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Dimethyl sulfoxide as an inducer of differentiation in preosteoblast MC3T3-E1 cells

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Abstract Osteoblastic differentiation is an essential part of bone formation. Dimethyl sulfoxide (DMSO) is a water miscible solvent that is used extensively for receptor ligands in osteoblast studies. However, little is known about its effects on osteoblastogenic precursor cells. In this study, we have used a murine preosteoblast cell line MC3T3-E1 cells to demonstrate that DMSO effectively induces osteoblastic differentiation of MC3T3-E1 cells via the activation of Runx2 and osterix and is dependent upon the protein kinase C (PKC) pathways. We further demonstrated that prolonged activation of PKC pathways is sufficient to induce osteoblastic differentiation, possibly via the activation of PKD/PKCμ.

Keywords: DMSO; Protein kinase C; PKD; Erk; Proliferation; Osteoblasts

1. Introduction

In normal bone remodeling or bone turnover, osteoblastic bone formation and osteoblastic bone resorption is coupled in a precise and orchestrated manner. Osteoblastic differentiation is an essential part of bone formation. Several groups of proteins are necessary for osteoblastic differentiation, such as bone morphogenetic proteins (BMPs) and transcription factors such as Runx2 and osterix (Osx) [1,2].

Dimethyl sulfoxide (DMSO; $(CH_3)_2SO$) is a water miscible solvent that serves as powerful solvents that dissolve most water-insoluble drugs [3]. DMSO possesses anti-inflammatory properties [4], as well as the ability to act as a free radical scavenger [5]. Thus, its properties have been exploited in the treatment of dermatological, rheumatic, and renal manifestations of amyloidosis. Depending on the cellular context and probably via the alteration of splice site selection, DMSO is capable of inducing or inhibiting cell proliferation, apoptosis and/or differentiation [6]. DMSO induces the differentiation of many cell types including leukemia cells and mammary adenocarcinoma LA7 cells [7,8]. DMSO induces differentiation of LA7 cells into mammary cells with milk production, which originally requires the supplementation of lactogenic hormones. Therefore, it is recently developed as an in vitro model system for studying mammary cells differentiation [8]. In osteoblast studies, DMSO is used extensively as a solvent for receptor ligands such as rosiglitazone for peroxisome proliferator-activated receptor gamma [9]. However, limited data is available on the effect of DMSO on the osteoblastic differentiation.

In this study, we investigated the effect of DMSO on the proliferation and differentiation of MC3T3-E1 cells, a non-transformed murine cell line derived from neonatal mouse calvariae. Treatment with BMPs, such as BMP2, induces MC3T3-E1 cells to undergo osteoblastic differentiation, which leads to the formation of bone nodules in vitro [10]. We demonstrated that DMSO effectively induces osteoblastic differentiation of MC3T3-E1 cells via the activation of Runx2 and Osx and is dependent upon the protein kinase C (PKC) pathway.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma–Aldrich Co. (St. Louis, MA, USA) unless otherwise stated.

2.2. Cell culture

MC3T3-E1 cells (clone 4; obtained from American Type Culture Collection, Rockville, MD, USA) were maintained in complete modified Eagle's medium (MEM) alpha medium (αMEM; Invitrogen, San Diego, CA, USA) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA) in a humidified 5% CO_2 atmosphere at 37 °C. Cells were passaged before reaching confluence.

2.3. Cell differentiation

During cell differentiation studies, MC3T3-E1 cells were plated at 5×10^4 cells per cm^2, cultivated to complete confluence and then treated with increasing concentrations of DMSO (0.02–1%). For bone nodules formation, MC3T3-E1 cells were cultured in osteogenic medium containing 400 μM ascorbic acid and 5 mM β-glycerophosphate to provide inorganic phosphate. PKC activator, phorbol 12-myristate 13-acetate (PMA) and inhibitor, GF 109203X (GFx), both obtained from Calbiochem-Novabiochem (San Diego, CA, USA) were employed to modulate the activity of PKC in MC3T3-E1 cells. Cells were normally pre-incubated with PMA (1 μM) or GFx (2 μM) for 1 h and then differentiated with DMSO for 6 days in phosphorylation and 15 days in bone nodules formation studies. As DMSO was used as solvent for PMA and GFx, equal volume of DMSO that contributed to less

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Abbreviations: DMSO, dimethyl sulfoxide; PKC, protein kinase C; BMP, bone morphogenetic protein; Osx, osterix; GFx, GF 109203X; PMA, phorbol 12-myristate 13-acetate; Tris, Trizma base; TX, Triton-X100; ALP, alkaline phosphatase; PNPP, *p*-nitrophenyl phosphate; MAPKs, MAP kinases

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than 0.05% (v/v) was included as solvent control. Fresh culture media containing PKC modulators and DMSO were replenished every 2 days. Cell differentiation experiments were repeated for three times and data from representative experiments are shown.

2.4. Cell proliferation assays

Cell proliferation studies were performed as previously described using MTT (GE Healthcare, Piscataway, NJ, USA) assays [11]. After treatment with DMSO (0.02–1%) for 2 days, MTT was added at a final concentration of 0.5 mg/ml for 3 h, cells were solubilized with isopropanol containing 0.1% sodium dodecyl sulfate and 0.04 N hydrochloric acid, and the absorbance measured at 570 nm. All experiments were repeated for three times.

2.5. Total protein extraction, Western blot analysis and antibodies

MC3T3-E1 cells were washed with PBS and then lysed with lysis buffer containing 50 mM Trizma base (Tris)–Cl at pH 8, 150 mM sodium chloride, 1% Triton-X100 (TX), 1× aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate, and 1× Complete™ protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) at 4 °C for 15 min. Western blot analysis was performed as previously described [12] using polyclonal antibodies against phosphorylated p44/p42 MAP kinase (MAPK) (p-Erk1/2), phosphorylated pan-PKC, phosphorylated PKCθ (p-PKCθ), phosphorylated PKD/PKCμ (p-PKD/PKCμ), as well as monoclonal antibodies against Erk2 and different isotypes of PKC that were obtained from Cell Signaling Technology (Beverly, MA, USA) and Transduction Laboratories (Lexington, KY, USA), respectively. Data presented are representative of independent experiments repeated twice with proteins extracted from duplicate treatment.

2.6. Total RNA extraction and gene expression analysis by real time quantitative PCR

Transcript expression of Runx2, Osx, and alkaline phosphatase (ALP) was determined using reverse transcription (RT) followed by real-time TaqMan quantitative PCR (qPCR) analysis performed in a ABI 7700 sequence detector (Applied Biosystems Inc., Foster City, CA, USA). Total RNA was isolated using TRI Reagent (Molecular Research Center Inc., Cincinnati, OH, USA). First-strand cDNA was synthesized using random hexamers and M-MLV reverse transcriptase (Promega Co., Madison, MI, USA). TAMRA labeled probes and PCR reagents were obtained from ABI. PCRs were performed in triplicate and data obtained were analyzed using the comparative CT method (ABI) with signals normalized to 18S signal for each sample. All experiments were repeated for 3 times.

2.7. Assessment of osteoblastic cell differentiation

Cells were incubated without or with DMSO (0.02–1%) in normal or osteogenic culture media for selective time periods. ALP activity was measured as *p*-nitrophenol produced from the hydrolysis of *p*-nitrophenyl phosphate (PNPP) [13]. In brief, cells were washed with ice-cold PBS, lysed with 50 mM Tris, pH 7.4, and 0.1% TX, and incubated with 2-amino-2-methyl-1-propanol buffer (0.5 M, pH 10.4) containing 10 mM PNPP at 37 °C for 15–30 min. The reaction was stopped by the addition of NaOH (final concentration, 0.5 M) and the absorbance measured at 410 nm. Enzyme activity was normalized to the protein content determined using Protein Assay Kit (Bio-Rad). Experiments were performed in triplicate wells and data shown are representative of two independent experiments.

2.8. Histochemical staining

Cells were washed twice with ice-cold PBS and then fixed in ice-cold fixative (10% formalin in PBS) for 15 min. After washed with deionized water, fixed cells were stained with Fast Blue RR Salt and Napthol AS-BI Phosphate for 30 min, and the reaction stopped by washing the cells with surplus deionized water. Mineralized matrix was detected using Von Kossa staining by treating fixed cells with 5% silver nitrate for 30 min, followed by subsequent washes with 5% sodium carbonate in 10% formalin for 1 min and 5% sodium thiosulfate for 5 min. The reaction was stopped by washing the cells thrice with deionized water. Alternatively, fixed cells were stained with 1% alizarin S solution, pH 4.2 for 15 min and then were washed thoroughly with deionized water.

2.9. Statistical analysis

Statistical analyses were performed using Two-way ANOVA. Bonferroni analyses were used for post hoc examination of the ANOVA results ($P < 0.0001$). All statistical analyses were performed using the Statview for Macintosh software package (SAS Institute Inc., NC, USA, version 5).

3. Result

3.1. Effect of DMSO on cellular proliferation of MC3T3-E1 cells

No significant difference was observed in cell proliferation in cells incubated with DMSO at 0.5% (v/v) or below (Fig. 1). However, 1% DMSO significantly reduced the proliferation of MC3T3-E1 to 93.3 ± 2.0% (Fig. 1; $n = 5$, $^{*}P < 0.001$). While osteogenic medium alone increased proliferation to 133.7 ± 4.2%, 1% DMSO significantly suppressed the enhancement to 123.7 ± 3.6% when compared to control. Viable cell count confirmed that osteogenic medium increased the cell number of MC3T3-E1 cells and DMSO significantly reduced the cell number in both normal and osteogenic media (data not shown).

3.2. DMSO-induced formation of bone nodules via increasing number of ALP expressing cells

At high concentrations of DMSO (0.5% and 1%), there were noticeable morphological changes and formation of mineralized bone-like nodules (Fig. 2A). The mineralization phenotype was also assessed and confirmed by alizarin red S staining (Fig. 2B). To determine whether DMSO-induced osteoblastic differentiation or only accelerated the rate of mineralization, samples were assayed for ALP activity. MC3T3-E1 cells cultured with DMSO resulted in a significant and dose-dependent increase in ALP activity (Fig. 3A). Maximal ALP activity was observed with 0.5% DMSO, which reached a plateau with 1% DMSO (Fig. 3A). ALP staining confirmed DMSO increased ALP-positive cells upon and was comparable to that of control treatment with 50 ng/ml BMP2 (Fig. 3B).

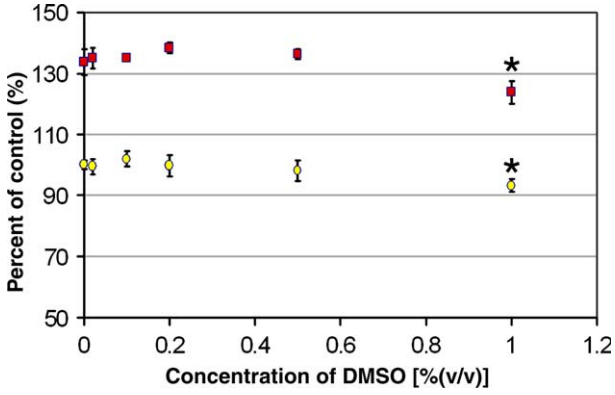

Fig. 1. Effect of DMSO on the proliferation of MC3T3-E1 cells. MC3T3-E1 cells were cultured for 2 days in normal (circle) or osteogenic medium (box) with increasing concentrations of DMSO. Cell proliferation was assessed by MTT reduction. All readings were calculated by comparing with the untreated control cells, which was set as 100%. $^{*}P < 0.01$ compared with no-DMSO treatment group, unpaired Student's *t* test. Results are the means ± S.E.M. of 2 independent experiments ($n = 5$).