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Diosgenin stimulates osteogenic activity by increasing bone matrix protein synthesis and bone-specific transcription factor Runx2 in osteoblastic MC3T3-E1 cells☆

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Abstract

Diosgenin, a steroid saponin extracted from the root of wild yam (*Dioscorea villossa*) is claimed to have osteogenic property. However, detailed studies providing evidence to this claim have not been fully undertaken. In this study, we investigated the effect of diosgenin on the osteogenesis of murine MC3T3-E1 osteoblastic cells. Cells were cultured with varying levels of diosgenin (0–10 μM) within 25 days of bone formation period. Diosgenin was found to stimulate proliferation within the range of 0.01–5 μM using MTT assay. The medium and cellular levels of Type 1 collagen and alkaline phosphatase (ALP), both of which are major bone matrix proteins, increased within the low range of diosgenin concentration (>0–3 μM), and this pattern was further confirmed by collagen and ALP staining of the extracellular matrix (ECM). The cellular protein expression of ALP and collagen Type 1 was also increased at 0.1–1 μM diosgenin treatment as analyzed by Western blot. Calcium deposition within the ECM also showed the same pattern as assessed by Alizarin Red S and Von Kossa staining. Bone-specific transcription factor runt-related transcription factor 2 (Runx2) and Runx2-regulated osteopontin protein expressions were induced at low concentration (0.1–1 μM) and again decreased with high diosgenin concentrations. Based on our findings, our study suggests that diosgenin can enhance bone formation by stimulating the synthesis and secretion of Type 1 collagen and ALP and bone marker proteins Runx2 and osteopontin expression. The increased levels of these marker proteins, in turn, can increase the formation of calcium deposits within the ECM thereby increasing bone formation.

Keywords: Diosgenin; MC3T3-E1 cells; ALP activity; Type 1 collagen; Osteopontin; Runx2; Mineralization

1. Introduction

Bone formation is characterized by proliferation and formation of a properly laid-out collagenous extracellular matrix. Once matrix synthesis begins, osteoblast marker genes are activated in a clear temporal sequence; alkaline phosphatase and the parathyroid hormone (PTH)/PTH-related protein receptor are induced at early times while osteopontin and osteocalcin appear somewhat later [1]. Once these marker genes are induced, mineralization of the collagenous extracellular matrix follows. Since bones are continually being destroyed and reformed to maintain constant bone volume and calcium homeostasis [2], there are many opportunities in which perturbations to these processes could take place therefore resulting in several bone-related diseases. The balance between bone formation and bone resorption must be delicately maintained to ensure the integrity of the skeletal system. An imbalance brought about by increased bone resorption over bone formation can lead to most adult skeletal diseases including osteoporosis [3,4]. Until recently, however, most therapies for skeletal disorders are focused mainly on the resorption side and far less attention has been paid to promoting bone formation [4]. Therefore, developing therapeutics favoring bone formation might be a useful approach in bone disease treatments.

Diosgenin, a furostanol saponin, is a major bioactive constituent in the seeds of fenugreek (*Trigonella foenum graecum*) as well as in the roots of wild yams (*Dioscorea villosa*) that are consumed by populations in parts of Latin America, Eastern Europe and Asia as a food ingredient or condiment [5]. Recently, numerous studies are being undertaken showing the anti-proliferative and proapoptotic effects of diosgenin against several cancer cell lines including human erythroleukemia, osteosarcoma, HER2-overexpressing breast cancer cells, and HT-29 human colon cancer cells [6–10]. In vivo studies also showed the ability of diosgenin to decrease the elevated levels of

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serum LDL in rats fed cholesterol [11]. While there is a plethora of researches focusing on the anticancer and antitumor properties of diosgenin, a very limited literature on the effects of diosgenin on bone formation is available. Previously, an in vivo experiment conducted by Higdon et al. [12] showed that bone loss can be significantly reduced by sustained diosgenin delivery in ovariectomized adult rat model. Moreover, 2 μM diosgenin up-regulates vascular endothelial growth factor-A (VEGF-A) and promotes angiogenesis in murine MC3T3-E1 preosteoblastic cells [13].

With these background studies, we hypothesized that diosgenin possesses osteogenic properties and could be a potential activator for bone formation which can be a treatment for bone-related diseases. To validate this, we evaluated the effect of varying levels of diosgenin on (1) the cell proliferation, (2) the levels of synthesized and secreted Type 1 collagen and ALP, two early osteoblast differentiation markers which are the major proteins for extracellular matrix maturation and calcification, respectively, and (3) the calcium deposits in ECM for matrix calcification in murine MC3T3-E1 osteoblastic cells. Moreover, the effect of diosgenin treatments on the protein expression of osteoblast differentiation markers (Type 1 collagen, ALP and osteopontin) and bone-specific transcription factor runt-related transcription factor 2 (Runx2), which controls osteoblast markers expression, was also analyzed. Findings from this study, therefore, could provide an insight in the potential use of diosgenin in enhancing bone formation.

2. Methods and materials

2.1. Reagents

Antibodies for target proteins (Type 1 collagen, ALP, osteopontin, and Runx2) were obtained from Santa Cruz while antibody for loading control (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) was obtained from Cell Signaling. All chemicals and cell culture reagents were obtained from Sigma (St. Louis, MO, USA) and Gibco, Invitrogen (Carlsbad, CA, USA), unless otherwise indicated.

2.2. Cell culture

Mouse osteoblastic MC3T3-E1 cells (ATCC CRL-2593) were seeded at a density of 1×10^4 cells/ml and cultured in regular growth culture media containing α-minimum essential medium (α-MEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco), 1 mM sodium pyruvate (Sigma, St. Louis, MO, USA), and 100 units/L penicillin and 100 mg/L streptomycin (Gibco) in a humidified atmosphere of 5% CO_2 at 37°C. At 80% confluence, the cells were cultured in differentiation media as growth media supplemented with 3 mM sodium phosphate (Sigma) and 50 μg/ml L-ascorbic acid (Sigma) as osteogenic differentiation medium. Cells were treated with 0–10 μM of diosgenin from a 1 mM diosgenin stock dissolved in dimethyl sulfoxide (DMSO) and the medium was changed every 2 days. The cells cultured in normal osteogenic differentiation medium without diosgenin treatment were used as osteogenic control (osteogenic medium [OSM]) and cells cultured in normal osteogenic medium with volume of DMSO similar to that with 10 μM diosgenin were used as vehicle control.

2.3. Cell proliferation assay

Cell proliferation was determined by following the reduction of the synthetic metabolic substrate resazurin to resorufin (Alamar Bule kit, Invitrogen) or MTT assay (Promega), according to manufacturer's instructions. Briefly, cells (1×10^4 cells/well in 96-well plate) were maintained in growth media for 24 h at 5% CO_2, 37°C. At 90% confluence, the cells were treated with various concentrations (0, 0.01, 0.1, 1, 2, 3, 5 and 10 μM) of diosgenin for 1, 3 and 6 days. Following the respective time treatment, resazurin reagent or MTT was added to the cell cultures (1:10) and samples were incubated at 37°C for 4 h. Absorbance was measured in an optical 96-well microplate reader (Sunrise Absorbance Reader; Tecan Austria) at 570 nm using 600 nm as reference wavelength.

2.4. Collagen measurement

2.4.1. Cellular and medium collagen concentration measurement

The amount of cellular and medium collagen was measured using the Picro-Sirius red method. Cells were washed with phosphate-buffered saline (PBS) and were hydrolyzed with 0.5 M acetic acid overnight at 4°C. Fifty micro liters each of the standards, cell fractions and medium samples were added on each well of 96-well plate and were allowed to dry at 37°C in humidified atmosphere for 24 hours. After drying, the wells were washed with 200 μl distilled water and 100 μl of 0.1% Picro-Sirius red dye in picric acid was added on each well. The samples and standards were then incubated with the dye for 1 hour at 37°C. After incubation, the samples and standards were washed three times with 200 μl of 10 mM HCl to remove the unbound dye. Finally, the bound collagen was dissolved by adding 200 μl of 0.1 M NaOH for 5 min. The samples and standards were then transferred to a clean 96-well plate and absorbance was read at 540 nm.

2.5. Cell matrix collagen staining

The synthesis of collagen was assessed by staining with Van Gieson. Cells were washed with distilled water and were allowed to dry completely. The cells were fixed with 2% formaldehyde at 4°C for 15 min. The fixed cells were then washed twice with distilled water and stained with Van Gieson reagent for 15 min. Excess dye was removed by washing with at least three changes of distilled water. Collagen in the ECM is stained red.

2.6. ALP activity

2.6.1. Cellular and medium ALP activity

Cellular and medium ALP activity were measured in cells treated with 0, 1, 3, 5 and 10 μM diosgenin and in control cells incubated for 5 and 15 days. Cells were washed with PBS and lysed using 25 mM Tris–Cl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS) and 1 mM phenylmethanesulfonyl fluoride (PMSF) as protease inhibitor. The lysates were sonicated on ice for 30s at 50% pulse. The sonicated lysates were centrifuged for 15 min at 4°C 12 000×*g*. The supernatant was stored at −80°C until analysis. Media were also collected for the measurement of the secreted ALP. The ALP activity in lysates and in media was measured using *p*-nitrophenyl phosphate as substrate and absorbance at 405 nm was measured as previously described. Protein concentration was estimated by BCA assay (Pierce Biotechnology, Rockford, IL, USA). The activity of cellular and medium ALP was expressed as nmol sodium paranitrophenyl-2-phosphate (PNPP)/mg protein/min and nmol PNPP/ml medium/min, respectively.

2.6.2. Cell matrix ALP staining

Cultured cells were rinsed with PBS and fixed in 2% formaldehyde. The cells were stained using Naphthol As-Mx phosphate disodium salt as a substrate, *N,N*-dimethyl formamide and fast red salt (Sigma) as dye for 30 min at 37°C, or until yellow color appeared. After washing with PBS, the cells were photographed using a phase-contrast microscope. The alkaline phosphatase activity was assessed as red stains indicating the products of enzyme activity.

2.7. ECM calcium deposit staining

2.7.1. Alizarin red S staining

To measure extracellular matrix Ca deposits for bone nodule formation, cellular matrix was stained using Alizarin red S dye which combines with Ca in the matrix. Cells were seeded (1×10^4 cells/ml) in 48-well plates and incubated with 0, 0.01, 0.1, 1, 3 and 10 μM diosgenin. At the differentiation days of 15 and 25 days, cells were washed with PBS twice and then fixed with 2.0% formaldehyde. The cells were stained with 40 mM of Alizarin red S solution (pH 4.4) for 45 min at room temperature and rinsed with deionized water twice.

2.7.2. von Kossa staining

Since Ca coprecipitates with the phosphate ion in the matrix, mineralization of the nodules in the cultures was assessed using von Kossa stain. Cells were washed with PBS and fixed with 2.0% formaldehyde for 15 min. After washing with deionized water three times, cells were incubated with 3% silver nitrate at room temperature under ultraviolet light for 1 h. After washing with deionized water, the images of the stained cells were also photographed using phase contrast microscope.

2.8. Western blot analysis

MC3T3 cells were cultured in 10 cm dishes and treated with 0, 0.01, 0.1, 1, 3 and 5 μM diosgenin for 3 and 10 days. Cells were harvested and lysed with lysis buffer (150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 25 mM Tris-HCl, pH 7.6, supplemented with 1% protease inhibitor). Whole cell lysates were separated by SDS-polyacrylamide gel electophoresis (SDS-PAGE) with 12% gels and transferred to PVDF membrane (Immobilon-P, Millipore, USA). The membranes were blocked with 5% nonfat milk and incubated with the primary antibodies for collagen Type 1 and ALP (Santa Cruz Biotechnology). The membranes were then incubated with secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and were developed using Super Signal West Pico Chemiluminescence detection reagents (Pierce Biotechnology).

2.9. Immunoprecipitation for Runx2 and osteopontin

Osteopontin and Runx2 were precipitated by incubating 100 μg of total proteins with 1 μg of anti-Runx2 and anti-osteopontin antibodies (Santa Cruz Biotechnology), respectively, overnight at 4°C. The antigen-antibody complex was then added to protein A/G agarose resin (Pierce Biotechnology) and incubated at room temperature for 2 h to facilitate binding. The bound proteins were washed five times with IP buffer (25 mM Tris, 150 mM NaCl, pH 7.2). Finally, the bound proteins were eluted using Ig elution buffer (0.2 M glycine-HCl, pH 2.8), resolved by SDS-PAGE, and transferred to PVDF membrane. Membranes were immunoblotted with antibodies as indicated, and proteins were visualized as previously described.

2.10. Statistical analyses

Data were analyzed using software SPSS 17.0. Values for cell proliferation, ALP activity and collagen synthesis are presented as mean±S.E. The data analysis was performed using one way analysis of variance (ANOVA) and Tukey's HSD test was used as post hoc to test if significance was detected among the treatments at the level of $P<.05$.

3. Results

3.1. Cell proliferation assay

The effects of serial concentrations (0–10 μM) of diosgenin on the proliferation of osteoblastic MC3T3-E1 cells for 1, 3 and 6 days treatment were examined using Alamar blue assay for cell proliferation. A preliminary assay was carried out to compare the viability/proliferation rate using DMSO and ethanol as solvents for diosgenin. Neither solvents showed any cytotoxicity in osteoblastic cells, however, DMSO-dissolved diosgenin exhibited higher efficiency for proliferation than ethanol-dissolved diosgenin; thus, DMSO was used as solvent for subsequent experiments (data not shown). There was no vehicle (DMSO) effect, compared to normal osteogenic control (OSM). Diosgenin exhibited stimulatory effects on osteoblastic cell proliferation in the range of 0.01–5 μM. A particularly prominent stimulation was observed within the range of 1–2 μM (Fig. 1A) on 1, 3 and 6 days of culture period. There was no observed cytotoxic effect on all levels of concentration within 1-day treatment; however, the cytotoxic effect by diosgenin at 10 μM became apparent during 3- and 6-day treatment periods. The protein synthesis of osteoblastic cells during bone matrix maturation period (5–10 days) was also measured (Fig. 1B). The observed stimulation of protein synthesis by diosgenin showed the same pattern of cell proliferation within the range of 2–4 μM diosgenin during 5 and 10 days treatment. During early matrix maturation at 5 days, an observed reduction of protein synthesis was observed in DMSO-treated cells. This temporal reduction might be possibly due to cellular adjustment during this period, since cells were able to recover at 10 days showing accelerated protein synthesis induced by diosgenin.

3.2. Collagen synthesis and secretion

Type 1 collagen is the major matrix protein and an integral part of the ECM which serves as scaffold in which hydroxyapaptite crystals are deposited during matrix mineralization. Thus, the formation of a collagenous ECM is a prerequisite for bone formation. As shown in the Fig. 2 A and B, cellular (synthesized) and medium (secreted) collagen levels increased in a time-dependent manner. Diosgenin significantly stimulated collagen synthesis and secretion within the concentration

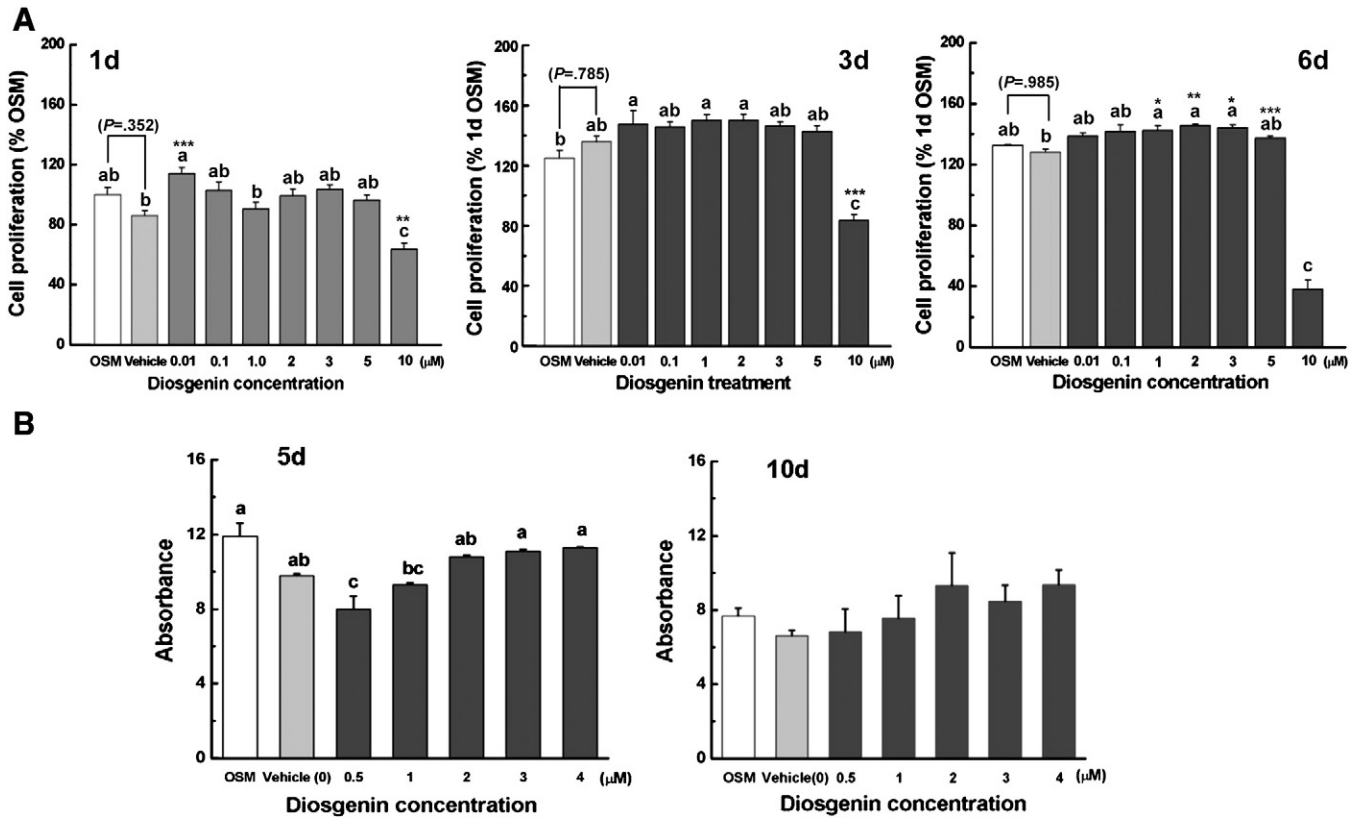
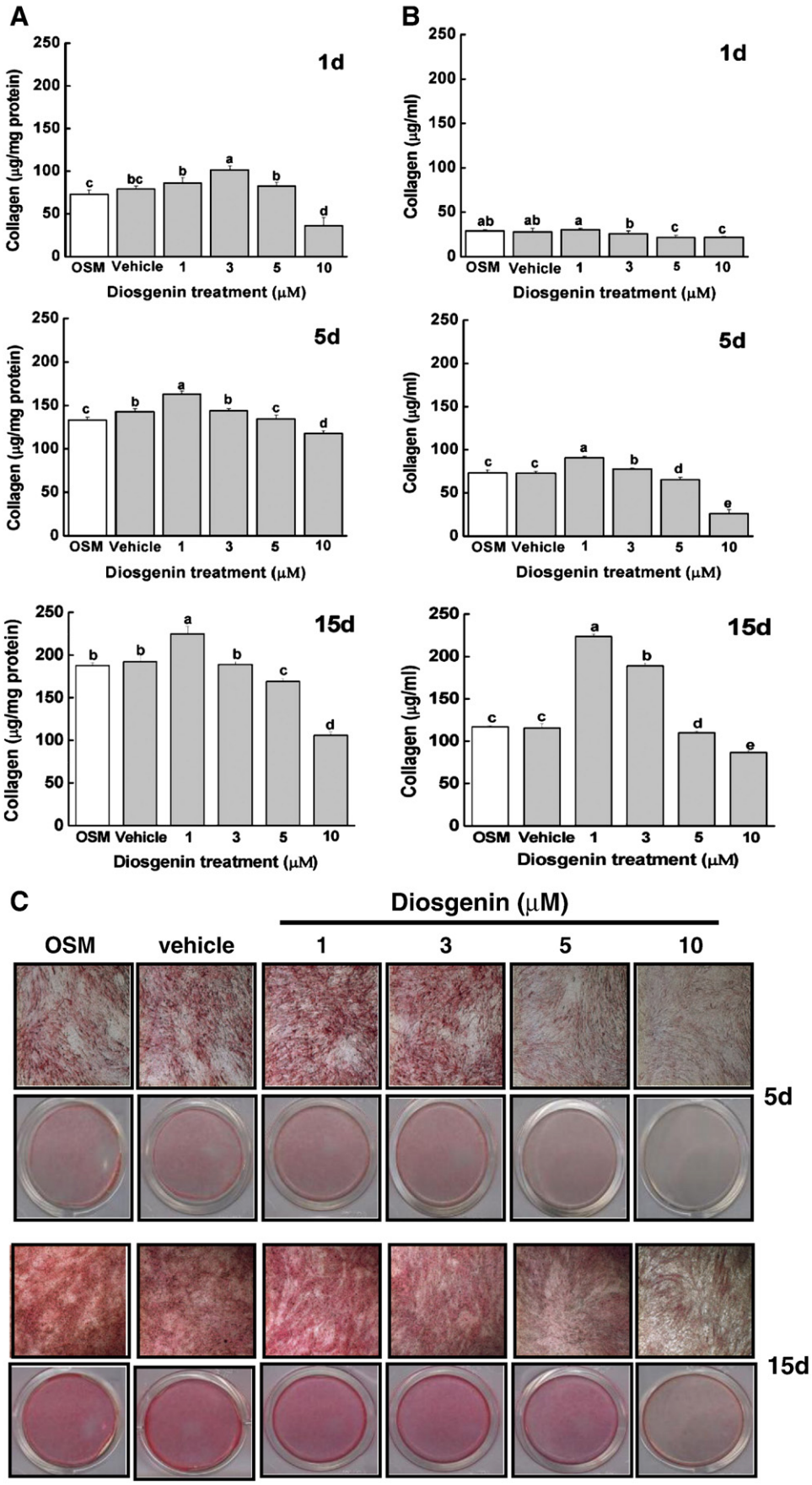


Fig. 1. Diosgenin effect on osteoblast proliferation (A) and protein synthesis (B) in MC3T3-E1 cells. Diosgenin was dissolved in DMSO (0.14 M) as vehicle and treated to the cells. (A) Cell proliferation was measured using AlamaBlue kit* (Invitrogen) or MTS assay (Promega). No vehicle cytotoxicity was observed, compared to normal osteogenic differentiation media (OSM). Diosgenin stimulated osteoblast proliferation up to 6 days within the range of 0.01–5 μM. Mean±S.E.M ($n=6$), abc: analyzed by one-way ANOVA, followed by Tukey test for mean comparison. As compared to vehicle. *$P<.05$; **$P<.01$; ***$P<.001$. (B) Cellular protein synthesis, measured by BCA assay (Promega), also showed the same pattern of cellular proliferation at Day 5 with significance and the same trend at day 10. Mean±S.E.M. ($n=4$), abc: Tukey test, one-way ANOVA, $P<.05$.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range of 1–3 μM compared to normal OSM group, and dose-dependently decreased up to 10 μM during 1, 5 and 15 days of treatment period.

The effect of various concentrations of diosgenin on extracellular matrix collagen was also measured by staining the cell matrix on 5 and 15 days culture. Cell matrix collagen level as indicated by the intensity of red dye in the cell layers, was higher in the treatment with 1–3 μM diosgenin than the normal OSM group (Fig. 2C). Collagen staining also showed a time-dependent stimulation in response to diosgenin treatment within 15 days of culture period.

3.3. Cellular and medium ALP activity

ALP is a major osteoblastic differentiation marker whose synthesis is largely dependent on the synthesis and processing of Type 1 collagen. We examined the activity of ALP during 1, 5, and 15 days of osteoblast proliferation and differentiation periods, both in cells (Fig. 2A) and in media (Fig. 2B). Cellular (synthesized) ALP activity was increased significantly by diosgenin treatment within the concentration range of 1–3 μM during 5–15 days diosgenin treatment, as compared to normal osteogenic differentiation media (OSM) or vehicle controls. There was no negative vehicle effect compared to the normal OSM control, and eventually, cellular ALP activity was further increased on day 15 (Fig. 3A). Since ALP is cell membrane-attached and is a secreted protein, medium ALP activity was also measured. Media ALP activity which is at extracellular secreted one also showed the same trend with marked increase by low concentrations of diosgenin treatment (1–3 μM) on 1, 5 and 15 days of treatment (Fig. 3B).

The influence of diosgenin treatment on cell layer ALP activity in extracellular matrix was measured by ALP staining. ALP activity showed a time-dependent stimulation in response to diosgenin treatment at 5 and 15 days of culture period (Fig. 3C). At 1 day treatment there was no significant difference on ALP activity by diosgenin, however, staining was barely visible at 10 μM diosgenin (data not shown). Proliferation data did not show any cytotoxic effect within 1-day treatment; however, based from this result it is clear that ALP activity was already compromised at this early stage. The diosgenin stimulated ALP activity was more pronounced in the concentration range of 1–3 μM on day 5 and 15, as compared to the OSM and vehicle controls.

3.4. Mineralization assay

To examine the effect of diosgenin on mineralization, we assessed whether diosgenin treatment could increase calcium deposits in the ECM of treated cultures. Extracellular matrix Ca deposits for mineralized nodule formation were both stained with Alizarin red S dye which combines with Ca ions (Fig. 4A) and von Kossa dye where silver nitrate combines with phosphate that are normally proportional to Ca deposits (Fig. 4B). The calcified nodules appeared bright red color by Alizarin red S staining and brown by von Kossa staining. Mineralization by MC3T3 cells occurred in a time-dependent manner within 25 days culture and low concentrations of diosgenin (0.01–1 μM) stimulated ECM mineralization in a dose-dependent manner whereas Ca deposits were not observed in cells treated with higher concentrations (>5 μM) diosgenin using either Alizarin Red S or Von Kossa staining.

3.5. Expression of bone related proteins

Similar to previous experiments, MC3T3 cells were treated with varying levels (0–5 μM) of diosgenin for 3 and 10 days. Cells were harvested and lysed in the indicated treatment period and the whole cell homogenates were assessed for bone matrix proteins Type 1 collagen and ALP, by Western blot analysis. ALP and collagen Type 1 protein expression patterns showed a dose-dependent increase at the low concentration range of diosgenin up to 1 μM, followed by a decrease at high concentrations (3–5 μM) on 3 and 10 days treatment (Fig. 5A). This pattern corresponds both with the cellular and synthesized collagen and ALP levels.

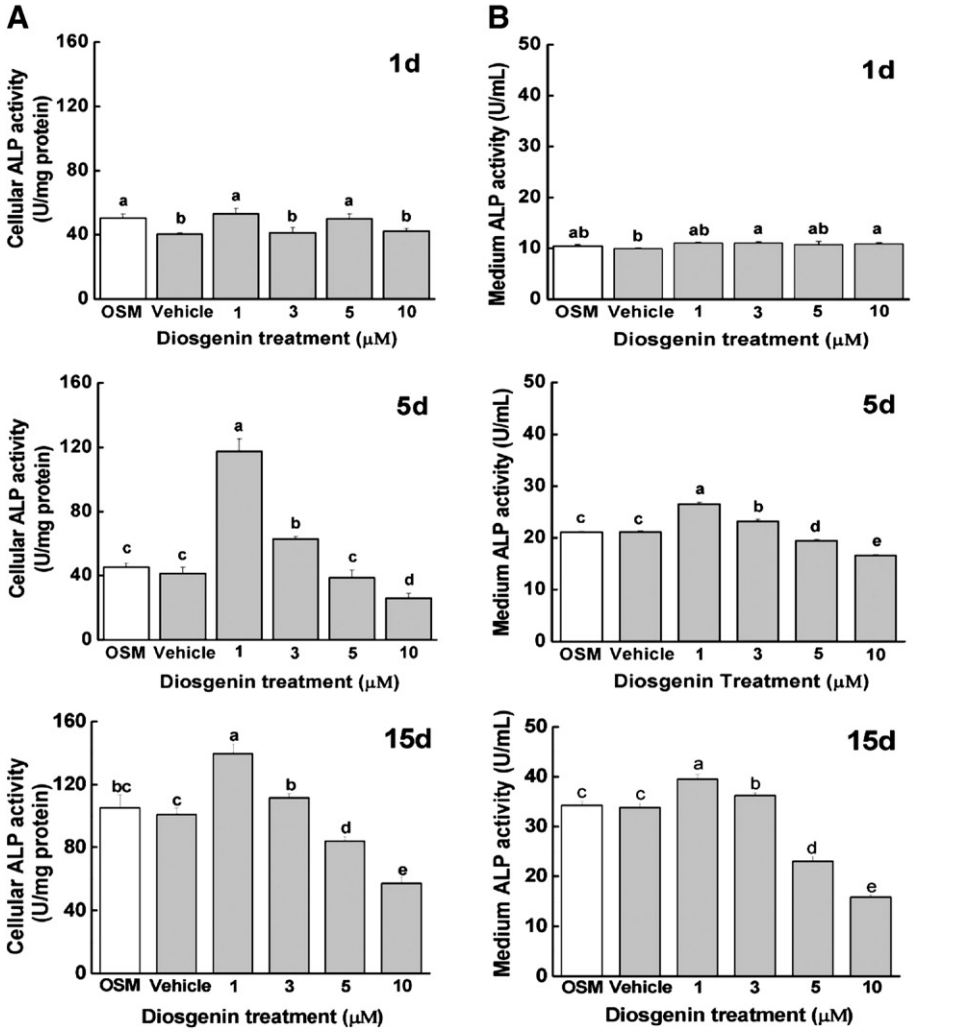
Since osteoblast marker proteins are regulated by Runx2, the major bone-specific transcription factor responsible for bone marker gene transcription for osteoblast differentiation, its expression as affected by diosgenin treatment was also assessed, along with osteopontin expression by blotting of immunoprecitated proteins (Fig. 5B). Osteoponin appears in the later part of differentiation, just before the onset of matrix mineralization. At 10 days treatment, immunoprecipitated Runx2 protein expression was slightly stimulated at low diosgenin concentration (up to 0.1 μM) and was then decreased at 1–3 μM. The protein expression of osteopontin, which is regulated by Runx2 and as another major osteoblast marker protein, showed the same pattern of Runx2 expression.

4. Discussion

Bone metabolism involves a delicate balance between bone formation and resorption. Recently, a number of studies reported that dietary components and herbal products can positively influence these processes, particularly by inhibiting bone resorption, thus having beneficial effects on the skeleton [14]. A wide variety of natural compounds with therapeutic effect on bone formation and skeleton construction have been reported and several natural compounds have been shown to enhance osteogenic differentiation [15–21], such as red yeast rice [16,19], black cohosh [17] and green tea [18]. Previously, diosgenin was found to enhance VEGF-A production and promote angiogenesis in MC3T3-E1 cells [13], both of which play a critical role in the process of bone growth, repair and remodelling [22]. Furthermore, sustained diosgenin delivery has been shown to lower bone loss in ovariectomy-induced osteoporosis in rats [12]. However, in vitro studies on the effect of diosgenin on bone formation have been limited. Thus, in the present study, we investigated the effect of diosgenin on the proliferation, differentiation and mineralization of murine MC3T3-E1 osteoblastic cells.

The problem that usually arises in using some bioactive compounds is the marked toxicity on the cell culture system. Initially, to optimize the solvent for diosgenin in this study, the proliferation of MC3T3-E1 cells was investigated with varying levels of diosgenin concentrations (0–10 μM) using DMSO and ethanol as vehicle. Since the viability and proliferation of cells is much higher and stable in DMSO compared to ethanol, we used DMSO as vehicle for subsequent experiments. To remove the possibility that any observed cytotoxicity could be due to the amount of DMSO introduced in the culture media, we used the same volume of DMSO in our vehicle control

Fig. 2. Cellular (A) and medium (B) collagen quantification and cell matrix collagen staining (C) by diosgenin treatment. Cells were treated with varying levels of diosgenin and DMSO as vehicle for 1, 5 and 15 days. (A and B): Cellular and medium collagen level were measured by colorimetric method using Picro-Sirius Red. Collagen synthesis was normalized with protein concentration. Means having different superscripts indicate significant difference between diosgenin treatments at $P<.05$ by ANOVA followed by Tukey's post hoc test ($n=8$, mean+8). OSM, osteogenic medium; vehicle, DMSO. (C) The effect of diosgenin on the formation of collagen scaffold for mineralization was assessed by staining and presented as the image of the phase contrast microscopic morphology (upper images) and the cellular layer on each culture dish (lower images). Matrix collagen shown as red stain intensity was stimulated by diosgenin treatment in a dose- and time-dependant manner and it was more prominent on Day 5 and 15. Representative image of four wells.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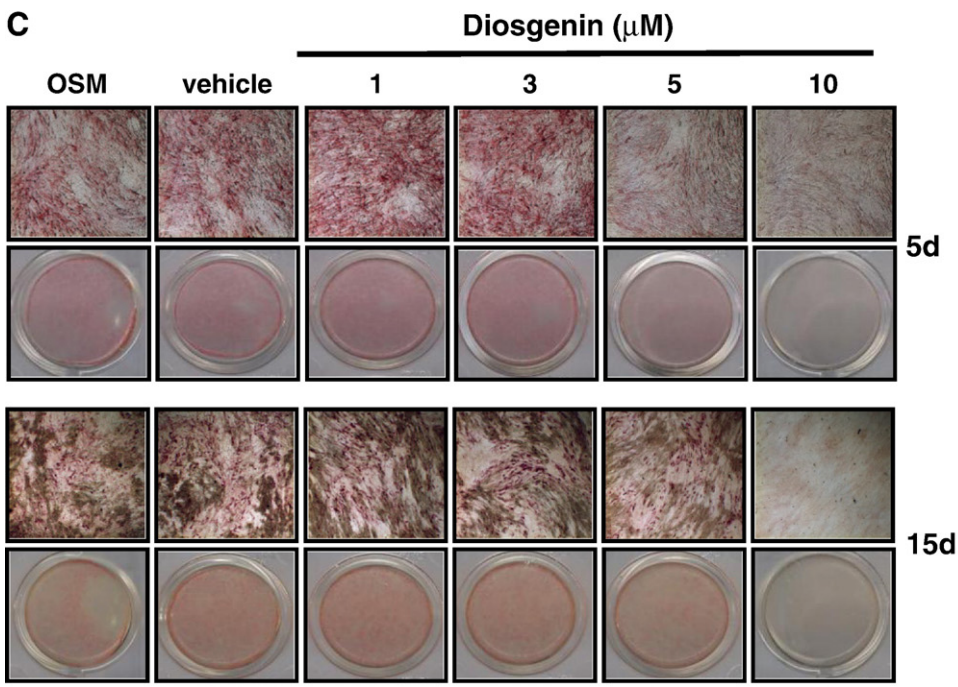
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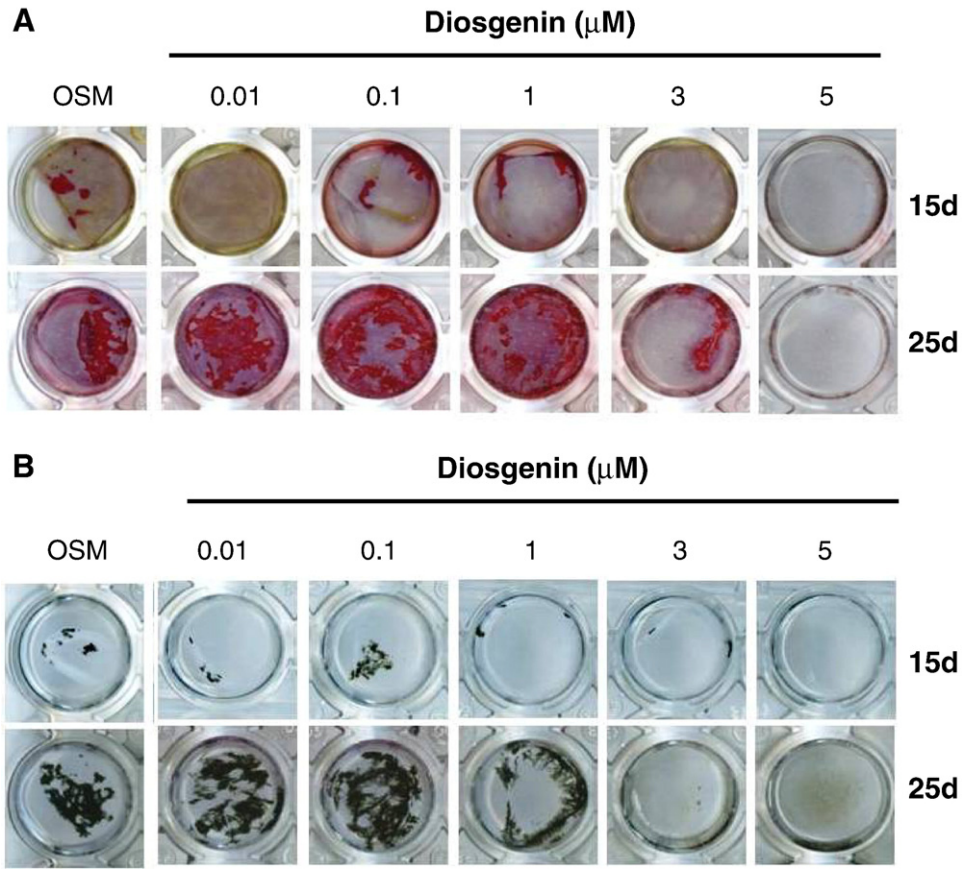

Fig. 4. Alizarin red (A) and von Kossa (B) staining for Ca deposits (bone nodule) by diosgenin treatment in MC3T3-E1 cells. Extra cellular matrix Ca deposits for matrix mineralization was measured using Alizarin red S dye which binds with Ca and silver nitrate dye which binds with P in cell layer matrix. Diosgenin treatment in low concentration (0.01–1 μM) increased mineralization as compared to control groups in 25-day culture.

corresponding to that in 10 μM diosgenin which was our maximum diosgenin concentration. Diosgenin was found to enhance proliferation dose-dependently up to 10 μM without any observed toxicity within 24-h treatment. However, subsequent proliferation assay showed an apparent toxicity at 10 μM during 3- and 6-day culture period. In addition, diosgenin also increased protein synthesis during matrix maturation period (10 days) within the range of 2–4 μM. Our proliferation results imply that determining the right diosgenin concentration is very critical in defining whether it would enhance bone formation or induce cell death on osteoblastic cells.

The formation of an efficient extracellular matrix is a prerequisite for mineralization. In vitro, collagen is the major synthetic product of osteoblast cultures and the formation of the collagenous matrix contributes to changes in cell structure, osteoblast differentiation and gene expression in multiple ways [23]. Moreover, the induction of ALP, the most widely recognized marker for osteoblastic activity, requires the initial processing of collagen components [24]. The levels of collagen synthesis in cells/media and in the ECM, as assessed by colorimetric determination and ECM staining, respectively, were stimulated by diosgenin in a time-dependent manner. The highest collagen stimulation was observed at 1 μM diosgenin treatment and dose-dependently decreased up to 10 μM, and this stimulation is consistent within the 15-day cell culture period. The effects of collagen on the developmental expression of the osteoblast phenotype are most likely mediated through interaction of collagen with heterodimeric integrin receptors [25]. In addition, the dependency of mineralization on a mature collagenous extracellular matrix can be further emphasized when osteoblastic cells are cultured in the absence of ascorbic acid, a cofactor for collagen synthesis and accumulation. In the absence of ascorbic acid, ALP levels are reduced fivefold and mineralization does not proceed [23]. Ascorbic acid stimulates procollagen hydroxylation, processing and fibril assembly, followed by the dramatic induction of osteoblast-associated genes including ALP, osteopontin, osteocalcin and PTH/PTH-related peptide receptors [26]. Similarly, an identical pattern of diosgenin effect was also observed with ALP secretion and synthesis. This result is somewhat expected since ALP induction is largely dependent on collagen synthesis [24]. ALP, a homodimeric metalloenzyme which catalyzes the hydrolysis of phosphomonoester with release of inorganic phosphate (P_i) and alcohol, is one of the most frequently used biochemical markers of osteoblast activity. It provides the P_i involved in crystal nucleation in the ECM [27] and degrades the ubiquitous mineralization inhibitor pyrophosphate (PP_i) [28], eventually inducing the formation of hydroxyapatite crystals within the collagen fibrils. In the in vivo osteoblast model by Murshed et al. [29],

Fig. 3. Cellular (A) and medium (B) ALP activity and cell matrix ALP activity staining (C) by diosgenin treatment. Cells at confluence were treated with varying levels of diosgenin and DMSO as vehicle for 1, 5 and 15 days. (A and B) Cellular and medium ALP were measured by colorimetric method using PNPP as substrate. ALP activity was normalized with cellular protein concentration. Means having different superscripts indicate significant difference between diosgenin treatments at $P<.05$ by ANOVA followed by Tukey's post hoc test. ($n=8$, mean±8). (C) The effect of diosgenin on extracellular matrix ALP activity was measured by staining the products of enzyme activity and presented as the image of the phase contrast microscopic morphology (upper images) and the cellular layer on each culture dish (lower images). The cells were cultured in six-well plates and incubated in AS-MX phosphate solution as substrate with Fast Red salt as stain. Matrix ALP activity shown as red stain intensity was stimulated by diosgenin treatment in a time- and dose-dependant manner and was more prominent on Days 5 and 15.

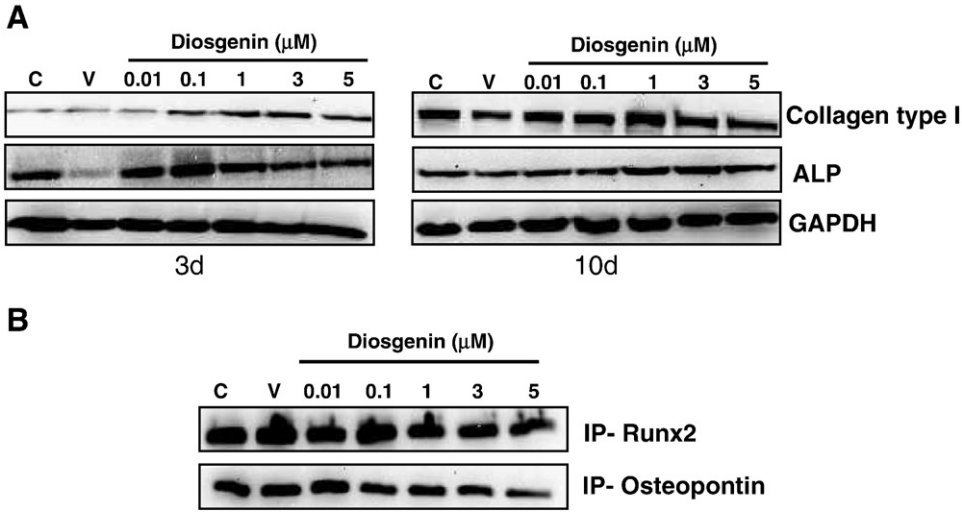

Fig. 5. Effect of diosgenin on bone matrix proteins, bone-specific transcription factor Runx2 and osteopontin. (A) The protein expressions of bone matrix and also early osteoblast differentiation marker proteins, collagen Type 1 and ALP, were analysed by Western blot analysis at 3 and 10 days diosgenin treatment (0–5 mM). Diosgenin increased the protein expression of collagen Type 1 and ALP within the low range of diosgenin (0.01–1 mM). (B) Runx2 and osteopontin were immunoprecipitated and their expressions were subsequently analyzed by Western blot analysis at 10-day diosgenin treatment. Representative image of two replicates with repeating twice for both immunoblotting and immunoprecipitated blotting.

coexpression of tissue non-specific alkaline phosphatase and Type 1 collagen is necessary and sufficient to induce ECM mineralization in bone. Thus, the inductive effect of low dose of diosgenin (1 μM) on the collagen Type 1 and ALP levels in MC3T3-El cells is a positive indicator of its ability to promote osteogenesis in this particular cell line. The ECM calcium deposits were also assessed up to 25-day culture period. Again, diosgenin enhanced formation of calcium deposits in a time-dependent and dose-dependent manner as previously observed, however, the effective range of dose is within 0.01–3 μM only. The protein expression of both collagen Type 1 and ALP also showed the similar pattern of the stimulatory effect of diosgenin within the concentration range of 0–5 μM. These two major bone matrix proteins, with respect to their quantity and ability to support calcification, are synthesized within the cells and are being secreted outside the osteoblasts and eventually contribute to construct the mature bone matrix.

Runx2 is a master transcription factor regulating osteoblast differentiation and mineralization. It is at the top of a genetic cascade controlling positively its own expression and the expression of the major osteoblast-specific and osteoblast-enriched genes, including Type 1 collagen, osteocalcin and osteopontin, through its binding to multiple osteoblast *cis* acting element 2 elements present in these genes [30]. To investigate whether bone-specific transcription factor Runx2 and osteopontin, which is the protein product of Runx2-regulated bone marker gene are stimulated by diosgenin, we analyzed their expression by immunoaffinity followed by immunoblotting. Runx2 protein expression was increased by diosgenin within the low range of 0–1 μM dose-dependently, and then decreased up to 5 μM diosgenin. The highest Runx2 expression was observed at 0.1 μM diosgenin level implying that the optimal concentration can be within the low range of diosgenin for osteogenicity. Consistently, the same expression pattern was also observed for osteopontin, another bone marker protein for osteoblast differentiation and mineralization whose expression is also regulated by Runx2.

Considering all the findings of the present study, our results suggest that defining the right diosgenin dose is critical in enhancing bone formation. With our cell culture system using osteoblastic MC3T3-E1 cells, it is clear that low doses of diosgenin (0–5 μM) could enhance osteoblastic cell differentiation. The positive effect of diosgenin on bone formation is mediated by its ability to increase cell proliferation and the synthesis of cellular and synthesized Type 1 collagen and ALP and eventually enhancing matrix calcification by Ca deposit. This study results may aid in the development of therapeutic approach utilizing diosgenin for the enhancement of bone health and prevention of bone-related disorders such as osteoporosis. Further study is also needed to elucidate the potential mechanism by which diosgenin affects osteogenesis to design a more effective treatment scheme.

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