Effects of the Dichloromethane Fraction of Dipsaci Radix on the Osteoblastic Differentiation of Human Alveolar Bone Marrow-Derived Mesenchymal Stem Cells

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Dipsaci Radix is the dried root of Dipsacus asper Wall. It has been used in Korean herbal medicine to treat bone fractures. In this study, we examined the effect of the dichloromethane fraction of Dipsaci Radix (DRDM) on the osteoblastic differentiation of human alveolar bone marrow-derived MSCs (ABM-MSCs). The ABM-MSCs were isolated from healthy subjects and cultured in vitro, followed by phenotypic characterization. They showed a fibroblast-like morphology and expressed CD29, CD44, CD73, and CD105, but not CD34. Calcified nodules were generated in response to both dexamethasone (DEX) and DRDM. There was a significant increase in the alkaline phosphatase (ALP) activity and protein expression of bone sialoprotein (BSP) and osteocalcin (OC) in response to DEX and DRDM as compared to control. These results provide evidence for the osteogenic potential of cultured ABM-MSCs in response to DEX and DRDM. Also, an active single compound was additionally isolated from DRDM. The single compound (hederagenin 3-O-(2-O-acetyl)-α-L-arabinopyranoside) also significantly increased ALP activity and the level of protein expression of BSP and OC. These results highlight the possible clinical applications of DRDM and hederagenin 3-O-(2-O-acetyl)-α-L-arabinopyranoside in bone regeneration.

Key words: mesenchymal stem cell; osteoblastic differentiation; Dipsaci Radix; dexamethasone; herbal medicine

Mesenchymal stem cells are a focus of intense investigation because of their potential in regenerative medicine. They have a number of characteristics, including self-renewal capacity and multipotentiality, i.e., ability to differentiate into diverse, though limited, cell lineages.1,2

FGF-2 has been found to have a significant effect on the self-renewal ability of MSCs, leading to an increased growth rate and life span of cells extracted from human bone marrow.3 Sugiyama et al.4 reported that murine primary bone marrow stromal cells transduced with BMP-2 or BMP-4 can induce osteogenesis in immunocompetent mice. Kawaguchi et al.5 reported that autotransplantation of MSCs isolated from bone marrow into an alveolar bone defect can promote the regeneration of periodontal tissues.

DEX is the primary stimulant used for in vitro induction of osteoblast differentiation from MSCs,6 but DEX has a number of drawbacks,3,7 including immunosuppressive effects, which limit its potential applications. Therefore, alternative inductive stimuli can serve as additional tools for examining MSCs for potential clinical applications.

Dipsaci Radix is the dried root of Dipsacus asper Wall. Oriental herbal medicines are used in complementary medicine with considerable research and clinical applications. Dipsaci Radix is one such herbal medication that is used for protection against degeneration of cartilage and bone, and against the regeneration of damaged hard tissues.8,9 A number of biochemical fractionations have been conducted in our laboratory in an effort to generate a better-defined product from DR. These studies suggest that the DRDM has the most potent osteo-inductive effects in vitro (some results have not yet been published). The aim of this study was to determine whether DRDM can be used for in vitro induction of osteoblastic differentiation by alveolar bone marrow-derived MSCs (ABM-MSCs), as an alternative to DEX. Also we attempted to measure the osteogenic activity of an active single compound from DRDM on osteoblasts.

Materials and Methods

ABM-MSC culturing. ABM-MSCs were isolated and expanded as described previously.30 Briefly, alveolar bone marrow aspirate (0.1 to 3.0 cc) was collected from osteotomy sites during dental implant surgery from four patients (ranging in age from 46 to 63 years), who were treated at the Wonkwang University Dental Hospital Department

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Abbreviations: ABM-MSC, alveolar bone marrow derived mesenchymal stem cell; ALP, alkaline phosphatase; BSP, bone sialoprotein; DEX, dexamethasone; DRDM, dichloromethane fraction of Dipsaci Radix
Calcium accumulation assay. Cells (1 x 10^6) were cultured in 6-well culture dishes. Extra-cellular mineralization was induced by adding 4 mM NaHPO_4 on day 23. The medium was removed and washed out with PBS on day 25. The cells were fixed with ice-cold 70% ethanol for 1 h at 4 °C. The ethanol was removed and the cells were stained with 40 mM alizarin red-sulfate (AR-S, Sigma) for 10 min at room temperature. The stained portions were photographed with a digital camera (Nikon E955, Nikon, Tokyo, Japan), and the AR-S concentration was determined at 562 nm by melting the stained portion using a solution in which 10% (w/v) cetylpyridinium chloride had been dissolved in 10 mM sodium phosphate (pH 7.0). The AR-S standard curve used the same solution. The experiment was repeated 3 times.

Western blot. In western analysis, cells were incubated in serum-free media. The proteins were concentrated from culture media by trichloroacetic acid (TCA) precipitation (final TCA concentration, 10%) and resuspended in sample loading buffer. The proteins separated by electrophoresis were transferred from the gel onto a PVDF membrane using a tank transfer system (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 5% non-fat-milk for 1 h at room temperature. The membrane was incubated with anti-BSP and OC polyclonal antibody at a dilution of 1:50 for 2 h at room temperature, the membrane was washed at room temperature for 30 min with three changes of 1× TBS containing 0.05% Tween 20 (TBST). Then, this was incubated for 1 h at room temperature with rabbit polyclonal anti-goat IgG coupled to horse-radish peroxidase at a dilution of 1:1,000. The membrane was washed as above with 1× TBST. Subsequently, it was reacted using an ECL kit (Amersham, Piscataway, NJ) for 1 min and exposed to Hyperfilm-MP (Amersham).

Results

Phenotypic characterization of ABM-MSCs

The ABM-MSCs, examined by optical microscopy, exhibited a spindle-shaped fibroblast-like morphology (Fig. 1), as in previously published studies of MSCs.12,13 Flow cytometry analysis of the immunofluorescently labeled ABM-MSCs demonstrated the expression of CD29, CD44, CD73, and CD105, but not CD34 (Fig. 2). These results are consistent with the phenotypic features of MSCs, such as those isolated from the femur and the iliac bone.13,14

Effect of DR(Dm) on alkaline phosphatase activity

The ALP activity of ABM-MSCs, cultured in the presence of DEX or DR(Dm), was determined to examine the ability of DR(Dm) to induce osteogenesis. The results indicated that the ALP activity of ABM-MSCs cultured in the presence of DEX or DR(Dm) was significantly higher than the negative control at each of the time points tested (Fig. 3). The highest ALP activity was observed among the P6 cultured cells in response to both stimulants. ALP activity in response to DR(Dm) was not significantly different from that of DEX for the P3 and P9 cells. Among the P6 cells, ALP activity in response to DEX was approximately 19% higher than DR(Dm) (p < 0.05). Among the P3 and P9 cells, DR(Dm) stimulated ALP activity equivalent to DEX.
Calcium accumulation assay

To investigate further the differentiation along the osteogenesis pathway, ABM-MSCs were cultured in the presence and the absence of DEX or DRDM, followed by a calcium accumulation assay. When the ABM-MSCs were cultivated for 25 d in the presence and the absence of DEX or DRDM, mineralized nodule formation was observed and quantified (Figs. 4, 5). Abundant calcium accumulation was observed during the entire incubation period with DEX and DRDM. There was little calcified nodule formation in the control groups during P3, P6, and P9. In P3, calcified nodule formation was established in specific regions when induced with either DEX or DRDM. The morphology of the calcium nodules forming in response to DEX and to DRDM showed a similar pattern. The P3 cultures exhibited a single large nodule in both induction cultures, whereas the P6 cultures contained many small nodules, and the P9 cells had a lower density of smaller nodules. The optical density of the calcium nodules was quantified and compared to determine the degree of mineralization. There was a significant increase in calcium accumulation in P3, 833 ± 6/C621 ± 6 with DEX and 718 ± 3/C617 ± 9 with DRDM, which was approximately 6 and 7 times higher than that observed for the control (114 ± 2/C66 ± 3).

The level of calcium accumulation in P6 in the control, DEX, and DRDM groups was 84 ± 3/C615 ± 0, 671 ± 2/C628 ± 2, and 631 ± 1/C630 ± 7 respectively. DEX and DRDM produced a 7-fold increase in calcium accumulation as compared to the control. The level of calcium accumulation in P9 was 5 and 3 times higher in the DEX (589.4 ± 12.0) and the DRDM group (449.1 ± 27.1) respectively than in the control group.

Fig. 1. Morphology of Human Mesenchymal Stem Cells from Alveolar Bone Marrow.
(A) Cultured MSC cells after initial seeding and (B) near-confluent ABM-MSC population (original magnification ×100). The cells exhibited a fibroblastic morphology.

Fig. 2. Phenotypic Characterization of ABM-MSCs.
FACS analysis indicated that the cells were negative for CD34 expression and positive for CD29, CD44, CD73, and CD105, which are characteristic phenotypes of MSCs. The solid histograms show cells with negative control IgG. Representative examples of three experiments.

Fig. 3. Alkaline Phosphatase (ALP) Activity.
One unit was defined as the activity producing 1 nmol of p-nitrophenol for 30 min. The ALP activity of ABM-MSC cultured in the presence of DEX or DRDM was significantly higher than the negative controls, and the highest ALP activity was observed among P6 cultured cells in response to both stimulants. *Statistically significant with control (p < 0.05). #Statistically significant with DEX (p < 0.05).

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Effects of DRDM on the expression of BSP and OC

BSP is a non-collagenous structural protein present in the bone matrix with high concentrations of sialic acid and acidic phosphoprotein. It is specifically expressed by fully differentiated osteoblasts in the bone and cementum.\(^{15,16}\) ALP is a marker activated during the early stages of osteoblast differentiation, whereas BSP and OC are expressed during the intermediate and late stages of differentiation.

The levels of BSP and OC, typical proteins of bone differentiation, increased in P3, P6, and P9 with DEX and DR\(_{DM}\) as compared to control (Fig. 6). In P3, the degrees of BSP and OC expression by DEX and DR\(_{DM}\) were similar in mode and higher than that of the control. Expression of BSP and OC in P6 appeared to be higher with DEX than with DR\(_{DM}\). In P9, unlike P3 and P6, expression of BSP and OC with DR\(_{DM}\) was lower than with DEX. The decrease in BSP and OC expression in P9 due to DR\(_{DM}\) and DEX showed a similar pattern in the reduction of calcium accumulation assay in P9.

Identification of the single compound

Repeated chromatographic separation and purification of DR\(_{DM}\) provided a single compound, the structure of which was determined by extensive MS, \(^1\)H NMR, and \(^{13}\)C-NMR spectral analysis as well as by a comparison of spectral data with previously reported values.\(^9\)

Compound 1: Hederagenin 3-O-(2-O-acetyl)-\(\alpha\)-L-arabinopyranoside. \(C_{37}H_{58}O_{9}, (-)-ESI-MS m/z\) (rel. int.): 645[M – H]-(100); \(1^{3}C\)-NMR (125 MHz, pyridine-d5) \(\delta\) 0.91, 0.92, 0.93, 0.99, 1.02, 1.22 (3H each, s, CH3), 2.10 (3H, s, CH3COO–), 4.90 (1H, d, J=7.1 Hz, anomeric H), 5.47 (1H, br.d, 12-H); \(1^{3}C\)-NMR (125 MHz, pyridine-d5) \(\delta\) 38.5(C-1), 26.0(C-2), 80.9(C-3), 43.2(C-4), 46.9(C-5), 17.9(C-6), 32.6(C-7), 39.6(C-8), 48.0(C-9), 36.6(C-10), 23.5(C-11), 122.4(C-12), 144.6(C-13), 41.8(C-14), 28.2(C-15), 23.7(C-16), 46.5(C-17), 42.0(C-18), 46.2(C-19), 30.8(C-20), 34.0(C-21), 33.0(C-22), 103.8(C-10), 74.2(C-20), 72.3(C-3), 69.6(C-4), 67.0(C-5), 21.1(CH3COO–), 169.9(CH3COO–).

Effect of the single compound on osteoblast differentiation

The single compound, hederagenin 3-O-(2-O-acetyl)-\(\alpha\)-L-arabinopyranoside, significantly increased ALP activity at concentrations ranging from \(10^{-7}\) M to \(10^{-11}\) M (Fig. 9A), but it did not show a significant effect on cell proliferation (Fig. 9B). Furthermore, the levels of protein expression of BSP and OC also increased under treatment with the single compound (\(10^{-8}\) M to \(10^{-9}\) M) at 14 d (Fig. 9C).

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**Fig. 4.** Alizarin Red Staining for Mineralized Nodule Formation. The mineralized matrix was stained with alizarin red and photographed. Abundant calcium accumulation was observed during the entire incubation period with DEX and DR\(_{DM}\). The P3 cultures exhibited a single large nodule in both induction cultures, whereas the P6 cultures contained many small nodules, and the P9 cells had a lower density of smaller nodules.

**Fig. 5.** Optical Density of Calcium Accumulation. There was a significant increase in calcium accumulation in P3, 833.6 ± 21.6 with DEX and 718.3 ± 17.9 with DR\(_{DM}\), which was approximately 6 and 7 times higher than that observed for the control (114 ± 6.3). *Statistically significant compared to the control group (\(p < 0.05\)). #Statistically significant compared to the DEX group (\(p < 0.05\)).

**Fig. 6.** Western Blot Analysis to Confirm Protein Expression of BSP and OC. The levels of BSP and OC increased in P3, P6, and P9 with DEX and DR\(_{DM}\) as compared to the control. In P9, unlike P3 and P6, expression of BSP and OC with DR\(_{DM}\) was lower than that with DEX.
In this study, there was significant evidence that DRDM, a derivative of an herbal medicine, can induce the differentiation of ABM-MSCs into osteoblast cells. The results indicate that incubation of ABM-MSC with DRDM induced ALP activity, expression of bone-specific proteins, including BSP and OC, and the formation of calcium nodules. Also, an active single compound, hederagenin 3-0-(2-O-acetyl)-C11-L-arabinopyranoside, supported the effect of DRDM by increasing ALP activity and the expression levels of BSP and OC. The magnitude of osteoblastic differentiation due to DRDM appeared to be equivalent to that of a well-studied glucocorticoid, dexamethasone.

This study provides the first evidence that a herbal extract can be used for osteoblastic differentiation from ABM-MSCs. In addition, MSCs can be cultured successfully using a small amount of blood from ABM, as reported by Matsubara et al. The iliac bone, tibia, and femur are used extensively as sources of MSCs. The drawbacks of using these donor sites include the morbidity associated with the procurement procedure, and the differences in the embryonic development of the long bones and jaw bone. One of the previous concerns was the limited quantity of cells that can be harvested from the alveolar bone. Our study indicates that up to 10^7 cells can be generated from the small bone marrow specimens collected, whereas other studies were able to generate up to 10^8 MSCs from the alveolar bone. The present study also indicates that ABM-MSCs have a higher proliferative capacity. This observation is corroborated by previous reports demonstrating the higher osteogenic potential of ABM-MSCs than iliac bone.

There are limited in vitro and in vivo data suggesting the application of certain herbal and natural products in bone regeneration. Soybean has been found to induce osteoblastic differentiation, and to decrease osteoclastic activity. Soybean extracts are applied as bone fillers and regenerative materials. Safflower seeds are commonly used as a natural remedy to treat bone fractures in traditional Korean medicine, and have recently been used in regenerative applications. Other data on safflower seeds include application to the treatment of periodontal 1-wall intrabony defects, and in the prevention of bone resorption caused by estrogen deficiency. Angelica sinensis has effects on osteoblast proliferation and differentiation. We have also studied some herbal medicines both in vitro and in vivo in relation to regenerative medicine in periodontal and bone tissues.

This study confirms that ABM-MSCs exhibit phenotypic features similar to stem cells derived from long bones. By analyzing the stages of MSC differentiation, it was shown that DRDM induced increases in ALP activity, calcium accumulation, and BSP and OC expression. Titorenco et al. reported that differentiation of osteoprogenitors is less effective at higher numbers of passages, and that gene expression of bone-specific markers is lower in cells cultured after many passages as compared to expression in primary culture. In the present study, biochemical activity and protein expression decreased progressively with each passage. These results suggest that cells from the early passages (before P9) should be used in therapy.

As reported by Matsubara et al., the bone marrow collected from the alveolar bone in this study was inevitably small in amount compared to the iliac bone or femur due to site specificity. However, a small volume of bone marrow was sufficient to induce a large number of MSCs, unlike in other studies, which employed a large volume of bone marrow. This means that a large amount of bone marrow is not always necessary for MSC proliferation.

Multipotent MSCs have the capacity for renewal, and some precursor cells can differentiate into osteoblasts under the influence of growth factors or stimuli. Many studies have used DEX to differentiate MSCs into osteoblasts. The primary purpose in searching for novel stimuli to induce osteogenic differentiation is to identify products with reduced side effects. Glucocorticoids are used to treat autoimmune and inflammatory diseases, including arthritis, asthma, multiple sclerosis, and systemic lupus erythematosis, but long-term administration of glucocorticoids has undesired side effects.

**Fig. 7.** Isolation of an Active Single Compound from Phlomidis Radix.

**Fig. 8.** Structure of the Compound from DRDM.
including immunosuppression and osteoporosis with severe bone loss, and increased risk of fracture. The precise mechanism of glucocorticoids is not completely understood, but Phillips et al. reported recently that the coordinate action of DEX and osteogenic transcription factor Runx2/Cbfal synergistically induces osteogenesis in primary dermal fibroblasts. Dipasic Radix, a regimen to treat bone fractures or diseases over long periods in traditional Korean medicine can be used in regenerative medicine without serious side effects like those of DEX. DEX and hederagenin 3-O-(2-O-acetyl)-α-L-arabinopyranoside increased important bone markers. Our results suggested that DEX and hederagenin 3-O-(2-O-acetyl)-α-L-arabinopyranoside were demonstrated to have osteoblast differentiation activity in vitro and in vivo.

**Conclusion**

This study examined the capacity of a herbal medicine, DR, to induce osteoblast differentiation in MSCs. MSCs harvested from the alveolar bone were used. Their morphologic (fibroblast-like) and phenotypic characteristics (CD34−, CD29+, CD44+, CD73+, and CD105+) were consistent with those of MSC. DR indicated significant ALP activity, calcium accumulation, and OC and BSP protein expression, in a magnitude similar to that obtained with DEX. Also, a single compound having osteoblast differentiation activity was successfully isolated and the compound proved to be hederagenin 3-O-(2-O-acetyl)-α-L-arabinopyranoside.

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**References**

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