA expression by inhibiting PGE2 production. It is worthy to note that the change in PCNA expression correlated with the phase of the cell cycle that the agents acted upon. Celecoxib suppressed cell proliferation at G1 phase with significant decrease of PCNA expression. These results indicate that PCNA expression is predominant at G1 and S phases.

4. Inhibitory Action of PGE2 Synthesis in Osteoclast Cell

Osteoclast cells produced a significantly high level of PGE2 (300 pg/10^6cells) after 12 h of incubation (Fig. 3-A). In the presence of UD, a significant inhibitory action of PGE2 production in osteoclast cells was observed even at a concentration of 5 μg/ml. At this concentration, no growth inhibitory action of cells was observed (Fig. 1-A), indicating that PGE2 reduction precedes the inhibition of cell growth, thus confirming the role of PGE2 in stimulating cancer cell growth. Specific inhibitory action of PGE2 in both cell types was additionally confirmed by time course study. In the absence of UD, there is a steady increase of PGE2 production, which reaches a plateau after 24 h (Fig. 3-B). However, PGE2 levels in osteoclast cells remained at baseline levels in the presence of 20 μg/ml UD. At such concentration, no cell growth inhibitory action was observed at 12 h incubation (data not shown), confirming the role of PGE2 in stimulating osteoclast cell growth. These results demonstrated that UD inhibits the production of PGE2 in osteoclast, which, in turn, contributes to its growth inhibitory activity.

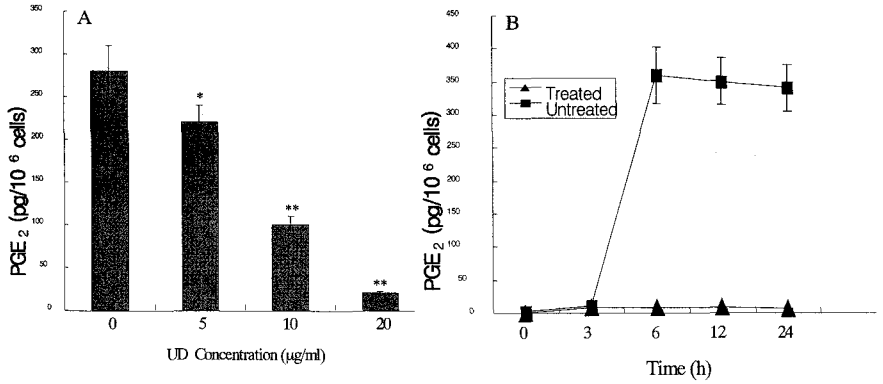

Fig. 3. Inhibitory Action of PGE2 Synthesis in Osteoclast Cell

The amount of PGE2 present in the supernatant of the culture medium was measured by an enzyme immunoassay and expressed as picograms/10^6 cells. (A) In the presence of UD at concentrations ranging from 5 to 20 μg, osteoclast cells demonstrated a dose-dependent suppression of PGE2 synthesis after 12 h incubation. (B) Time-dependent inhibition of PGE2 synthesis was also observed in osteoclasts at concentration of 20 μg of UD. Suppression of PGE2 synthesis was observed through the course of treatment. A delayed inhibitory action (12 h) was observed for UD. Results are mean values ± SD (bars) of independent experiments performed in triplicate (A). Student's t-test was used to analyze the data.
*. $P < 0.05$; **. $P < 0.001$.

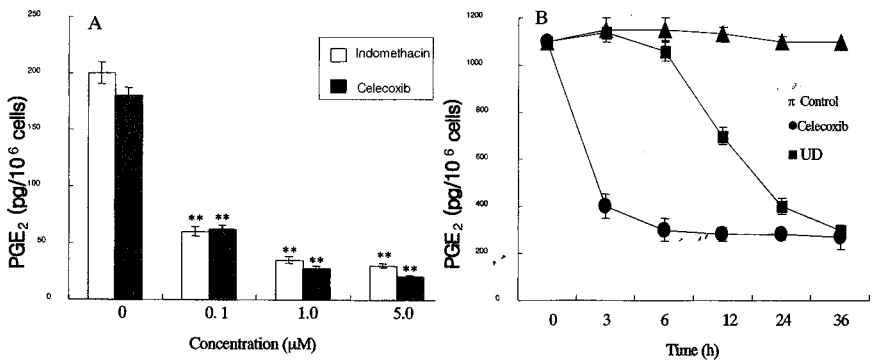

Fig. 4. Comparative Inhibitory action of PGE2 Synthesis

The amount of PGE2 present in the supernatant of the culture medium was measured by an enzyme immunoassay and expressed as pg/10^6 cells. (A) Both celecoxib and indomethacin inhibited PGE2 synthesis even at a concentration of 0.1 μM. B, suppression of conversion of arachidonic acid to PGE2 by UD (20 μg/ml) and celecoxib (5 μM). A rapid inhibition (2 h) was observed in the presence of celecoxib. Results are mean values ± SD (bars) of independent experiments performed in triplicate (A). Student's t-test was used to analyze the data. *, $P < 0.05$; **, $P < 0.001$.

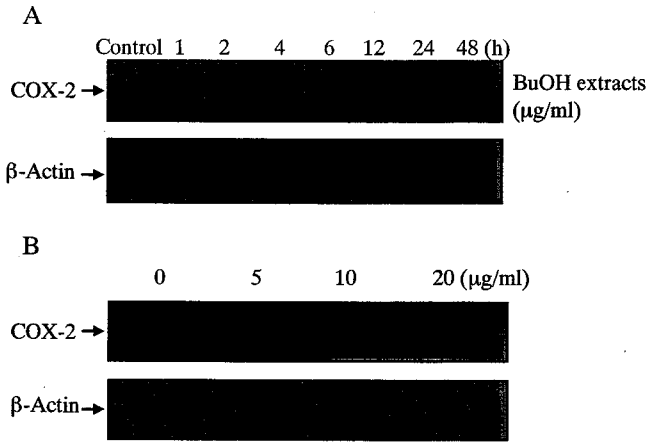

Fig. 5. Inhibitory Action of COX-2 Protein Expression

(A) Osteoclast cells were treated with UD at 20 μg/ml for 2, 4, 6, 8, 12, 24 and 48 h, and proteins were extracted from cells and separated through SDS-PAGE. A significant decrease of COX-2 protein expression was observed after 48 h treatment with UD (top panel). ß-Actin expression was used as an internal control (bottom panel).

(B) Osteoclast cells were treated with UD at concentrations of 0, 5, 10 and 20 μg/ml for 72 h, and the amount of COX-2 proteins was determined as above. A dose-dependent decrease of COX-2 protein expression was observed after treatment with UD (top panel). ß-Actin expression was used as an internal control (bottom panel).

Indomethacin and celecoxib exhibited dose-dependent inhibition of PGE2 synthesis in osteoclast cells at 12 h (Fig. 4-A). These results confirmed that the increased level of PGE2 was attributable to the increase in COX-2 activity and/or COX-2 expression in both cells. Furthermore, no reduction of cell growth at even 1 μM indomethacin or celecoxib was observed at 12 h incubation (data not show), confirming that PGE2 reduction precedes the cell growth inhibitory action. The effect of UD and celecoxib on COX-2 activity was examined by cell arachidonic acid conversion assay. These results show that there is a significant decrease of PGE2 level in the

presence of both UD and celecoxib at IC50 doses. However, inhibition of COX-2 activity by celecoxib occurred earlier than by UD: COX-2 activity decreased after 12 h for UD and 2 h for celecoxib (Fig. 4-B). The delayed inhibitory action of UD suggest that it inhibits PGE2 synthesis through a decrease of COX-2 expression, instead of direct inhibition of COX-2 activity.

5. Inhibitory Action of COX-2 Protein Expression

The effect of UD on COX-2 protein expression in osteoclast cells was investigated by Western blot analysis. Osteoclast cells were chosen for this assay because they produce a higher level of PGE2. The cells were treated with UD at concentration of 300 μg/ml for 4, 8, 12, 24, 48, and 72 h. Western blotting showed that a decrease of COX-2 expression did not occur until 48 h of incubation with UD (Fig. 5-A). These results may explain the delayed inhibitory action of PGE2 synthesis by UD (Fig. 5-B). There was a dose-dependent decrease of COX-2 expression on Western blot (Fig. 5-B).

Ⅳ. Discussion and Conclusion

UD has been shown to have a broad spectrum of biological activities, including anti-inflammatory and anticancer activity based on its long history in clinical applications. UD is an extract developed to have therapeutic effects in inflammatory diseases involving cartilage destruction, such as RA. According to published work that is well accepted by the traditional Korean medicine community, UD was formulated to facilitate blood circulation as well as to reduce anti-inflammatory activity. UD have been used for hundreds of years in this oriental region, and their safety and efficacy are well established through a long history of human use, but their use still lacks scientific support[8,13]. Although the barks of U. davidiana stem and root have been used in traditional Korean medicine for inflammatory diseases, the action mechanisms of this species are not nearly understood. It may be important to understand how this plant extract performs anti-inflammatory action in vivo.

Recently, Glycoprotein of UD was shown to have strong scavenging activities against oxygen free radicals. The glycoprotein has inhibitory actions on protein kinase C alpha (PKC alpha) translocation, nuclear factor-kappa B (NF-kappaB) DNA binding activity, nitric oxide (NO) production, and apoptosis in 12-O-tetradecanoylphorbol 13-acetate (TPA)-stimulated NIH/3T3 cells. Interestingly however, it could not regulate the DNA binding activity of AP-1. Therefore, UD glycoprotein, a natural anti-oxidant, was believed is a potential modulator of apoptotic signal pathways in NIH/3T3 cells[14]. Four lignan xylosides and two neolignan glycosides were isolated from the stem and root barks of UD. Investigation of the constituents of the stem and root barks of UD resulted in the isolation of five new triterpene esters named ulmicin A-E[15]. These compounds showed significant neuroprotective activities against glutamate-induced neurotoxicity in primary cultures of rat cortical cells[16].

This study confirmed that UD has strong dose-dependent anti-inflammatory activity, with the IC50 at 10 μg/ml. Inhibition of cell growth is because of its ability to cause G0-G1-phase arrest. Inhibition of cell proliferation is confirmed by its ability to reduce PCNA expression that correlates with decreased cell proliferation[17]. We also investigated the effect of UD, as well as COX-specific inhibitors on apoptosis using flow cytometry, TUNEL (terminal deoxynucleotidyl transferase-mediated nick end labeling) assay, and DNA ladder assay. No significant induction of apoptosis was observed at all concentrations of UD (data not shown). However, an increased apoptosis was observed at higher concentration(40 μM) of celecoxib. These results indicate that

inhibitory action of osteoblast cell growth by UD is through the G0-G1-phase arrest rather than apoptosis. The mode of action of UD is similar to that of COX-2 inhibitors such as indomethacin and celecoxib, which also cause G0-G1 arrest.

The anti-inflammatory activity of UD may result, at least in part, from the reduction of PGE2. These observations are additionally supported by these results showing that, indomethacin and celecoxib, because of their specific inhibition of COX-reduced PGE2 production in osteoblast cells. Both agents also inhibit osteoblast cell growth in vitro. These results show that doses required to suppress PGE2 production as well as PCNA expression for UD and other agents was 10-fold less than those for cell growth inhibition, indicating that decrease of PGE2 and PCNA precedes the growth inhibition. Alternatively, a COX-2-independent pathway may also play a role.

The molecular mechanism linking PGE2 level and cell proliferation remains unclear. It has been shown that PGE2 may regulate cancer cell proliferation in an autocrine and/or paracrine manner via the PG receptors, especially EP2[18]. The stimulation of the receptors results in an increase of cyclic AMP level in cancer cells that triggers the signal transduction pathway leading to uncontrolled cell proliferation[19]. One of the possible mechanisms is that inactivation of PG receptors by reducing the PGE2 synthesis by UD may lead to reduced expression of cyclin D1 and cyclin E proteins, key factors that control the G0-G1 cell cycle[20]. These results show that osteoblast cell, although with different PGE2 levels, had equal sensitivity to UD and other COX-specific inhibitors. Furthermore, UD inhibited PGE2 synthesis. Taken together, these results support the notion that there is a COX-2-independent pathway also responsible for anti-inflammatory activity of UD.

Because COX-2 is the key enzyme in converting arachidonic acid to PGs, it is plausible that UD may act on this enzyme by either reducing its activity or inhibiting its expression, leading to the decrease of PGE2. Increased COX-2 protein expression has been found in various inflammatory cells. The effect of UD on the COX-2 activity was initially examined because a decreased PGE2 level was also seen in the presence of COX-2-specific inhibitor celecoxib. These results show a significant decrease of COX-2 activity after 12 h of the treatment, whereas it was sharply decreased only 2 h after treatment with celecoxib. These results indicated that UD may affect COX-2 expression rather than its activity. The COX-2 mRNA expression was then assayed by real-time reverse transcription-PCR, and insignificant decrease of mRNA expression was observed (data not shown). The COX-2 protein expression was examined by Western blot analysis and found that there was a significant decrease of COX-2 protein in osteoblast cells after UD treatment. These results indicate that reduced COX-2 protein expression is mainly because of the increase of the protein turnover rate (i.e., decreased stability). It is interesting to note that a decrease in PGE2 level does not parallel with COX-2 activity, i.e., PGE2 synthesis is suppressed throughout the course of treatment, whereas COX-2 activity decreased at a later time(12 h) and COX-2 expression became suppressed at 24 h, indicating that there are at least two independent mechanisms responsible for the decrease of PGE2. We hypothesize that initial inhibition of PGE2 synthesis is through the inhibition of arachidonic acid release from cell membrane (i.e., phospholipase A2) and the later inhibition is caused by reduction of COX-2 protein expression. These results suggest that UD inhibits PGE2 production at multiple levels along the PGE2 synthesis pathway.

It is apparent that UD exert its anti-inflammatory activities by multiple mechanisms. These results show that the extract can inhibit PGE2 production. However, the extract inhibits cell growth in vitro. There may be a synergistic effect of various components in the herb, which act on different anti-inflammatory pathways such as COX-dependent

and COX-independent pathways. These results strongly support the traditional use of raw herbs in decoction. Furthermore, because of the poor water solubility of pure flavonoids, the use of herb extract is more attractive given its excellent water solubility and bioavailability.

We have demonstrated, for the first time, that UD extract exhibits a strong anti-inflammatory activity in vitro, especially on osteoclast that is usually resistant to chemotherapy. The anti-inflammatory activity of UD can be attributed, in part, to its inhibitory effect on PGE2 production via suppression of COX-2 expression and arachidonic acid release from cell membrane. Future studies will focus on the relationship between increased PGE2 and cell cycle regulation and will determine its efficacy in clinical trials in patients with RA and osteoporosis. The information generated from this study will help to support the development of human clinical trials and to develop UD herbal acupuncture solution in the future.

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